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Assessment of pollution of river Ganges by tannery effluents using genotoxicity biomarkers in murrel fish, *Channa punctatus* (Bloch)

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River pollution due to rapid industrialization and anthropogenic activities adversely affects the aquatic organisms, especially fish. Here, we assessed the genotoxicity, mutagenicity and bioaccumulative aspects of tannery effluents in freshwater murrel, *Channa punctatus*, an inhabitant of river Ganges. Test specimens were collected from three different polluted sites of the river within and nearby Kanpur area during different seasons and blood samples of these specimens were processed for comet assay and micronucleus test as genotoxicity biomarkers. A significantly (P <0.05) higher micronuclei induction, nuclear abnormalities and % tail DNA was observed in the specimens collected from the polluted sites. Bioaccumulation studies in the muscle (1.202 µg/g) and gill tissues (<0.300 µg/g) of the specimens revealed the concentration of chromium (core component of tanning industry) above the maximum permissible limits as prescribed by World Health Organization (WHO). The findings of the present analysis indicated contamination of river Ganges with tannery effluents which induce genotoxicity in fish with seasonal variation.

Keywords: Aquatic pollution, Bioaccumulation, *Channa punctatus*, Ganga, Leather industry, Spotted snakehead.

Industrial effluents containing toxic and hazardous substances, including heavy metals considerably pollute the aquatic ecosystem[1]. Among the different industrial units, the leather tanning industries pose a major problem as their treated/untreated tannery effluents containing heavy metals, especially chromium, cause genotoxic, mutagenic and carcinogenic effects in aquatic organisms and thereby to humans[2]. Tannery effluents remain as one of the highest pollutants among all the industrial wastes[3].

Chromium (Cr) is a scarce metal and its presence in the aquatic ecosystem is generally low[4]. However, natural water receives Cr from anthropogenic sources viz. industrial effluents, gets polluted, and thus become harmful to aquatic organisms[5]. Chromium toxicity is affected by species, body size and life stage of the organism as well as the pH of the water and, to a lesser extent, by hardness, salinity and temperature[6]. Chromium (VI) passes readily through the gill membrane and accumulates rapidly in various tissues at higher levels than in the gills[7], including the brain, gall bladder, gastro-intestinal tract, intestine, kidney, opercular bone, spleen and stomach[8].

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Hexavalent chromium is one of the trace elements in biological system necessary for glucose tolerance in mammals[9] and serum cholesterol level suppression[10]. However, above the permissible limits, it affects physiological performance of the body. Hexavalent chromium penetrates into the cells in the surface transport system, gets reduced to trivalent chromium and further, induces genotoxic effects in the cell[11,12]. Several studies have revealed that heavy metal chromium and its compounds lead to DNA damage through DNA single- and double-strand breaks resulting in chromosomal aberrations, sister chromatid exchanges, micronuclei & DNA adducts formations, as well as alterations in DNA replication & transcription[13-15].

The evaluation of genotoxic effects of metals in terrestrial and aquatic ecosystems by studying such effects on the animals from the respective habitats has been an established procedure among the researchers[16]. Studies on metal pollution in different edible fish species are not uncommon[17,18]. Industrial effluents, agricultural runoffs, and domestic waste pollute the water bodies with heavy metals which thereby enter into the food chain and the bioaccumulation processes (http://www.eoearth.org/view/article/152839/; http://www2. epa.gov/nutrientpollu-

tion/sources-and-solutions-fossil-fuels). The toxic effects due to bioaccumulation of heavy metals have been reported already[19], and such metal accumulation in fish tissues serve as effective indicators of environmental contamination[20].

Techniques such as micronucleus test, chromosomal aberrations and DNA damage assays are used for evaluation of genotoxicity of chemicals in animals[21]. The comet assay is an easy, more consistent and cost efficient technique to examine the genotoxic potential of toxicants in the environment[22]. Micronucleus assay has also been extensively used for detection of clastogenic and aneugenic effects of chemicals.

Several biological as well as eco-toxicological characteristics, such as wide geographical distribution, freshwater habitat, availability throughout the year, maintenance and acclimatization to laboratory conditions, commercial importance, ease of blood collection, etc., make *Channa punctatus* a successful model species for toxicological studies[23]. Here, we attempted quantification of accumulation of heavy metals especially chromium in different tissues of *C. punctatus* inhabiting the polluted areas affected by tannery effluents and correlate the concentration of metals with respect to their genotoxic effects on other murrel species.

