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In vitro analysis of the phytotoxic and genotoxic potential of Aligarh wastewater and Mathura refinery wastewater

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ABSTRACT

Present report deals with the phytotoxicity and genotoxicity of Mathura refinery wastewater and Aligarh wastewater of Northern India. The IC_{50} value in *Allium cepa* root growth inhibition test was recorded to be 0.14*X* and 0.10*X* for Mathura refinery and Aligarh industrial wastewaters, respectively. Significant decline in the survival of various *Escherichia coli* K12 DNA repair defective mutants was observed when the tester strains were exposed to the aforementioned samples. The order of sensitivity was invariably as: AB1157 (*wild type*) < AB2494 (*lexA mutant*) < AB2463 (*recA mutant*) < AB2480 (*uvrA recA double mutant*). These results suggested a significant amount of DNA damage within the bacterial cells exposed to test wastewaters. *A. cepa* genotoxicity test also demonstrated a considerable amount of chromosomal damage of *A. cepa* brought about by the test samples. The aberration index (A.I.) for Aligarh wastewater and refinery wastewater was recorded to be 11.2% and 14.7%, respectively, whereas the aquaguard mineral water serving as negative control displayed the A.I. value to be 2.6%. Interestingly, genotoxicity of both industrial wastewaters was reduced to a remarkable extent in presence of mannitol, the hydroxyl radical scavenger. Present study clearly indicated a distinct pattern of the chromosomal aberrations showing predominantly stickiness and stray chromosomes in case of AWW while clumping and stickiness in case of RWW, thereby affirming the genotoxicity of both test waters.

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1. Introduction

Industrial wastes and effluents containing heavy metals are undesirable by products of economic development and technological advancement. Among the inorganic pollutants, heavy metals are of primary concern because of their ubiquitous presence in the global environment [1]. Marine contamination by heavy metals in the gulf of Oman primarily containing arsenic, cobalt and nickel as a result of atmospheric inputs has been found [2]. A high concentration of heavy metals in the sediments collected from the Gulf of Gemlik (Turkey) has been reported, which is primarily due to increasing levels of pollution as a result of industrialization [3]. Moreover, seawater and sediment samples from East London and Port Elizabeth harbours were found to contain high concentrations of Cu, Mn, Zn and Fe [4]. It was also demonstrated that the stream water and the sediment in the ToLich and KimNgu rivers were heavily polluted with heavy metals exceeding the Vietnamese surface water standards [5]. Aligarh wastewater has been reported to contain various heavy metals in our previous investigations [6,7]. Among them Pb and Cd were of special mention due to their relatively higher concentrations in the wastewater samples.

Abbreviations: AWW, Aligarh wastewater; DNA, deoxyribonucleic acid; MI, mitotic index; MMS, methyl methane sulphonate; ROS, reactive oxygen species; RWW, refinery wastewater.

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Tannery wastewater was reported to cause induction of gene conversion and point mutation in Yeast D7 strain [8]. The genotoxic effect of wastewaters coming from pharmaceutical production processes of cotrimoxazole B and piriton was also reported [9]. These effluents caused various types of chromosomal aberrations including disturbed spindle, vagrant and chromosome bridges and also showed dose dependent reduction in the number of dividing cells. The genotoxic effect of wastewater sludges from Danish municipal wastewater using *Allium cepa* genotoxicity test was studied by Rank and Nielson [10], and it was found to induce significant chromosomal aberrations at anaphase–telophase stage in *A. cepa* cells.

Petroleum refinery wastewater contains various kinds of chemicals which include oil and greases, phenols (cresols and xylenols), ammonia, suspended solids, cyanides, nitrogen compounds and heavy metals like chromium, iron, nickel, copper, molybdenum, selenium, vanadium and zinc [11]. Significant increase in chromosomal aberrations, formation of micronuclei and DNA damage (measured in peripheral leukocytes) in petroleum refinery workers have been reported by Roma et al. [12].

