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In situ assessment of genotoxic and mutagenic potential of polluted river water in *Channa punctatus* and *Mystus vittatus*

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Abstract

River Gomti, a tributary of river Ganga in northern India, is being polluted due to indiscriminate disposal of domestic sewage and industrial wastes that contain genotoxic chemicals. The study was conducted to evaluate the genotoxic potential of polluted water of river Gomti in two fish species, namely *Channa punctatus* and *Mystus vittatus*. The fishes were exposed *in situ* in nylon cages to the polluted water of river Gomti fixed near a distillery outlet located in Lucknow. The induction of DNA damage and micronuclei were determined in blood erythrocytes using comet assay and micronucleus test, respectively. The induction in micronuclei frequencies and DNA damage were found to be significantly elevated ($p < 0.01$) in exposed specimens after 3 days post-exposure as compared to the control, i.e. from laboratory-acclimatized fish specimens. The comparison of DNA damage between the two species indicated that *C. punctatus* is more sensitive to aquatic pollutants. Thus, this fish could be used as a bio-indicator of genotoxicity for bio-monitoring of water bodies. The results further revealed that the river Gomti is being contaminated with potential genotoxic and mutagenic chemicals produced from industrial and domestic activities; therefore, immediate measures are needed to reduce the inflow of pollutants in the river.

Keywords: *Channa punctatus*, DNA damage, genotoxicity, micronucleus test, *Mystus vittatus*

Background

Majority of river systems are polluted due to admixture of domestic waste, industrial effluents, and many other pollutants that are adversely affecting the human health and the ecosystem of the water bodies. The major sources of contamination include waste from paper, PVC plastic, pigments and ceramic industries, battery, mining and smelting units, and other modern industries (Gupta et al. 2003). The pollutant enters into the aquatic bodies through sewage and with the runoff from agricultural wastes (Cherian and Goyer 1989). Many of these polluting agents contain a number of chemicals that are highly persistent and have mutagenic and/or clastogenic properties (Waters et al. 1991, 1999). Several studies have shown that a wide range of chemical pollutants in aquatic ecosystem affect essential physiological functions in various

aquatic organisms and causes adverse effects at cellular and molecular levels (Mishra and Mohanty 2009; Li et al. 2010, 2011).

In situ toxicity tests have been increasingly used with cladocerans, amphipods, and fishes as an effective tool in monitoring environmental quality. Furthermore, exposing test organisms *in situ* may reduce the uncertainties of extrapolation from the laboratory (Hoffman et al. 1995). *In situ* standard toxicity assessment in various aquatic organisms has been used extensively in river environments to assess water and sediment toxicity, and this method is recognized as a useful tool for assessing non-point source contaminants (Chappie and Burton 1997; Tucker and Burton 1999). According to Burton (1991), this technique is relatively simple and eliminates possible errors induced by the sampling and manipulation of sediments in the laboratory. Further, *in situ* tests with zooplankton species constitute a rapid and efficient way of evaluating environmental contamination and validating laboratory experiments (Pereira et al. 1999).

Genotoxic pollution of aquatic ecosystem refers to the introduction of contaminants with mutagenic, teratogenic, and/or carcinogenic potentials into its principal media and genome of the resident organisms (Fagr et al. 2008). Genotoxicity is a deleterious action, which affects a cell's genetic material affecting its integrity (Smith 1996). Genotoxicants include certain chemical compounds like heavy metals (Matsumoto et al. 2005; Igwilo et al. 2006), microbial toxins (Smith 1996), and polycyclic aromatic hydrocarbons (Fernandez and L'Haridon 1992; Germain et al. 1993). These genotoxicants have been reported to cause mutations because they form strong covalent bonds with the DNA, resulting in the formation of DNA adducts preventing accurate replication (Hartwell et al. 2000).

Since there is a growing concern over the presence of genotoxins in the aquatic environment, the development of sensitive biomarkers for detection of genotoxic effects in aquatic organisms has gained importance (Hayashi et al. 1998). The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays, but the comet assay is gaining popularity over others since its advantages include sensitivity for detecting low levels of DNA damage (Gedic et al. 1992) and the short time needed to complete a study. The micronucleus (MN) assay is another useful and popular technique for showing clastogenic and aneugenic effects (Norppa and Falck 2003) and has been extensively used *in situ* (Al-Sabti and Metcalfe 1995). Several studies have shown that the MN test and comet assay (CA) are the two sensitive, rapid, and extensively used methods in the detection of mutagenicity and genotoxicity of chemicals and xenobiotics under field and laboratory conditions (Jha 2004; Pandey et al. 2006; Nagpure et al. 2008; Ventura et al. 2008; Ali et al. 2009; Kumar et al. 2010; Nwani et al. 2010).