Material and Methods

Study area, *sampling and fish species*—The river Ganga near Kanpur, Uttar Pradesh, India receives a huge amount of tannery effluents all through the year. The location of sampling stations is shown in Fig. 1. Water samples were collected from river Ganga during winter (Nov. 2009), spring (Feb. 2010) and summer (April 2010) at three locations, i.e. one being up stream at Nanarao Ghat, from where river Ganga enters towards Kanpur City (Site A), one at the tannery effluent discharge site at Dapka Ghat (Site B) and another 300 m downstream of the effluent discharge site (Site C). The specimens of test species *Channa punctatus*, a freshwater air-breathing murrel fish (Bloch; Family: Channidae, order: Perciformes), were collected from the above three sites and were processed for comet assay, micronuclei test and bioaccumulation estimation.

For the control, the specimens of *C. punctatus* with similar age and relatedness were procured from Chinhat Farm, Aquaculture Research and Training Unit, National Bureau of Fish Genetic Resources, Lucknow, India. The procured specimens were

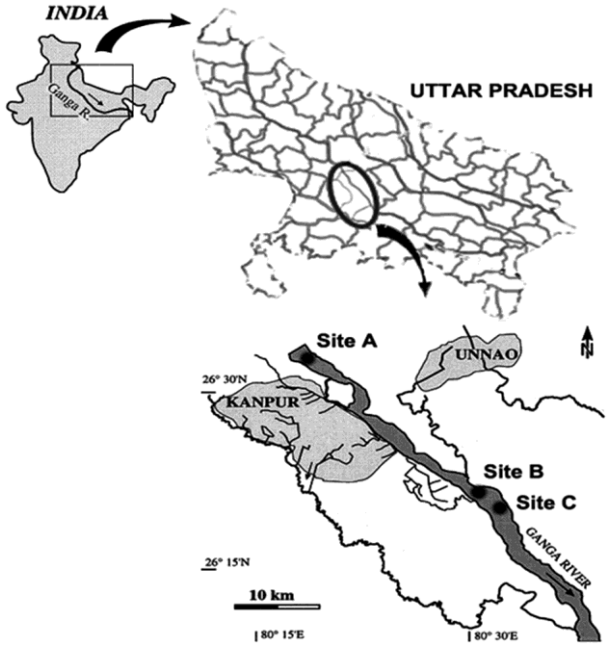

Fig. 1—Geographical location of the three sampling areas of Ganga River at Kanpur. Sites A, B and C.

subjected to prophylactic treatment at the laboratory by immersing them twice in 0.05% $KMnO_4$ solution for 2 min to avoid any dermal infections and acclimatized for one month under laboratory conditions following Bennett and Dooley[24] before the start of the experiment. The average (±SD) wet weight and length of collected specimens was 15.70±1.25 g and 13.06±2.07 cm, respectively. The specimens were fed with commercial fish feed throughout the acclimation process. For each sampling duration and control, the experiment was replicated twice and performed in accordance with the OECD[25] guideline No. 203.

Physicochemical properties of water samples—The estimation of physicochemical parameters of the water, namely temperature, pH, dissolved oxygen, conductivity, and total hardness, were analyzed by standard methods of APHA[26]. Respective WHO and BIS (http://www.indiawaterportal.org/sites/indiawaterportal.org/files/indian_standard_for_drinking_water_as_per_bis_specifications_2010.pdf) permissible values were taken as references values to compare.

Estimation of heavy metals in river water and fish tissues—Water samples collected in clean bottles from the three sampling sites were acidified with 1% of concentrated nitric acid (HNO_3) and analyzed according to the standards of APHA[26]. Analysis of heavy metals chromium (Cr), cadmium (Cd), lead (Pb)

and copper (Cu) in river water samples and in the muscle and gill tissues of test specimens, collected from the polluted sites in Summer, was performed by Flame Atomic Absorption Spectrometer (Perkin Elmer, Analyst 300) according to AOAC[27] and the concentration of heavy metals was expressed as µg/g fresh weight in each fish tissues. These values were compared with the permissible limits of World Health Organization[28].

