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RESEARCH ARTICLE

Variation in genotoxic susceptibility and biomarker responses in *Cirrhinus mrigala* and *Catla catla* from different ecological niches of the Chenab River

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Abstract A large number of methods have been applied to evaluate genotoxic damage in different aquatic species. Comet assay, as a method for detecting DNA alterations, and micronucleus test, as an index of chromosomal damage are the most widely used and authentic methods in laboratory and field studies. The primary objective of the study was to evaluate the genotoxic effects of heavy metals generated by toxic industrial effluents and various kinds of pollutants from urban and agricultural areas and domestic waste on *Catla catla* and *Cirrhinus mrigala* due to water pollution in the Chenab River, Pakistan. The heavy metals Cd, Cu, Mn. Zn, Pb, Cr, Sn, and Hg were detected by atomic absorption spectrophotometry from water samples collected from predetermined sampling sites. All the physicochemical parameters and heavy metals were found to exceed the upper limits recommended by various agencies. Comet assays showed significant ($p < 0.05$) DNA damage in *C. mrigala* compared to *C. catla* for tail length and olive tail moment from three different sites. Significant ($p < 0.05$) differences were reported between fish collected from polluted sites and farmed fish, but only non-significant ($p > 0.05$) findings were observed between fish collected from farmed and non-polluted upstream waters. Micronucleus assays showed similar findings for single and double micronucleus induction in *C. catla* and *C. mrigala*. A significantly ($p < 0.05$) higher micronuclei induction and percent tail DNA was observed in *C. mrigala* specimen collected from the polluted site. These findings infer that DNA damage could be used as a biomarker of pollution load and its early monitoring by using simple and reliable techniques such as the comet and micronucleus assays, expedient methods for toxicity screening of aquatic environments. Regular monitoring is necessary to assess eco-health of the Chenab River by choosing perhaps *C. mrigala*, being a bottom feeder, as a bioindicator that could provide more reliable information to determine the status of the environmental quality of the river.

Keywords DNA fragmentation · Biomarker · Heavy metals · Micronucleus · Genotoxicity

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Introduction

The Chenab River is a major river of India and Pakistan. It forms in the Upper Himalayas in the Lahaul and Spiti district of Himachal Pradesh, India, and flows through the Jammu region of Jammu and Kashmir into the plains of the Punjab, Pakistan. The waters of the Chenab are allocated to Pakistan under the terms of the Indus Waters Treaty. It flows from the Jammu region of Jammu and Kashmir into the plains of the Punjab. It is joined by the Jhelum River at Trimmu and then by the Ravi River at Ahmedpur Sial. It then merges with the Sutlej River near Uch Sharif, Pakistan, to form the Panjnad. The Chenab then joins the Indus at Mithankot. The total length of the Chenab is approximately 960 km (Qadir and Malik 2011). Industrial waste and domestic sewage are dumped through surface ditches (drains) into the water bodies

of the country. A recently conducted nationwide wastewater assessment showed that the total wastewater supply in Pakistan is 4.6 × 106 m^3/day, and a total of 7.85 million cubic meters per day of wastewater (30 % of the total) is used for irrigating an area of 32,500 ha. It has also been estimated that 64 % of the total wastewater is disposed of either into rivers or into the Arabian Sea. Similarly, 400,000 m^3/day wastewater is additionally added into canals. These practices threaten both human health and the environment at downstream and, more importantly, reduce the effective availability of Pakistan's already short water supplies (Ensink et al. 2004). Indiscriminate disposal of sewage and industrial effluent has seriously affected the quality of surface water.

Aquatic animals have an important role as bioassays to monitor water bodies for pollution. Changes in the genome caused by genotoxic pollutants can lead to mutations, and DNA-damaging agents require continuous monitoring and detection (Villela et al. 2006). There is a need to develop the molecular basis that can mark the effects of environmental pollutants for a wide range of organisms. Ostling and Johanson (1984) introduced a microgel electrophoresis technique for the detection of DNA damage in a single cell. Singh et al. (1988) used the same technique under alkaline conditions (pH>13) in order to detect the DNA damage in individual cells, the so-called comet assay. This assay presents advantages in comparison to other genotoxicity assays in that it is sensitive enough to detect low levels of DNA damage and requires only a small number of cells per sample (Tice et al. 2000). Another of the most promising and popular assays is the micronucleus (MN) test. This technique is a marker of cytogenetic damage mainly caused by aneugenic or clastogenic compounds. The assessment of cytogenetic damage is a very important assay for the identification of pollution threats in aquatic environments (Dixon et al. 2002).

Genotoxic pollution in aquatic environments has led to the development of many different test systems. Water and sediment samples could be tested for water quality parameters and biological systems such as bacteria, yeast, aquatic animals, and plants. Unfortunately, the river system in Pakistan has been dangerously exposed to toxic industrial effluents and various kinds of pollutants from urban and agricultural areas. These toxicants adversely affect not only the ecosystems of water bodies but also human health. Recently, research interest on the use of bioindicators and biomarkers for investigation of the mutagenic effects of pollution (environmental monitoring) is developing (El-Shehawi et al. 2007). For this purpose, fishes are suitable organisms (Landolt and Kocan 1983) because they are susceptible to a variety of pollutants and play different and important roles in heterotrophic web undergoing bioaccumulation. They respond to a variety of mutagens even at very low concentrations (Goksoyr et al. 1991). The micronucleus assay was initially developed for application in mammals, but has now been subsequently modified for use in fish. Micronucleus assay is one of the best biomarkers that clearly correlate with pollution load (Baršienė et al. 2013). The aim of this study was to assess the genotoxic potential of *Catla catla* and *Cirrhinus mrigala* as data regarding the genotoxic and mutagenic nature of composite heavy metals on fish, which is lacking, from natural aquatic ecosystem. Furthermore, the study aimed to evaluate the suitability and sensitivity of blood cells through the comet and micronucleus assays of two selected fish species indwelling a heavy metal-contaminated aquatic ecosystem as a reliable indicator of DNA damage and water pollution load.

Materials and methods

Study area

The polluted water of the Chenab River has led to the extinction and decrease in population of different species of fishes in almost 190 km of its length. The industrial and sewage wastes of Faisalabad city are disposed into the Rivers Chenab and Ravi. Chenab River receives a vast amount of these industrial and municipal wastes from the eastern and southern parts of Faisalabad city through the Chakbandi Drain. This polluted water contains large amounts of toxic chemicals from a variety of industries, such as textiles, chemical and pharmaceutical industries, tanneries and sugar mills, etc., well sufficient to reduce the water productivity of Chenab River by changing the water quality parameters necessary for the growth of aquatic flora and fauna. This detrimental change in water quality has reduced the population of many fish species including Indian major carps. The freshwater fish species *C. mrigala* and *C. catla* collected from highly polluted areas were analyzed for DNA damage.

