Fish eco-genotoxicology: Comet and micronucleus assay in fish erythrocytes as in situ biomarker of freshwater pollution

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A B S T R A C T

Owing to white meat production Labeo rohita have vast economic importance, but its population has been reduced drastically in River Chenab due to pollution. Atomic absorption spectrophotometry showed a merciless toxicity level of Cd, Cu, Mn, Zn, Pb, Cr, Sn and Hg. Comet assay results indicated significant (p < .05) DNA fragmentation in Labeo rohita as 42.21 ± 2.06%, 31.26 ± 2.41% and 21.84 ± 2.21% DNA in comet tail, tail moment as 17.71 ± 1.79, 10.30 ± 1.78 and 7.81 ± 1.56, olive moment as 13.58 ± 1.306, 8.10 ± 1.04 and 5.88 ± 0.06, respectively, from three different polluted sites on the river. Micronucleus assay showed similar findings of single micronucleus induction (MN) as 50.00 ± 6.30‰, double MN 14.40 ± 2.56‰, while nuclear abnormalities (NA) were found as 150.00 ± 2.92‰. These higher frequencies of MN induction and NA were found to be the cause of reduction of 96% of the population of this fish species in an experimental area of the River Chenab. This fish species has been found near extinction through the length of the river Chenab and few specimens in rainy seasons if restored by flood, may die in sugarcane mill season. Due to sweeping extinction Labeo rohita showed the highest sensitivity for pollution and could be used as bioindicator and DNA fragmentation in this column feeder fish species as a biomarker of the pollution load in freshwater bodies.

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1. Introduction

Waste disposal from industry and urban structures in Asian rivers has resulted in deposition of a variety of new toxic chemicals and organic compounds. Such activities have endangered the existence of ecosystems and their inhabitants. Changes in genome caused by genotoxic agents led to mutations and pose a burden to the populations of fish species. Toxicants those induce genetic damage involve everlasting monitoring and before time detection (Villela et al., 2006). The unremitting input of toxicants into the freshwater bodies has led to the advancement in techniques for evaluation and monitoring the fate of such ecosystems (Rand, 1995). Fishes are marvelous model animals for genotoxicological studies and provide early warnings for toxicants induced environmental alterations and degradations (Pawar, 2012). According to Harshbarger and Clark (1980) fish species may be used to estimate the possible effects of toxicants to produce carcinogenic and teratogenic effects in human.

Singh et al. (1988) founded a most economical and sensitive technique under alkaline (pH > 13) conditions for the detection of genetic damage at cellular level, the comet assay having sensitivity for detecting minimum intensity of DNA fragmentation and require a small number of blood cells per fish specimen (Tice et al., 2000). Other most promising and accepted method used for cytogenetic damage is the micronucleus (MN) assay. Measurement of cytogenetic damage by MN presented an incredibly important assay in detection of pollution stress and load in aquatic ecosystems resulting in the decline of populations of particular species (Dixon et al., 2002; Baršiene et al., 2013). Micronucleus test along with nuclear abnormalities is extensively applied method among currently available assays due to its proven suitability for fish species (Cavas and Ergene-Gozukara, 2003, Kirschbaum et al., 2005).
2. Materials and methods

2.1. Study area

River Chenab receives vast amount of toxic industrial and domestic wastes disposed (31.57°N & 72.53°E Bhawana, Faisalabad, Punjab, Pakistan) by Chakbandi Main Drain (Fig. 1). This waste water holds genotoxic and cytotoxic chemicals from a variety of industries situated in Faisalabad city and is well sufficient for disarranging change in water productivity by changing the physicochemical parameters of River Chenab. This habitat degradation has resulted in retarded growth of aquatic organisms, including fish species like *Labeo rohita*. 170 km stretch of the river was selected for the estimation of pollution at downstream Chakbandi Main Drain. For this purpose, water analysis and fishing were performed from three experimental sites (R1, R2 and R3) along the river. Two sites U1 and U2 upstream Chakbandi Main Drain was selected as a control and samples were polled and designated as U.