Experimental procedure—On each sampling day, about 50 µl of peripheral blood was collected from caudal vein of each test specimens (n=5) using heparinized 1-ml disposable syringes and transferred to 1-ml eppendorf tube containing 450 µl of chilled Ca^{++} and Mg^{++} free phosphate buffer saline (PBS) for single cell gel electrophoresis assay (comet assay, CA) and micronucleus (MN) test. After blood collection, the specimens were transferred to separate water tanks, while gill and muscle tissues were sampled from other specimens for bioaccumulation studies. The tube containing blood and PBS was then placed on ice for further viability test, MN test and CA.

Micronucleus test—For MN test, peripheral blood samples obtained from the caudal vein were immediately smeared onto the pre-cleaned slides. After fixation in methanol for 10 min, the slides were allowed to air-dry for 1 h at room temperature (25-28 °C) and finally stained with 6% Giemsa solution in Sorenson's phosphate buffer (pH 6.9) for 25 min. The slides were then thoroughly washed in tap water; air dried and mounted in DPX for permanent slide preparations. The slides were observed under a light microscope (Leitz Wetzlar Germany; Type 307-083.103; oil immersion lens, 100/1.25). Two slides were prepared from each specimen, and a total of 2000 erythrocyte cells were examined from each slide under 100X magnification. Small, non-refractive, circular or ovoid chromatin bodies, displaying the same staining and focusing pattern as the main nucleus were scored as micronuclei[29].

Comet assay—DNA damage including single-strand breaks and alkali-labile sites were detected by CA as a three layer procedure[30] with minor modifications[31]. The whole blood was kept in phosphate buffer saline (PBS, *p*H 7) and the gill tissue (~75 mg) was homo-genized in PBS (*p*H 7) followed by centrifugation at 4000 rpm at 4 °C for 5 min. Cell viability of both the erythrocytes and gill cells was evaluated by the trypan blue exclusion test[32]. The samples showing more than 85% cell viability were further processed for CA[33].

Two slides per specimen procured from each sampling site were prepared and 20 cells per slide (200 cells per sampling site) scored randomly and analyzed using an image analysis system (Komet–5.5 Kinetic Imaging, U.K) affixed to florescent microscope (Leica) equipped with suitable filters. DNA damage was measured in terms of % Tail DNA (=100 - % Head DNA) as determined by the software.

Data analysis—One-way analysis of variance (ANOVA) was used to compare the mean difference in %Tail DNA between the tissues and the three sites. The %MN frequencies were compared between the three sites by Mann-Whitney test. The *P* values <0.05 were considered statistically significant.

Results

Physicochemical properties and chemical analysis of water samples—During the experimental period, the physicochemical properties of the water samples varied as shown in Table 1. The water temperature recorded 23.48, 21.25 and 22.0 °C at site A, B and C, respectively. Among the 3 selected locations, site B had maximum total dissolved solids (873.2 mg/l) and the least dissolved oxygen (6.57 mg/l). The estimated concentration of heavy metals (Cr, Cd, Pb and Cu) in water samples revealed a higher value of Cr (Table 2), when compared with World Health Organization[28].

Bioaccumulation studies—The bioaccumulation of heavy metals (expressed as µg/g dry weight) in the

Table 1—Physico-chemical characteristics of river water samples collected from different sites.

Parameters	Control	Site A	Site B	Site C	CETP treated tannery effluent
Air temperature (°C)	36.4±0.04	31.83±0.10	22.75±1.0	24.25±1.0	32.1±0.02
Water temperature (°C)	28.6±0.02	23.48±0.05	21.25±0.5	22.0±0.82	26.4±0.12
pH	7.4±0.05	8.63±0.08	8.13±0.06	8.55±0.06	7.0±0.02
Total Dissolved Solids (mg/L)	152.1±2.6	282.0±9.09	873.2±11.6	335.0±17.0	930±2.4
Dissolved Oxygen (mg/L)	6.6±0.02	8.56±0.03	6.57±0.022	6.80±0.14	1.4
Total hardness (mg/L)	160±0.21	170.8±0.33	176.8±0.37	171.6±0.22	-
Total alkalinity (mg/L) as $CaCO_3$	220±0.32	271.8±0.34	281.4±0.45	276.8±0.33	-

gills and muscle tissues of the test specimens was estimated. The muscles of *C. punctatus* revealed a higher concentration of Cr (1.202 µg/g), while in gill tissues it was found to be <0.300 µg/g. The Cd concentration was found to be 0.068 µg/g in muscle tissue, while in gill tissues it was 0.031 µg/g, while Pb was found to be 1.233 µg/g in muscle and 0.653 µg/g in gills. The concentration of Cu in muscle tissue was found to be 0.699 µg/g and 14.48 µg/g in gills.

