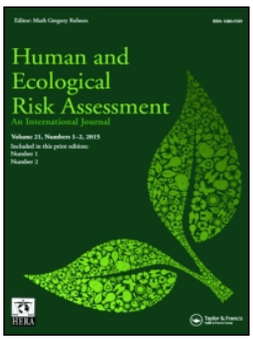

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Mutagenic, genotoxic and bioaccumulative potentials of tannery effluents in freshwater fishes of River Ganga

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ABSTRACT

The tannery industries are the reason of major environmental concerns as they release toxic heavy metals, like chromium, in rivers posing risks of genotoxicity and mutagenicity in aquatic organism and indirectly in humans through food chain. In the present analysis, the freshwater inhabitant fishes of River Ganges, viz., *Labeo calbasu, Puntius sophore*, and *Mystus vittatus*, were examined for assessing the genotoxic, mutagenic, and bioaccumulative potentials of tannery effluents. For genotoxicity assessment, the blood and gill samples of fishes prevailed from polluted sites of River Ganges adjoining Kanpur city were utilized for comet assay and micronucleus test. The present investigation revealed the presence of significantly ($p < 0.05$) higher micronuclei induction and % tail DNA in erythrocytes and gill cells of the fishes collected from the polluted sites. The bioaccumulation studies revealed chromium concentration in muscle (0.89 μg/g) and gill tissues (0.24 μg/g) of *L. calbasu*; muscle (0.44 μg/g) and gills (1.23 μg/g) of *P. sophore*; and muscle (0.9617 μg/g) and gills (0.3628 μg/g) of *M. vittatus*, quite higher than the permissible limits of the World Health Organization. Consequently, the present study indicates strongly that River Ganges is contaminated with harmful tannery pollutants causing genotoxicity and mutagenicity in freshwater fishes.

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KEYWORDS
DNA damage; fishes; genotoxicity; micronuclei; tannery effluent

Introduction

The booming tanning industries in India are turning the River Ganges into a disposal ground by discharging the effluents containing organic compounds, inorganic salts like sulfides, and toxicity-causing heavy metal chromium, which is its main component (Alam *et al.* 2009, 2010; Balusubramanian and Pugalenthi 2000; Matsumoto *et al.* 2006; Nagpure *et al.* 2015b; Reemste and Jekel 1997). Among all the other industrial wastes, the tannery wastes are one of the main pollutants (Camargo *et al.* 2003). The problem is aggravated due to the dumping of untreated effluents into River Ganges through some drains (Biswas 2002; Consortium of 7 IITs 2010; Ganga Action Plan I; Tare and Bose 2009). Although some common effluent treatment plants are recognized for the management of tannery wastewater through treatment, studies undertaken from time to time pointed out the presence of higher

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chromium concentrations in treated tannery effluents (CPCB 1984, 2010; NRCD 2009). Generally, tanning is a chemical process during which semisoluble protein called "collagen" is converted into highly durable leather in a sequence of many complex stages after consuming large quantities of water (Landgrave 1995). Later, the tannery effluents are discharged per kilogram of skin or hide processed, apart from other solid and gaseous wastes (Nariagu and Pacyna 1989). A cumulative amount of genotoxic pollutants are released into the aquatic environment. Hence, in view of the potential dangers of these pollutants to aquatic fauna and flora, attempts were made to explore the feasibility of application of micronucleus test and comet assay in fish biomonitoring from the polluted sites of River Ganga.

Among the other aquatic resources, fish equally serves as an important portion of the human nutrition and an important indicator of aquatic pollution; hence, it is an obvious choice for studying metal pollution in aquatic environments (Erdogrul and Ates 2006; Karadede and Unlu 2000; Prudente *et al.* 1997; Unlu *et al.* 1996; Velma *et al.* 2009). The contributing factors for heavy metals contamination in aquatic environs are industrial effluents, agricultural runoffs, geological weathering, and other anthropogenic activities (Adnano 1986; Tchounwou *et al.* 2012). Interestingly, these heavy metals have a tendency to bioaccumulate easily by uptake via the food chain (Beijer and Jernelov 1986; El-Moselhy *et al.* 2014). Hexavalent chromium, Cr (VI), is an important trace element in biological system that is essential for glucose tolerance in mammals (Schwarz and Mertz 1959) and acts as a serum cholesterol suppressor (Schroeder 1968). However, when present in levels exceeding the permissible limits, chromium upsets the body's physiological performance abnormally. The hexavalent chromium enters the cells through surface transport system, reduces to trivalent chromium in the cell, and thus induces genotoxicity (Bianchi *et al.* 1983; Sugiyama 1992). It has been well established that chromium compounds incite DNA damage along with DNA single- and double-strand breaks causing chromosomal aberrations, DNA adducts and micronucleus formation, sister chromatid exchanges, and alterations in DNA replication and transcription (Matsumoto and Marin-Morales 2004; Medeiros *et al.* 2003; O'Brien *et al.* 2001; Velma and Tchounwou 2010; Wu *et al.* 2000; Zhitkovich *et al.* 1996).