2.2. Sampling of fish species

Specimens of *Labeo rohita* were collected by using gill nets and drag nets from highly polluted water of the River Chenab from the area of Thatta Muhammad Shah (Site R1), Bela Reta (Site R2), and Bandimahni Beg (Site R3). Sampling campaigns were performed though out the year twice a month. Farmed fish were also used as a reference for wild (polluted) and wild (non-polluted) for the estimation of genotoxicity. Farmed fish was collected from the Fish Seed Hatchery, Faisalabad and divided into two groups. One group of farmed fish was treated with colchicine and designated as “positive control” and untreated group as a control (negative control).

Fig. 1. Joining of the River Jhelum (left) and the River Chenab (right) at Head Trimu Jhang (31.56°N, 72.65°E). There is clear difference in the water of both rivers. The dark black color of the River Chenab is due to the polluted industrial and sewage wastes (Google map source).

The weight of the fish specimens collected from each point ranged from 800 to 1150 g. Fish blood (2cc) was collected just after catch from the caudal vein near the ventral fin of each specimen in heparinized tubes. After bleeding each wild fish was released to the river. Four years were spent collecting data regarding ecogenotoxicity and population dynamics.

2.3. Water analysis

River and the drain water samples were collected in polypropylene bottles and analyzed for selected heavy metals (Sb, Pb, Cr, Mn, Zn, Cd, Cu and Hg) and other water quality parameters (Boyd, 1981). The concentration of each metal was detected by heavy metal kits (Merck) and atomic absorption spectrophotometry (APHA, 1998).

2.4. Comet assay

Two µl of fresh blood was spread and sandwich between two layers, one of low melting agarose (0.5%) and other layer of normal melting agarose (0.6%) on frosted microscopic slides. The gel was then polymerized on ice. After solidification of agarose slides were dipped in lysis buffer (100 mM Na2EDTA, 10 mM Tris-HCl, 2.5 M NaCl, 1% sodium sarcosinate, 1% Triton X-100 and 10% Dimethyl Sulphoxide) for one hour at 4°C. For DNA unwinding slides were placed in the electrophoresis buffer (pH 10, 1 mM Na2EDTA and 0.3 M NaOH) for 20 min and then placed for electrophoresis (20 V and 300 mA) for 30 min. Slides were then placed in Tris-HCl buffer at 25°C for neutralization. Slides were stained with ethidium bromide (10%) and visualized by fluorescent microscopy (Dhawan et al., 2009).

2.5. Micronucleus assay

Fish blood was smeared on clean and oven dried microscopic slides. These blood smear slides were air dried at 25°C for two hours and then fixed in cold Corney’s fixative for five minutes and were again fixed in methanol for ten minutes and left to air dry at 25°C for 1 h. Slides were stained for 30 min in 10% aqueous Giemsa and washed in double distilled water and again let them air dry. 35 fish specimens were analyzed for each experimental site for a total of 35,000 erythrocytes/fish sample. For positive control, blood from the farmed specimens was subjected to colchicine treatment. For each fish specimen five slides were prepared. The frequencies of micronucleus induction in erythrocytes were scored at T1200x magnification. Erythrocytes in fish blood with intact nuclear abnormalities were also scored by following protocol adopted by Alink et al. (2007) and Obiakor et al. (2010).

2.6. Statistical analysis

Data were statistically analyzed by the one-way analysis of variance while variance was considered significant at P < .05. The results represent mean along with standard error. Duncan’s multiple range test was used to compare the means. Statistical analyses were executed by using the program SPSS 9 for the PC. Image analyses for DNA damages were performed by using TriTek Comet Score™ Freeware 1.6.1.13.

3. Results

Water quality parameters (WQPs) analyzed in this study proved the acute level of toxicity and high pollution load in this section of...
the River Chenab indicating that water is not even suitable for the irrigation purposes (Table 1). All such WQPs were found in the normal range in the controls used in this study. Four-year study of fish population showed that there are a few specimens of this species in 170 km stretch of River Chenab. In the months of April and August some more specimens of this species were reported. This increase in the population of adult specimens during these two months is only due to the migrating individuals due to the rain and floods from River Jhelum or upstream areas of River Chenab desolately leading to die in pollution load (Fig. 2).

