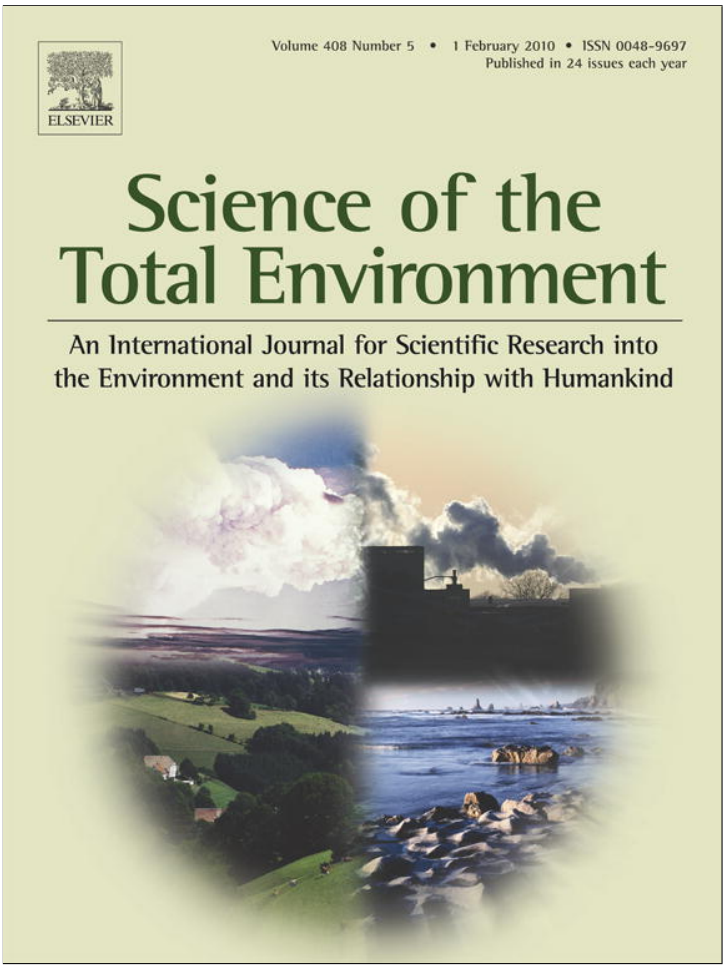

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The evaluation of surface and wastewater genotoxicity using the *Allium cepa* test

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ABSTRACT

Screening for mutagens in complex environmental mixtures, such as surface water or industrial wastewater, is gradually being accepted as a routine method in environmental monitoring programs. In the present work, the simplified *Allium cepa* root assay was utilized to evaluate the possible cyto- and genotoxic effects of surface and wastewaters collected near the Sava River (Croatia) over a three-month monitoring period. Physicochemical characterization of the water samples included measurements of conductivity, chemical and biological oxygen demand, levels of suspended matter and salts, nitrate, nitrite, ammonium, total nitrogen and total phosphorus. Morphological modifications of the *A. cepa* roots, inhibition of root growth, cell division and induction of mitotic and chromosomal aberrations were observed. The most highly polluted water samples (industrial effluents) caused an inhibition of root growth of over 50%, a decrease in the mitotic index of over 40%, and a considerable increase in chromosomal aberrations compared to the control. The measured biological effects of some water samples appeared related to the physicochemical characteristics. Therefore, mutagenicity/genotoxicity assays should be included, along with conventional chemical analysis, in water quality monitoring programs. Their use would allow the quantification of mutagenic hazards in surface and wastewaters.

1. Introduction

The pollution of water resources is a worldwide problem (Vargas et al., 2001; Ohe et al., 2003; Monte Egito et al., 2007). In addition to the direct health effects, pollutants also pose subtle dangers in that they may be mutagenic or toxic and lead to human afflictions such as cancer, atherosclerosis, cardiovascular diseases and premature aging. To evaluate the toxic/genotoxic risks of such complex mixtures, toxicity and genotoxicity tests employing microorganisms, plant cells and mammalian cells have been used alone or in combination with chemical analysis (Smaka-Kincl et al., 1996; Ohe et al., 2003; Žegura et al., 2009). Plant bioassays have several advantages over microbial and mammalian systems. Advantages include the similarity in the chromosomal morphology of plants and mammals, as well as the fact that plants and mammals have a similar response to mutagens. In addition, plant systems are less expensive and less time consuming than mammalian systems. Because of the large size and small number of their chromosomes, *A. cepa* root-tip cells are used to measure a variety of morphological and cytogenetic parameters that can serve as toxicity indicators, including the induction of micronuclei and chromosomal aberrations (Rank and Nielsen, 1998; Leme and Marin-Morales, 2009).