Sampling of fish species

Fish were harvested from Chenab River in Pakistan through its 190-km length upstream of Trimu Head. This part of the river is highly polluted due to the disposal of sewage and industrial waste through the Chakbandi Drain at latitude 31.570° and longitude 72.534° (Fig. 1). Three polluted sites, viz. Wara Thatta Muhammad Shah (R1), Bela Reta (R2), and Bandimahni Beg (R3), were selected along the length of Chenab River after receiving polluted water from the Chakbandi Drain. Two upstream sites, Libhan Wala (U1) and Thali (U2), were selected as the upstream sites before entering the drain into Chenab River in Tehsil and District Jhang for the comparison of wild fish. Apart from during the rainy season, this polluted water flows through Chenab River

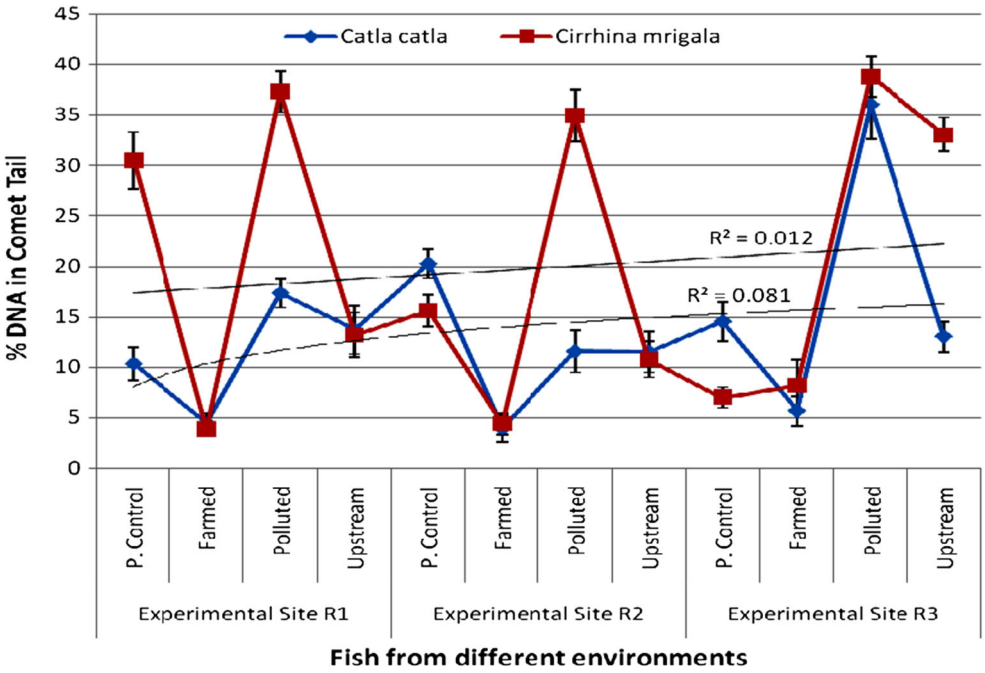

Fig. 1 Percent DNA damage in *Catla catla* and *Cirrhina mrigala*, type and site interactions

up to Trimu Head. Dragnets and gill nets were used to harvest seven fish of each species from three different sites (R1, R2, and R3) of the river, upstream to this area, with farmed fish being used as the control. Farmed fish with toxicants supplied to consumers was designated as the "positive control" and toxicant-free fish was designated as the control fish. The weight of the recovered fish ranged from 500 to 880 g. All the fish specimens were freshened out in running dechlorinated tap water. Venous blood was collected from the caudal vein of each fish in heparin-coated tubes.

Water analysis

Water samples were taken from the river at every point from which fish were harvested, and these were then analyzed for selected heavy metals and other water quality parameters, as defined by the Environmental Protection Agency of Pakistan and by Boyd (1981). The selected heavy metals analyzed were tin (Sb), chromium (Cr), lead (Pb), zinc (Zn), manganese (Mn), cupper (Cu), cadmium (Cd), and mercury (Hg). The concentration of each metal was detected by using heavy metal kits and according to APHA (1998) by using Hitachi polarized Zeeman Atomic Absorption Spectrophotometer 2000 series. The blanks and calibration standard solution were also analyzed in the same way as for the samples. The instrument calibration standards were made by diluting standard (1000 ppm) supplied by Merck, Germany. A known 1000-mg/L concentration of all the abovementioned metal solutions was prepared from their salts. All reagents used were of analytical grade. The percent recoveries in all the cases were within the acceptable limits of 70–120 %, as per regulatory guidelines.

Comet assay

Immediately after blood sampling, a small amount of blood (40 μL) was diluted with phosphate-buffered 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). Lysis: 1 h, at 4 °C, in a lysis buffer. DNA unwinding: 30 min, in the dark, in an electrophoresis buffer. Electrophoresis: 20 min, 300 mA, 25 V. Neutralization: three washes for 5 min each in buffer. Slides were fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses. Slides were stained with ethidium bromide and analyzed under a fluorescent microscope. The length of DNA migration measured DNA damage, which was visually determined in 250 randomly selected cells as 50 per slide for each fish. DNA damage was by Comet Score V5 and classified into five classes (head diameter, DNA in tail, tail length, tail moment, and olive tail moment) based on the comet tail length and DNA damage.

Micronucleus assay

Fresh blood was smeared on the slides, which were then air-dried before being fixed in cold Corney fixative for 5 min. After fixing, the slides were stained in aqueous 10 % Giemsa for 30 min. Five fish were analyzed for a total of 25,000 erythrocytes/fish sample. The frequencies of micronuclei in the erythrocytes were detected using a binocular microscope under T1200x magnification. Erythrocytes with intact cellular and nuclear membranes were scored using the same criteria as in previous studies (Bombail et al. 2001; Serrano-Garcia and

Montero-Montoya 2001). Nuclear abnormalities (NAs) in the erythrocytes were scored according to Cavas and Ergene-Gozukara (2005) and classified as in Carrasco et al. (1990).

Statistical analysis

Data were statistically analyzed by one-way analysis of variance. The results are represented as the mean ± standard deviation. Variance was considered significant at $p < 0.05$. All statistical analyses were performed using the program SPSS 9 for the PC.

