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Genotoxicity Assessment of Water Samples from the Sungai Dua River in Pulau Pinang, Malaysia, Using the *Allium cepa* Test

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Abstrak: Kesan sampingan yang tidak diingini daripada sumber air tercemar bukan hanya terhad kepada flora dan fauna, tetapi boleh juga dipindahkan kepada organisma melalui rantai makanan yang sama. Empat sampel air yang diambil sebaik sahaja hujan berhenti dan lima hari selepas itu daripada dua lokasi daripada Sungai Sungai Dua (SGD) diuji ketoksikannya menggunakan asai *Allium cepa*. Sampel air dianalisis untuk kandungan metal dan juga dinilai secara makroskopik dan mikroskopik. Sampel mengandungi natrium (Na^+) dan kalsium (Ca^{2+}) melebihi air paip yang digunakan sebagai kawalan, dan menunjukkan perencatan ke atas pertumbuhan akar dan mitosis dalam *A. cepa*. Walau bagaimanapun, kesan perencatan adalah tidak bergantung kepada dos. Tiada aberasi kromosom diaruh pada kepekatan 100.00% (sampel yang tidak dicairkan). Keputusan ini mungkin melambangkan kesan mitodepresif dan genotoksik yang lemah oleh sampel air daripada SGD ke atas sel *A. cepa*.

Kata kunci: Mitosis, Sampel Air, Pencemaran, Logam, Aberasi Kromosom

Abstract: Unwanted side effects from a polluted water body may not be limited to the flora and fauna, they may also be transferred to the organisms along the food chain. Four water samples collected immediately and five days after rainfall from two locations inside the polluted Sungai Dua River (SGD) were tested for toxicity using the *Allium cepa* assay. The samples were analysed for metal content and were both macroscopically and microscopically evaluated. The water samples contained more sodium (Na^+) and calcium (Ca^{2+}) than the control tap water, and they showed root growth and mitotic inhibitions (MI) in *A. cepa*. However, the inhibitory effects were not dose-dependent. No chromosomal aberration (CA) was induced at 100.00% (undiluted water sample). These results suggest the water samples from SGD had weak mitodepressive and genotoxic effects on the *A. cepa* cells.

Keywords: Mitosis, Water Samples, Pollution, Metals, Chromosome Aberration

INTRODUCTION

Water is an indispensable natural product that finds its use in virtually all aspects of human life. Thus, there is a great need for ensuring that water used by humans does not contain hazardous substances. The pollution of water bodies is

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a global problem because of the danger that polluted waters may contain mutagenic and carcinogenic substances that may cause or promote the occurrence of human diseases such as cancer, atherosclerosis, cardiovascular disease and premature aging (Radić *et al*. 2010). Several tests have used microorganisms, diatoms, plant and mammalian cells, the presence and distribution of aquatic organisms, and chemical analysis to ascertain the quality of water sources (Smaka-Kincl *et al*. 1996; Wan & Mashhor 2002; Ohe *et al*. 2003; Zegura *et al*. 2009). The plant assay of *Allium cepa* L. (onions) (2n=16) has been used extensively to evaluate the cytotoxicity and genotoxicity of water samples (Rank & Nielsen 1998; Monarca *et al*. 2003, 2005; Fatima & Masood 2006; Radić *et al*. 2010). This assay is low cost, is easy to use and produces similar results to animal tests because of similarity in their genetic compositions, hence same response to mutagens. The presence of metacentric chromosomes in *A. cepa* cells allows easier and better microscopic assessment.

Sungai Dua in Bahasa Malaysia means two rivers. The Sungai Dua River (SGD) is found in the town of the same name in Pulau Pinang, Malaysia. SGD's course extends to the Hamna area of Gelugor. The regular pollution of the river through wastewater discharge and refuse by residents poses dangers not only to the fauna and flora in it but also to higher animals, including humans along the food chain. This is because the river has as its primary producers the plants being watered by this polluted water. The gravity of the hazards caused by a polluted river increases when heavy metals are the major contaminants. Many hazardous organic chemicals have also been detected in wastewaters, and some of these, such as nitrosamines, polychlorinated biphenyls (PCB), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/F), and polychromatic hydrocarbons (PHA) are known to induce damage to DNA (Connor *et al*. 1992; Klöpffer 1996; Rogers 1996; Webber *et al*. 1996; Tørsløv *et al*. 1997).

