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Mutagenic and genotoxic effects of wastewater detected by the *Allium* test

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Abstract: Screening for mutagens in complex environmental mixtures, such as industrial wastewater, is gradually being accepted as a routine method in environmental monitoring programs. The present study was carried out to evaluate the potential toxic and genotoxic effects of Marrakesh's CMR wastewater, collected from Draa Lasfar mine located about 12km south-West of the city of Marrakech, Morocco. The results from the *Allium* test indicate that wastewater at 100 % concentration inhibits root growth and mitotic indices; induces binucleated cells as a function of the proportion, but is not toxic at very low concentrations. Marrakesh's wastewater CMR leads to a decontrol in the cellular division, bringing about polyploid cells. In this work, we show the evidences that the exceeding genetical material of theses polyploidizated cells tend to be eliminated from the nucleus in the form of micronucleus. Our analyses prove this fact, both by the presence of a number of cells carrying a micronucleus, and by the evidences of the elimination of the exceeding material itself.

The wastewater sample was analyzed by flame atomic absorption spectrometer for Zn, Pb, Cu and Cd, whose presence could partly be responsible for the toxicity of wastewater. The study concludes that the classical *Allium* test can give a more comprehensive data when done in combination with analysis by flame atomic absorption spectrometer. Also, when wastewater is used for other purposes in combination with soils, it should be judiciously used at very low concentrations in order to protect the ecosystem health from any potential adverse effects.

Keywords: Wastewater; CMR; Mitosis; Binucleated cell; Genotoxicity.

Introduction

Industrial wastewater can cause serious environmental hazards in developing countries, especially around the Mediterranean basin. It has been estimated that 3,180m^3 of industrial wastewater are produced per year[1]. In Morocco, the volume of industrial wastewater produced annually is estimated at 180,000m^3 during the production season[2]. Most frequently, industrial wastewater is pumped and discharged into evaporation ponds or directly dumped in rivers or spread on soil[3,4]. The effect on the environment is negative, leading to a saturation of the soil, causing pollution of superficial groundwater and of the water table itself. This unfavorable effect of industrial wastewater deals with analysis and identification of CMR (Cancerogenic and mutagenic and reprotoxic) in industrial wastewater[5,6]. It is well known that CMR are major contributors to the toxicity of industrial wastewater. The major elemental constituents of wastewater are K^+, Na^+, Ca^{2+}, SO_4^{2-}, PO_4^{3-} and Mn^{2+} and nearly all naturally occurring elements can be found in wastewater in trace quantities, including As, Mo, Se, Cd and Zn[7].

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Genotoxic compounds in wastewater can have deleterious effects on human health in an exposed population through irrigation by wastewater and ingestion of plants that uptake the compounds from wastewater. Standard chemical analyses are limited in their ability to characterize the genotoxic potential of wastewater because of its chemical complexity. Bioassays, however, provide means of assessing the toxicity of complex mixtures like watewater even without prior knowledge about its chemical composition[8].

However, standard targeted chemical analyses are rather inadequate for evaluating the toxic and genotoxic potential of the complex mixtures found in wastewaters. These standard analyses do not provide information about the biological effects of micropollutants that occur in concentrations too low to be determined analytically[9]. Therefore, in this study, the *Allium cepa* aberration assay was utilized as a short-term and cost-effective indicator of toxicity in the routine monitoring of wastewater pollution. The screening would provide valuable information about the presence of genotoxic and/or mutagenic substances in surface waters by demonstrating the potential of such substances to induce chromosomal aberrations in *Allium cepa* root cells. Mitotic frequency and chromosome breaks were evaluated in mitotic cells while micronucleus formations and binucleate cells were scored in interphase cells.

Results and Discussion

Physicochemical characterization

The levels of the physicochemical parameters are presented in table 1.

Table 1: Physicochemical analysis of wastewater samples collected monthly over a 3 month period.