Fish serve as useful genetic model for the evaluation of pollution in aquatic ecosystems (Mitchell and Kennedy 1992) and can play significant roles in assessing potential risk associated with contamination in aquatic environment since they are directly exposed to chemicals and mutagens resulting from agricultural production via surface runoff or indirectly through the food chain of the ecosystem (Cavas and Ergene-Gözükara 2005). Therefore, the use of fish biomarkers as indices of the effects of pollution are of increasing importance and can permit early detection of aquatic environmental problems (Lopez-Barea 1996; Van Der Oost et al. 2003).

Gomti River is one of the major tributaries of river Ganga in northern India; its ecosystem is being polluted seriously due to industrialization and urbanization occurring

during the past few decades (Singh et al. 2005; Sarkar et al. 2010). The contaminants in the river are increasing day by day, thereby causing genotoxicity in the fishes and adversely affecting their diversity; hence, they are of significance. Keeping view of the facts, the present study was conducted with the aim to investigate the genotoxic effect of polluted river water, containing various types of agricultural and industrial wastes, based on the induction of micronuclei and DNA damage in the blood erythrocytes using MN and CA tests in two native fish species, namely *Channa punctatus* (Bloch) and *Mystus vittatus* (Bloch), exposed to the most polluted river water.

Methods

Experimental site

The river Gomti, a tributary of river Ganga, originates from Gomat Taal, near Madho Tanda, Pilibhit district, Uttar Pradesh (UP). It flows nearly 800 km through UP and meets the river Ganga near Saidpur in the Ghazipur district of UP. In the middle of its course, the river passes through the Lucknow urban area. The river receives a large amount of domestic and industrial pollutants as it flows through the highly populous areas of Uttar Pradesh. Various industries, namely distillery, sugar units, carpet, aircraft accessories, and yeast manufacturing, located on the riverbank ultimately release pollutants like suspended solids, oil and grease, dyes, cyanide, chromium, nickel, etc. into the river through different channels. The site selected for the study, i.e. Daliganj (N36°53'2210", E080°54'0270") in Lucknow (Figure 1), receives maximum pollutants, as per the UP Pollution Control Board report (personal communication), through different channels.