Apart from the reports (Wan and Mashhor 2002) which established sources and confirmed the occurrence of pollution in the Pinang river basin, there is no information on the adverse effects from polluted SGD waters on cellular organisms. This study, therefore, seeks to assess the toxic effects of water samples from SGD on root growth, cell division and chromosome morphology in *A. cepa*.

An evaluation of water samples for toxicity on cell division and chromosomes in a eukaryotic organism like *A. cepa* will be a useful for predicting likely side effects on humans from agricultural production in farmlands watered by the polluted SGD, with full knowledge that the produce is being sold to market for consumption. We hope that our findings will reveal the extent of pollution in this river.

MATERIALS AND METHODS

Study Area and Sampling Sites

SGD is in the town of Sungai Dua (latitude 5.45° longitude 100.333333°) in eastern Pulau Pinang, Malaysia. The Hamna area of Gelugor is one of the residential locations where the SGD extends and where the water samples were

collected for this study. The sampling site was divided into two locations: A and B. Location A is the part of the river that receives domestic wastewaters from the houses in the area, and location B is the place where farmers along the river take water to irrigate their farmlands (Fig. 1).

Sampling Technique

The water samples were collected immediately and five days after rainfall from our chosen locations (A and B) using a plastic pail tied to a rope. The samples were kept separately inside thoroughly washed 5 litre plastic bottles. The samples were labelled as follows. Water sample A1 (WSA1) was collected at location A immediately after rainfall on 28 December 2009. Water sample B1 (WSB1) collected at location B immediately after rainfall on 28 December 2009. Water sample A2 (WSA2) was collected at location A five days after rainfall on 3 January 2010. Water sample B2 (WSB2) was collected at location B five days after rainfall on 3 January 2010. At each sampling time, the samples were quickly transferred to the laboratory and stored at 4°C for metal analysis and toxicity screenings.

(a)

(b)

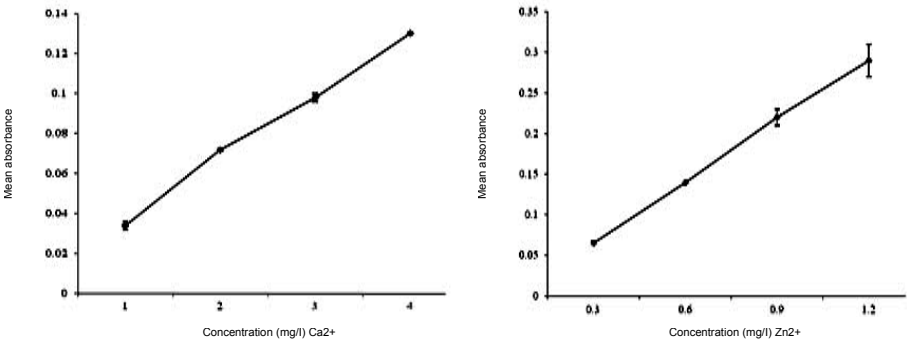
Figure 1: Sungai Dua course showing the sampling locations; (a) represents the sampling location in SGD that receives refuses and wastewaters A and (b) represents the sampling location where farmers along SGD fetch water to water their plants B.