Parameters	Values (Mean±SD)	Values by WHO
pH	5.69±0.03	6.5-8.4
EC (ms/cm)	20.3±0.1	12
COD (g/L)	101±3	-
BOD (g/L)	44.5±0.4	-
SS (mg/L)	19.93±1.38	5
Na^+ (mg/L)	5825±1,5	175
K^+ (mg/L)	10.45±0.21	12
Ca^{2+} (mg/L)	1468.9±0.53	100
Cl^- (mg/L)	6914.55±0.4	350
Mn^{2+} (mg/L)	224±0.25	200
SO_4^{2-} (mg/L)	810±0.5	250
NO_3^- (mg/L)	13.7±0.1	25
NO_2^- (mg/L)	1.59±0.3	0.5
NH_4^+ (mg/L)	0.73±1.6	0.5
PO_4^{3-} (mg/L)	10.9±0.2	25
Zn (mg/L)	15.42±4.56	2
Pb (mg/L)	7.91±3.53	0.3
Cu (mg/L)	4.27±1.99	0.2
Cd (mg/L)	0.14±0.89	0.01

SD: Standard Deviation, each number is the mean of three individual values measured monthly over a 3 month period

The pH levels of the wastewater samples were slightly acidic. Electrical conductivity of wastewater is a simple and useful indicator of its salinity or total salt content. This result is not surprising as wastewater from the city dump often contains high levels of dissolved salts. COD and BOD values of the wastewater samples were higher, most likely due to the discharge of organic matter. The highest concentrations of the other chemical indicators (SS, sodium, potassium, calcium, magnesium, chloride, sulfate, nitrate, nitrite, ammonium, orthophosphate) were detected in the wastewater sample from Draa Lasfar mine than WHO (World Health Organization). The estimated concentration of Zn was highest followed by Pb, Cu and Cd.

Allium test

Based on the analysis carried out in roots from *Allium cepa*, after treatment with distinct concentrations of wastewater, inhibition of mitotic index was observed, as well as the presence of the several cells with micronuclei, mini cells, irregular nuclei and chromosomal alterations, such as polyploid cells and chromosomal losses.

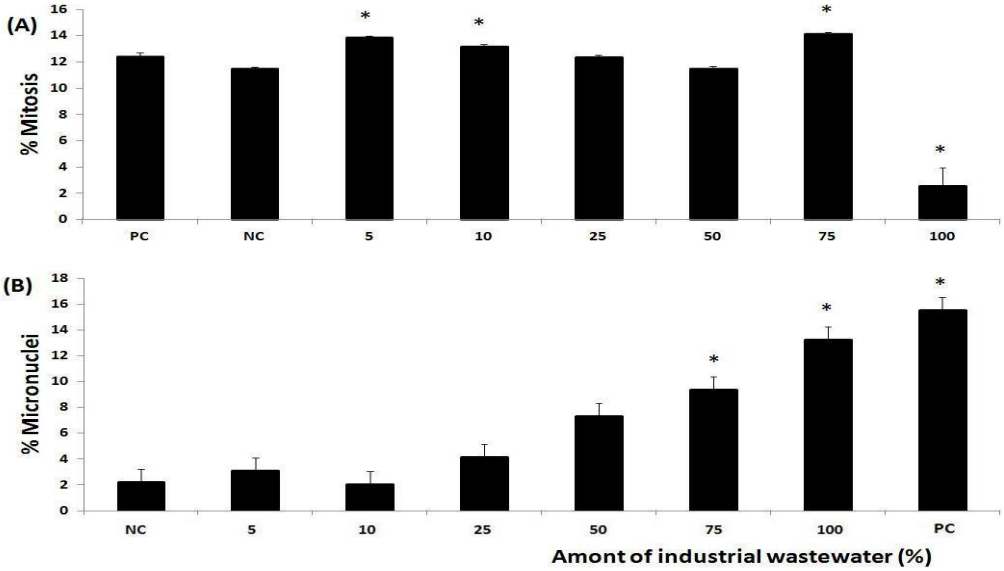

Fig 1: Mitotic index (A) and micronucleus frequency (B) values in *Allium cepa* roots exposed to different industrial wastewater concentrations. PC: positive control; NC: negative control (*$p < 0.05$ against the NC).