The *Allium* test has been utilized for monitoring the potential synergistic effects of a mixture of pollutants including heavy metals, and hydrophilic and lipophilic chemicals (Fiskesjö, 1985; Grover and Kaur, 1999; Rank et al., 2002; Caritá and Marin-Morales, 2008). Unlike physico-chemical analysis, genotoxicity tests are currently not an integral part of the water quality monitoring program conducted by Croatian Waters (the legal entity for water management in Croatia). 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 (Kungolos et al., 2006). Therefore, in this study, the *A. cepa* aberration assay was utilized as a short-term and cost-effective indicator of toxicity in the routine monitoring of water 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 *A. cepa* root cells.

2. Materials and methods

2.1. Sampling sites

The chosen sampling sites (Fig. 1) are from the Sava River basin and are part of a systematic water quality monitoring program performed on

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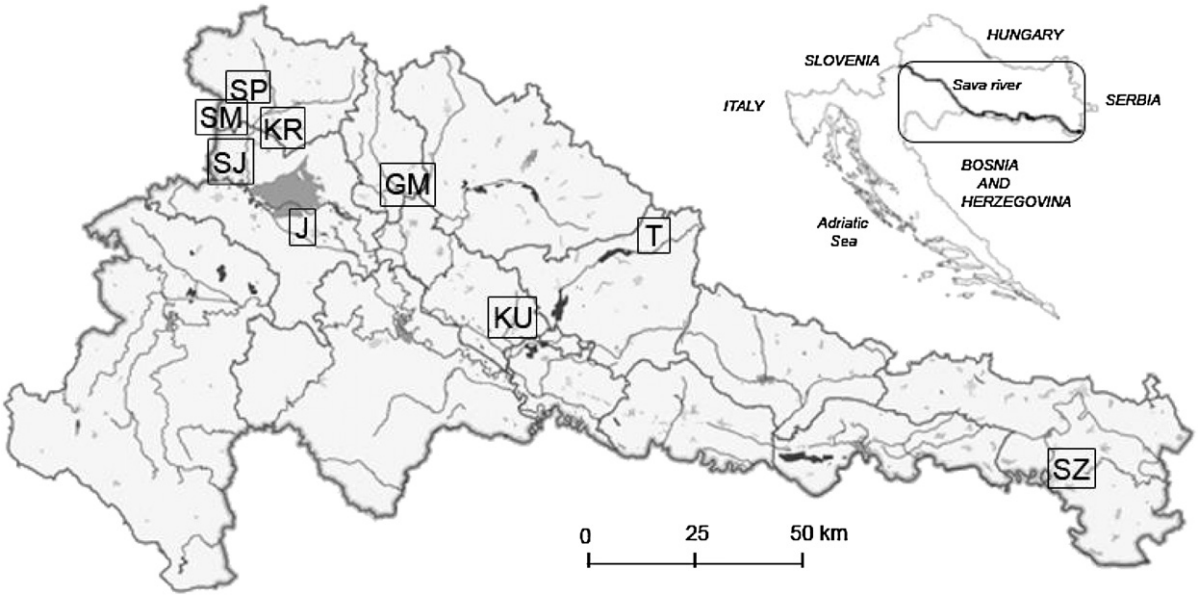

Fig. 1. Geographic location of the sampling sites: SJ, SZ, SP, KR, T, GM — river water; SM, KU — wastewater from chemical industry; J — wastewater from the Jakuševac stream near the Zagreb city dump.

a monthly basis. The monitoring stations were as follows: Sava Jesenice (SJ) — the Sava River downstream of the Slovenian town, Jesenice, that produces municipal wastewater; Sava Županja (SZ) — the Sava River downstream from a sugar factory near the Serbian border; Sutla Prišlin (SP) — downstream from a lead smelter; Krapinica Krapina (KR) — municipal wastewater, unlined open dumps of different wastes; Toplica (T) — downstream from the town of Daruvar and containing municipal wastewater and runoff from soil contaminated by agricultural practices; Kutinica (KU) — sampled before the mouth of the Ilova River and downstream of an artificial fertilizer plant (producing nitrogenous fertilizers, mineral NPK fertilizers, carbon black, bentonites, additives for foundries, and cattle feed additives); Glogovnica Mostari (GM) — municipal wastewater and leaching from soil contaminated by agricultural use; Savski Marof (SM) — wastewater from the Gorjak stream near a pharmaceutical plant (producing azithromycin) and the food industries (baker's yeast fermentation facility) of Savski Marof, before the mouth of the Sava river; and Jakuševac (J) — wastewater from the Jakuševac stream near the main dump of the city of Zagreb. Prior to discharge, industrial effluent (SM) was treated mechanically (using a sieve and a sedimentation tank) and biologically (via oxidation with activated sludge). Each surface or wastewater sample was collected monthly over a three-month period (from March to May 2008).