In view of the above, the phytotoxicity and genotoxicity testing of Aligarh wastewater (AWW) and Mathura refinery wastewater (RWW) was carried out as Aligarh city houses numerous lock manufacturing plants obviously releasing certain heavy metals and Mathura refinery wastewater might be containing some genotoxicants.

2. Materials and methods

2.1. A. cepa

A. cepa (onion) red variety was purchased from local market of Aligarh. Methyl methane sulphonate (MMS) was procured from Sigma–Aldrich, USA. Cadmium chloride, lead nitrate and Tris buffer were obtained from Sisco Research Laboratories (SRL). Acetocarmine, iron allum and ethanol were obtained from Bangalore Genei, India. Glacial acetic acid, N-butyl alcohol and mannitol were purchased from Qualigens, India. Nutrient agar and Nutrient broth were purchased from Hi-media, India. Aligarh wastewater (AWW) and Mathura refinery wastewater (RWW) samples were collected from industrial effluents of Aligarh and Mathura refinery, respectively. *Escherichia coli* K12 strains were a kind gift from Dr. Mary K. Berlyn, Yale University, USA.

2.2. A. cepa phytotoxicity test

A. cepa phytotoxicity test was carried out as per the basic protocol of Fiskesjo. [13] for the toxicity bioassay of the industrial wastewaters i.e. AWW and RWW. Equal sized, small onion bulbs (red variety) were taken. Using a sharp knife, the yellowish brown scales/outer hard layer and the bottom plates were removed carefully, slightly exposing the root primordial. Boiling tubes were filled with serial dilutions of AWW and RWW. Aquaguard mineral water served as the negative control. One onion bulb was placed on top of each tube, with root primordial downward dipped in the liquid. The boiling tubes were incubated for 2 days at $25 \pm 5\,^{\circ}C$ in a dark chamber, refilling the liquid every morning and evening, ensuring that there was no free space between the onion bulb and the sample present in the tube. After terminating the experiment, the roots from each onion bulb were removed using knife. The roots were then soaked on filter paper before the length measurement. At least 3 long roots were taken for measurement from each onion bulb and five replicates of each dose was run. Inhibition in the growth of *A. cepa* roots is, in fact, considered as an index of the degree of toxicity [13].

2.3. E. coli survival assay

E. coli survival assay was carried out in which *E. coli* K12 strains were treated with varying concentrations of industrial wastewater namely AWW and RWW. The survival of DNA repair defective single and double mutants along with wild type strains of *E. coli* was determined by the established procedure [14]. The bacterial cells were then harvested by centrifugation from exponentially grown cultures. The pellets so obtained were then suspended in 0.01 M $MgSO_4$ solution and treated with an equal volume of test samples. Aliquots were withdrawn at regular intervals from 0 to 6 h, suitably diluted and plated to assay the colony forming ability of the cells.

2.4. Chromosomal aberration assay

The same onion bulbs exposed to the test samples at varying concentrations in *A. cepa* test were used for chromosomal aberration test. Aquaguard water was used as negative control and MMS (methyl methane sulphonate) as positive control. Elongated roots from the onion bulbs were allowed to grow for 48 h. Root tips were then harvested and fixed in absolute alcohol and glacial acetic acid (3:1) for about 30 min. After this root tips were kept in 1% iron alum solution for 3–12 h. This was followed by slide preparation using acetocarmine as the stain. After the preparation of permanent slides the chromosomal aberrations were observed through microscope calculated by the established procedure [10].

3. Results and discussion

The phtotoxicity test with *A. cepa* as system was carried out for Mathura refinery wastewater (RWW) and Aligarh wastewater (AWW). The dose response relationships of the above-mentioned wastewaters following 2 days exposure have been depicted in Fig. 1. The IC_{50} values of RWW and AWW were recorded to be 0.14*X* (i.e. 0.14 times concentration of the test water) and 0.10*X*, respectively.