Fishes are important bioindicators of aquatic pollution. Hence, they facilitate in assessing the impending perils of contamination owing to the fact that they remain directly exposed to aquatic pollution for longer durations (Kushwaha *et al.* 2012). In general, many fish species have been utilized for the assessment of the mutagenic, clastogenic, and teratogenic effects of environmental contaminants (Kushwaha *et al.* 2012; Matsumoto *et al.* 2006; Nagpure *et al.* 2015b; Talapatra and Nandy 2014). In the present investigation, freshwater fishes such as *Labeo calbasu, Puntius sophore*, and *Mystus vittatus* have been preferred for the assessment of genotoxicity, mutagenicity, and bioaccumulation status of tannery effluents. The selection of the fish species during the study was based on both the prevalence and live captivity of these fishes near the polluted sites. Additionally, some other important characteristics such as freshwater habitat, consumer value, ease of blood and gill tissue collection, and adaptation to laboratory conditions also contribute well to pursuing the present analysis. On the other hand, the selection of sampling locations was established on the grounds of elevated pollution levels of River Ganga around the Kanpur city (Alam *et al.* 2009, 2010; Nagpure *et al.* 2015b; Singh *et al.* 2003; Sinha *et al.* 2006; Tare *et al.* 2003). The analysis of polluted waters of River Ganga near Kanpur revealed an increase in chromium levels, pointing out the reckless disposal of untreated tannery effluents in the holy river. Hence, the present study aims at assessing the genotoxic, mutagenic, and bioaccumulative potentials of

tannery effluents in certain fishes of River Ganga that may contribute to the groundwork of establishing preventive methodologies for treating tannery effluents and assuring the safety of river water for survival of aquatic organisms along with human health.

Methods and materials

Sampling sites and chemical analyses

The Ganga River near Kanpur in the state of Uttar Pradesh, India, receives a colossal amount of tannery effluents. Water samples of River Ganga were collected during April, 2010, from three different locations, first being the upstream at Nana Rao Ghat, i.e., Site A, the second being the tannery effluent discharge site at Dapka Ghat, i.e., Site B, and downstream of the effluent discharge site as Site C. The geographical whereabouts of the three sampling sites are presented in Figure 1. The water samples taken from the three selected sites were kept in clean bottles and relocated to the laboratory for metal analysis. The specimens of test species, i.e., *L. calbasu, P. sophore*, and *M. vittatus*, were collected from the aforementioned three sites, and the tissue samples were processed for comet assay, micronuclei test, and bioaccumulation estimation.

Physicochemical properties and chemical analysis of water samples

The temperature, pH, dissolved oxygen, conductivity, total dissolved solids (TDS), total hardness, and total alkalinity of the river water samples were analyzed using the standard methods of APHA (2005). For the estimation of certain heavy metals such as cadmium (Cd), chromium (Cr), Copper (Cu), and lead (Pb) in polluted water samples, the test water samples were first acidified with 10 ml of concentrated nitric acid (HNO_3; pH1) and analyzed on atomic absorption spectrometer (Perkin Elmer, AAnalyst 300). The concentrations of these heavy metals were compared with the approved limits set by Bureau of Indian Standards (BIS) (1991) and the World Health Organization (WHO) (2003) in drinking water.

Heavy metal bioaccumulation in fish tissues

For bioaccumulation studies of the heavy metals, the muscle and gill tissues weighing 2 g each of the three fish species (*L. calbasu, P. sophore*, and *M. vittatus*) were processed for tissue digestion and further analysis on atomic absorption spectrophotometer (AAS). The estimation of heavy metal, especially chromium, cadmium, copper, and lead, concentrations for bioaccumulations studies in fish tissue samples was performed on AAS.