For 100% of industrial wastewater, an important loss of mitosis was observed (Figure 2A). A significant increase in micronucleus frequency was observed in *Allium cepa* roots exposed to 100% industrial wastewater. The figure 3 shows a possible sequence of events that precedes the formation of buds and their elimination either as micronuclei or as mini cells induced by industrial wastewater.

The results obtained for the concentrations of industrial wastewater tested show a presence of polyploid metaphases in the lowest concentrations (Figure 2C). In the highest concentration, there was an increase of polyploid cells ($p<0.05$). Our analyses revealed nuclear morphological alterations, characterized by the presence of irregular nuclei and nuclear buds as shown in Figures 2 (D–F). Nuclear buds were observed in all concentrations. The frequencies were significant at 5% level in highest concentrations. In our analyses, the nuclear buds appeared to be caused by polyploidization events, whose extra material is

released from the cells (Figure 2D). Irregular nuclei were also observed and they were characterized by a larger size and altered morphology (Figure 2F). Such nuclear alteration seems to occur prior to nuclear formation of buds. Under these conditions, the number of micronuclei was not significantly different from the positive control and underlines the genotoxic potential of this effluent.

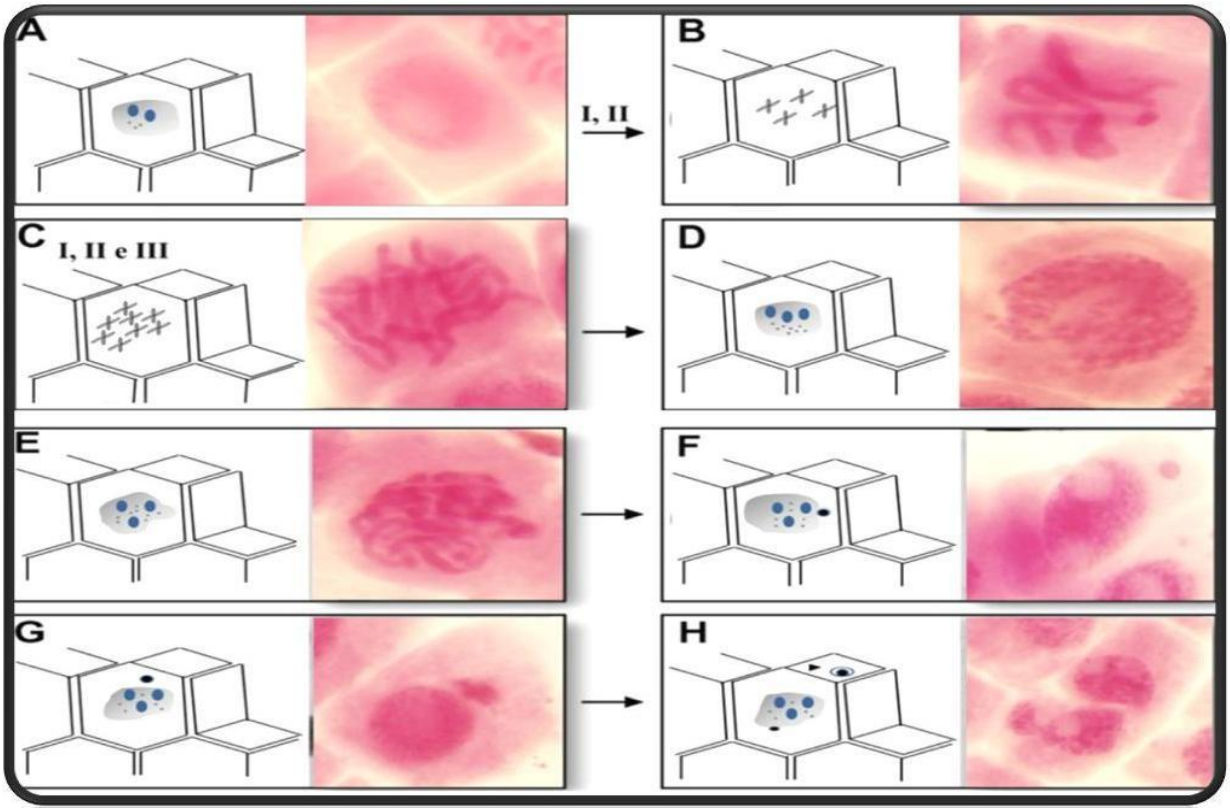

Fig 2: Sequence of events leading to the formation of nuclear bud with the elimination of the micronucleus. Micronucleus formed are eliminated out of the cell, giving rise to mini cell. **(A)** Cell exposed to the herbicide solution; **(B)** C-metaphase; **(C)** polyploid C-metaphase; **(D)** polyploid nucleus; **(E)** irregular nucleus; **(F)** nuclear bud formed by extra material due to a possible heterochromatinization; **(G)** cell with irregular nuclear morphology and micronucleus presence; **(H)** cell with irregular morphology and adjacent mini cell; Arrows indicate extra genetical material being eliminated from the nuclear buds; arrow head indicates mini cell; **I**-Total spindle mitotic absence; **II**-citocinese absence; **III**-replication of the genetical material.