E. coli survival assay was done to assess the genotoxic effect of RWW and AWW on various *E. coli strains*. The survival pattern of *E. coli* K12 strains exposed to 1× concentration of RWW up to 6 h is shown in Fig. 2. The maximum survival was shown by AB1157 and it was recorded to be 77% after 6 h treatment. AB2494 strain exhibited 20% survival whereas AB2463 strain showed only 4% survival following 6 h exposures. The minimum survival was recorded for AB2480 and that was 1% with the test sample under the same conditions. Survival of *E. coli* K12 strains

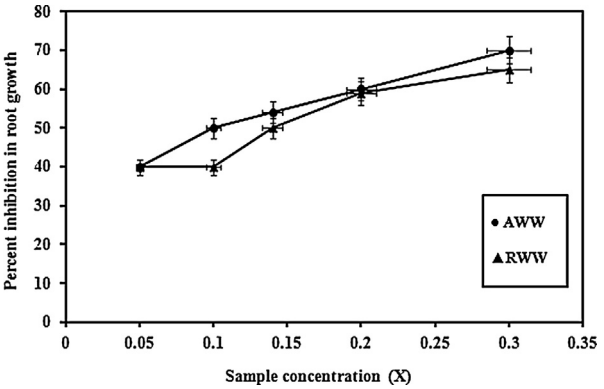

Fig. 1. Percent inhibition of *Allium cepa* roots following two days exposure to different concentrations of Aligarh wastewater and Mathura refinery wastewater.

exposed to 1× concentration of AWW up to 6 h is depicted in Fig. 3. The maximum survival was displayed by AB1157 strain and that was recorded to be 55% after 6 h treatment. AB2494 strain exhibited 19% survival while AB2463 strain showed only 11% survival after 6 h exposure. The minimum survival was exhibited by AB2480 to be 3% after 6 h treatment.

Chromosomal aberration test was also performed to analyze the genotoxic potential of RWW, AWW and test heavy metals. Changes in the mitotic index (MI) and abnormality pattern in the *A. cepa* system caused by Mathura refinery wastewater (RWW) are listed in Table 1. A lower MI value (39.1) for RWW treated *A. cepa* cells compared with untreated control (44.7) was recorded which attained a value of 42.8 when the treatment was given in the presence of mannitol exhibiting a recovery of 8.6%. The aberration index of RWW was 14.7% as compared to negative control to be 2.6% and it showed around 50% decline in presence of the $OH^{\bullet}$ radical scavenger. Chromosomal aberration test depicted the changes in the mitotic index (MI) and abnormality pattern caused by Aligarh

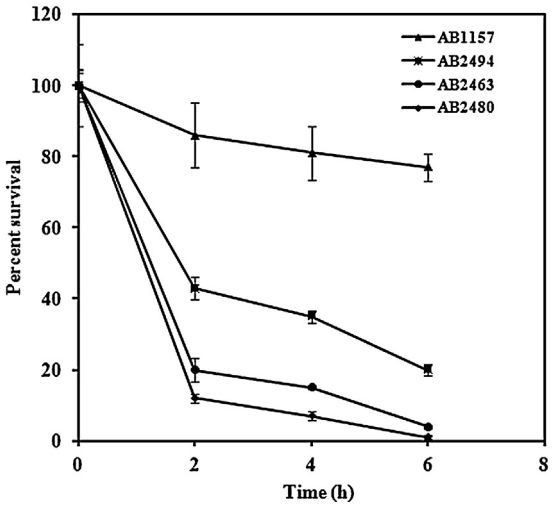

Fig. 2. Percent survival of *E. coli* K12 strains exposed to 1× concentration of RWW.