Metal Analysis

Water samples A1, A2, B1 and B2, together with controls tap water 1 (TW1) and 2 (TW2), were analysed for the presence and concentration of sodium (Na^{+}), calcium (Ca^{2+}), lead (Pb^{2+}), cadmium (Cd^{2+}) and zinc (Zn^{2+}) ions using an atomic absorption spectrometer (AAS) (PerkinElmer A Analyst 100, USA). The metal standards were prepared to known concentrations (Table 1), labelled, and kept inside plastic bottles that were pre-cleansed with concentrated nitric acid and distilled water (DW). The water samples and controls were filtered using Whatman® filter paper (No. 1, Qualitative Circles 110 mm Ø, Cat No 1001 110) that had been rinsed with DW. This method necessitated analysis of the DW for the presence of the above metals to remove any possible influence on the results of the analysed metals in the water samples and controls. The absorbance of the standards, water samples and controls was taken in triplicates. Graphs of the concentrations against the absorbance of each of the standards for the metals were plotted [Fig 2(a) – (e)]. Thereafter, the concentrations of the five metals in water samples A1, A2, B1, B2, TW1 and TW2 were interpolated from their respective graphs.

Table 1: Concentrations of standards for the analysed metals.

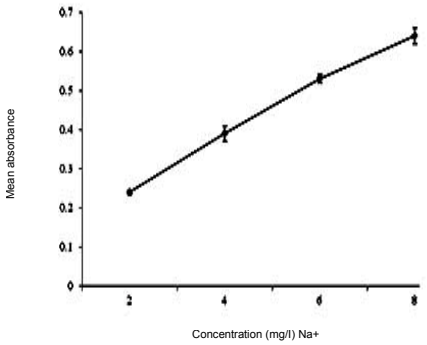
Metal (ion)	Concentration (mg/l)			
Sodium (Na^{+})	2.0	4.0	6.0	8.0
Calcium (Ca^{2+})	1.0	2.0	3.0	4.0
Lead (Pb^{2+})	2.0	4.0	6.0	8.0
Zinc (Zn^{2+})	0.3	0.6	0.9	1.2
Cadmium (Ca^{2+})	0.4	0.8	1.2	1.6



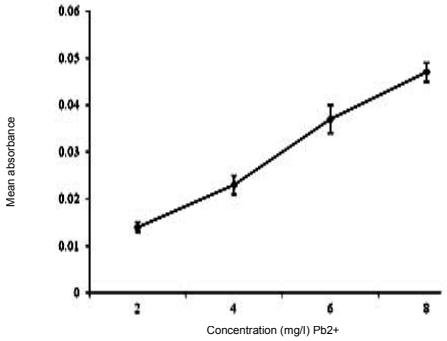
(a) (b)

Figure 2: Standard curves of the analysed metals: (a) calcium; (b) sodium; (c) lead; (d) zinc; (e) cadmium *(continued on next page).*

Figure 2: *(continued)*



(c)



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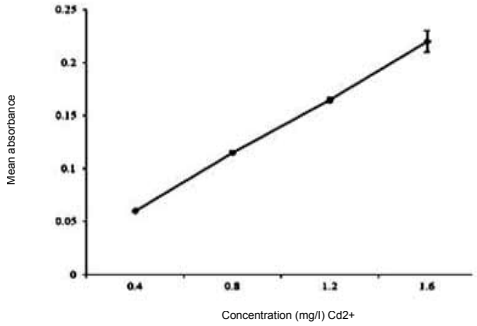

(e)

Allium cepa Test

Procurement and preparation of onions

A. cepa L. (2n=16), were purchased at the Jusco shopping complex, Pulau Pinang, Malaysia. The onions were sun dried for a week and those attacked by fungi were discarded at the beginning of the experiment. The outer scales were carefully removed, without tampering with the primordial root ring.

Macroscopic evaluation

Each water sample was diluted with tap water to obtain 6.25%, 12.50%, 25.00%, 50.00% and 100.00% (undiluted) concentrations. Ten equally sized *A. cepa* per concentration were suspended on the water sample inside 50 ml beakers and kept in the dark for 48 h, but with the renewal of the previous water sample after 24 h. The negative control was set up with tap water only. After 48 h, the root

lengths of at least 20 roots from each of 7 *A. cepa* per concentration were measured using a ruler. The effective concentration at 80% root growth of the control was determined as opposed 50% root growth of the control reported by Akinboro and Bakare (2007) and Yildiz *et al*. (2009).