Table 2 shows that wastewaters at 100% concentration inhibit root growth, decrease divisional frequency, and increase binucleated cells that were statistically significant when compared to the control. In the wastewater mixtures the frequency of mitosis was higher than that of the control. The proportions of dividing cells in prophase were the highest, whereas, the number of cells in metaphase, anaphase, and telophase was low in the wastewater-mixtures (in 50% or more). All concentrations of wastewater used in the experiment influenced changes in the percentage of a particular phase's distribution in comparison to the control. The mitotic indices for the negative control were 10.47±0.41% for prophase and 2.80±1.12% for metaphase, anaphase and telophase. In 100% of wastewater, the values were 4.27±0.94%, 0.69±0.09%, respectively.

Table 2: Effects of different concentrations of wastewater on the root meristems of *Allium cepa* after 5 days of exposure.

Con[a] (%)	Root Length (cm) Mean±SD	Mitotic Index Mean±SD	Mitotic Phases (%) Mean±SD		BN Cell[d]/ 1.000 Mean±SD	MN[e]/1.000 Mean±SD	CHR[f] Breaks and Bridges/1000 Mean±SD
			Prophase (%)[b] Mean±SD	MP-AP-TP (%)[c] Mean±SD			
0[g]	3.98±0.07	11.48±0.10	10.47±0.47	2.80±1.12	0.63±0.31	2.20±1.10	0.00±00
5	3.87±0.03	13.85±0.09*	11.07±012	2.23±1.04	2.58±0.88*	3.07±0.57	0.71±0.75
10	3.99±0.01	13.17±0.15*	12.28±0,21*	1.3±0.08	9.14±1.01*	2.04±0.11	1.45±1.0
25	3.88±0.17	12.34±0.20	12.04±0.05*	2.2±1.02	10.2±0.32*	4.1±0,98	2.0±0.05*
50	3.18±0.01*	11.46±0.18	12.07±0.09*	1,48±0.73	10.42±0.37*	7.28±0.61*	2.25±0.10*
75	3.34±0.05*	14.15±0.12*	12.31±0.03*	0.85±0.14	9.00±1.00*	9.38±0.45*	1.46±0.13
100	1.84±0.08*	2.52±1.39*	4.27±0.94*	0.69±0.09*	11.00±1.00*	13.23±0.75*	4.40±1.40*
EMS (2mM)[h]	_	12.38±0.31	10.24±0.24	2.32±1.20	0.73±0.14	15.54±1.29	5.38±0.43