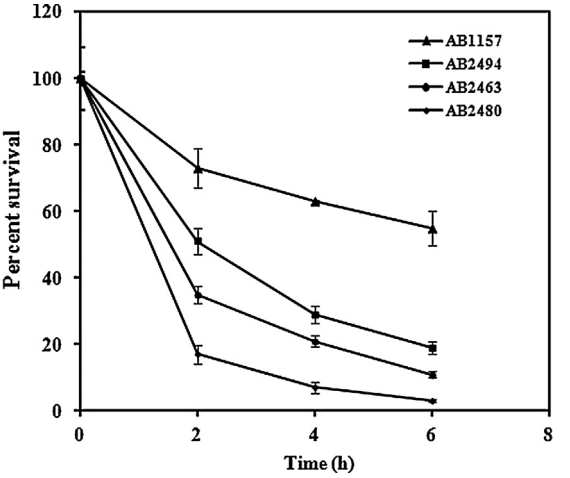

Fig. 3. Percent survival of *E. coli* K12 strains exposed to 1× concentration of AWW.

wastewater (AWW) and test heavy metals (cadmium chloride and lead nitrate) as indicated in Table 2. The MI value of AWW showed a decline from 44.7 to 37.8 to the extent of 15.4% whereas the presence of mannitol in the treatment tubes brought it up to 41.2 exhibiting a recovery of 8.25%. Chromosomal aberration based on its index showed its value for AWW treated *A. cepa* to be 11.2% compared to 2.6% for the aquaguard water. The aberration index showed 52% reduction in the presence of mannitol. Interestingly, lead nitrate also exhibited the MI value, the chromosomal aberrations and aberration index very close to those of AWW.

Water pollution has attracted a lot of interest in recent years due to its multidimensional hazardous effects. Disposal of the treated as well as untreated disposal of industrial waste material leads to serious problems for human health and survival. Oil refineries as well as other industries generate huge amount of sludge containing both organic and inorganic toxicants which pollute the nearby sites. Many of the constituents in the wastes are carcinogenic and potent immunotoxicants [15].

To monitor the harmful effects of pollutants, a number of toxicity bioassays have been developed. *A. cepa* test introduced by Levan [16] has been used frequently and validated by several workers for testing chemical pollutants posing hazardous environmental effects ([13,17–19]). Root growth inhibition of *A. cepa* was used as an indicator of toxicity of the refinery wastewater and Aligarh wastewater. The IC_{50} value in *A. cepa* system was recorded to be 0.14× for RWW and 0.10× for Aligarh wastewater in the year 2008. Since water bodies represent a highly dynamic system, the degree of toxicity induced by industrial effluents can surely change over a period of time. Recent study in our lab [20] on the phytotoxic potential of RWW suggested the IC_{50} value to be 0.75×. Thus, it can be concluded that from the year 2008 to 2011, there was a definite hike in the IC_{50} value from 0.14*X* to 0.75×. This increase in the IC_{50} value from 2008 to 2012 signifies a reduced toxic potential of RWW, which might be the outcome of the installation of treatment plants in the refinery. These treatment plants must have detoxified or blocked the release of certain

Table 1
Mitotic index and chromosomal abnormalities brought about by Mathura refinery wastewater.

Sample	Abnormalities					Aberration index
	MI ± SD	Stickiness	Clumping	Stray chromosomes	Fragments	
Negative control (aquaguard water)	44.7 ± 1.9	1	0	0	0	2.6
Positive control (MMS)	56.1 ± 3.9	8	1	1	1	16.9
RWW(1×)	39.1 ± 2.7[a]	2	3	0	0	14.7
RWW(1×) + Mannitol	42.8 ± 1.6[a]	1	2	0	0	7.1

Results are mean ± SEM of three different experiments.
[a] Significantly different from both controls at $p < 0.05$ by one way-ANOVA.

Table 2
Mitotic index and chromosomal abnormalities brought about by Aligarh wastewater.