Micronuclei induction—Micronuclei and nuclear abnormalities such as blebbed, notched and lobed nuclei, have been observed in the erythrocytes of the specimens collected near the tannery effluents discharge site (Figs. 2 a-f). At the effluent discharge site, the micronuclei frequency was significantly higher in summer (April) as compared to the winter (November) and spring (February) seasons. The micronuclei frequency in specimens sampled from the site B was not significantly different from that of site C specimens (Fig. 3a). The micronuclei frequency in specimens from site A as well as the laboratory acclimatized specimens was almost identical. A similar trend was observed for the frequency of nuclear abnormalities which was higher in the summer month at effluent discharge site as compared to the other two months (Fig. 3b). The nuclear abnormality and micronuclei data indicated that the Cr concentrations >0.05 mg/L induce genotoxic effects in aquatic organisms of river Ganges (Table 2 and Fig. 3).

DNA damage—The extent of DNA damage in the erythrocytes and gill tissues of specimens of site B was higher as compared to sites A and C. Specimens sampled during summer month had higher damage rather than November and February (Fig. 4a and b). The DNA damage in the erythrocytes and gill cells of the test specimens collected from site B and control is presented in Fig. 5a-d. The specimens procured from the tannery effluent discharge site exhibited significantly (P <0.05) higher DNA damage in both, the tissues as well as gills, compared to the control. In addition, a higher DNA damage was observed in the gill tissues as compared to the erythrocytes showing a tissue specific variation.

Table 2—Chemical analysis of water samples collected from different sampling sites of River Ganga at Kanpur during November 2009; February and April 2010.

Seasons and sites	Heavy metal concentration (mg/L)			
	Cr	Cd	Pb	Cu
November (2009)				
Site A	<0.05	<0.01	<0.05	<0.05
Site B	0.098*	<0.01	<0.05	<0.05
Site C	0.057*	<0.01	<0.05	<0.05
February (2010)				
Site A	<0.050	<0.02	<0.05	<0.05
Site B	0.308*	<0.02	<0.05	0.063
Site C	0.107*	<0.02	<0.05	<0.05
April (2010)				
Site A	<0.05	<0.02	<0.05	<0.02
Site B	<0.05	<0.02	<0.05	<0.02
Site C	<0.05	<0.02	<0.05	<0.02
WHO (2003) limits	0.05	0.003	0.01	-
BIS (1991) limits	0.05	0.01	0.1	0.05

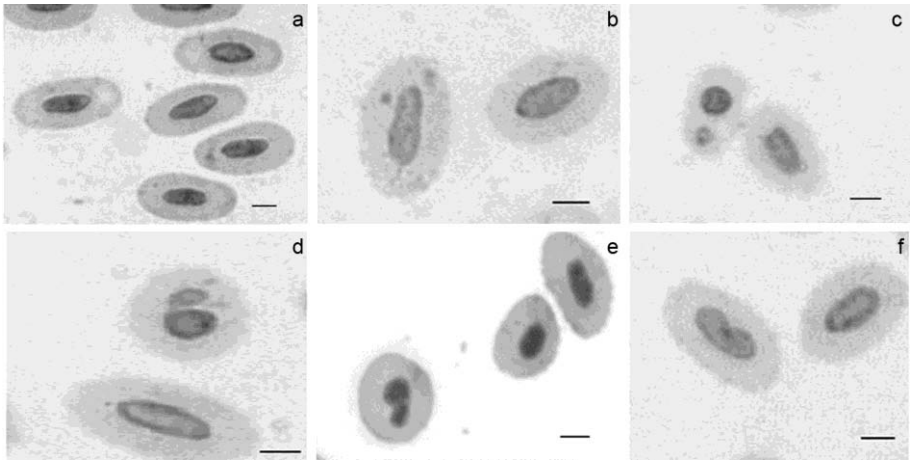

Fig. 2—Blood cells of *Channa punctatus* specimens: (a) Control; and specimens collected from site B showing (b) micronuclei; (c) abnormal nuclei; (d) bi-nucleated nuclei; (e) lobed nuclei; and (f) notched nuclei. Bar represents 10 µ.