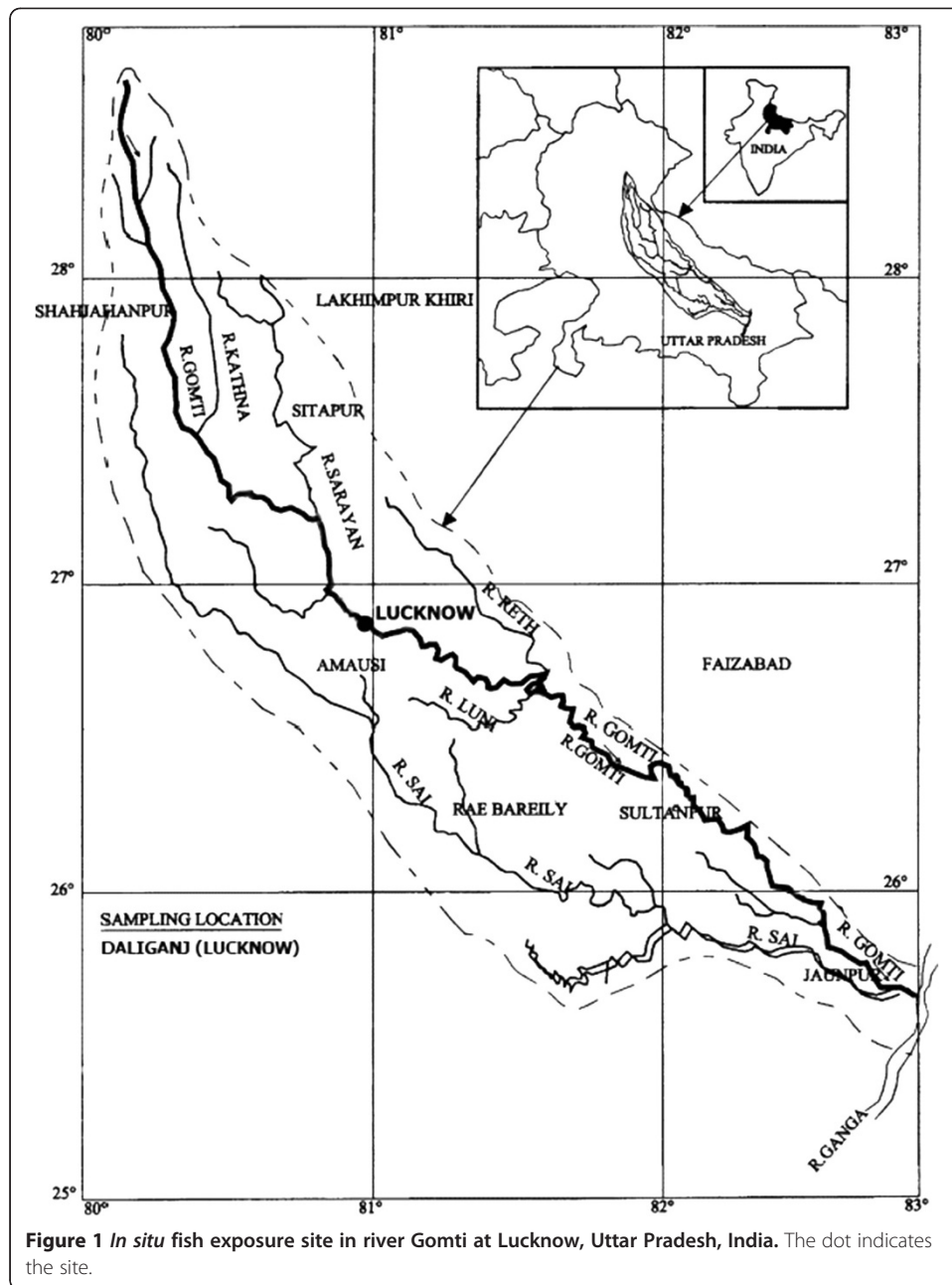
Water quality assessment

River water was sampled at the 1st, 3rd, 6th, 10th, and 15th days of fish exposure. The estimation of physicochemical parameters of the water, namely temperature, pH, dissolved oxygen, conductivity, and total hardness, were analyzed by standard methods (APHA et al. 2005). In addition, concentrations of heavy metals cadmium and mercury, and pesticide endosulfan were also estimated.

Experimental animals and *in situ* exposure conditions

For *in situ* exposure, the specimens of freshwater air-breathing murrel fish *C. punctatus* (Bloch; Family: Channidae; Order: Perciformes) and freshwater catfish *M. vittatus* (Bloch; Family: Bagridae; Order: Siluriformes) were caught from nearby ponds with the help of local fishermen. After transportation to the laboratory, the specimens were given prophylactic treatment with 0.05% KMnO₄ solution for 2 min to avoid any dermal infection and were properly maintained and acclimatized well under laboratory conditions for a period of more than a month. During acclimatization, they were fed with fish feed and boiled eggs at 5% of their body weight. The specimens of *C. punctatus* had an average wet weight and length of 20.46 g (range 12 to 25) and 13.53 cm (range 11 to 16), respectively, whereas the values for *M. vittatus* were 15.59 g (range 11 to 19) and 10.41 cm (range 9 to 13).

After acclimatization, the specimens of similar length and weight were transported to selected polluted site, randomly placed in nylon cages, and transplanted in the polluted river water. The blood samplings were done at day 1, 3, 6, 10, and 15 post-exposure for



MN test and CA. Four specimens per species were sampled at each sampling time. Two replicate slides per specimen were prepared for MN test and CA. Similarly, four acclimatized specimens of each species, along with *in situ* exposure, were used for MN test and CA, and average data of all exposure duration were considered as baseline values for statistical comparisons.

Blood collection

About 50 μ l of peripheral blood was collected from the caudal vein of the specimens on each sampling time using heparinized 1-ml disposable syringes and transferred to

1-ml eppendorf tube containing 450 μ l of chilled Ca^{++} - and Mg^{++} - free phosphate buffer saline. The tube was placed on ice for its further use in MN test, viability test, and for CA. After blood collection, the specimens were transferred to separate water tanks.