Results

Comet assay

The comet assay or single-cell gel electrophoresis is a rapid and sensitive technique that detects DNA strand breaks, measuring the migration of DNA from immobilized individual cell nuclei (Fairbairn et al. 1995). DNA damage was by Comet Score V5 and classified into five classes (head diameter, DNA in tail, tail length, tail moment, and olive tail moment) based on the comet tail length and DNA damage. Two fish species, viz. *C. catla* (a surface feeder) and *C. mrigala* (a bottom feeder), were analyzed for DNA damage. All water quality parameters were found to be far beyond the permissible limits (Table 1) and significant ($p < 0.05$) DNA damage was observed in *C. mrigala* (Fig. 1). In respect to the comet assay of *C. catla* specimens taken from sites R1, R2, and R3, significant differences in head diameter were reported between fish collected from polluted waters and those collected upstream, indicating the extent of damage. The differences found between farmed and upstream fish were not significant, however. In respect to tail length, for the polluted sites R1 and R3, significant differences in tail length were reported compared to farmed and upstream fish. For the polluted site R2, however, these differences were not significant. In the case of DNA in the tail, for site R1, significant differences were reported between farmed and polluted fish (4.423 and 17.330 %, respectively), whereas the differences between farmed and upstream fish were not significant (Fig. 2). For site R2, significant differences were obtained for the positive control and all other types, but only non-significant differences were reported between fish collected from farmed, polluted, and upstream waters (3.914, 11.529, and 11.477 %, respectively). For site R3, significant differences were obtained for fish collected from polluted and upstream waters (35.991 and 13.026 %, respectively), but the differences between fish collected from farmed and upstream waters were not significant (5.63 ± 1.44 and 13.023 %, respectively; Table 2). In the case of tail moment, significant differences were reported only for fish from polluted and upstream waters, whereas the differences between fish from farmed and upstream waters were non-significant.

In the case of head diameter in the comet assay of blood from *C. mrigala* collected from site R1, there were significant ($p < 0.05$) differences between the farmed, polluted, and upstream area fish (Fig. 3). Similar findings were observed in the case of the comet tail in the comet assay (Table 3).

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

Sites				
	Cadmium (mg/L)	Copper (mg/L)	Manganese (mg/L)	Zinc (mg/L)
R1	0.139 ± 0.012C	0.907 ± 0.212E	01.59 ± 0.150C	00.215 ± 0.036E
R2	0.135 ± 0.013C	0.863 ± 0.211EF	01.53 ± 0.138C	00.207 ± 0.036 F
R3	0.130 ± 0.014CD	0.826 ± 0.203 F	01.36 ± 0.139D	00.206 ± 0.035 F
	Lead (mg/L)	Chromium (mg/L)	Tin (mg/L)	Mercury (mg/L)
R1	1.501 ± 0.151C	0.349 ± 0.051D	0.304 ± 0.037D	00.996 ± 0.033BC
R2	1.348 ± 0.120D	0.289 ± 0.040E	0.273 ± 0.030DE	01.013 ± 0.017BC
R3	1.298 ± 0.121D	0.246 ± 0.032 F	0.261 ± 0.030E	00.893 ± 0.012CD
	Phenols (mg/L)	Sulfates (mg/L)	BOD (mg/L)	COD (mg/L)
R1	01.67 ± 0.145E	264.79 ± 47.230D	70.64 ± 2.33 F	146.43 ± 13.61 F
R2	01.48 ± 0.121 F	250.36 ± 47.271E	61.70 ± 1.88G	135.00 ± 13.40G
R3	01.32 ± 0.135G	246.07 ± 45.679E	50.88 ± 1.44H	124.07 ± 13.87G
	pH	TDS (mg/L)	Salinity (mg/L)	Conductivity (mS/m)
R1	10.37 ± 0.053CD	1597.64 ± 221.95E	1392.86 ± 153.16E	02.25 ± 0.258E
R2	10.28 ± 0.019D	1475.43 ± 220.16 F	1250.00 ± 145.16 F	02.11 ± 0.269 F
R3	10.06 ± 0.044E	1214.43 ± 237.61G	921.43 ± 137.15G	01.70 ± 0.309G

Means sharing the same 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

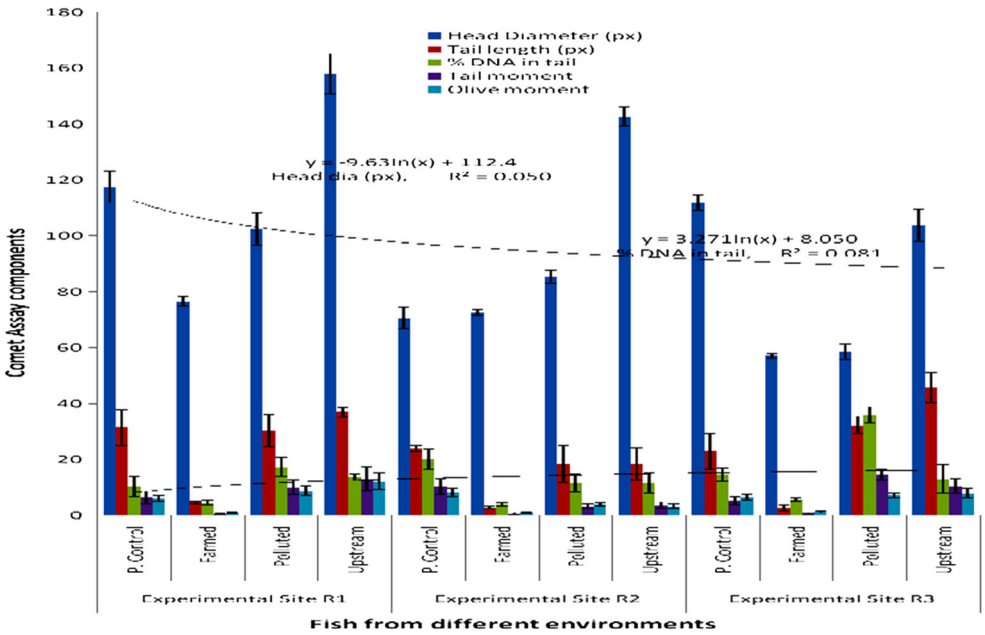

Fig. 2 Comparative analyses of the comet assays of blood from *Catla catla* collected from different environments

In the case of the percent DNA in the tail, a significant difference ($p < 0.05$) was reported for farmed and polluted area fish; a significant difference was also found in respect to polluted and upstream area fish, but the differences between the farmed and upstream area fish were not significant. Fish from farmed and upstream waters exhibited a non-significant difference in pollution load ($p > 0.05$). For site R2, however, significant differences were obtained when comparing the fish from the polluted site and upstream waters, but non-significant differences were observed between fish from farmed and upstream waters. For site R3, meanwhile, significant differences were obtained for fish from farmed, polluted, and upstream waters. The maximum DNA damage was exhibited by the fish collected from site R3, followed by those from sites R1 and R2.

In the case of tail moment, significant differences were reported between polluted and upstream fish. Fish from site R2 showed non-significant differences ($p > 0.05$) across all comparators: positive control (farmed fish with toxicants), polluted, and upstream. For site R3, significant differences were obtained for fish from farmed and polluted waters, but only non-significant differences were found between polluted and upstream fish. In the case of olive moment for fish from site R1, a significant difference ($p < 0.05$) was reported between the fish from the farmed and polluted waters and between the fish from the polluted and upstream waters. In the case of *C. mrigala*, tail length showed a highly significant correlation to DNA in the tail and head diameters (Table 4). Fish from site R2 showed significant differences observed in polluted and farmed fish, while non-significant differences were reported between fish from farmed and upstream sites (Fig. 4). For site R3, significant differences were obtained between fish from farmed and polluted sites.