Micronucleus test

For *in vivo* study of mutagenicity, peripheral blood obtained from the caudal veins of fish samples, collected from the three selected sites, was smeared on the precleaned slides. After fixation in methanol for 10 min, the smeared slides were air-dried for 1 h at room temperature and finally stained with 6% Giemsa solution in Sorenson's phosphate buffer (pH 6.9) for 25 min. Subsequently after the staining process, the slides were then carefully washed in tap water, air-dried, and finally mounted in DPX (a mixture of distyrene, a plasticizer, and

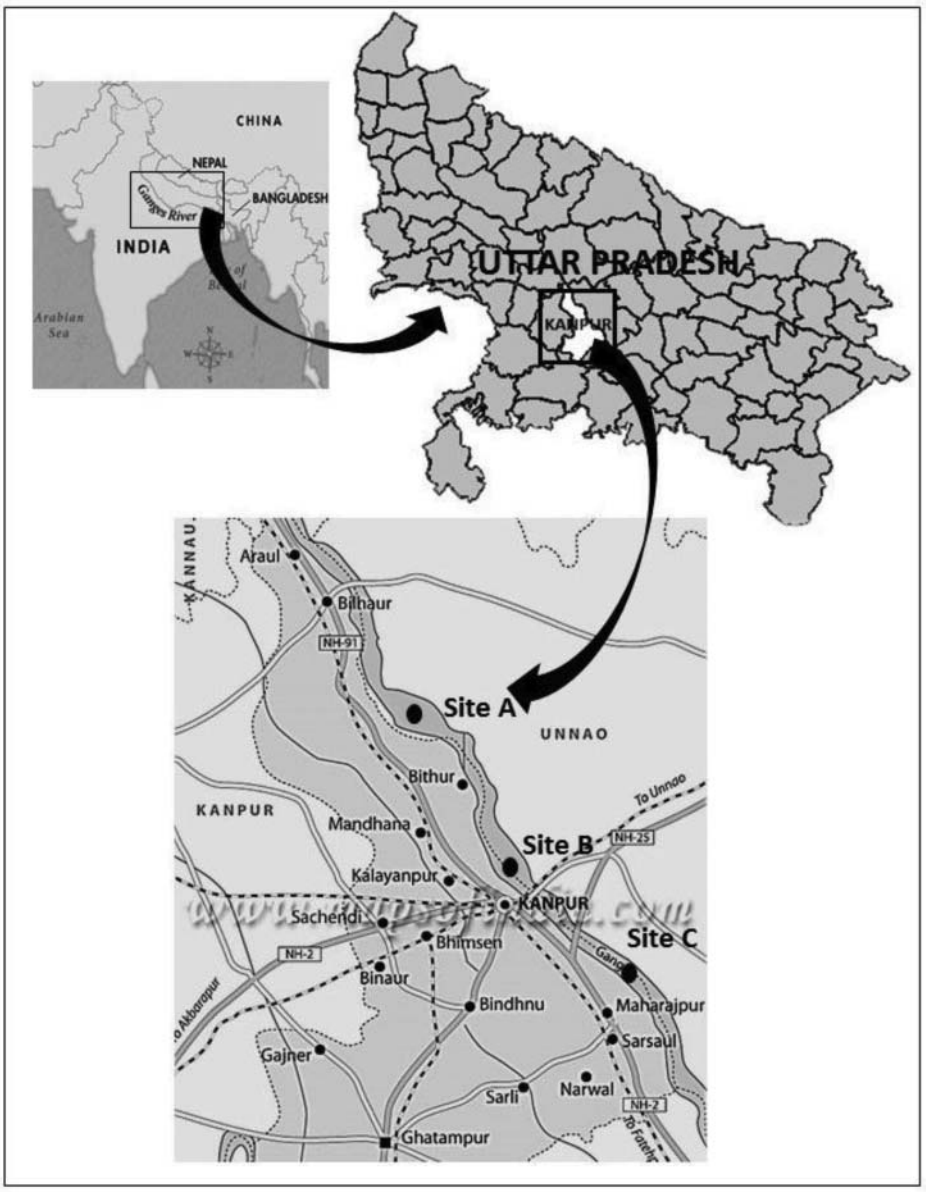

Figure 1. The geographical location of the three sampling regions, i.e., Site A, Site B, and Site C of River Ganga at Kanpur city, India. The three sampling sites A, B, and C are indicated by three large dots.

xylene) for obtaining permanent slide preparations. Finally, these permanent slide preparations were observed under a light microscope (Leitz Wetzlar Germany; Type 307–083.103; oil immersion lens, 100/1.25) for micronuclei analysis. During the experimental procedure, from each specimen, two slides were prepared, and 2,000 erythrocyte cells from each slide were examined under 100× magnification. The small, circular or ovoid chromatin bodies, nonrefractive, showing the same staining and focusing pattern as the main nucleus were

counted as micronuclei, similar to and as previously been recommended by Al-Sabti and Metcalfe (Al-Sabti and Metcalfe 1995).