Micronucleus test

A very thin smear of peripheral blood was made onto the pre-cleaned slide, separately from each blood samples. After fixation in methanol for 20 min, the slides were allowed to air-dry and then stained with 6% Giemsa solution for 25 min. A total of 2,000 erythrocytes from each slides were examined at $\times 100$ magnification under a Leica microscope equipped with a digital camera. Small, non-refractive, circular or ovoid chromatin bodies displaying the same staining and focusing pattern as the main nucleus were scored as micronuclei (Al-Sabti and Metcalfe 1995). The MN frequency (%) was calculated as

$$\text{MN\%} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells scored}} \times 100.$$

Comet assay

DNA damage including single-strand breaks and alkali-labile sites was detected by comet assay as a three-layer procedure (Singh et al. 1988) using conventional microscopic slides (Klaude et al. 1996). Cell viability test for each blood sample was carried out using the trypan blue extrusion test (Anderson et al. 1994; Henderson et al. 1998). The samples showing >85% cell viability were further processed for comet assay. In brief, 20 μ l of blood sample was mixed with 80 μ l of 0.5% low-melting-point agarose (LMPA) prepared in Ca^{++} - and Mg^{++} -free phosphate buffered saline (PBS) and layered on one end of a frosted glass slide, which was pre-coated with a layer of 200 μ l of 1% normal agarose in PBS. The slide was again covered with a third layer of 100 μ l of 0.5% LMPA and covered with cover slip. After gel solidification, the cover slip was removed, and the slide was immersed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris, pH 10, and 10% DMSO and 1% Triton X-100 added fresh) and refrigerated at 4°C for 2 h. After lysis, the slide was placed on a horizontal electrophoretic chamber. The chamber was filled with fresh electrophoresis buffer (1 mM Na EDTA, 300 mM NaOH, 0.2% DMSO, and pH 13.5) to a level of approximately 0.25 cm above the slides. The slides were left in the buffer solution for 20 min for unwinding. Electrophoresis was performed using the same solution at 18 V and 300 mA for 18 min. The slides were then neutralized gently with 0.4 M Tris buffer at pH 7.5 and stained with 75- μ l ethidium bromide (concentration, 20 μ g/ml). Two slides per specimen were prepared, and 25 cells per slide, i.e., 200 cells per exposed duration, were scored randomly and analyzed using an image analysis system (Komet-5.5 Kinetic Imaging Ltd., Nottingham, UK) attached to a florescent microscope (Leica) equipped with appropriate filters. The parameter selected for quantification of DNA damage was percentage of tail DNA (=100 - % head DNA) as determined by the software.

Statistical analysis

The mean percentage of micronucleus frequencies was compared among species and sampling times using Mann-Whitney test. One-way analysis of variance was applied to

compare the difference in mean percentage of tail DNA among the species and sampling times. p values less than 0.01 were considered statistically significant.

Results

Physicochemical parameters of river water

The physicochemical characteristics of the test water are presented in Table 1. The average values of water temperature and pH were 21.8°C and 8.5, respectively. The average values of dissolved oxygen concentration and conductivity were 7.6 mg/l and 441 $\mu\text{M}/\text{cm}$, correspondingly. The mean total alkalinity and total hardness were 315 and 205 mg/l as CaCO_3 , respectively, during the experimental period. The estimated quantities of metals, like mercury and cadmium, and pesticide, like endosulfan and chloride, were 1.19, 3.00, 0.006, and 37.82 $\mu\text{g}/\text{l}$, respectively, observed at a level higher than the permissible limits of the BIS guideline (Indian Standard 1982) for a non-polluted river.

Induction of micronuclei

The observations of micronucleus present in erythrocytes of *C. punctatus* and *M. vittatus* are summarized in Table 2. A significant ($p < 0.01$) higher micronuclei induction was observed in exposed specimens of both species from day 3 onwards as compared to the baseline values and frequency of micronuclei on day 1. A time-dependent gradual increase in the micronucleus frequency was observed in both species, whereas the induction in micronuclei in exposed specimens from day 3 to 15 was nonsignificant (Figure 2). The maximum frequency of MN in the subject species was observed on day 15, with comparatively higher micronuclei induction in *C. punctatus* than in *M. vittatus*.