Micronucleus assay

The frequency of the micronucleus in polluted and control *C. catla* was recorded as 23.20 ± 4.19 and 2.20 ± 0.58 (frequency calculated in thousand cells). In *C. catla* collected from the upstream and farmed sites, the MN frequency was observed as 8.0 ± 1.05 and 2.20 ± 0.58, respectively, with non-significant differences ($p < 0.05$). MN frequency exhibited a significant difference ($p < 0.05$) between *C. catla* collected from polluted site and the positive control (Table 5). Analysis of variance and comparison of the means for the frequency of single micronucleus analysis (frequency calculated in thousand cells) and species interaction were found to be highly significant ($p < 0.01$). Species and type interactions among all the sampling sites (polluted, upstream, farmed (control), and positive control) were significantly different from each other, but non-significant results were found when comparing fish species, except in polluted sites (Table 5 and Fig. 5). *C. catla* collected from polluted sites (and control (farmed)) exhibited significant differences ($p < 0.05$). There was a significant increase in MN frequency (thousand cells) in *C. mrigala* collected from polluted sites (44.80 ± 3.73) of Chenab River compared to the farmed control (8.20 ± 2.20). Induction of micronuclei showed a remarkable increase in MN frequency (thousand cells) in *C. mrigala* collected from the polluted (44.80 ± 3.73) and upstream (20.60 ± 4.02) sites. MN frequency and other abnormalities showed non-significant differences ($p < 0.05$) in *C. mrigala* from upstream compared to the reference/control site (Table 5 and Fig. 6). Non-significant differences for MN frequency were also noted between fish from polluted and positive control sites. In the double micronucleus analysis (frequency calculated in

Table 2 Comet assay results for *C. catla* and species, site, and type interactions

Sites	Components				
	Head diameter (px)	Tail length (px)	DNA in tail (%)	Tail moment	Olive moment
Species and site interaction (mean ± SE)					
Site 1	113.59 ± 3.68	25.87 ± 2.87	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	96.37 ± 1.91A	22.53 ± 1.30B	13.50 ± 0.78B	6.50 ± 0.62B	5.56 ± 0.41B
Species and type interaction (mean ± SE)					
Control +ve	99.89 ± 3.41bc	26.09 ± 2.44bc	15.00 ± 1.46cd	7.25 ± 1.28bc	6.90 ± 0.71bc
Farmed	68.70 ± 2.08 fg	3.27 ± 0.71e	4.66 ± 0.73e	0.50 ± 0.14d	1.02 ± 0.18d
Polluted	82.07 ± 3.47de	26.98 ± 2.33bc	21.62 ± 1.78b	9.29 ± 1.19bc	6.59 ± 0.72bc
Upstream	134.83 ± 3.78a	33.78 ± 3.43ab	12.72 ± 1.72d	8.98 ± 1.69bc	7.71 ± 1.19bc
Species, site, and type interactions (mean ± SE)					
R1. +ve	117.46 ± 5.57cde	31.46 ± 5.63c–i	10.31 ± 1.69d–j	6.30 ± 2.23d–h	5.98 ± 1.10c–h
R1. F	76.50 ± 4.87 h–l	4.54 ± 1.72lmn	4.42 ± 1.04hij	0.34 ± 0.16 h	0.93 ± 0.25gh
R1. P	102.42 ± 7.48d–h	30.46 ± 5.87c–i	17.33 ± 2.42d–g	10.06 ± 2.71b–h	8.85 ± 1.84b–e
R1. U	157.98 ± 6.18a	37.00 ± 7.23b–h	13.66 ± 2.42d–j	13.09 ± 4.12a–g	12.06 ± 2.92abc
R2. +ve	70.50 ± 3.16j–m	23.98 ± 3.84e–l	20.21 ± 3.40cde	10.24 ± 2.76b–h	8.18 ± 1.53b–f
R2. F	72.58 ± 2.94i–m	2.68 ± 0.98mn	3.91 ± 1.28j	0.53 ± 0.33 h	0.92 ± 0.34gh
R2. P	85.40 ± 4.47f–k	18.26 ± 2.23 g–n	11.53 ± 2.14d–j	3.11 ± 0.74gh	3.83 ± 0.76e–h
R2. U	142.78 ± 6.01abc	18.56 ± 3.52 g–n	11.48 ± 4.07d–j	3.50 ± 1.17fgh	3.21 ± 0.96e–h
R3. +ve	111.70 ± 6.21def	22.84 ± 2.66e–m	14.50 ± 1.98d–j	5.21 ± 1.47d–h	6.54 ± 1.01c–h
R3. F	57.02 ± 1.66lmn	2.60 ± 0.75mn	5.63 ± 1.44 g–j	0.62 ± 0.24 h	1.22 ± 0.35gh
R3. P	58.40 ± 3.72lmn	32.22 ± 2.81c–i	35.99 ± 3.43a	14.70 ± 1.89a–e	7.11 ± 0.73c–g
R3. U	103.72 ± 4.88d–g	45.78 ± 5.92a–d	13.02 ± 2.13d–j	10.34 ± 2.61b–h	7.85 ± 1.65b–f

Means sharing the same letter in a row or in a column are statistically non-significant ($p > 0.05$). Small letters represent comparisons among interaction means; capital letters are used for the overall mean. R1–R3: polluted experimental sites in the river)

F farmed, *P* polluted, *U* upstream, *+ve* positive control (fish types)

thousand cells), species interaction was found significant ($p < 0.05$). *C. catla* collected from polluted, upstream, control, and positive control showed non-significant differences ($p < 0.05$) for the double micronucleus assay. The double micronucleus frequency in *C. catla* from polluted, upstream, control, and positive control was recorded as 2.80 ± 1.70, 1.40 ± 0.75, 0.00 ± 0.00, and 8.60 ± 3.67, respectively. Non-significant differences for double MN frequency were also observed in *C. mrigala* collected from different sampling locations (Table 5). *C. mrigala* from upstream showed the highest frequency for double micronucleus. Nuclear abnormality significantly increased in *C. catla* and *C. mrigala* collected from polluted sits compared to the control fish. Statistical analysis indicated the sensitivity of the two fish species and the impact of habitat with varying degrees of pollution load on these fish species.

Overall, fish collected from the polluted area of Chenab River showed the highest frequency of micronucleus induction and nuclear abnormalities (Table 5). *C. catla*, however, showed lower frequencies of micronucleus induction indications compared to *C. mrigala* (Fig. 5). *C. mrigala* showed a considerable amount of micronucleus induction (Fig. 6), even from the area of lower pollution intensity (upstream of the Chakbandi Drain). This may be because *C. mrigala* is a bottom feeder and, thus, has maximum exposure to the polluted sediments. Farmed (control) fish showed a negligible amount of such DNA damage.