Microscopic evaluation

The root tips from the remaining three *A. cepa* were cut and fixed in ethanol/acetic acid (3:1) inside universal bottles and kept at 4°C for 24 h before use. The fixed root tips were hydrolysed in 1N HCl at 60°C for 5 minutes. The hydrolysed root tips were washed several times with DW. Three root tips were crushed on each slide and stained with aceto-orcine for 10 minutes. Excess stains were removed, and the cover slip's edges were sealed as suggested by Grant (1982). A total of 5000 cells from 5 slides per concentration were observed (at 1000 x magnification) for different mitotic stages and chromosomal aberrations using a Nikon Eclipse E400 (Japan) light microscope. The mitotic index (MI) and the frequency of chromosomal aberrations (CA) was calculated (Fiskesjo 1997; Bakare *et al*. 2000).

$$MI = \frac{\text{Number of dividing cells}}{\text{Total number of cells counted}} \times 100$$

$$\text{Frequency of CA (\%)} = \frac{\text{Number of aberrant cells}}{\text{Total number of cells counted}} \times 100$$

Statistical Analysis

Quantitative data were summarised as means ± standard deviation and percentages, which were then subjected to Duncan multiple comparison and Dunetts tests in a one-way ANOVA, using SPSS version 15.0 for Windows (SPSS Inc., Chicago, USA). The effects of the water samples and controls on the root growth, cell division and chromosomes of *A. cepa* were compared. Significant differences were set at $p \leq 0.05$.

Table 2: Concentrations (ppm) of the analysed metals in the water samples and controls.

Metal (ion)	Sample	Mean absorbance	Concentration (mg/l)
Na^+	TW1	0.399	3.70
	TW2	0.452	4.30
	A1	0.281	1.80
	B1	0.256	1.60
	A2	0.298	1.94
	B2	0.286	1.82
Ca^{2+}	TW1	0.077	1.80
	TW2	0.072	1.75

(continued on next page)

Table 2: *(continued)*

Metal (ion)	Sample	Mean absorbance	Concentration (mg/l)
	A1	0.068	1.70
	B1	0.064	1.50
	A2	0.064	1.50
	B2	0.054	1.08
Zn^{2+}	TW1	0.009	ND
	TW2	0.014	ND
	A1	–0.001	0.0
	B1	0.008	ND
	A2	0.010	ND
	B2	0.005	ND
Pb^{2+}	TW1	0.001	ND
	TW2	0.013	ND
	A1	0.001	ND
	B1	0.001	ND
	A2	0.001	ND
	B2	0.002	ND
Cd^{2+}	TW1	0.002	ND
	TW2	–0.001	0.0
	A1	0.002	ND
	B1	0.002	ND
	A2	0.002	ND
	B2	0.003	ND

Note: ND; non detectable.

RESULTS

Metal Analysis

Table 2 shows the concentrations of Na^{+}, Ca^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} in the water samples. The amount of Na^{2+} and Ca^{2+} ions in the four water samples was lower than that of TW1 and TW2. The highest (4.30 mg/l) and lowest (1.60 mg/l) concentrations of Na^{+} were recorded for TW2 and B1, respectively; Ca^{2+} highest in TW1 (1.80 mg/l) and lowest in B2 (1.08 mg/l). There was 0.0 ppm of $Zn^{2+}$and Cd^{2+} in A1 and TW2. The three analysed (Zn^{2+}, Pb^{2+} and Cd^{2+}) were not detected by the AAS in the other samples.