Sample	Abnormalities					Aberration index
	MI ± SD	Stickiness	Clumping	Stray chromosomes	Fragments	
Negative control (aquaguard water)	44.7 ± 1.9	1	0	0	0	2.6
Positive control (MMS)	56.1 ± 3.9	8	1	0	1	16.9
AWW (1×)	37.8 ± 2.7[a]	2	0	2	0	11.2
AWW (1×) + Mannitol	41.2 ± 3.1[a]	1	0	1	0	5.4
Cadmium chloride (100 ppm)	38.6 ± 2.9[a]	1	2	0	1	9.7
Lead nitrate (100 ppm)	37.2 ± 2.5[a]	2	0	2	0	12.5

Results are mean ± SEM of three different experiments.
[a] Significantly different from both controls at $p < 0.05$ by one way-ANOVA.

toxicants into water bodies. Toxicity of several other industrial waste samples have been determined in terms of IC_{50} values employing *A. cepa* system [21].

In addition to significant phytotoxicity of the RWW, present study also establishes its genotoxic potential in terms of significantly decreased survival of the DNA repair defective mutants of the *E. coli* K12 (Figs. 2 and 3). The efficacy of the *E. coli* 12 repair defective mutants of *E. coli* K12 in assaying the genotoxicity of wastewater has been well established [22–24]. Differences in the sensitivity pattern of mutants towards the samples suggest the induction of specific lesions in DNA owing to the different chemicals composition of test samples. In addition to *E. coli* survival assay, chromosomal aberration test involving *A. cepa* system was also employed for the genotoxicity testing of the test samples [10].

Chromosomal aberrations are seen as a variation in the normal pattern of chromosomes at the metaphase–anaphase stage. It was found that the *A. cepa* cells exposed to Aligarh wastewater, refinery wastewater and the test heavy metals exhibited a high percentage of chromosomal aberrations as compared to control. Moreover, it was seen that these samples caused a mitodepressive effect as there was a decrease in the MI value when the cells were exposed to the test samples. This mitodepressive effect got reverted back in presence of the ROS scavenger, mannitol, as it might be helpful in the clearance of $OH^{\bullet}$ radicals. Our results are consistent with the report of Rathore et al. [25] wherein myrobalan having scavenging properties reverted the mitodepressive effect caused by Pb in *A. cepa* root tip cells.

All test samples invariably caused the induction of chromosomal aberrations (Tables 1 and 2). Rank and Nielson [10] reported the induction of chromosomal aberrations as a result of exposure to industrial wastewater. Moreover, chromosomal abnormalities in the bone marrow cells of mice were also demonstrated to be caused by untreated wastes from silk industries [26].

It is interesting to note that the *E. coli* survival assay as well as *A. cepa* chromosomal aberration assay both led us to suggest a significant genotoxicity of the test samples. Moreover, chromosomal aberration pattern seems to serve as a valid biomarker for the detection of pollution caused by certain test industrial wastewaters. For instance, the aberration pattern of AWW in *A. cepa* system was similar to that of lead nitrate which suggests the significant role of lead and similar heavy metals in the genotoxicity of AWW.

In the year 2008, AB1157 strain upon exposure to RWW for 6 h showed the mean survival to be about 77% which was increased to 81% in our recent study in 2011, highlighting the reduced bacteriotoxicity of refinery waste. However, there was little or no variations in the survival pattern of other mutant strains like AB2494, AB2463 and AB2480 from 2008 to 2011.

4. Conclusion

Present findings on the phytotoxicity and genotoxicity strongly suggest the highly toxic nature of the liquid wastes from Aligarh and Mathura refinery. Contamination of water bodies would render them unsuitable for irrigation purposes and recreation activities rather consuming such waters in any way. Thus, there is an immediate need for the adoption of proper treatment and bioremediation strategies to alleviate the pollution hazards caused by these wastewaters. Interestingly, the cytotoxic/genotoxic potential of the test wastewaters has shown a declining trend presumably because of the additional treatment strategies employed in the industries concerned during 2008 and 2011, even then the efforts are not sufficient and