Discussion

With increased focus about the genotoxicity of water pollutants, sensitive bioassays are considered an important tool to screen the genotoxicity of contaminated rivers (Scalon et al. 2010). Urban and industrial discharges are still responsible for the high concentrations of toxic contaminants in aquatic environments (Richards et al. 2000), yet the potential genotoxic effects in fish exposed to these contaminants are poorly understood. Many contaminants present in these environments induce genetic alterations, leading to lethal mutations (Russo

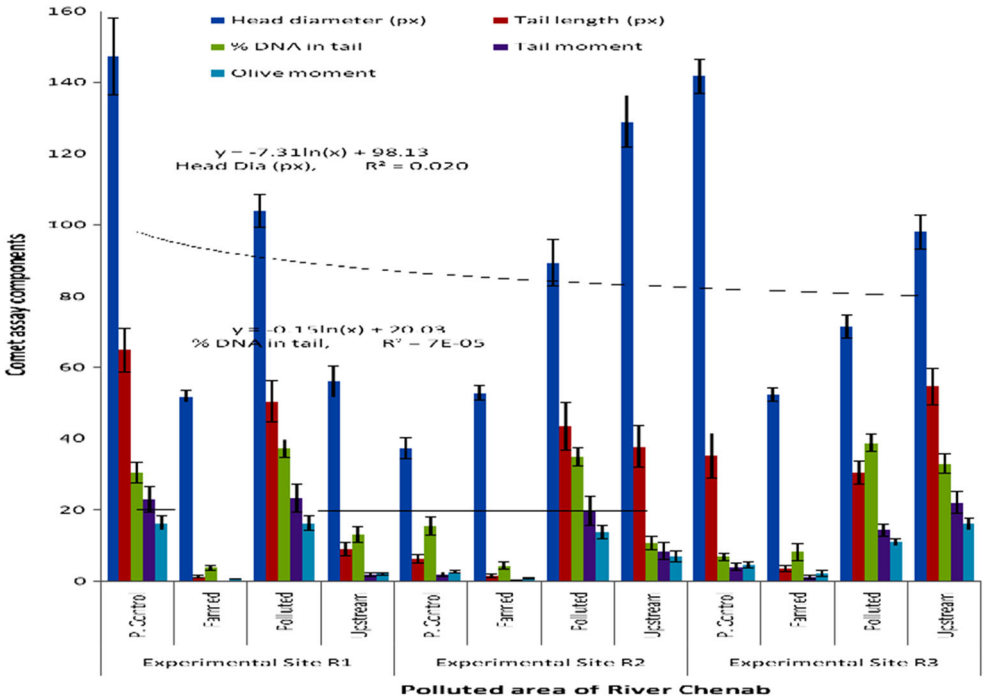

Fig. 3 Comparative analyses of the comet assays of blood from *Cirrhina mrigala* collected from different environments

et al. 2004). Fish can be used as useful genetic models for the evaluation of pollution in freshwater ecosystems (Elliott et al. 1988). Marcon et al. (2010) reported that a high concentration of some metals in the water could be one of the main reasons that might induce genetic damage in various fish species. In this context, the comet assay has been successfully used to identify the effects of genotoxic toxicants on the unity of DNA (Cok et al. 2011; Scalon et al. 2010). The three parameters of genotoxicity—tail moment, tail length, and tail intensity—have been widely used by different investigators for the assessment of DNA damage. With an increase in quantum of damage in a cell, more DNA migrates into the tail region and is quantified in terms of an increased amount of determined fluorescence in the tail region, as well as by tail length. The ratio of the DNA in the tail region (tail intensity) is commonly used for quantifying DNA strand breakage and represents the most reliable parameter (Mitchelmore and Chipman 1998).

This project sought to estimate such effects on *C. mrigala*'s genetic makeup and to use fish DNA fragmentation as a biomarker of freshwater pollution. For this study, we have used a simple and more comprehensive comet assay technique for the direct measurement of DNA damage in fish caught from a highly contaminated area of Chenab River. Five parameters (head diameter, tail length, DNA in tail, tail moment, and olive moment) were studied in *C. catla* and *C. mrigala*. The results of the comet assay analysis are presented in Tables 2 and 3. The results showed increased levels of genotoxic damage in fish collected from polluted sites when compared to the control. These results are in concordance with the results from other comet assay data of *C. mrigala* in the context of the environmental biomonitoring of genotoxicity in fish (Pavlica et al. 2011; Zohra et al. 2014). All five parameters exhibited significant differences when comparing the polluted site and the control site of Chenab River. In respect to the comet assay of *C. catla* and *C. mrigala* specimens taken from sites R1, R2, and R3, significant differences in head diameter were reported between fish collected from polluted waters and those collected upstream, indicating the extent of damage. In the case of percent DNA in the tail, a significant difference ($p < 0.05$) was reported for farmed and polluted area fish and also found in respect to polluted and upstream area fish, but the differences between the farmed and upstream area fish were not significant. The comet assay showed significantly ($p < 0.05$ higher damage in *C. mrigala* in terms of head diameter, DNA in tail, tail length, and olive moment from polluted sites compared to *C. catla*. Similarly, different authors have also reported high DNA damage in fish from natural water bodies (Rajaguru et al. 2003; Liney et al. 2006). Significant DNA damage was reported in this study in *C. mrigala* collected from the polluted area of Chenab River (Bentivegna et al. 2013). In the present study, all water quality parameters and selected heavy metals were found far higher in concentration in the water samples collected from the polluted sites of Chenab River than the permissible limits defined by WHO, indicating a higher intensity of pollution in this area of Chenab River. The present findings were also in line with the findings of Nkuranga (2007) who also found that different dissolved solids were from the different industries located in Kigali along the Rwanzekuma and Ruganwa Rivers, increasing the electrical conductivity of the water. Abdul-Razak et al. (2009) also reported high biochemical oxygen demand (BOD) and chemical oxygen demand

Table 3 Comet assay results for *C. mrigala* and species, site, and type interactions