DNA damage

The DNA damage measured as percentage of tail DNA in the erythrocytes of the subject species is presented in Table 3, which indicated that both fish species exposed to polluted river water exhibited significantly higher DNA damage ($p < 0.01$) as compared to baseline values. A time-dependent gradual and linear increase in DNA damage was found in both species with the advancement of the experiment. Significant ($p < 0.01$)

Table 1 Physicochemical characteristics of Gomti River water collected from *in situ* exposure site at Lucknow

Serial number	Characteristics	Unit	Mean value	Ranges
1.	Air temperature	°C	24.5	23.9 to 25.1
2.	Water temperature	°C	21.8	21.2 to 22.3
3.	pH	-	8.5	8.2 to 8.8
4.	Conductivity	$\mu\text{M}\cdot\text{cm}^{-1}$	441	420 to 458
5.	Total alkalinity	mg/l as CaCO_3	315	307 to 324
6.	Total hardness	mg/l as CaCO_3	205	192 to 216
7.	Dissolved oxygen	mg/l	7.6	7.2 to 7.9
8.	Chloride	mg/l	37.82	36.12 to 39.08
9.	Mercury	$\mu\text{g}/\text{l}$	1.19	1.15 to 1.23
10.	Cadmium	$\mu\text{g}/\text{l}$	3.00	2.60 to 3.36
11.	Endosulfan	$\mu\text{g}/\text{l}$	0.006	0.004 to 0.007

Table 2 Mean percentage of micronucleus frequencies in erythrocytes of exposed fish

Species	Baseline	Duration (days)				
		1	3	6	10	15
<i>C. punctatus</i>	0.026 ^{1a} (±0.01)	0.038 ^{1a} (±0.02)	0.12 ^{1b} (±0.03)	0.13 ^{1b} (±0.02)	0.14 ^{1b} (±0.01)	0.17 ^{1b} (±0.04)
<i>M. vittatus</i>	0.03 ^{1a} (±0.01)	0.03 ^{1a} (±0.02)	0.09 ^{1b} (±0.01)	0.11 ^{1b} (±0.02)	0.12 ^{1b} (±0.02)	0.13 ^{1b} (±0.03)

Mean percentage of micronucleus frequencies (±SE) in erythrocytes of *C. punctatus* and *M. vittatus* exposed to polluted water of river Gomti. Values with different numeric superscript differ significantly between species within duration. Values with different alphabet superscript differ significantly between durations within species.

increase in DNA damage was observed among all sampling intervals in *C. punctatus*, whereas nonsignificant increase was observed in *M. vittatus* from day 3 to 15. Among the two species, significant variation ($p < 0.01$) in DNA damage was also observed on the 1st, 3rd, and 15th day post-exposure (Figure 3).

Discussion

In recent years, increasing concern about genotoxicity due to pollution in land and water has led to development of many mutagenesis test systems in bacteria, yeast, plants, and animals including fishes. Fishes are reported to be suitable organisms for ecotoxicological studies because they play different roles in the heterotrophic web, undergo bioaccumulation, respond to mutagens at low concentration such as environmental pollutants, and activate xenobiotics through the cytochrome P-450-dependent oxidative metabolism like mammals (Goksoyr et al. 1991; Cavas and Ergene-Gözükara 2005). *In situ* investigation of mutagenic pollution effects in terms of environmental bio-monitoring has been reported by many researchers (De Flora et al. 1993; Sanchez-Galan et al. 2001). Furthermore, Hayashi et al. (1998) evaluated the monitoring systems that use aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. *In situ* cage studies or transplantation provide precise information of the place and duration of exposure, but it may be difficult to establish in surveys of native populations (Oikari 2006; Pellacani et al. 2006, Klobučar et al. 2010). Transplanted animals used from the same unpolluted source can also potentially reduce inter-individual variability and decrease the influence of adaptive mechanisms (Klobučar et al. 2003; Pellacani et al. 2006). Nagpure et al. (2008) have also reported *in situ* bio-monitoring