SD: standard deviation; *****: Significant when compared to negative control at $p<0.05$ (Dunnett's Multiple comparisons test); [a] : Amount of wastewater in mixture; [b] : Percent of cells in Prophase among total number of cells counted; [c] : Percent of cells in Metaphase, Anaphase and Telophase among total number of cells counted; [d] : Binucleated cells; [e] : Micronucleated cells; [f] : Chromosome breaks and bridges; [g] : Negative control, distilled water; [h] : Positive control, 2 day old *Allium cepa* roots treated with 2 mM EMS for 1 h and 24 h recovery in distilled water

The characteristic effect caused by tested preparations was an increase of prophase indices and simultaneous decrease of metaphase, anaphase and telophase index when compared to the control. Dunnett's multiple comparisons test shows that the number of binuleated cells was significantly high in wastewater-mixtures. The frequencies of micronucleated cells and chromosome breaks in the *Allium* test were higher but also showed a correlation with the proportion of wastewater in the mixes.

A correspondence analysis was done to determine the association between toxicology parameters and isolate distinct groups (Figure 3). Two significant factors were obtained. Factor 1 explained 70.6% of the variance and was comprised among other parameters, by Root Length, Mitotic Index, Percent of cells in Prophase and Percent of cells in Metaphase, Anaphase and Telophase. Factor 2, explained 15.3% of the variance and was comprised among other parameters by Binucleated cells, micronucleated cells and chromosome breaks and bridges. From its parametric composition, factor 1 could be considered to represent the carcinogenic component of the wastewaters and factor 2 to represent the mutagenic component of the wastewaters. The greater importance of factor 1 lies in the fact that this is

comprised of parameters that presented very high values and showed very similar trends. Representation of factor 2 versus factor 1 produced two groups, one comprised by the carcinogenic parameters with the lowest concentrations of wastewater and another by mutagenic parameters with the highest concentrations of wastewater

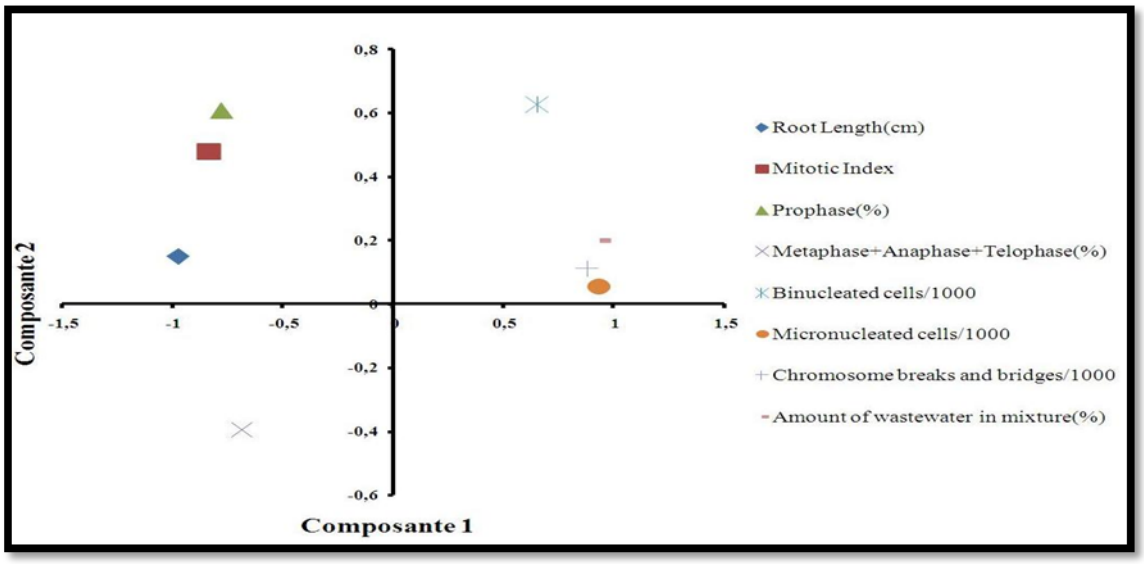

Fig 3: Factor analysis of corresponds genotoxic parameters of industrial wastewater