Macroscopic Evaluation

The effects of the water samples on the root growth of onions suggest their levels of toxicity using growth inhibition as a determining factor. All the water samples reduced the root growth of the *A. cepa,* but not in a dose-dependent manner (Table 3). Neither water samples A nor B could induce 50% root growth inhibition

of the negative control; hence EC_{20} was determined for the water samples. The concentration at which 20% root growth of negative control was inhibited refers to EC_{20}. The order of toxicity to the root growth, based on the values of EC_{20} induced by the water samples was B1 (4.0%) > A2 (5.2%) > B2 (50.0%) > A1 (95.2%). At 6.25%, 12.50% and 25.00% concentrations of A1 and B2, there was promotion of growth above the controls, but they were not significantly different. The inhibition of root growth by the undiluted water samples (100.00%) was reduced between the two sampling times, with higher inhibitions from A1 and B1. A2 and B2 induced more root growth, amounting to lower inhibitions. However, the inhibition of root growth caused by B1 at 50.00% and 100.00% was significantly different from the control ($p \leq 0.05$).

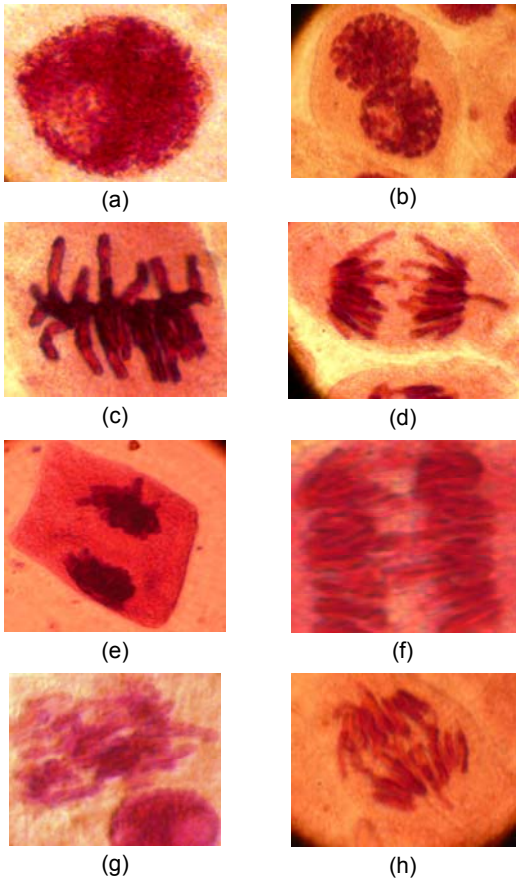

Figure 3: Normal stages of mitosis and chromosomal aberrations induced in the cells of onions suspended in the water samples from SGD: (a–e) interphase, prophase, metaphase, anaphase, telophase; (f) multiple anaphase bridge; (g) sticky chromosome, chromosome breaks; (h) disturbed spindles.

Microscopic Evaluation

The cytotoxicity and genotoxicity of A1, A2, B1 and B2 were determined based on their impacts on cell division and chromosome behaviour in *A. cepa.* Higher but insignificantly different (from control) MI values were recorded at all concentrations except 100.00% (A1), 25.00% and 100.00% (B1), and 6.25% (B2). The mitotic activity in the test organism was suppressed at various concentrations of the water samples. Lower and significantly different ($p \leq 0.05$) MI values were obtained at 6.25% (A2), 25.00% and 50.00% (B2) as well as 100.00% for A1, B1 and B2. It is worth noting that the induction of lower MI values by these water samples was not concentration dependent (Table 4).