Sites	Components				
	Head diameter (px)	Tail length (px)	DNA in tail (%)	Tail moment	Olive moment
Species and site interaction (mean ± 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	85.96 ± 2.11B	28.21 ± 1.54A	19.78 ± 0.83A	10.08 ± 0.77A	07.82 ± 0.44A
Species and type interaction (mean ± SE)					
Control +ve	108.82 ± 5.74b	35.43 ± 3.54ab	17.66 ± 1.53bcd	09.72 ± 1.46bc	07.92 ± 0.87bc
Farmed	52.31 ± 1.07 h	02.20 ± 0.38e	05.45 ± 0.93e	00.57 ± 0.20d	01.22 ± 0.31d
Polluted	88.33 ± 3.08cd	41.41 ± 3.14a	37.01 ± 1.43a	19.19 ± 2.03a	13.68 ± 1.00a
Upstream	94.37 ± 4.05cd	33.79 ± 3.06ab	19.00 ± 1.52bc	10.86 ± 1.45b	08.44 ± 0.87b
Species, site, and type interactions (mean ± SE)					
R1. +ve	147.32 ± 10.7ab	64.80 ± 6.24a	30.47 ± 2.85abc	23.12 ± 3.52a	16.40 ± 1.95a
R1. F	51.86 ± 1.65lmn	01.32 ± 0.34n	03.81 ± 0.64j	00.12 ± 0.04 h	00.65 ± 0.13 h
R1. P	104.02 ± 4.66def	50.34 ± 5.71abc	37.29 ± 2.51a	23.48 ± 3.90a	16.22 ± 2.04a
R1. U	55.94 ± 4.35lmn	09.02 ± 1.75j–n	13.20 ± 2.18d–j	01.92 ± 0.39 h	02.06 ± 0.38fgh
R2. +ve	37.42 ± 2.84n	06.36 ± 1.23 k–n	15.58 ± 2.55d–j	01.97 ± 0.59 h	02.63 ± 0.44e–h
R2. F	52.76 ± 2.06lmn	01.62 ± 0.44n	04.35 ± 1.08ij	00.28 ± 0.10 h	00.79 ± 0.19gh
R2. P	89.44 ± 6.53f–j	43.42 ± 6.61b–e	34.96 ± 2.53a	19.78 ± 4.26ah	13.83 ± 1.96ab
R2. U	129.10 ± 7.22bcd	37.78 ± 5.83b–g	10.73 ± 1.80d–j	08.55 ± 2.34c–h	07.12 ± 1.42c–g
R3. +ve	141.72 ± 4.76abc	35.12 ± 6.27b–h	06.93 ± 0.99f–j	04.06 ± 1.06e–h	04.72 ± 0.87d–h
R3. F	52.32 ± 1.87lmn	03.66 ± 0.98lmn	08.19 ± 2.48e–j	01.31 ± 0.57 h	02.23 ± 0.88fgh
R3. P	71.54 ± 3.39i–m	30.48 ± 3.13c–i	38.80 ± 2.42a	14.30 ± 1.82a–f	10.99 ± 0.90a–d
R3. U	98.08 ± 4.84e–i	54.56 ± 5.19ab	33.06 ± 2.66ab	22.12 ± 3.02a	16.14 ± 1.64a

Means sharing the same letter in a row or in a column are statistically non-significant ($p > 0.05$). Small letters represent comparisons among interaction means; capital letters are used for the overall mean. R1–R3: polluted experimental sites in the river

F farmed, *P* polluted, *U* upstream, *+ve* positive control (fish types)

Table 4 Correlation matrix for the comet assay results of blood from *C. catla* and *C. mrigala*

	Catla catla					*Cirrhina mrigala*				
	T.DNA.	Type	H.dia	T.length	T.mome	T.DNA.	Type	H.dia	T.length	T.mome
Type	0.137					0.299				
	0.672					0.345				
H.dia	−0.132	0.428				0.283	−0.022			
	0.683	0.166				0.373	0.945			
T.length	0.569*	0.392	0.480			0.733**	0.175	0.835**		
	0.050	0.208	0.114			0.007	0.587	0.001		
T.mome	0.818**	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	0.572*	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 indicate Pearson's correlation coefficient. Lower values indicate level of significance at 5 % probability

*$p < 0.05$ (significant); **$p < 0.01$ (highly significant)

T.DNA % DNA in tail, *H.dia* head diameter, *T.length* tail length, *T.mome* tail moment, *O.mome* olive moment

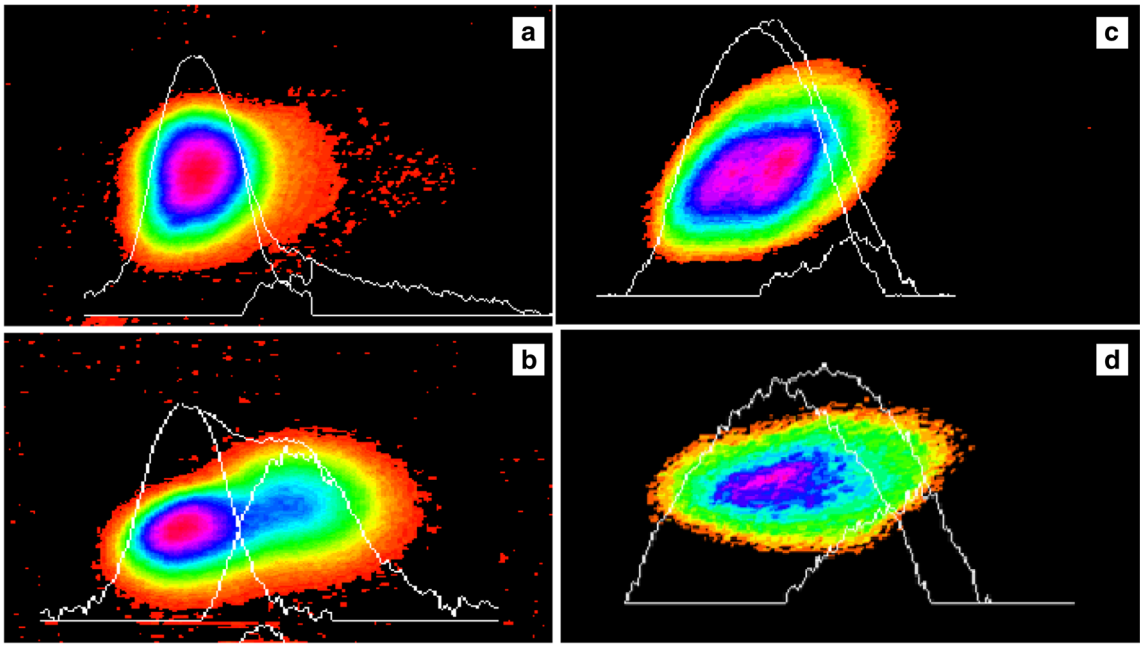

Fig. 4 Photomicrographs of the comet assays. **a**, **b** *Cirrhina mrigala*. **c**, **d** *Catla catla*. TriTek Comet Score™ Freeware 1.6.1.13

(COD), high organic matter, and the highest dissolved solids, indicating a higher level of water pollution by effluents from the industry and domestic sewage. All the heavy metal concentrations decrease along the length of the drain.