Five components of comet assay showed significant DNA damage in *Labeo rohita* specimens harvested from three sites (R1, R2, R3) of the polluted areas of the River Chenab (Fig. 3). Fish from site R1 showed significant (p < .05) DNA damage in comet tail when compared to the farmed and upstream area fish which showed negligible amount of DNA in the comet tail (Fig. 4). Non-significant differences were found among upstream area fish and farmed fish (Fig. 5; Table 2). 

Table 1

| Sites | Physicochemical parameters of river water |
|-------|------------------------------------------|
|       | Cadmium mg L⁻¹ | Copper mg L⁻¹ | Manganese mg L⁻¹ | Zinc mg L⁻¹ |
|       | D: 0.01 mg/L, P: 0.01 mg/L | 1.5 mg/L | D: 0.1 mg/L, P: 0.3 mg/L | D: 5 mg/L, P: 15 mg/L |
| R1    | 0.183 ± 0.009b  | 1.670 ± 0.021a | 2.12 ± 0.025a   | 0.344 ± 0.003a |
| R2    | 0.182 ± 0.001b  | 1.622 ± 0.038a | 2.02 ± 0.017ab  | 0.339 ± 0.002b |
| R3    | 0.180 ± 0.003b  | 1.557 ± 0.020c | 1.86 ± 0.040c   | 0.331 ± 0.003c |
|       | D: 0.05 mg/L, P: 0.001 mg/L | 0.002 mg/L | D: 0.01 mg/L, P: 0.001 mg/L |
| R1    | 0.527 ± 0.023a  | 0.431 ± 0.011b | 0.379 ± 0.008c  | 1.079 ± 0.044a |
| R2    | 0.527 ± 0.023a  | 0.431 ± 0.011b | 0.379 ± 0.008c  | 1.079 ± 0.044a |
| R3    | 0.375 ± 0.013c  | 0.366 ± 0.011b | 0.366 ± 0.011b  | 0.912 ± 0.020b |
| Phenols mg L⁻¹ | Sulfates mg L⁻¹ | BOD mg L⁻¹ | COD mg L⁻¹ |
| D: 0.001 mg/L, P: 0.002 mg/L | D: 200 mg/L, P: 400 mg/L | D: 30 mg/L, P: 100 mg/L |
| R1    | 435.00 ± 2.717a | 78.56 ± 1.22a | 195.43 ± 1.48a |
| R2    | 420.71 ± 1.409b | 67.47 ± 1.90b | 183.00 ± 2.88b |
| R3    | 410.57 ± 4.407c | 55.43 ± 1.04c | 174.00 ± 1.40c |
| pH    | TDS mg L⁻¹ | Salinity mg L⁻¹ | Conductivity mS/m |
| D: 6.5–8.5, P: 6.5–8.5 | D: 500 mg/L, P: 2000 mg/L | P: 100 mg/L, D: 250 mg/L |
| R1    | 2397.86 ± 121.24a | 1942.86 ± 20.20a | 3.17 ± 0.061b |
| R2    | 2269.00 ± 111.31b | 1771.43 ± 18.44b | 3.08 ± 0.041b |
| R3    | 2071.14 ± 90.26c | 1414.29 ± 14.29c | 2.81 ± 0.061c |

Means sharing similar letter in column belonging to particular parameter are statistically non-significant (P > .05). R1-R3; Three different polluted experimental sites of River Chenab upstream to Trimu Head. COD; Chemical Oxygen demand, BOD; Biochemical Oxygen demand. Values were determined in the summer season when there is considerable dilution of the river water by rain and glacier waters. **No relaxation. D; Desirable limits. P; Permissible limits. ¹ Effluent inland surface water quality standards.

Fig. 2. Reduction in the *Labeo rohita* population in 170 km length of the River Chenab due to the pollution. Population restoration in rainy seasons and Bandi (April) to some extent but again reduced due to pollution load.
No DNA damage was observed in the blood cells of farmed *Labeo rohita* (Fig. 6).