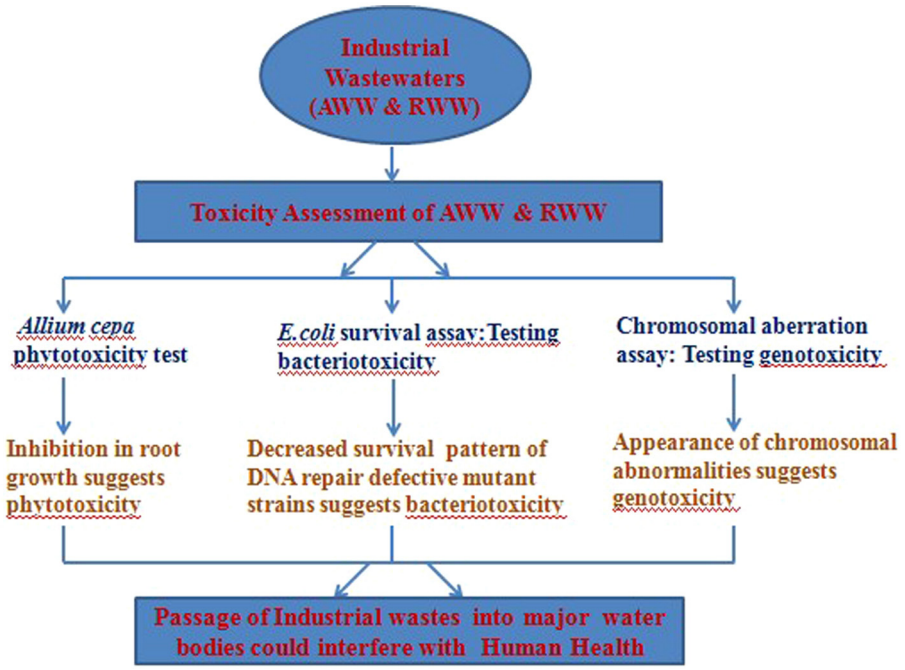

Fig. 4. The overall schematic pathway representing the toxicity of AWW and RWW.

we recommend that more effective treatment strategies should be adopted for a fool proof decontamination of the effluents before they are released into the major water bodies. Fig. 4 shows the schematic representation of the overall toxic potential of RWW and AWW.

Conflict of interest

The authors declare no financial or commercial conflicts of interests.