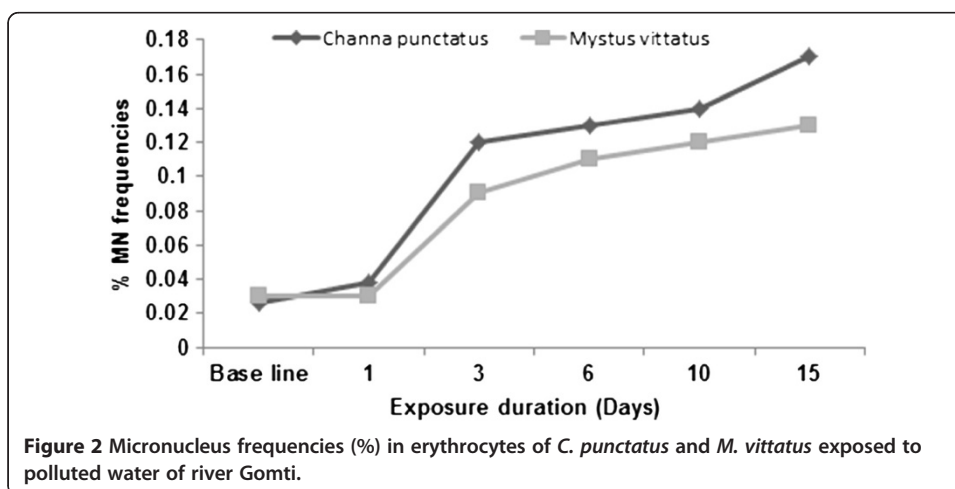


Table 3 Mean percentage of tail DNA in the erythrocytes of the subject species

Species	Baseline	Duration (days)				
		1	3	6	10	15
<i>C. punctatus</i>	3.63 ^{1a} (±0.54)	6.05 ^{1b} (±0.36)	9.05 ^{1c} (±0.48)	9.72 ^{1c} (±0.54)	12.88 ^{1d} (±0.59)	15.88 ^{1e} (±0.64)
<i>M. vittatus</i>	6.01 ^{2a} (±0.84)	7.95 ^{2a} (±0.06)	11.91 ^{2b} (±0.63)	10.47 ^{1b} (±0.70)	10.84 ^{1b} (±0.77)	12.76 ^{2b} (±0.70)

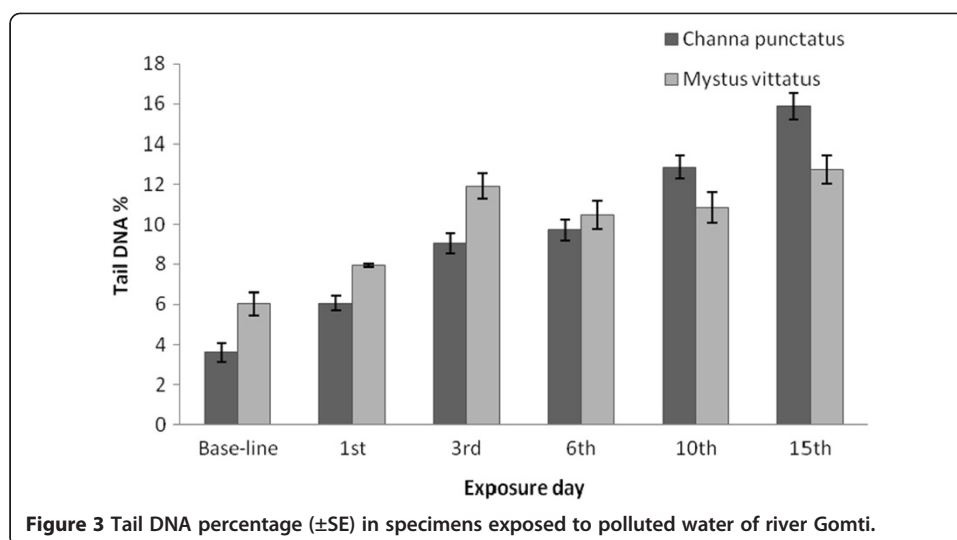
Mean percentage of tail DNA (±SE) in the blood cells of *C. punctatus* and *M. vittatus* exposed to polluted water of river Gomti. Values with different numeric superscript differ significantly between species within duration. Values with different alphabet superscript differ significantly between durations within species.

studies through genotoxicity assessment in some carp species that provided important information on the increasing pollution status of the river waters.