Alkaline single cell gel electrophoresis or comet assay

For the analysis of genotoxicity, the DNA damage was identified by using comet assay as a three-layer procedure (Singh *et al.* 1988) with slight modifications (Mckelvey *et al.* 1993). The peripheral blood obtained from the test specimen was kept in phosphate-buffered saline (PBS, pH 7), and the gill tissue (~75 mg) was homogenized in PBS (pH 7) and centrifuged at 4,000 rpm at 4°C for 5 min. Additionally, cell viability of both the erythrocytes and gill cells was evaluated through the trypan blue exclusion test (Anderson *et al.* 1994), and the samples exhibiting over 85% of cell viability were processed for comet assay (Kushwaha *et al.* 2012). During the experimental procedure, two slides per specimen were prepared, and 25 cells per slide (250 cells per concentration) were scored arbitrarily and analyzed using an image analysis system (Komet 5.5 Kinetic Imaging, the United Kingdom) attached to florescent microscope (Leica) equipped with suitable filters. As per the software analysis, the factor selected for quantification of DNA damage was % Tail DNA (= 100 − % Head DNA) for genotoxicity assessment.

Data analysis

The statistical evaluation including the one-way analysis of variance was applied to compare the mean difference in %tail DNA between tissues and the three sites. The %MN (micronucleus) frequencies were compared between the three sites using Mann–Whitney test. The *p*-values less than 0.05 were statistically significant.

Results and discussion

Physicochemical properties and chemical analysis of water samples

The physicochemical factors analyzed during the experimental process revealed that the test water temperature varied from 22°C to 26°C; the pH ranged from 6.5 to 8.5; and the concentration of dissolved oxygen ranged from 6.5 to 8.5 mg/L. The other physicochemical parameters during the experimentation, *viz*. the TDS, total hardness, and total alkalinity of the test water ranged from 273 to 888 mg/L, 170 to 176 mg/L, and 271 to 282 mg/L, as $CaCO_3$, respectively. The observations of the chemical analysis of polluted water samples reveal the presence of chromium concentration higher than the permissible limits set by WHO (2003) and BIS (1991).

Estimation of bioaccumulation in fish tissues

The bioaccumulation studies (expressed as μg/g dry weight each) in the muscle of *L. calbasu* revealed a 0.8870 μg/g concentration of Cr, while in gill tissues the Cr concentration was 0.2391 μg/g, which is much higher than the maximum permissible limits set by WHO in fish tissues. The concentration of Cd in muscle tissues of *L. calbasu* was 0.1497 μg/g, while in gill tissues the concentration of the same was <0.05 μg/g. The

presence of Pb concentration was 0.9642 μg/g in muscles of *L. calbasu*, while in gills it was 0.7126 μg/g. The concentration of Cu in muscle tissues of *L. calbasu* was found to be 1.0980 μg/g, while in gills it was 0.3741 μg/g.

The estimation of bioaccumulation in muscle tissues of *P. sophore* relates a higher concentration of Cr, i.e., 0.4411 μg/g, while in gill tissues it was found to be 1.2268 μg/g. The concentration of Cd in muscle tissues of *P. sophore* was <0.05 μg/g, while in gill tissues it was 0.2169 μg/g. The Pb concentration in *P. sophore* muscles was 0.7671 μg/g; however, in gills it was 1.4483 μg/g. The concentration of Cu in muscle tissues of *P. sophore* was observed to be 0.7040 μg/g, while in gills it was 0.9896 μg/g.

In the muscles of *M. vittatus*, the bioaccumulation estimations reveal a higher concentration of Cr, i.e., 0.9617 μg/g, while in gill tissues, it was detected as 0.3628 μg/g. The Cd concentration present in muscle tissues of *M. vittatus* was 0.1046 μg/g, while in gill tissues it was 0.0599 μg/g. The Pb concentration in muscle tissues of *M. vittatus* was observed to be 0.0378 μg/g, while in gills, it was present at 0.7823 μg/g. The concentration of Cu in muscle tissue of *M. vittatus* was detected as 0.3603 μg/g, whereas in gills it was 0.2330 μg/g.