Table 3: Effects of the water samples and control on root growth of *A. cepa.*

Conc. (%)	A1			B1			A2			B2		
	AV ± SD root length (cm)	Root growth (%)	Root growth inhibition (%)	AV ± SD root length (cm)	Root growth (%)	Root growth inhibition (%)	AV ± SD root length (cm)	Root growth (%)	Root growth inhibition (%)	AV ± SD root length (cm)	Root growth (%)	Root growth inhibition (%)
TW (0.00)	3.15 ± 0.25	100.00	0.00	3.15 ± 0.25	100.00	0.00	1.83 ± 0.49	100.00	0.00	1.83 ± 0.49	100.00	0.00
6.25	4.08 ± 0.98	129.52	0.00	2.20 ± 0.85	69.84	30.16	1.37 ± 0.28	74.86	25.14	2.38 ± 0.71	130.06	0.00
12.50	3.90 ± 1.60	123.81	0.00	2.76 ± 1.03	87.62	12.38	1.49 ± 0.92	81.42	18.58	1.49 ± 0.37	81.42	18.58
25.00	3.58 ± 0.31	113.65	0.00	2.21 ± 0.61	70.16	29.84	1.49 ± 0.52	81.42	18.58	1.97 ± 0.65	107.65	0.00
50.00	3.10 ± 0.64	98.41	1.59	1.56 ± 1.01*	49.52	50.48	1.74 ± 0.25	95.08	4.92	1.46 ± 0.70	79.78	20.22
100.00	2.49 ± 0.61	79.05	20.95	1.68 ± 0.76*	53.33	46.67	1.56 ± 0.39	85.25	14.75	1.74 ± 0.54	95.08	4.92

Notes: Conc., concentration; TW, tap water; AV, average; SD, standard deviation.
*Values are significantly different from control ($p \leq 0.05$).

Table 4: MI and frequency of CA induced by the water samples and control in *A. cepa* cells.

Conc. (%)	A1		B1		A2		B2	
	MI ± SD	CA ± SD	MI ± SD	CA ± SD	MI ± SD	CA ± SD	MI ± SD	CA ± SD
TW	2.34 ± 0.21	0.19 ± 0.10	2.34 ± 0.21	0.19 ± 0.10	3.11 ± 0.56	0.14 ± 0.05	3.11 ± 0.56	0.14 ± 0.05
6.25	3.06 ± 0.48	0.24 ± 0.10	3.08 ± 0.52	0.36 ± 0.17	1.88 ± 0.50*	0.18 ± 0.07	3.22 ± 0.15	0.20 ± 0.10
12.50	3.00 ± 0.30	0.28 ± 0.09	2.40 ± 0.72	0.32 ± 0.14	2.65 ± 0.75	0.22 ± 0.08	2.98 ± 0.56	0.22 ± 0.14
25.00	2.74 ± 0.70	0.37 ± 0.14	1.84 ± 0.36	0.16 ± 0.04	2.46 ± 0.45	0.32 ± 0.12*	1.92 ± 0.23*	0.26 ± 0.15
50.00	2.62 ± 0.40	0.50 ± 0.24*	2.84 ± 0.23	0.60 ± 0.26*	2.44 ± 0.62	0.26 ± 0.10	1.68 ± 0.43*	0.40 ± 0.19*
100.00	1.36 ± 0.50*	0.40 ± 0.22	1.40 ± 0.24*	0.22 ± 0.10	2.20 ± 0.25	0.24 ± 0.09	1.54 ± 0.37*	0.08 ± 0.04

Notes: MI; mitotic index, CA; chromosomal aberration.
*Values are significantly different from control ($p \leq 0.05$).

Furthermore, the tested water samples and controls induced different types of CA, such as disturbed spindles, chromosome lag, sticky chromosomes, anaphase bridges and chromosome fragmentation (Fig. 3). These aberrations were not also dose related, but they were significantly different from the controls at 25.00% for A2 and 50.00% for A1, B1 and B2 (Table 4).