The *Allium* test is one of the best-established test systems to detect environmental genotoxic and mutagens[10-15]. Dose-dependent significant inhibition on *Allium*'s root growth signified the cytotoxic potential of wastewater. The inhibition of root growth generally related to apical meristematic activity[16], cell elongation during the differentiation[17], and the inhibition of protein synthesis[18]. Thus, the appearance of stunted roots indicates the retardation of growth and cytotoxicity[19]. The cytotoxicity levels of an agent can be determined by the variation in the Mitotic Index (MI)[20]. MIs lower than the negative control may indicate that the growth and development of exposed organisms have been affected by test compounds. On the other hand, MIs above those of the negative control are a result of the induction of increased cell division, which may be characterized as an event detrimental to cells, leading to uncontrolled proliferation and even tumor formation[21]. In the present study mitotic index (MI) value was low (2.52±1.39) in wastewater (100%) exposed root tips which is 21.95% of the control value (11.48±0.10). This was associated with the decrease in root length. The cytotoxicity level can be determined by the decreased rate of the mitotic index. A mitotic index decreases below 22% of the control causes sub lethal effects on the test organisms[22], while a decrease below 50% usually has lethal effects[23]. This inhibition of mitotic activities may be correlated to the presence of cytotoxic substances Pb, Cd, Zn, and Cu detected in wastewater. But it needs to be mentioned that for wastewater at 100% some part of the positive result could also be due to physical factors such as the lack of aeration and physical hindrance to growth. Interestingly, the results of the mitosis show that the proportion of cells (among total number of cells counted) in metaphases, anaphases and telophases taken together steadily decreased from 2.52±1.02 at 25% of wastewater to as low as 0.69±0.09 at 100% concentration of wastewater. This most likey could be due to the arrest in the cell cycle before metaphase to restore the integrity of DNA. It has been proposed in previous works that damaged DNA increase the time they stay at G2 and prophase, as a consequence of the

working of checkpoints for unrepaired DNA, where post replication repair takes place[24]. ANOVA test and Factor analysis of corresponds showed that the number of micronuclei in the interphase cells was not significant and there were few chromosome breaks recorded in metaphases. The frequencies of micronuclei and chromosome breaks also show correlation with the proportion of wastewater in the mixtures. The complex role of difference in mitotic index and action of cellular checkpoints is the possible reason for such anomaly. The number of binuleated cells scored in interphase cells was significantly high in wastewater mixtures. Wastewater thus seems to have an effect on phragmoplast. Phragmoplast a plant cell specific structure forms during late cytokinesis and serves as a scaffold for cell plate assembly and subsequent formation of a new cell wall separating the two daughter cells. It is a complex assembly of microtubules, microfilaments, and endoplasmic reticulum[25].

A positive correlation between genotoxic potential and chemical analysis, at least to some extent, was observed. However, such a strong toxic effect demonstrated by the wastewater samples can hardly be explained by the relatively low chemical levels measured in the study. It is more likely that these effects are caused by CMR substances (Zn, Pb,Pb, Cu and Cd) identified by the typical chemical analysis performed as a part of wastewater quality monitoring. Therefore, this demonstrates that the effects of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone.

Conclusion

In conclusion, the consistency of the results during the long monitoring periods, the minimum facility requirements, and the simplicity and low cost of the procedure make the *Allium cepa* assay desirable for environmental monitoring. This study also demonstrated that the toxicity/genotoxicity bioassays should be an integral tool in the evaluation of wastewater toxicity prior to its release into the environment. This study showed the usefulness of combining physicochemical analysis with cytogenetic methods to better understand the toxicity of chemical pollutants and their influence on health.