2.2. Physicochemical parameter analysis

Conductivity (μS/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, mg/L), nitrate (mg/L), nitrite (mg/L), ammonium (mg/L), total nitrogen (N, mg/L) and total phosphorus (P, mg/L). The analyses were carried out according to recommended ISO methods (ISO 7888, 1985; ISO 6060, 1989; ISO 10523, 1994; ISO 11923, 1997; ISO/TR 11905, 1997; ISO 14911, 1998; ISO 5815, 2003; ISO 6878, 2004; ISO 10304, 2007). These routinely measured water quality indicators are presented as the mean of three individual values measured monthly over a three-month period (Table 1).

2.3. Allium test

Small bulbs (1.5–2.0 cm in diameter) of the common onion, *A. cepa*, ($2n = 16$) were purchased at a local supermarket. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. For each water sample, a series of six bulbs were placed in distilled water for 48 h. Several of the newly formed root tips were then cut from each bulb and examined for any visible morphological abnormalities. The bulbs with satisfactory root lengths (2–2.5 cm) were used in the study, while those with exceptionally long or short roots were discarded (on average 2–3 bulbs). Therefore, individual sets of three bulbs were used for each water sample. Tap water (pH 6.5) was used as a negative control (Fiskesjö, 1993, 1997). Elemental analyses of the tap water were conducted using an atomic absorption spectrophotometer (Perkin Elmer AA 600), while Cl content was determined using ion chromatography. The following values were obtained: Ca 45.1 mg/L, Mg 10.4 mg/L, Cu 0.010 mg/L, Al 0.004 mg/L, Fe 0.034 mg/L, Zn 0.010 mg/L Cl 18 mg/L. The heavy metals Cd, Hg, Cr and V were below the detection limits. Hydrogen peroxide (300 mM, Merck) was used as a positive control mutagen. After 24 h of exposure, several root tips were removed from the bulbs, fixed in 3:1 ethanol:glacial acetic acid and stored overnight at 4 °C. The next day they were rinsed in tap water and stained in aceto-carmine. Microscope slides were prepared by squashing the stained root tips in 45% (v/v) glacial acetic acid. One slide was prepared per bulb, and each slide was examined using bright-field microscopy (Zeiss Standard 20) at a total magnification of 1000×. For each water sample, microscopy was performed on three replicate slides which contained 3–4 root tips. To obtain mitotic indices (MI), approximately 6000 cells (2000 cells in each of the three slides) were observed for each water sample. The number of chromosomal aberrations was recorded in approximately 300 dividing cells (preferably 100 per slide). Types of aberrations scored include c-mitosis, laggards, chromosome breaks, anaphase bridges and stickiness. After 72 h of exposure to the water samples, the root lengths were measured and used as an index of general toxicity. The results for mitotic index and root length are expressed as percent of the negative control. Visible morphological modifications, such as changes in root consistency and color as well as the presence of swelling (c-tumors), hooks or twists in the roots were also observed.

2.4. Statistical analysis

Statistical analyses were performed using the STATISTICA 7.1 (StatSoft, Inc., USA) software package. Data on physicochemical

Table 1
Physicochemical analysis of surface and wastewater samples collected monthly over a 3-month period (1–3).

Parameter	pH	Conductivity	COD	BOD	SS	Nitrate	Nitrite	Ammonium	Total	*N* total P
		μS/cm	mg O_2/L	mg O_2/L	mg/L	mg N/L	mg N/L	mg N/L	mg N/L	mg P/L
Sample										
J1-3	7.66	1642*	112.70*	37.53*	5.3	4.67*	0.02	1.64	6.32*	0.62
SM1-3	7.95	653	48.05*	16.9*	15.5	2.37	0.06	0.07	3.12	0.23
KU1-3	7.71	641	9.35	6.70	45.6*	6.82*	0.35*	9.73*	18.09*	3.51*
SP1-3	8.01	558	2.98	2.48	2.0	1.10	0.02	0.29	1.73	0.21
GM1-3	7.88	531	7.50	3.35	11.5	1.60	0.03	0.39	2.69	0.40
T1-3	7.82	467	5.00	4.85	10.7	1.39	0.07	0.47	3.46	0.52
KR1-3	8.03	560	4.65	4.05	4.6	1.02	0.03	0.64	2.50	0.30
SJ1-3	8.09	546	2.48	1.30	6.0	1.13	0.01	0.06	1.44	0.09
SZ1-3	7.98	431	2.15	1.65	5.8	1.09	0.02	0.07	1.33	0.10

Each number is the mean of three individual values measured monthly over a 3-month period. Numbers in each column labeled with an asterisk are significantly different from other values at $p<0.05$ according to DMRT.

parameters, root length and mitotic index were compared using analysis of variance (ANOVA) to confirm the variability of the data and validity of results. Duncan's multiple range (DMRT) test was performed to determine the significant differences between treatments ($p<0.05$). Chromosomal aberrations were analyzed using the Mann–Whitney *U* test. Differences between corresponding controls and exposure treatments were considered statistically significant at $p<0.05$.