Thus, the present study revealed the amount of the aforesaid heavy metal concentrations, especially chromium, to be above the maximum permissible limits set by the Food and Agricultural Organization (FAO)/WHO (1984), WHO/FAO (1989), and FAO (1983) for dietary allowances, fish, and fishery products as represented in Table 1.

Micronuclei induction

The micronucleus test performed in the peripheral blood cells of *L. calbasu, P. sophore*, and *M. vittatus*, collected from the polluted sites of the River Ganga at Kanpur, shows the presence of micronuclei (Figure 2). This indicates that the waters from polluted sites induced genotoxicity in the inhabitant fishes. There was significantly higher induction of micronuclei in the fish specimens attained from the highly polluted tannery effluent discharge site, i.e., Site B as compared to the comparatively less polluted Sites B and C as shown in Figure 3. Higher induction of micronuclei frequencies was observed in the specimens of *P. sophore* from Site B (0.17 ± 0.052) than those from Site A (0.04 ± 0.021) and site C (0.15 ± 0.02). Similarly, *M. vittatus* collected from site B also showed higher micronuclei induction (0.1263 ± 0.026) than that from Site A (0.032 ± 0.011) and Site C (0.09 ± 0.012).

Table 1. Heavy metal concentrations (μg/g dry weight) in tissues of fishes collected from polluted sites.

Fish (tissue)	Chromium[a]	Cadmium[b]	Lead[c]	Copper[d]
L. calbasu (muscle)	0.89	0.15	0.96	1.1
L calbasu (gill)	0.24	<0.05	0.71	0.37
P. sophore (muscle)	0.44	<0.05	0.77	0.70
P. sophore (gill)	1.23*	0.22*	1.45	0.99
M. vittatus (muscle)	0.96	0.10	0.04	0.36
M. vittatus (gill)	0.36	0.06	0.78	0.23

*Values represent a higher concentration of the heavy metals in animal tissues as set by FAO/WHO (1984, 1989). WHO, World Health Organization; FAO, Food and Agricultural Organization.
[a]FAO/WHO chromium permissible limit in tissues is 1 μg/g.
[b]FAO/WHO cadmium permissible limit in tissues is 0.2 μg/g.
[c]FAO/WHO lead permissible limit in tissues is 1.5 μg/g.
[d]FAO/WHO copper permissible limit in tissues is10 μg/g.