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Experimental Section

Study site

Industrial wastewater was taken from Draa Lasfar mine located about 12km south-West (Fig. 4) of the city of Marrakech, Morocco and are part of a systematic wastewater quality monitoring program performed on a monthly basis. Each wastewater sample was collected monthly over a three-month period (from April to June). It was transported in 1L bottles and refrigerated at 4°C until required for analysis and exposure experiments. The sample was mixed in varying proportion with distilled water to obtain the different percent of wastewater in mixtures (0, 5, 10, 25, 50, 75, and 100%) for the *Allium* test.

Fig 4: Geographic location of the sampling sites.

Physicochemical parameter analysis

Conductivity (EC, mS/cm) and pH were measured in situ. The samples were maintained at 4°C until the bioassays were carried out. Chemical analyses included chemical oxygen demand (COD, mg of O_2/L), biological oxygen demand (BOD, mg of O_2/L), suspended solids (SS, g/L) and the concentrations of sodium (Na^+, mg/L), potassium (K^+, mg/L), calcium (Ca^{2+}, mg/L), magnesium (Mn^{2+}, mg/L), chloride (Cl^-, mg/L), sulfate (SO_4^{2-}, mg/L), nitrate (NO_3^-, mg/L), nitrite (NO_2^-, mg/L), ammonium (NH_4^+, mg/L) and orthophosphate (PO_4^{3-}, mg/L). The analyses were carried out according to recommended AFNOR methods[26-38]. The mineralization of industrial wastewater was determined according to AFNOR X 31-15[39] by analyzing for Zn, Pb, Cu and Cd by flame atomic absorption spectrometer (UNICAM 929 AA, System). These routinely measured water quality indicators are presented as the mean of three individual values measured monthly over a three-month period.

***Allium* test**

Equal-sized bulbs were chosen from a population of a local market variety of the common onion *Allium cepa* (2n = 16). A series of six bulbs were placed in distilled water for 24h and afterwards the best growing five bulbs, exposed for 4 days to varying aqueous concentrations of wastewater solutions (100, 75, 50, 25, 10 and 5%) at room temperature (21°C ± 4°C), were used. The test concentrations were renewed every 24h during the experiments. On the 5th day, the bulbs were removed and thoroughly washed in running tap water[40]. The root length was recorded (longest five roots per bulb). Root meristems (10–15), chosen at random from 5 bulbs per exposure, were excised and fixed immediately for 3h in acetic acid/ethanol (1/3). The root tips (2–4cm) were hydrolyzed and stained in 9 parts of 2% aceto-orcein and 1 part of HCl mixture. Slides were prepared from each of the root meristem following the squash technique of Sharma and Sharma[41], and coded to prevent observer bias. For positive control, five onion bulbs were subjected to 1h treatment in 2 mM EMS, washed thoroughly in running tap water and transferred to distilled water for a recovery period of 24h. The number of cells in mitosis, binucleated cells, chromosome breaks, and cells with micronucleus were scored[42-45]. At least 10,000 cells from about 10 root meristems per exposure were scored. The mean values for mitotic index, root length, binucleated cell, micronucleus and chromosome breaks at each point were scored and the standard deviation was calculated accordingly. The experiments were repeated at least once in order to establish the reproducibility of the results.

Statistics

Statistical analyses were performed using the statistical programme SigmaStat 3.0 (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago, IL 60606-6307) software package. Data were compared using analysis of variance (ANOVA) to confirm the variability of the data and validity of results. Dunnet's multiple comparisons test was performed to determine the significant differences between treatments. The genotoxic potentials were analyzed using the correspondence analysis test. Differences between corresponding controls and exposure treatments were considered statistically significant at 0.05.

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