3. Results

3.1. Physicochemical characterization

The levels of the physicochemical parameters are presented in Table 1. The results correlate with the degree of loading of the tested water samples. The pH levels of the water samples were slightly alkaline and varied between 7.66 and 8.09. Electrical conductivity of water is a simple and useful indicator of its salinity or total salt content. The highest conductivity values (in comparison to other samples ($p<0.05$, DMRT)) were detected in leachate from the city dump. 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 SM and J were significantly higher compared to other water samples, most likely due to the discharge of organic matter. The highest concentrations of the other chemical indicators (SS, nitrate, nitrite, ammonium, total N, total P) were detected in the water sample from the wastewater channel from the artificial fertilizer plant (KU).

3.2. Allium test

Onion (*A. cepa*) roots exposed to tap water for 72 h (negative control) had an average length of 5.3 cm and showed normal morphology. Samples SJ and KR collected after the first month had root lengths reduced by 20 and 25% compared to the negative control ($p<0.05$), respectively. Significant reductions in root length over the three-month monitoring period were recorded in *A. cepa* exposed to water samples SP (4.2 cm), GM (4.1 cm), KU (3.6 cm), J (3.6 cm) and SM (2.7 cm) (Fig. 2A). In addition, the water samples GM and SM induced the presence of tumors and a brown coloration in roots while the water samples J and SP caused morphological abnormalities in the form of hook-shaped roots. Twisted roots with an average length of 2 cm were noticed in response to the positive control (300 mM H_2O_2). The mitotic index of *A. cepa* meristematic cells treated with the mutagen was significantly decreased (52% in comparison with negative control). Significant inhibition of cell division in the onion roots over the three-month period were recorded in water samples SP, J and SM (30%, 36% and 48% compared to the negative control, respectively) (Fig. 2B). Water samples from the remaining sites showed no significant cytotoxic effects. The positive control (300 mM aqueous solution of H_2O_2) induced the highest number of aberrations; the main effect observed was stickiness followed by laggards and c-metaphase (c-mitosis). The most polluted water samples increased the number of aberrant cells in the order J<SP<KU<SM. The most frequent abnormalities were stickiness, anaphase bridges and c-mitosis (Table 2, Fig. 3). The water sample GM produced significant mitotic damage in the *A. cepa* root-tip cells, mainly in the form of c-mitosis, while other effects such as breaks or chromatin abnormalities were observed at the same frequency as in the negative control. Although the water samples from the remaining sites (SJ, SZ, KR and T) showed slightly increased numbers of chromatin and mitotic abnormalities overall, none were statistically significant.

4. Discussion

A variety of bioassays has been used to demonstrate the mutagenic activity of industrial effluents and surface waters (Kungolos et al., 2006; Žegura et al., 2009). In this study, toxic effects were evaluated by analyzing root growth and root morphology. Cyto- and genotoxicity were estimated by observing cytological parameters such as the mitotic index and the number of chromosome abnormalities, including c-mitosis, laggards, chromosome breaks, anaphase bridges and stickiness. In the present study, water samples SP, J and SM were cytotoxic and SP, J, KU and SM were genotoxic. The strongest (phyto) toxic, cytotoxic and genotoxic effects in the root meristem cells of *A. cepa* were induced by wastewaters collected near the chemical industries SM (azithromycin production, baker's yeast fermentation facility) and KU (artificial fertilizer plant). The most likely reason for the high genotoxicity and cytotoxicity of these industrial water samples is the complex assortment of chemicals produced in the factories, the release of which is not controlled by limited targeted chemical analyses. Antibiotics have attracted special attention due to their serious impact on the ecosystem and connections to the emergence of drug-resistant bacteria (Isidori et al., 2005; Koch et al., 2005). According to a study by Terzić et al. (2008), the most prominent representative of macrolide antibiotics in Croatian wastewater was azithromycin. This may be due to the fact that one of the most important world manufacturers of azithromycin, the pharmaceutical company PLIVA, is located in Croatia. Due to its poor degradation, significant amounts of active azithromycin may be introduced into wastewater treatment plants and therefore build up in the environment (Koch et al., 2005). At the artificial fertilizer plant, the major portion of the disposed waste is phosphogypsum (the primary byproduct from phosphoric acid production), which contains high concentrations of fluoride and elevated levels of heavy metals including Fe, Pb, V, Cr (VI), Mn, Ni, Cu and Zn (Durgo et al., 2009).