Chronic exposure to heavy metals in water bodies caused nuclear damage; thus, for confirmation, the micronucleus assay was performed. Significant relationships were observed among the MN frequencies and nuclear abnormalities (Table 5). The present results are in agreement with earlier studies, which have detected elevated MN frequencies in fish inhabiting contaminated environments. The MN frequency was higher in *C. mrigala* than in *C. catla* from the polluted

Table 5 Analysis of variance and micronucleus test of *C. catla* and *C. 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 ± 4.19bc	02.80 ± 1.07b	06.00 ± 0.84cd	
Upstream	08.00 ± 1.05cd	01.40 ± 0.75b	03.20 ± 0.37d	
Control (farmed)	02.20 ± 0.58d	00.00 ± 0.00b	01.00 ± 0.32d	
Positive control	43.60 ± 5.35a	08.60 ± 3.67ab	17.80 ± 2.92a	
Mean	19.25 ± 4.00B	03.20 ± 1.17B	07.00 ± 1.65B	
Fish type	Micronucleus assay (*Cirrhina mrigala*)			
	Single micronucleus	Double micronucleus	Nuclear abnormalities	
Polluted	44.80 ± 3.73a	06.20 ± 0.97ab	09.60 ± 1.72a–d	
Upstream	20.60 ± 4.02bcd	05.20 ± 1.53b	10.00 ± 1.05a–d	
Control (farmed)	08.20 ± 2.20cd	00.80 ± 0.37b	06.20 ± 1.85bcd	
Positive control	37.40 ± 3.92ab	08.40 ± 2.80ab	15.20 ± 2.06ab	
Mean	27.75 ± 3.66A	05.15 ± 1.00AB	10.25 ± 1.08A	

Means sharing the same letter in a row or in a column are statistically non-significant ($p > 0.05$). Small letters represent comparisons among interaction means; capital letters are used for the overall mean. Frequency calculated per thousand cells

*$p < 0.05$ (significant); **$p < 0.01$ (highly significant)

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

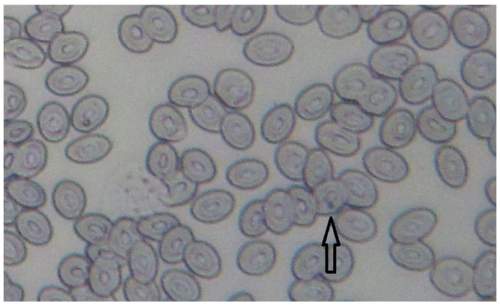

Fig. 5 Micronucleus assay of fish (*Catla catla*) blood harvested from the polluted area of the Chenab River indicating nuclear abnormalities and micronucleus induction

sites of Chenab River compared to the control site. This indicates that *C. mrigala* is more sensitive to water toxicants, and it could be affected by other factors such as interspecies sensitivity, metabolic capacity, DNA repair, and defence mechanisms (Rodriguez-Cea et al. 2003). Previous laboratory investigations have shown that fish exposed to cyclophosphamide, cypermethrin, and textile mill effluent produced higher MN frequencies in gill cells than in erythrocytes. Cavas and Ergene-Gozukara (2003) have also reported that the gill cells of the fish *Oreochromis mossambicus* are more sensitive to genotoxic exposure than the cells from other tissues, such as the kidney or liver. Many studies, in fact, have indicated the sensitivity of erythrocytes to aquatic pollutants. Furthermore, the use of the kidney, liver, or other such tissues for genotoxicity biomonitoring has many limitations due to the low mitotic index of the cells from such tissues, while the use of connective tissues such as blood erythrocytes has been demonstrated to be advantageous for such studies (Cavas and Ergene-Gozukara 2005). In recent decades, morphological NAs, together with micronucleus induction, have received considerable attention. Although the mechanisms responsible for the induction of morphological NAs have not been yet fully understood, several previous studies have suggested that NAs are induced in response to exposure to genotoxic agents (Serrano-Garcia and Montero-Montoya 2001). Our results confirm earlier claims of high MN values for different fish species from polluted water bodies, which confirms fish being a suitable model for the detection of genotoxic damage for

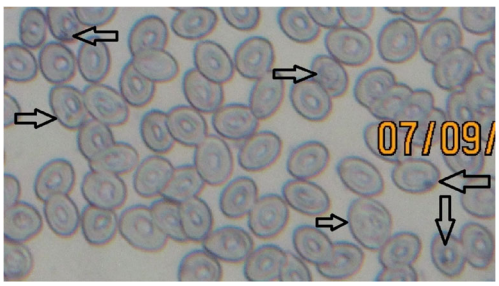

Fig. 6 Micronucleus assay of fish (*Corrhina mrigala*) blood harvested from the polluted area of Chenab River indicating nuclear abnormalities and micronucleus induction

water monitoring (Sanchez-Galan et al. 2001; Betancur et al. 2009; Omar et al. 2012).

Austin (1998) elaborated that potentially harmful genotoxic substances, e.g., heavy metals, pesticides, and hydrocarbons, are often released into aquatic environments, whereas Boettcher et al. (2010) reported large-scale mortalities due to the release of large quantities of pollutants into aquatic environments. It was observed in present investigations that fish kills were reported by contamination of waterways, and due to this reason, *C. catla* is also under threat of extinction. Austin (1998) reported that a lower level of discharge might result in the bioaccumulation of pollutants in organisms living in aquatic ecosystems. This bioaccumulation of pollutants ultimately results in the reduction of metabolism, immune suppression, and damage to the epithelia and gills, and the present study corroborates the findings of Austin (1998) on the cause of the reduction in the number of fishes in Chenab River. Ramesh and Nagarajan (2013) concluded that DNA damage within the cells can serve as the earliest sign of a whole range of health problems, including diseases and exposure to environmental toxins. A similar study performed by Bombail et al. (2001) showed higher erythrocyte MN and NA induction frequencies in *Pholisgunellus* specimens captured from highly polluted areas compared to those collected from clean areas, as here in our study on the comparison of polluted area fish to that of upstream area fish. These findings by previous studies verify the genotoxicity of the bottom feeder fish *C. mrigala* and prove that DNA damage could be used as a biomarker of pollution load and its early monitoring by using simple and reliable techniques such as the comet and micronucleus assays.

Conclusion

Water from Chenab River was found to be highly polluted by organic and inorganic pollutants. Genotoxic damage was studied through the micronucleus assay which indicates cell injuries, which have undergone at least one mitotic cycle, and measures chromosome loss. On other hand, the comet assay detects reparable injuries (alkali-labile sites) by direct measurement of DNA strand breakage. Hence, we recommend both tests together for genotoxic monitoring to understand the underlying mechanisms of genetic damage/break. The findings of the present study indicate the potential of the measurement of DNA damage in bottom-dwelling species, such as *C. mrigarala*, as a biomarker of pollution for early warning and monitoring of water bodies.