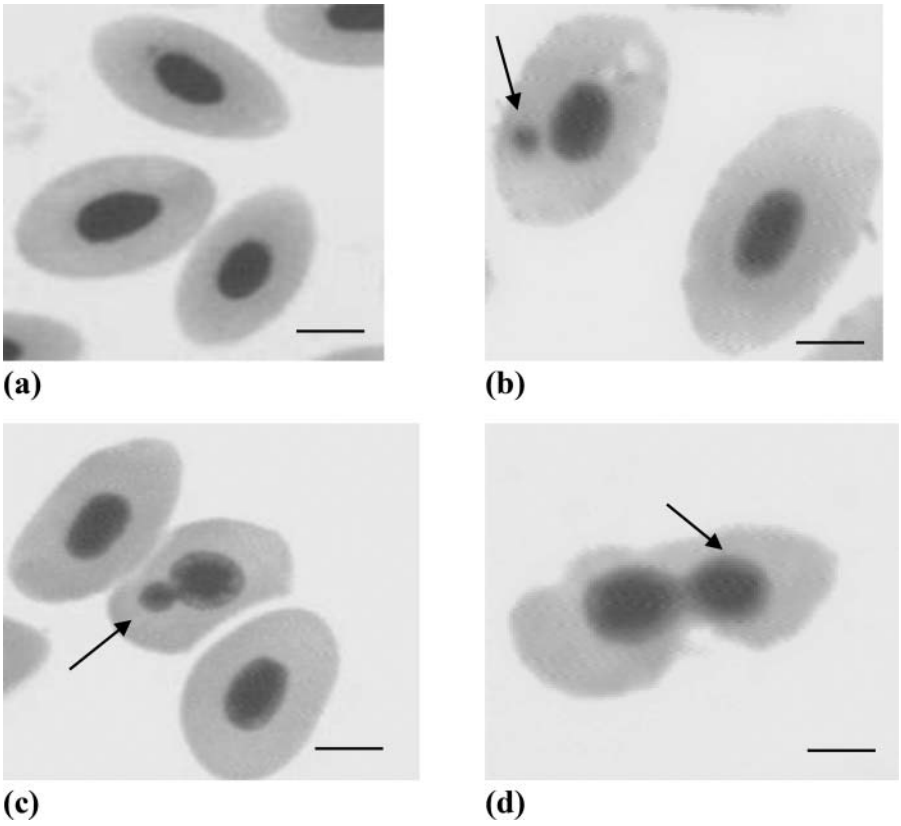

Figure 2. The cytological visualization of micronuclei in the red blood cells of *Puntius sophore, Labeo calbasu*, and *Mystus vittatus* specimens collected from tannery effluent discharge site, i.e., Site B. Red blood cell isolated from fishes of Site B (A) showing no micronuclei, (B) showing micronuclei in *P. sophore*, (C) micronuclei in *L. calbasu*, and (D) micronuclei in *M. vittatus*. Micronuclei are marked by an arrow, and the bar represents 10μ.

In *L. calbasu*, higher micronuclei induction was observed in specimens collected from Site B (0.17 ± 0.031) than those from Site A (0.039 ± 0.011) and Site C (0.11 ± 0.02). A significantly ($p < 0.05$) higher micronuclei frequency was revealed in fishes acquired from tannery effluent discharge Site B (Figure 3).

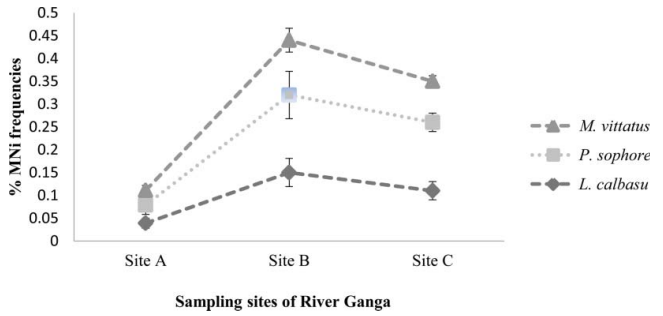

Figure 3. The %MN frequencies in erythrocytes of *P. sophore, L. calbasu*, and *M. vittatus* collected from the three different sites A, B, and C of River Ganga.

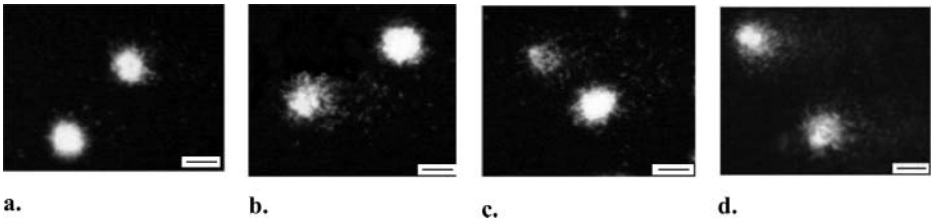

Figure 4. The visualization of DNA damage in red blood cells of specimens of *Puntius sophore, Labeo calbasu*, and *Mystus vittatus* collected from site B through fluorescence microscopy. The red blood cells showing (A) normal erythrocyte, (B) DNA damage in erythrocytes of *P. sophore*, (C) DNA damage in erythrocytes of *L. calbasu*, and (D) DNA damage in erythrocytes of *M. vittatus*. The DNA damage in the three fish specimens collected from the polluted site was confirmed by the presence of "comet tail" observed in ethidium bromide–stained nuclei following electrophoresis steps of comet assay. Bar represents 10μ.

The micronuclei data suggest that concentrations of chromium lower than 0.05 mg/L found in polluted waters of tannery effluent discharge site induce genotoxic effects in the effluent-exposed fish, which is correspondingly supported by the chemical analysis of effluent discharge site water samples. However, the micronuclei frequency data also revealed that river water from the upstream and downstream sites was less genotoxic than the effluent discharge site water.