DISCUSSION

The *A. cepa* test is an easy and fast genetic assay to elucidate cytotoxic and genotoxic effects of polluted water and other chemical substances on mitosis and chromosome structure in *A. cepa* cells (Rank & Nielsen 1998; Carruyo *et al*. 2008; Teerarak *et al*. 2009; Yildiz *et al*. 2009; Radić *et al*. 2010). The extent of cell proliferation and differentiation in the apical meristems could directly correlate with the rate of root growth in *A. cepa*. Thus, MI can be used to determine the rate of root growth. In the present investigation, the results of metals analysis established that SDG water body contained non heavy metals ions (Na+ and Ca2+), while the heavy metals ions (Zn2+, Pb2+ and Cd2+) were not detected in the water samples. Therefore, water samples A1, B1, A2 and B2 may have inhibited root growth in A. cepa due to the presence of Na+ in SGD water body (Table 2). Previously, the inhibitory effects of NaCl on the root growth of Chrysanthemum morifolium Ramat, red raspberry and four vegetables were reported (Hossain et al. 2004; Jamil et al. 2006; Neocleous & Vasilakakis 2007). The mechanism of NaCl root growth inhibition may be via osmotic stress or salt toxicity, and consequently leads to the loss of turgor pressure, thus decreasing cell division and growth (Nilsen & Orcutt 1996). However, the inhibition of *A. cepa* root growth in a non concentration dependent manner by the water samples implies weak toxic effects. This could have been due to the absence of heavy metals in SDG. Some heavy metals have been implicated in the root growth reduction of *A. cepa* (Arambasić *et al*. 1995; Samardakiewick & Woźny 2005; Carruyo *et al*. 2008). It can be said further that the inability of water samples A1, A2, B1 and B2 to decrease root lengths of *A. cepa* suspended in them by 50% of the controls, rather by 20% was also in support of weak toxicity of the evaluated water body (SDG).

In *A. cepa* test, results of macroscopic and microscopic studies usually support each other (Akinboro & Bakare 2007). Therefore, the inhibition of root growth by Na^+ in the water samples could be adduced for the lower MIs induced by the water samples compared to the controls, hence, cytotoxicity of SDG water body was evident. MI has been used to measure the level of toxic effects that any tested substances have on cell division (Yildiz *et al*. 2009). The induction of lower MI values by the water samples A1, B1, A2 and B2 at 100.00% suggests inhibition of cell division in the apical meristematic cells of *A. cepa*, supporting the results of macroscopic evaluation about the reduction of root growth. However, the cytotoxicity of the water samples to the test organism was more inducible by the water samples A2 and B2 collected five days after rainfall. The obtainment of higher MI, than the control, at concentrations below 100.00% suggests a weak cytotoxic effect which was also confirmed by the reduction of

cell division in a non-dose related manner. This result is in accord with the earlier assertion that SGD water body did not contain highly toxic substances, as revealed by the effects of the water samples on root growth. Induction of CA which were not significantly different from the control by the undiluted water samples A1, B1, A2 and B2 implies weak genotoxic effects. Similar effects of sodium chloride and calcium propionate on *A*. *cepa* cells have been reported (Türkoğlu 2008; Teerarak *et al*. 2009). Sodium chloride and calcium propionate reduced number of dividing cells, as well as inducing various chromosomal damages in *A*. *cepa* cells. It is therefore logical to say that the observed effects of the SGD water samples A1, B1, A2 and B2 in this study could have been caused by the presence of Na^+ and Ca^{2+} (Table 2).

Inhibition of mitosis by Na^+ and Ca^{2+} might result from blockage of DNA and nuclear proteins synthesis in the G_2 phase of the cell cycle, preventing the cell from entering mitosis (Türkoğlu 2008). The cause of chromosomal abnormalities such as disturbed spindles or c-mitosis, chromosome bridge, sticky chromosome, chromosome fragmentation and chromosome lag shown in Figure 3 may not be unconnected with the presence of Na^+ and Ca^{2+} ion in the water samples and controls. It is possible that Na^+ and Ca^{2+} might have interfered with the process of mitotic spindle formation and chromatin organisation to induce the aforementioned aberrations.

CONCLUSION

The assessments of water samples from the two locations in SGD suggest that the water body did not contain highly toxic pollutants that might have found their way in through the indiscriminate disposal of refuse and wastewater discharge. Nevertheless, our findings do not support the continuation of the pollution of the SGD because of the health implications of these unhygienic habits on the people who consume agricultural produce from the farmlands being watered by this polluted water. It is therefore recommended that the authorities responsible for protecting the environment provide proper and adequate places for people to dump their refuse and that they educate the people on the dangers of disposing wastes into flowing rivers.

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