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References

Abdul-Razak A, Asiedu AB, EntsuaMensah REM, Degpaft-Johnson KAA (2009) Assessment of the water quality of the Oti River in Ghana. West Afr J Appl Ecol 15(2):1–12

APHA (American Public Health Association) (1998) Standard methods for the examination of water and waste water, 20th edn. American Public, New York

Austin B (1998) The effects of pollution on fish health. J Appl Microbiol 85:234S–242S

Baršienė J, Rybakovas A, Lang T, Andreikėnaitė L, Michailovas A (2013) Environmental genotoxicity and cytotoxicity levels in fish from the North Sea offshore region and Atlantic coastal waters. Mar Pollut Bull 68:106–116

Bentivegna A, Miloso M, Riva G, Foudah D, Butta V, Dalprà L, Tredici G. (2013) DNA methylation changes during in vitro propagation of human mesenchymal stem cells: implications for their genomic stability? Stem Cells Int 2013, 192425. Retrieved 03 Jan. 2014 from http://www.hindawi.com/journals/sci/2013/192425/

Betancur IP, Palacio Baena JA, Guerrero MC (2009) Micronuclei test application to wild tropical ichthyic species common in two lentic environments of the low zones in Colombia. Actual Biol 31:67–77

Boettcher M, Grund S. Keiter S, et al. (2010) Comparison of in vitro and in situ genotoxicity in the Danube River by means of the comet assay and the micronucleus test. Mutat Res 700:11–17

Bombail W, Aw D, Gordon E, Batty J (2001) Application of comet and micronucleus assays to butterfish (*Pholis gunnellus*) erythrocytes from the Firth of Forth, Scotland. Chemosphere 44:383–392

Boyd EC (1981) Water quality in warm water fish ponds, 2nd edn. Craftmaster Printers Inc., Opelika

Carrasco K, Tilbury KL, Myers MS (1990) Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Can J Fish Aquat Sci 47:2123–2136

Cavalcante DGSM, Martinez CBR, Sofia SH (2008) Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. Mutat Res 655:41–46

Cavas T, Ergene-Gozukara S (2003) Evaluation of the genotoxic potential of lambda-cyhalothrin using nuclear and nucleolar biomarkers in fish cells. Mutat Res 534:93–99

Cavas T, Ergene-Gozukara S (2005) Micronucleus test in fish cells: a bioassay for in situ monitoring of genotoxic pollution in the marine environment. Environ Mol Mutagen 46:64–70

Cok I, Ulutaş OK, Okuşluk O, Durmaz E, Demir N (2011) Evaluation of DNA damage in common carp (*Cyprinus carpio* L.) by comet assay for determination of possible pollution in Lake Mogan (Ankara). Scientific World Journal 11:1455–1461

Dixon DR, Pruski AM, Dixon LRJ, Jha AN (2002) Marine invertebrate ecogenotoxicology: a methodological overview. Mutagenesis 17:495–507

Elliott M, Griffiths AH, Taylor CJL (1988) The role of fish studies in estuarine pollution assessment. J Fish Biol 33:51–61

El-Shehawi MA, Ali KF, Seehy MA (2007) Estimation of water pollution by genetic biomarkers in tilapia and catfish species shows species-site interaction. Afr J Biotechnol 6:840–846

Ensink JHJ, Mahmood T, van der Hoek W, Raschid-Sally L, Amerasinghe FP (2004) A nation-wide assessment of wastewater in Pakistan: an obscure activity or a vitally important one? Water Policy 6:197–206

Fairbairn DW, Olive PL, O'Neill KL (1995) The comet assay: a comprehensive review. Mutat Res 339:37–59

Goksoyr A, Anderson T, Buhler DR, Stegerman JJ, Williams DE, Forlin L (1991) Immunochemical cross-reactivity of β-naphhtoflavone inductible cytochrome P450 (P450 IA1) in liver microsomes from different fish species and rat. Fish Physiol Chem 9:1–13

Landolt ML, Kocan RM (1983) Fish cell cytogenetics: a measure of the genotoxic effects of environmental pollutants. In: Nriagu J (ed) Aquatic toxicology. Wiley, New York, pp 335–353

Liney KE, Hagger JA, Tyler CR, Depledge MH, Galloway TS, Jobling S (2006) Health effects in fish of long-term exposure to effluents from wastewater treatment works. Environ Health Perspect 114:81

Marcon AE, Ferreira DDM, Moura DDM, de Moura MFV, et al. (2010). Genotoxic analysis in aquatic environment under influence of cyanobacteria, metal and radioactivity. Chemosphere 81:773–780

Mitchelmore CL, Chipman JK (1998) DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat Res 399:135–147

Nkuranga E (2007) Heavy metal removal and accumulation by an urban natural wetland: the Nyabugogo swamp, Rwanda. MSc thesis, UNESCO-IHE Institute for water education, Delft, the Netherlands

Omar WA, Zaghloul KH, Abdel-Khalek AA, Abo-Hegab S (2012) Genotoxic effects of metal pollution in two fish species, *Oreochromis niloticus* and *Mugil cephalus*, from highly degraded aquatic habitats. Mutat Res 746:7–14

Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages inindividual mammalian cells. Biochem Biophys Res Commun 123:291–298

Pavlica M, Štambuk A, Malović L, Mladinić M, Göran I, Klobuˇ VC (2011) DNA integrity of chub erythrocytes (*Squalius cephalus* L.) as an indicator of pollution-related genotoxicity in the River Sava. Environ Monit Assess 177:85–94

Qadir A, Malik RN (2011) Heavy metals in eight edible fish species from two polluted tributaries (Aik and Palkhu) of the river Chenab, Pakistan. Biol Trace Elem Res 143:1524–1540

Rajaguru P, Suba S, Palanivel M, Kalaiselvi K (2003) Genotoxicity of a polluted river system measured using the alkaline comet assay on fish and earthworm tissues. Environ Mol Mutagen 41:85–91

Ramesh F, Nagarajan K (2013) Evaluation of DNA damage of the fish *Clarias batrachus* exposed to Sago effluent using the comet assay. Eur Int J Sci Technol 2:12–16

Richards JP, Glegg AG, Cullinane S (2000) Environmental regulation: industry and the marine environment. J Environ Manage 58:119–134

Rodriguez-Cea A, Ayllon F, Garcia-Vazquez E (2003) Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. Ecotoxicol Environ Saf 56:442–448

Russo C, Rocco L, Morescalchi MA, Stingo V (2004) Assessment of environmental stress by the micronucleus test and the comet assay on the genome of teleost populations from two natural environments. Ecotoxicol Environ Saf 57:168–174

Sanchez-Galan S, Linde AR, Ayllon F, Garcia-Vazquez E (2001) Induction of micronuclei in eel (*Anguilla anguilla* L.) by heavy metals. Ecotoxicol Environ Saf 49:139–143

Scalon MCS, Rechenmacher C, Siebel AM, Kayser ML, Rodrigues MT, Maluf SW, Rodrigues MAS, Silva LB (2010) Evaluation of Sinos River water genotoxicity using the comet assay in fish. Braz J Biol 70:1217–1222

Serrano-Garcia L, Montero-Montoya R (2001) Micronuclei and chromatin buds are related genotoxic events. Environ Mol Mutagen 38:38–45

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantification of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221

Villela IV, Oliveira IM, Silva J, Henriques JAP (2006) DNA damage and repair in haemolymph cells of golden mussel (*Limnoperna fortunei*) exposed to environmental contaminants. Mutat Res 605:78–86

Zohra B, Nicolas C, Elise G, Pierre-Emmanuel B, Habib A, Lotfi A (2014) First evidence of fish genotoxicity induced by heavy metals from landfill leachates: the advantage of using the RAPD-PCR technique. Ecotoxicol Environ Saf 101:90–96