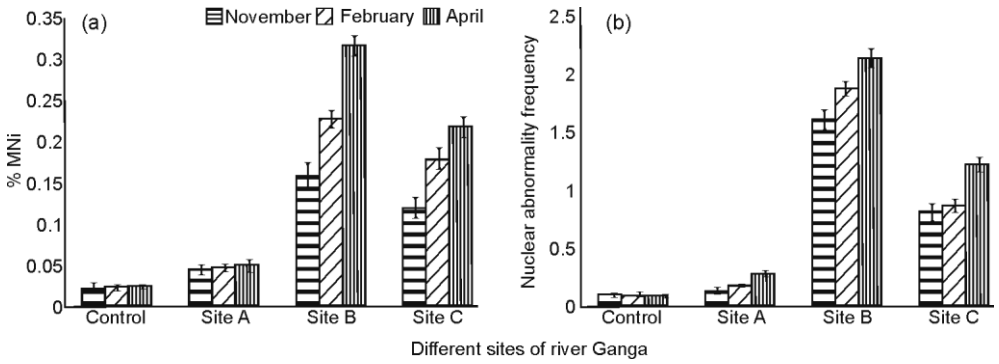

Fig. 3—(a) % MN frequencies; and (b) Nuclear abnormality frequencies in erythrocytes of *Channa punctatus* collected from three different sampling sites (A, B & C) during Nov. 2009, Feb. and April 2010.

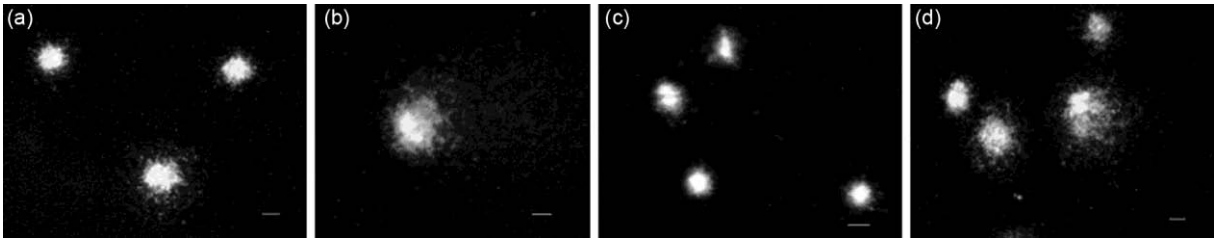

Fig. 4—Erythrocytes after comet assay from (a) control and (b) exposed specimens; and gill cells after comet assay from (c) control and (d) exposed specimens of *Channa punctatus* collected from site B. Bar represent 10 μ.

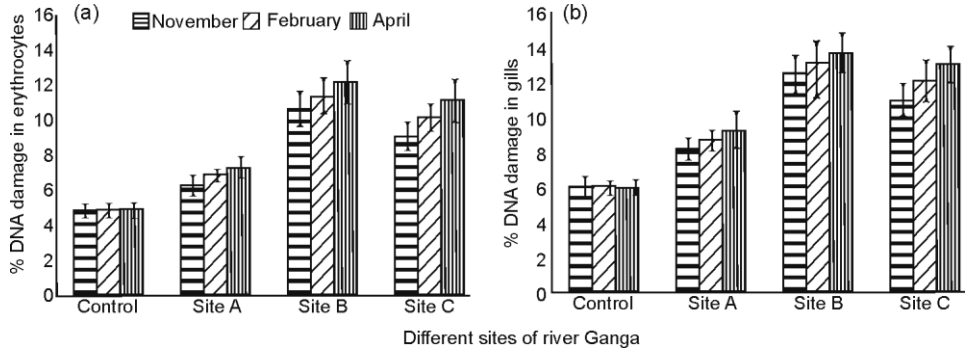

Fig. 5—% DNA damage (±S.E.) in (a) erythrocytes; and (b) gills of *Channa punctatus* collected from three sampling sites during Nov. 2009, Feb. and April 2010.