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References

[1] P.C. Nagajyoti, K.D. Lee, T.V.M. Sreekanth, Heavy metals, occurrence and toxicity for plants: a review, Environ. Chem. Lett. 8 (2010) 199–216.
[2] M. Stephen, W.F. Scott, Distribution of heavy metals in marine bivalves, fish and coastal sediments in the Gulf and Gulf of Oman, Mar. Pollut. Bull. 49 (2004) 410–424.
[3] S. Unlu, S. Topcuoglu, B. Alpar, C. Kirbasoglu, Y.Z. Yilmaz, Heavy metal pollution in surface sediment and mussel samples in the Gulf of Gemlik, Environ. Monit. Assess. 144 (2008) 169–178.
[4] O.S. Fatoki, S. Mathabatha, An assessment of heavy metal pollution in the East London and Port Elizabeth harbours, Water SA 27 (2001) 233–239.
[5] N. Thilan Huong, O. Masaim, H. Takahero, Mobility of heavy metals in the sediment of the To Lich river and the Kim Nguu river in Hanoi city, J. Fac. Agric. Kyushu Univ. 51 (2007) 141–146.
[6] R.A. Fatima, M. Ahmad, Certain antioxidant enzymes of *Allium cepa* as biomarker for the detection of toxic heavy metals in wastewater, Sci. Total Environ. 15 (2005) 256–273.
[7] S. Tabrez, M. Ahmad, Oxidative stress-mediated genotoxicity of wastewaters collected from two different stations in northern India, Mutat. Res. 726 (2011) 15–20.
[8] J. Moretton, P. Baro, M.D. Aquino, Genotoxicity of leachates from highly polluted lowland river sediments destined for disposal in landfill, Waste Manage. 28 (2008) 2134–2139.
[9] A. Lateef, P.M. Ufuoma, T.A. Yekeen, Bacteriology and genotoxicity of some pharmaceutical wastewaters in Nigeria, Int. J. Environ. Health 1 (2007) 551–562.
[10] J. Rank, M.H. Nielson, Genotoxicity testing of wastewater sludge using the *Allium cepa* anaphase–telophase chromosome aberration assay, Mutat. Res. 418 (1998) 113–119.
[11] R.P. Cote, NRC Associate Committee on Scientific Criteria for Environmental Quality, Ottawa, Ontario, Canada K1AOR6. 15021, 1976, pp. 17.
[12] J. Roma-Toress, J.P. Teixeira, S. Silva, Evaluation of genotoxicity in a group of workers from a petroleum refinery aromatics plant, Mutat. Res. 604 (2006) 19–27.
[13] G. Fiskesjo, The *Allium* test as a standard in environmental monitoring, Hereditas 102 (1985) 99–112.
[14] Z. Rehana, A. Malik, M. Ahmad, Mutagenic activity of the Ganges water with special reference to the pesticide pollution in the river between Kachla to Kannauj (U.P.) India, Mutat. Res. 343 (1995) 137–144.
[15] T.L. Propost, R.L. Lochmiller, C.W. Qualls Jr., K. McBee, In situ (mesocosm) assessment of immunotoxicity risks to small mammals inhabiting petrochemical waste sites, Chemosphere 38 (1999) 1049–1067.
[16] A. Levan, The effect of colchicines on root mitosis of *Allium cepa*, Hereditas 24 (1938) 471–486.
[17] G. Fiskesjo, The *Allium* test in wastewater monitoring, Environ. Toxicol. Water Qual. 8 (1993) 291–298.
[18] H.N.B. Gopalan, Ecosystem health and human well being: the mission of the international programme on plant bioassays, Mutat. Res. 426 (1999) 99–102.
[19] D.S. de Lima Moraes, B.Q. Jordao, Evaluation of the genotoxic potential of municipal wastewater discharged into the Paraguay River during periods of flood and drought, Environ. Toxicol. 16 (2001) 113–116.
[20] A.K. Gupta, Studies on Certain Phytotoxicity and Genotoxicity Biomarkers of Mathura Refinery Waste (Ph.D. thesis), Aligarh Muslim University, Aligarh, 2012.
[21] J. Rank, M.H. Nielson, Evaluation of the *Allium* anaphase–telophase test in relation to genotoxicity screening of industrial wastewater, Mutat. Res. 312 (1994) 17–24.

[22] ISGE, Integrated Study of Ganga Ecosystem Between Kachla to Kanauj, Ganga Project, Department of Environment and Forest, Government of India, New Delhi, 1990.
[23] A. Malik, M. Ahmad, Genotoxicity of some wastewaters in India, Environ. Toxicol. Water Qual. 10 (1995) 287–293.
[24] Z. Rehana, A. Malik, M. Ahmad, Genotoxicity of the Ganges water at Narora (U.P.) India, Mutat. Res. 367 (1996) 187–193.
[25] H. Rathore, R. Punyasi, P. Joshi, D. Rathore, D. Bhatnagar, Studies on the reversal of lead induced mitostatic effect in *Allium cepa* root tip cells with myrobalan (fruit of *Terminalia chebula*, Retz Combretaceae), Int. J. Altern. Med. 4 (2007) 3.
[26] O.M. Chaurasia, A. Kumar, M. Kumari, Genotoxic effect of silk dyeing wastes in bone marrow cells of mice, *Mus musculus*, Int. J. Cytogen Cell Biol. 70 (2005) 381–384.