In a field study, micronucleus assay was shown to be applicable to micronucleus-inducing agents in frogs (Saleh and Zeytinoğlu 2001). The micronucleus test is simple and successful among many mutagenesis assays, which are both dependent on any karyotypic characteristics and less time consuming (Heddle et al. 1991). According to Hartwell et al. (2000) and Fagr et al. (2008), the incidence of micronuclei in fish and other aquatic life serves as an index of these types of damage, and counting of micronuclei is much faster and less technically demanding than scoring of chromosomal aberrations. The comet assay has been considered as a sensitive, rapid, and reliable method of quantitatively measuring DNA damage in eukaryotic and prokaryotic cells (Bajpayee et al. 2005). It is increasingly being used in testing of substances such as industrial chemicals, biocides, agrochemical, food additives, and pharmaceuticals for genotoxicity testing (Brendler-Schwaab et al. 2005). Thus, for these reasons, comet assay and micronuclei test in fish erythrocyte seem to be promising tests in environmental genotoxicity and mutagenesis investigations (Andrade et al. 2004).

According to our findings, duration-dependent linear increase in DNA damage was found in both species. The DNA damage detected in this study could have originated from DNA single-strand breaks, DNA double-strand breaks, DNA adduct formations, and DNA-DNA and DNA-protein cross-links (Mitchelmore and Chipman 1998) resulting from the interactions with the toxic substance in polluted water of river Gomti with DNA (Fairbairn et al. 1995). Furthermore, environmental contaminants are known to modulate antioxidant defensive systems and to cause oxidative damage in aquatic organisms by the production of reactive oxygen species (Risso-de Facerney et al. 2001; Liu et al. 2006). A significant difference observed between *C. punctatus* and *M. vittatus* on days 1, 3, and 15 and between baseline values of DNA damage indicated that some other factors like species, age, and sex may have some influence to cause these minor variations. Similar variation in genotoxicity patterns was documented for other fish species such as cyprinids (Smith 1990; Lemos et al. 2001; Vigano et al. 2002). The significant increase in DNA damage at different exposure durations shows that *C. punctatus* is more sensitive for assessing the water quality in aquatic ecosystem.

The present investigation revealed significant increase in micronucleus frequencies observed after 3 days post-exposure in comparison to baseline values of micronuclei in unexposed fishes. The maximal MN was observed on day 15 in both fish species. A nonsignificant effect of micronucleus induction was observed between the two fish species. The present study indicated that *C. punctatus* and *M. vittatus* are sensitive fish species and could be successfully used for determination of the effect of pollutants present in river water. Similarly, they could also be used in periodical bio-monitoring



and river conservation programs by *in situ* exposure. Nagpure et al. (2008) reported similar observations in commonly available fishes, namely *Labeo rohita* and *Puntius puntius*, of the river Gomti. These findings are also in accordance with the studies conducted for basal-level range-finding for other fish species, as reviewed by genotoxicity studies using fish (Al-Sabti and Metcalfe 1995). The significantly higher DNA damage, as well as induction in MN frequencies in both fish species, was observed 3 days post-exposure as compared to baseline frequencies, which indicated that a minimum of 3-day, or a week, *in situ* exposure of fishes could be used to evaluate the status of a body of water for bio-monitoring purpose.

Our results are also supported by some other investigators' findings that assess the *in situ* and *ex situ* genotoxic and mutagenic potential of various toxic chemicals including heavy metals and pesticides in various fish species (Mitchelmore and Chipman 1998; Pandrangi et al. 1995; Sumathi et al. 2001; Lee and Steinert 2003). The results of this study emphasized the importance of comet assay and MN test and suggest its broader application as an early biological marker of fish exposure to genotoxic and clastogenic pollutants in aquatic environments.

Conclusions

The results of the present investigation on the genotoxic and mutagenic potential of the polluted water of river Gomti suggested a serious concern about its potential danger to aquatic organisms, especially to fish, and indirectly to human beings. However, further studies are needed to explore the biological consequences of DNA damage in aquatic organisms due to deleterious effects of polluted water of river Gomti and to formulate future strategies for safeguarding aquatic organisms and environment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NSN conceived and coordinated the study. BK designed the experiment and carried out the *in situ* exposure studies. SP carried out the micronucleus test. SS carried out the comet assay. SKS conducted the *in situ* exposure studies. RS participated in the comet assay. RK performed the statistical analysis. AD performed the statistical analysis. BK, RS, and AD wrote the manuscript. NSN and RK edited the manuscript. All authors read and approved the final manuscript.

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