A positive correlation between growth retardation and certain chromosomal aberrations was observed; water samples that did not

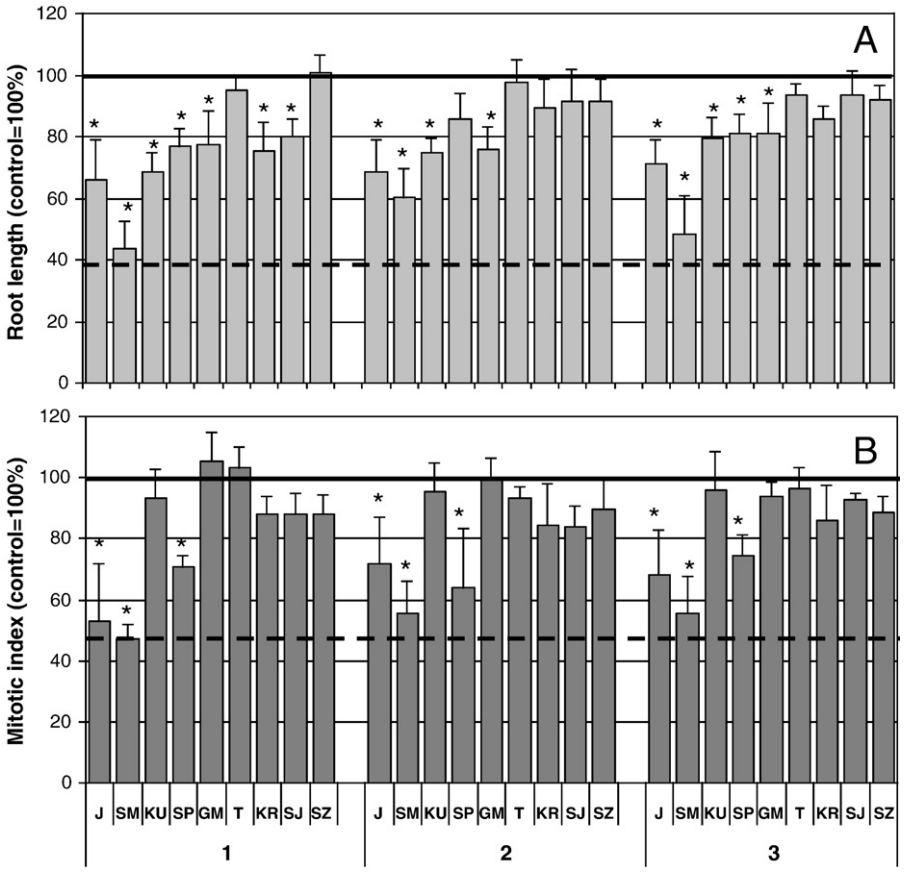

Fig. 2. (A) Root length (%) and (B) Mitotic index (%) of *Allium cepa* root-tip cells exposed for 24 h to water samples collected monthly over a three-month period (1–3). Solid line represents negative control (tap water) and broken line positive control (300 mM H_2O_2). Standard deviations were presented by error bars. Bars labeled with an asterisk are significantly different from negative control values (solid line) at $p<0.05$ (DMRT).

induce any obvious toxic or cytotoxic effects (SJ, SZ, T, KR), also did not produce any genotoxic effects. However, water samples that caused both an inhibition of root growth and a decrease of the mitotic index (SP, KU, J and particularly SM) showed significant genotoxic potential. It may be noted however, that cytotoxicity was not strictly correlated to genotoxicity in the case of water samples collected near the artificial fertilizer plant (KU). The unaffected mitotic activity in those water samples might be due to temporary stimulatory effects of nitrate, nitrite, ammonium and phosphate on the proliferation of *A. cepa* root-tip cells. The mitotic index is considered to reliably identify the presence of cytotoxic pollutants in the environment (Smaka-Kincl et al., 1996; Grover and Kaur, 1999; Chandra et al., 2005). Trace metals, pesticides and other pollutants were considered responsible for the diminished mitotic index of the *A. cepa* roots exposed to industrial wastewaters (Caritá and Marin-Morales, 2008; Fatima and Ahmad, 2006). Glińska et al. (2007) suggested that the decreased mitotic index in *A. cepa* roots treated with metal is probably due to either disturbances in the cell cycle or chromatin dysfunction induced by metal–DNA interactions.