Fish harvested from this polluted experimental site of the river indicated highest frequencies for single micronucleus induction (50.00 ± 6.30), double micronucleus (MN) induction (14.40 ± 2.56) and even nuclear abnormalities as 150.00 ± 2.92 calculated in a thousand cells (Table 4; Fig. 7). *Labeo rohita* showed significant (p < .05) amount of MN induction harvested upstream to the entrance of Chakbandi Main Drain into the river (Tables 3 and 4) indicating sensitivity of the species to the even lower intensity of the pollution. Control fish (farmed and upstream area fish) indicated negligible amount of such type of DNA damages.

### 4. Discussion

Recent literature regarding metal toxicity in fishes mainly comes from histopathological and physiological studies. Research in respect of potential cyto-genotoxic effects of metals and other genotoxic agents on these animals in respect to the population and habitat degradation is still insufficient (Galindo et al., 2010). Untreated industrial and municipal discharge is still responsible for environmental contamination, especially in aquatic ecosystems (Richards et al., 2000). The literature clearly indicated that potential genotoxic effects leading to staid mutations and population decline (Russo et al., 2004) in fishes rendering to such toxicants are not well understood. This project is planned to estimate such type of effects of pollution on *Labeo rohita*’s genetic makeup and
ultimately its populations. This will allow early detection and warning of habitat contamination leading to the extinction of particular species as the case here. The findings of this research project corroborate the findings of Van-Der-Oost et al. (2003) using fish biomarkers (DNA damage) as indices of effects of habitat contamination by genotoxic agents. For genotoxicity assessments we used a novel, reliable and most sensitive technique comet assay. This technique was applied on fish erythrocytes. The results obtained were correlated with the population of this fish species in this area of the river. Results indicated elevated levels of genotoxic damage when compare to the control (farmed) fish and fish was found almost extinct in this area of the river. Only some migrating individuals were found in rainy seasons and when water was released into the river from dams and heads (locally so called bandi) perhaps leading to die in this area of high intensity pollution load. The highest fish kill was reported in sugarcane mill seasons when the majority of the aquatic fauna was destroyed by wastes (locally called chitta pani) from such industries perhaps due to the suffocation. In the context of environmental biomonitoring for genotoxicity our results are in concordance with the findings of Pavlica et al. (2011) in respect of fishes as biondicator. Significant interactions were noted among the DNA damage, micronucleus induction and nuclear abnormalities. A study by Pietripiana et al. (2002) also demonstrated that heavy metal pollution induce micronucleus in erythrocytes of fish with higher frequencies. Results from this project are in agreement with previous studies regarding elevated micronucleus frequencies in fishes living in contaminated habitats. Recently in genotoxicity and cytotoxicity studies nuclear abnormalities along with micronucleus induction have attained substantial attention even yet mechanisms involved in the introduction of

| Sites       | Comet assay components          | Head diameter (px) | Tail length (px) | DNA in tail (%) | Tail moment | Olive moment |
|-------------|---------------------------------|-------------------|-----------------|-----------------|-------------|--------------|
| Site R1     |                                 | 63 ± 2.2a         | 16.66 ± 1.65a   | 19.14 ± 1.38a   | 6.46 ± 0.79a | 5.31 ± 0.51a |
| Site R2     |                                 | 83 ± 4.38bc       | 16.20 ± 1.63ab  | 16.38 ± 1.26b   | 4.72 ± 0.69b | 5.14 ± 0.52bc|
| Site R3     |                                 | 66 ± 2.13a        | 19.07 ± 1.81c   | 19.95 ± 1.33a   | 7.14 ± 1.08c | 6.01 ± 0.49b |
| Mean        |                                 | 71 ± 1.56C        | 17.31 ± 0.98C   | 18.49 ± 0.77A   | 6.11 ± 0.50B | 5.49 ± 0.29B |

Table 2
Comet assay for fish species, site and type interaction analyzed for Labeo rohita. Fish species and site interaction (mean ± SE).

Fig. 6. Erythrocyte from farmed Labeo rohita indicating normal blood cells having no DNA damage.

Fig. 7. Micronucleus test for Labeo rohita collected from the polluted experimental area of the River Chenab indicating micronucleus induction and nuclear abnormalities.
micronucleus assay of blood from *Labeo rohita*. Analysis of variance for single micronucleus frequency (Table 3) showing the source of variation, degrees of freedom, and F-value for MNs, MNd, and NAs. Frequency was calculated in thousand cells.

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