Discussion

Tannery discharges are ranked as one of the highest pollutants among all the industrial wastes[3]. Tanning is a chemical process in which semi-soluble protein "collagen" is renewed into tough, flexible, insoluble and highly durable leather in a sequence of many complex stages, consuming elevated quantities of water[34]. About 20-30 L effluents are discharged per kilogram of skin/hide processed[35], while solid and gaseous wastes are also discharged. Although, the major component of tannery effluents discharged from leather tanning industries is Chromium, yet some other pollutants of concern from the tanning industry include antimony, arsenic, azodyes, barium, cadmium compounds, cobalt, copper, lead, formaldehyde resins, mercury, nickel, pesticides residues, polychlorinated biphyenls, selenium and zinc[36,37].

Further, among other organisms, fishes accumulate pollutants either directly from polluted water or indirectly by intake of contaminated aquatic organisms during the process of food chain, and consequently threaten the entire ecosystem[38,39]. In the present study, we analyzed cadmium, copper and lead in addition to chromium for water and bioaccumulation

parameters. Also, we assessed the genotoxic and mutagenic effects of tannery effluents in the fresh-water fish *C. punctatus* through *in vivo* investigations.

A significantly higher micronuclei frequencies and nuclear abnormalities observed in water contaminated with the tannery effluents indicate the genotoxic effects of chromium present in the tannery waste released into the river. Abnormally blebbed, notched and lobed nuclei observed in the erythrocytes of the fish collected from the tannery effluents discharge site in concurrence to earlier investigations [2,40-42] reveal the extent of heavy metal pollution in the sites studied.

Similarly, comet assay has shown the higher DNA damage in these specimens. The results have clearly demonstrated that higher levels of chromium in the habitat, here from the tannery effluents, induced DNA damage similar to the recent genotoxic and mutagenic observations of Nagpure *et al*. in *Labeo rohita*[43]. The differential toxicity of Cr (VI) in erythrocytes and gills was further supported with the genotoxicity evaluation[4]. The extent of DNA damage was comparatively more in the gills than the erythrocytes exhibiting an organ-specific toxic potential of Cr (VI), particularly due to differential sensitivity, expression of receptors and cellular components of erythrocytes and gills cells that interact with the metal individually[16].

Further, the seasonal variation observed in the induction of micronuclei and formation of DNA damage, frequency of micronuclei and nuclear abnormalities i.e., higher in summer month (April) compared to the two winter and spring (Nov. and Feb.), and higher scale of DNA damage in the erythrocytes and gill tissues during summer is in alignment with similar investigations by earlier researchers[44,45].

In this study, we observed Cr concentration of <0.300 and 1.202 µg/g in the gills and muscle tissue, respectively of murrels sampled from habitat polluted with tannery effluents. Raja *et al*.[46], who studied heavy metal concentration in four marine edible fish species from Parangipettai Coast, Tamil Nadu, India reported a concentration range 0.65-0.92 µg/g. Higher levels of Cr concentration in water samples and fish tissues are often attributed to the tannery waste discharged into the such water bodies[36]. The increasing concentration of Pb in fish tissues, a point of major concern, indicates the gross discharge of domestic/ industrial wastes into water bodies[47]. The estimated levels of Cr and Pb in muscle and gill tissues of *C. punctatus* have been shown to be more than the permissible limits of WHO/FAO[48] and FAO[49]. Also, the chemical analysis of polluted water samples has proved the presence of Cr above the limits set by WHO[28] and BIS[50]. Accumulation of more Cr and Pb in muscles than the gill tissues is in agreement with the reports of Mohamed and Osman[51].

The present study has demonstrated that the river Ganga is considerably polluted by the discharges from tannery industries clustered on the bank of river in Kanpur. Significant results with regard to comet tail length and micronuclei observed in murrels exposed to tannery effluents in fresh water system have shown that the tannery effluents might induce genotoxicity in fishes. The comet assay and micronuclei test proved to be a dependable monitoring tool to measure the genotoxic potential of tannery effluents. There is an immense potential to utilize these assays for assessing detailed information on cell-specific genotoxic and mutagenic effects, inter-individual variability, seasonal variation and adaptability which would in turn help formulating strategies and measures for remediation of pollution, and conservation and sustainable management of fish diversity.

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