The cytogenetic analysis revealed the presence of genotoxic compounds in the tested water samples. Over the three-month period, samples collected at each site showed consistent results for genotoxicity. The industrial effluent, SM, as well as wastewater from the artificial fertilizer plant, KU, showed the strongest genotoxic effects in the root meristem cells. The most frequent abnormalities were due to chromatin dysfunction (stickiness and anaphase bridges) or spindle failure (c-metaphase). Chromosome bridges result from chromosome and/or chromatid breaks, indicating the clastogenic effect, whereas vagrant chromosomes and c-metaphases increase the risk for aneuploidy (Leme and Marin-Morales, 2009). Root-tip cells exposed to 300 mM H_2O_2 demonstrated a similar distribution of abnormalities (the most common type of abnormality was sticky chromosomes, while the least common type of abnormalities was fragments) and that was the reason H_2O_2 was chosen as the positive control mutagen. The first choice for a positive control in this study was methylmethanesulfonate (MMS) because it is widely recommended in mutagenicity testing (Fiskesjö, 1993, 1997; Rank and Nielsen, 1998; Caritá and Marin-Morales, 2008). However, MMS was considered unsuitable due to the types of abnormalities it induced. MMS induced fragments (24.8% of aberrant cells), followed by anaphase bridges (6% of aberrant cells) and vagrants (5% of aberrant cells), while sticky chromosomes represented only 3% of the total aberrations. In comparison, the percentage of sticky chromosomes, as the main effect of H_2O_2, was 48.9%. H_2O_2 was also used as a positive control mutagen in the evaluation of the genotoxic potential of surface water from the Pitimbu River (Monte Egito et al., 2007), most likely for this same reason.

Pronounced stickiness of the chromatin matrix often resulted in atypical metaphase and anaphases. The increased stickiness also leads to the formation of sticky bridges in anaphase and telophase, and thereby prevents normal cytokinesis. Sticky chromosomes indicate that the pollutant is affecting the organization of the chromatin. This effect is related to a disturbed balance in the quantity of histones or other proteins responsible for controlling the proper structure of nuclear chromatin (Kurás, 2004). Stickiness is considered a common sign of toxic effects on chromosomes probably leading to cell death (Fiskesjö, 1997). Sticky chromosomes have been reported in *Allium* roots after treatment with various heavy metals such as Hg, Ni and Cu (Fiskesjö, 1993, 1997; Monte Egito et al., 2007). The other effect frequently observed, c-metaphase, suggests that compounds present in industrial (SM, KU) and municipal wastewater samples (GM)

Table 2
Chromosome aberrations in *Allium* root meristem cells after 24 h exposure to surface and wastewaters collected monthly over a 3-month period (1–3).

Sample	No. dividing cells	No. C-mitosis	No. laggards	No. breaks	No. anaphase bridges	No. sticky chromosomes	% Aberrant cells
C	301	1	1	1	2	1	2.0
H_2O_2	176	18**	20**	7*	12**	86**	81.3
J1	209	3	6	5*	4	10*	13.4
J2	204	3	5	1	4	7	9.8
J3	212	2	8*	2	7*	8	12.7
SM1	173	14**	5	2	15**	18*	31.2
SM2	209	11**	4	2	9*	19*	21.5
SM3	177	11**	5	3	12**	22*	29.9
KU1	269	12**	6	2	11**	27*	21.6
KU2	280	9*	9*	1	9*	19*	16.8
KU3	254	5	4	2	9*	11*	12.2
SP1	219	4	4	1	9*	20*	17.8
SP2	202	3	3	0	9*	12*	13.4
SP3	200	2	8*	2	6	12*	15.0
GM1	307	10*	1	1	3	1	5.2
GM2	292	6	1	0	2	2	3.8
GM3	266	9*	1	0	1	0	4.1
T1	308	3	2	1	4	4	4.5
T2	234	2	1	0	3	3	3.8
T3	274	2	2	1	4	3	4.4
KR1	244	2	3	1	5	3	5.7
KR2	210	1		1	4	3	4.8
KR3	236	2	2	1	3	4	5.1
SJ1	230	3	2	1	0	1	3.0
SJ2	207	2	0	0	1	0	1.5
SJ3	229	2	1	0	1	0	1.7
SZ1	236	1		1	1	0	1.7
SZ2	243	4	2	1	1	1	3.7
SZ3	219	4	2	1	1	4	5.5

Means in each column labeled with asterisks are significantly different from negative control (*$p<0.05$, **$p<0.001$) according to Mann–Whitney test.

disturb the mitotic spindle, most likely the kinetochore function (Seth et al., 2008). According to White and Rasmussen (1998) municipal wastewaters, in general, show low genotoxic potential. However, such wastewaters can achieve loading values that are several orders of magnitude greater than wastes from industries. For this reason, domestic wastewater can constitute a significant genotoxic hazard to the aquatic environment. The occurrence of chromosome breaks was low and observed as significant only in the wastewater samples collected from the city dump. In summary, four of the nine sites demonstrated genotoxic potential that ranged from low to high depending on their origin (vicinity of the chemical industry, smeltery or city dump).