DNA damage

The DNA damage was expressed as % tail DNA in the erythrocytes, and gill cells of the specimens collected from polluted sites and the comet tail signify the DNA damage induced in the three fishes procured from the polluted sites (Figure 4). In *P. sophore*, the amount of DNA damage in specimens of Site B (13.62 $\pm$ 1.52) was also higher as compared to Site A (7.62 $\pm$ 0.81) and Site C (12.33 $\pm$ 1.06) specimens. In *M. vittatus*, the amount of DNA damage was higher in erythrocytes of specimens collected from Site B (10.21 $\pm$ 0.98), as compared to Site A (5.58 $\pm$ 0.64) and Site C (8.82 $\pm$ 0.77) specimens, while in gill cells, the amount of DNA damage was comparatively higher in specimens collected from Site B (12.21 $\pm$ 0.98), as compared to Site A (7.58 $\pm$ 0.64) and Site C (10.82 $\pm$ 0.82). Similarly, in *L. calbasu*, higher DNA damage was reported in erythrocytes of specimens collected from Site B (12.62 $\pm$ 1.12) as compared to Site A (7.12 $\pm$ 0.61) and Site C (10.63 $\pm$ 1.12) specimens, and in gill cells, it was also higher in specimens collected from Site B (Figures 5 and 6, respectively). The DNA damage noticed was comparatively more in the gills than in the erythrocytes, proposing the organ-specific toxic potential of Cr (VI) due to the differential sensitivity of erythrocytes and gills cells owing to the differential expression of receptors and cellular components that interact with the metal and metal-produced reactive oxygen species (Kumar *et al.* 2013; Nagpure *et al.* 2015a).

Genetic toxicology plays a twofold role in safety evaluation programs: one of its role is in enactment of risk assessment methods to define the impact of genotoxic agents on the environment that causes alterations in gene integrity; while the second role is in the application of genetic methodologies for the detection and mechanistic understanding of carcinogenic chemicals (Bhattacharya *et al.* 2011; Brusick 1987; Cimino 2006; Lorge *et al.* 2007). Several studies that unravel the genotoxic potential of Cr (VI) in humans and rodents have been

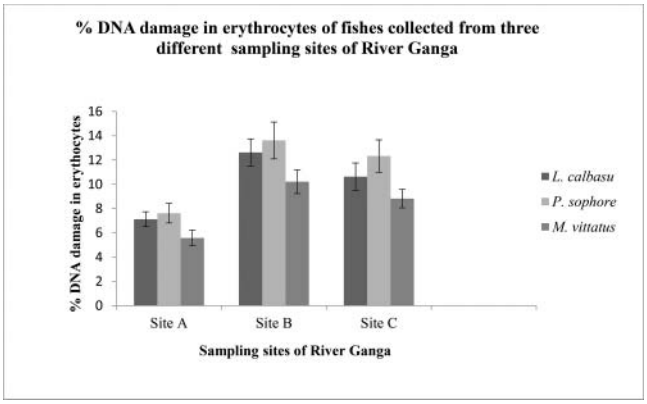

Figure 5. % DNA damage (±SE) in erythrocytes of *P. sophore, L. calbasu*, and *M. vittatus* collected from the three different sites A, B, and C of Ganga River.

widely reported (Bagchi *et al.* 2001; Kanojia *et al.* 1998; Maeng *et al.* 2004; Park *et al.* 2004; Patlolla *et al.* 2008; Thompson *et al.* 2013; Wise *et al.* 2006). While several assays including the micronucleus test, chromosomal aberrations, and DNA damage assays have been used for evaluating genotoxicity of toxic chemicals in different animals (Ahmad *et al.* 2006; Cavas and Ergene-Gozukara 2005; Cavas and Konen 2007; De Lemos *et al.* 2001; Farag *et al.* 2006; Patlolla and Tchounwou 2005), the comet assay has been extensively accepted as one of the sensitive, reliable, and cost-effective methods that is frequently utilized to examine the environmental genotoxicants and known to detect low levels of DNA damage even in short exposure durations (Andrade *et al.* 2004; Ateeq *et al.* 2005; Dhawan *et al.* 2009).