A positive correlation between genotoxic potential and chemical analysis, at least to some extent, was observed. However, such a

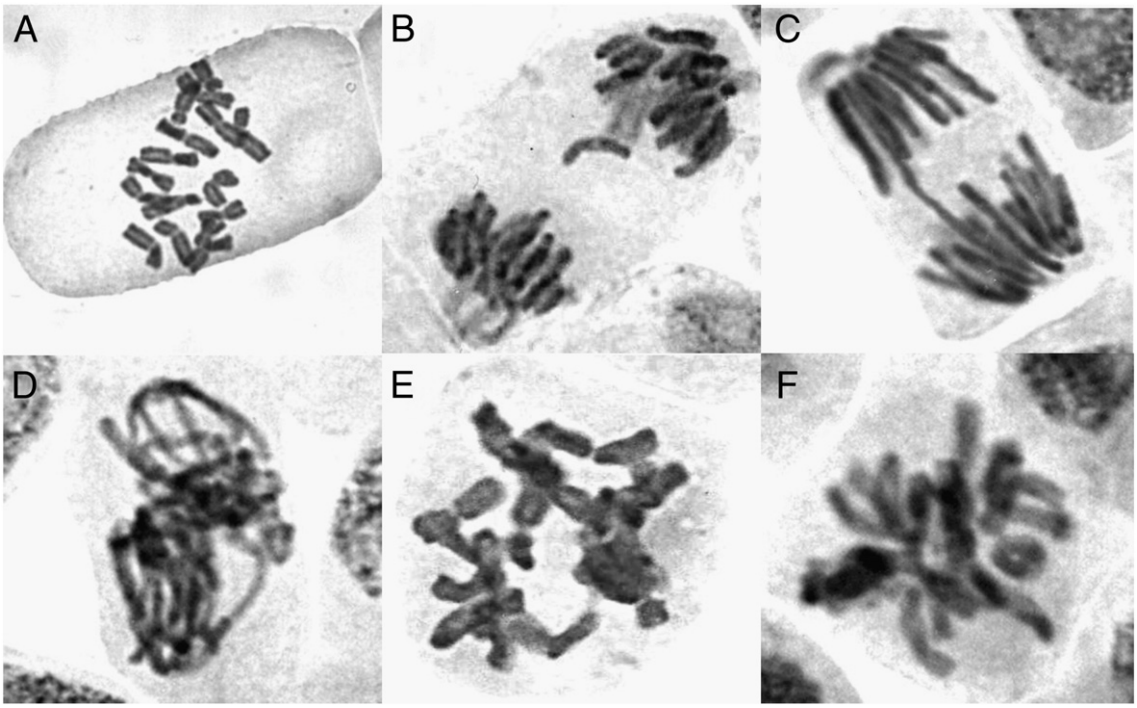

Fig. 3. The cytogenetic effects of surface and wastewaters on root meristem cells of *A. cepa* ($2n = 2x = 16$): (A) c-metaphase, (B), lagging chromosomes, (C) anaphase bridge, (D–F) sticky chromosomes.

strong toxic effect demonstrated by several of the water 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 substances not identified by the typical chemical analysis performed as a part of water 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.

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 *A. 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. It should also be used for monitoring surface water quality, as it would provide data useful in risk assessment. 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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References

Caritá R, Marin-Morales MA. Induction of chromosome aberrations in the *Allium cepa* test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes. Chemosphere 2008;72:722–5.

Chandra S, Chauhan LK, Murthy RC, Saxena PN, Pande PN, Gupta SK. Comparative biomonitoring of leachates from hazardous solid waste of two industries using the *Allium* test. Sci Total Environ 2005;347:46–52.

Durgo K, Oreščanin V, Lulić S, Kopjar N, Želježić D, Franekić Čolić J. The assessment of genotoxic effects of wastewater from a fertilizer factory. J Appl Toxicol 2009;29:42–51.

Fatima RA, Ahmad M. Genotoxicity of industrial wastewaters obtained from two different pollution sources in northern India: a comparison of three bioassays. Mutat Res 2006;609:81–91.

Fiskesjö G. The *Allium* test as a standard in environmental monitoring. Hereditas 1985;102:99-112.

Fiskesjö G. The *Allium cepa* in wastewater monitoring. Environ Toxicol Water 1993;8:291–8.

Fiskesjö G. *Allium* test for screening chemicals; evaluation of cytological parameters. In: Wang W, Gorsuch JW, Hughes JS, editors. Plants for Environmental Studies. New York: Lewis Publishers; 1997. p. 308–33.

Glińska S, Bartczak M, Oleksiaka S, Wolska A, Gabara B, Posmyk M, et al. Effects of anthocyanin-rich extract from red cabbage leaves on meristematic cells of *Allium cepa* L. roots treated with heavy metals. Ecotoxicol Environ Safe 2007;68:343–50.

Grover IS, Kaur S. Genotoxicity of wastewater samples from sewage and industrial effluent detected by the Allium root anaphase aberration and micronucleus assays. Mutat Res 1999;426:183–8.

Isidori M, Lavorgna M, Nardelli A, Pascarella L, Parrella A. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci Total Environ 2005;346:87–98.

ISO 7888. Water quality — Determination of electrical conductivity; 1985.

ISO 6060. Water Quality — Determination of chemical oxygen demand; 1989.

ISO 10523. Water quality — Determination of pH; 1994.

ISO 11923. Water quality — Determination of suspended solids by filtration through glass-fibre filters; 1997.

ISO/TR 11905. Water quality — Determination of nitrogen — Part 2: Determination of bound nitrogen, after combustion and oxidation to nitrogen dioxide, chemiluminescence detection; 1997.

ISO 14911. Water quality — Determination of dissolved Li^+, Na^+, $NH4^+$, K^+, Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} using ion chromatography — Method for water and wastewater; 1998.

ISO 5815. Water quality —Determination of biochemical oxygen demand after n days (BODn) — Part 1: Dilution and seeding method with allylthiourea addition; 2003.

ISO 6878. Water quality — Determination of phosphorus — Ammonium molybdate spectrometric method; 2004.

ISO 10304. Water quality — Determination of dissolved anions by liquid chromatography of ions — Part 1: Determination of bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulfate; 2007.

Koch DE, Bhandari A, Close L, Hunter RP. Azithromycin extraction from municipal wastewater and quantitation using liquid chromatography/mass spectrometry. J Chromatogr A 2005;1074:17–22.

Kungolos AG, Brebbia CA, Samaras CP, Popov V. Environmental Toxicology. Southampton: WIT Press; 2006. 362 pp.

Kurás L. Characterization of protein–DNA association in vivo by chromatin immunoprecipitation. In: Dickson RC, Mendenhall MD, editors. Signal Transduction Protocols, Methods in Molecular Biology, 284. Totowa: Humana Press Inc.; 2004. p. 147–62.

Leme DM, Marin-Morales MA. *Allium cepa* test in environmental monitoring: a review on its application. Mutat Res 2009;682:71–81.

Monte Egito LM, das Gracas Medeiros M, Batistuzzo de Medeiros SR, Agnez-Lima LF. Cytotoxic and genotoxic potential of surface water from the Pitimbu river, northeastern/RN Brazil. Gen Mol Biol 2007;30:435-431.

Ohe T, White PA, DeMarini DM. Mutagenic characteristics of river waters flowing through large metropolitan areas in North America. Mutat Res Genet Toxicol Environ Mutagen 2003;534:101–12.

Rank J, Nielsen MH. Genotoxicity testing of wastewater using the *Allium cepa* anaphase-telophase chromosome aberration assay. Mutat Res 1998;418:113–9.

Rank J, Lopez LC, Nielsen MH, Moretton J. Genotoxicity of maleic hydrazide, acridine and DEHP in *Allium cepa* root cells performed by two different laboratories. Hereditas 2002;136:13–8.

Seth CS, Misra V, Chauhan LKS, Singh RR. Genotoxicity of cadmium on root meristem cells of *Allium cepa*: cytogenetic and Comet assay approach. Ecotoxicol Environ Safe 2008;71:711–6.

Smaka-Kincl V, Stegnar P, Lovka M, Toman MJ. The evaluation of waste, surface and ground water quality using the *Allium cepa* test procedure. Mutat Res 1996;368:171–9.

Terzić S, Senta I, Ahel M, Gros M, Petrović M, Barcelo D, et al. Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. Sci Total Environ 2008;399:66–77.

Vargas VMF, Migliavacca SB, de Melo AC, Horn RC, Guidobono RR, Ferreira I, et al. Genotoxicity assessment in aquatic environments under the influence of heavy metals and organic contaminants. Mutat Res Genet Toxicol Environ Mutagen 2001;490:141–58.

White PA, Rasmussen JB. The genotoxic hazards of domestic wastes in surface waters. Mutat Res 1998;410:223–36.

Žegura B, Heath E, Černoša A, Filipič M. Combination of in vitro bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples. Chemosphere 2009;75:1453–60.