The studies on micronuclei test were initiated by Manna and colleagues in India, who reported the incidence of micronuclei in erythrocytes and chromosomal aberrations in *Oreochromis mossambicus* when treated with aldrin, cadmium chloride, and X-rays (Manna *et al.* 1985). Later, Manna and Sadhukhan also witnessed micronuclei in gills when fishes were exposed to five chemicals (anisole, cobalt chloride, lithium chloride, Rogar 30E, and Zn SO_4) by developing a detection method of micronucleated cells from the gills of *Tilapia* (Manna

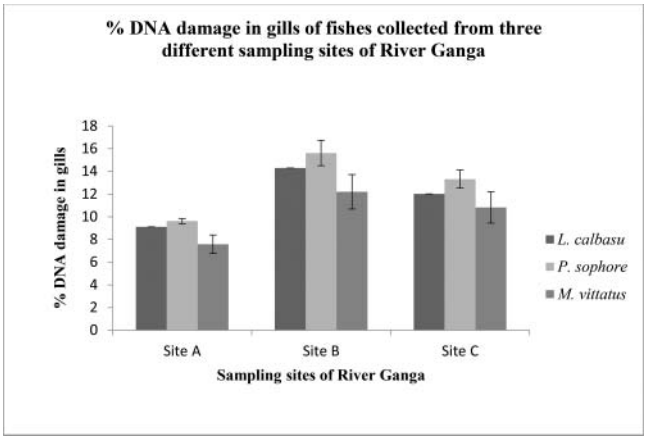

Figure 6. % DNA damage (±SE) in gill tissues of *P. sophore, L. calbasu*, and *M. vittatus* collected from the three different sites A, B, and C of Ganga River.

and Sadhukhan 1986). Another study related with the environmental pollution assessment revealed an upsurge in micronuclei frequency in the erythrocytes of *Heteropneustes fossilis* when exposed to mitomycin C and paper mill effluent (Das and Nanda 1986). Micronucleus assay is used for finding out the clastogenic and aneugenic effects of chemicals because it is formed by chromosome fragment or whole chromosome lagging at cell division due to the lack of centromere or damage (OECD 1997). It is also used broadly for *in situ* detection of aquatic pollutants (Kushwaha *et al.* 2012).

The erythrocyte micronuclei frequencies experiential in peripheral blood of fishes collected from the tannery effluents discharge site were in accordance with the reports of Ayylon and Garcia-Vazquez (2000), Carrasco *et al.* (1990), and Cavas and Ergene-Gozubara (2003). The water from effluent discharge and downstream site instigated significantly higher micronuclei frequency in the fishes as compared to the water from the upstream site, specifying that these abnormalities are the outcome of the genotoxic effects of chromium-containing tannery wastes discharged into the river stream. Similarly, higher DNA damage has also been detected in these specimens using comet assay, indicating that the tannery effluents induce genotoxicity in fishes, and the observations are in agreement with Von Burg and Liu (1993), Blasiak and Kowalik (2000) and Matsumoto *et al.* (2006), who proposed the potential genotoxic effects of chromium via inducing DNA damage in animals. These chromium-containing tannery residues are genotoxic pollutants posing a substantial risk to the environment (Matsumoto *et al.* 2006).

Paradoxically, pollution of water resources is a severe problem. Despite the existence of relevant legislation in the pollution of aquatic environment, it continues to occur through toxic chemicals being dumped into the water resources in the form of industrial wastes. The fish tissues collected from polluted sites revealed comparatively higher DNA damage and micronuclei frequencies as compared to nonpolluted sites. The results of the present study would help in guarding against the genetic hazard to human population, guide future environmental pollution studies, and to make policies toward reduction in genotoxic damage through judicious and careful use of these chemicals in agricultural and nonagricultural arenas. Moreover, it becomes also indispensable for the new chemicals to be released to test their genotoxic prospective using suitable biomarker(s).

Conclusion

Fishes accumulate toxic chemicals from ingesting contaminated water or aquatic organisms, posing a hazard to the entire ecosystem through the food chain. The present study has shown that the River Ganga is seriously polluted by the discharges from tannery industries clustered on the bank of river in Kanpur. Significant comet tail length and micronuclei detected in fishes continuously exposed with tannery effluents in freshwater system indicate that the tannery effluents are liable for inducing genotoxicity in freshwater fishes. Once again, the comet assay and micronuclei assay have served as sensitive monitoring tools to determine the genotoxic characteristics of tannery effluents and to investigate the health impacts of DNA damage, repair, and recovery in species of environmental concern. The use of these techniques in aquatic toxicology helped immensely in *in vivo* studies of toxicity studies in fish tissues as they are constantly being exposed to environmental pollutants. Further studies will provide detailed information on cell-specific genotoxic and mutagenic effects, interindividual variability, and adaptability, contributing to formulation of strategies and measures for the conservation of fish biodiversity.

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