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CYTOGENETIC BIOMONITORING OF ALMADINAH ALMUNAWARAH MUNICIPAL WASTEWATER TREATMENT PLANT USING THE *ALLIUM CEPA* CHROMOSOME ABERRATION ASSAY

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Abstract

This study aims to find out if the cytotoxic and genotoxic responses of *Allium cepa* are valuable biomarkers of the current conventional wastewater treatment protocols, used in the municipal wastewater plant located in Almadinah Almunawarah city (Kingdom of Saudi Arabia). Wastewater samples were collected at two different times, winter (February 2016) and summer (July 2016) seasons from the municipal wastewater plant. *Allium cepa* bulbs were exposed to different concentrations (100, 50, 25, and 12.5%) of treated wastewater for 12 h. In addition, *Allium cepa* bulbs were exposed to tap water, which was used as a negative control. The Feulgen reaction was used to stain three root tips from each onion bulb. Subsequently the frequency of different types of chromosome aberrations (CA), percentage of aberrant cells (%Abc), and mitotic index (%MI) were determined and compared to tap water, treated wastewater induced CA and decreased MI in a dose dependent manner. In addition, the %MI and %Abc in winter and summer were similar. To conclude, the treated wastewater may contain toxic compounds leading to mutagenicity and CA. According to these findings, the biomonitoring studies and the treatment of wastewater in Almadinah Almunawarah are needed.

Key words: Biomonitoring, Toxicity, *Allium cepa*, Chromosome aberrations, Micronuclei, Saudi Arabia.

Introduction

AlMadinah Almunawarah city has the following water resources: groundwater, surface runoff and valleys, desalinated water and treated wastewater (Gutub, 2013). The wastewater is the main non-conventional recourse, and it is obviously will increase with the population increase. Consequently, wastewater has to be effectively treated, and then it will directly or indirectly save ground and desalinated water (Kajenthira *et al.,* 2012). It has documented that approximately 45% of Almadinah city are served by sewer collection systems. This percentage is expected to be 100% by 2030 (Saudi Gazette, 2010). The tertiary treatment plant located in Al-Khulail area in the northern side of AlMadinah Almunawarah is used to treat the wastewater. The water then discharged in the downstream Alhamed valley.

Treated wastewater can be considered as a great solution to meet the demands for water. This water can be used for industrial and agricultural requirements (Miller, 2006). Regarding health issues, the reuse of this water can cause problems for human, soils and crops (Martı´nez *et al.,* 2013).

In general, the wastewater produced in Almadinah city mostly contains various pollutants that come from domestic and industrial sources. Despite the fact that the composition of wastewater possibly differ from community to community, organic and inorganic compounds can be easily found in wastewater and it may hardly degrade during treatment (Rank & Nielsen, 1998; Hussain *et al.,* 2002). Irrigation and other ecosystem services can benefit from the urban wastewater (Hussain *et al.,* 2002). Moreover, wastewater can be helpful in different ways including, recharge of groundwater and landscaping such as golf courses, playgrounds and schoolyards. In addition to that it can be used in industry, construction, and many other ways.

It was documented that, the plant located in Al-Khlail area in the northern side of Almadinah Almunawarah operates at a total capacity of 300,000 $m^3 day^{-1}$ (Shraim *et al.,* 2017). In addition to domestic sewage, the plant sometimes receives partially-treated industrial and medical wastewater. The wastewater is treated in the tertiary treatment. Most of treated wastewater is discharged in the downstream nearby Alhamed valley, which recharges nonportable ground water aquifers, and used mainly for agricultural and landscape irrigation. Approximately, 15% of this water is transported in trucks and are used for watering of green areas and trees in public parks and streets of Almadinah Almunawarah (Shraim *et al.*, 2017; Gutub, 2013).

Treated wastewater from Al-Madinah treatment plant may have good quality to meet international standards. Therefore, it will be a valuable source for many usages such as irrigation and industrial. What is more important is that treated wastewater can be essentially used as drinking water (Gutub, 2013). However, the results published. Shraim *et al.,* 2017 have indicated that Almadinah treatment plant has an inefficient protocol (Shraim *et al.,* 2017). Therefore, various hazardous pollutants may not be completely eliminated.

In this study, we used *Allium cepa* assay to evaluate the cytogenetic toxicity of treated wastewater. The onion, *Allium cepa L*. posses many advantages to be commonly used in experiments. *A. cepa L*. has very low cost and easy to handle. Additionally, it has suitable chromosomal features. The onion possesses large and few chromosomes (2n = 16). Therefore, it is a great model to assess chromosome damages and/or disruption of cell division, including aneuploidy (Fiskesjö, 1985, Leme & Marin-Morales, 2008). The onion also has a large data available and does not require more space in the lab (Grant, 1994; Khalil *et al.,* 2009), and may be done with a minimum training. The *A. cepa* approach is a more sensitive test system than other tests. It has been widely used to

examine chemical effects. Moreover, it is a great way to monitor the toxic and genotoxic effects of dangerous environmental contaminants (Nunes *et al.*, 2011; Roa *et al.*, 2012).

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.

Materials and Methods

Samples collection and pre-treatment: Treated wastewater samples were collected from the only municipal sewage treatment plants (STP) in Almadinah Almunawarah in glasswares that were cleaned by soaking overnight in 10% nitric acid and rinsing with distilled water. Wastewater samples were collected in February and July 2016 (representing winter and summer) from the outlet after chlorination (tertiary treated, effluents). Composite samples were collected for 24 h using a portable water sampler (WS750 dual bottle sampler from Global Water, CA, USA) at a rate of 100 ml h^{-1}. Samples were transported to the laboratory, the pH was adjusted to 7.2–7.6, filtered using vacuum filter funnel (porosity 25–50 µm, Aldrich) and stored at room temperature (18-20ºC).

Preparation of root tips: *Allium cepa* L bulbs (5-6 cm diameter) were obtained commercially. They did not have any leaves or roots. Dried epidermis and dry roots were eliminated. The bases of the bulbs were submerged in tap water. The water was changed daily. When the tips reach a length of 1-1.5 cm, while intact on the bulb, they were placed in a vial containing one of the following five effluent concentrations: (100%, 50%, 25%, 12.5% and 0%) for 8 h. Treated bulbs were placed in distilled water for 24 h (2-3 bulbs used for each treatment). The tips were cut and pretreated with 0.05% colchicine solution for 2.5 h in a dark place. Fixation and chromosome preparation were done according to a standard squash protocol described by Khalil *et al.*, 2009. Carnoy's fixative (3:1, absolute alcohol: acetic acid) was used for 24 hours to fix the selected tips. Fixed roots were kept in 70% ethyl alcohol in a refrigerator. Mitotic study was carried out using the Feulgen squash technique. Distilled water was used to wash treated roots. Roots then were hydrolyzed in 1 N HCl at 60ºC for 10 minutes, washed with distilled water, stained in leuco-basic fuchsin stain for at least 15 minutes at 25ºC in the dark. Each treatment was preformed independently. Three roots in triplicates were used for scoring.

Mitotic index determination: To calculate mitotic index, 2,000 cells per slid were examined (6,000 cells for three slides). Cells were observed under the microscope. All different stages in the cell cycle were quantified (i.e. interphase, prophase, metaphase, anaphase and telophase). Therefore, cell toxicity and genotoxicity were evaluated. The following formulae were used for mitotic and phase index (Seth *et al.*, 2008).

$$\text{Mitotic index (\%)} = \frac{\text{Number of cells in mitosis}}{\text{total number of cells}} \text{ X } 100$$

$$\text{Phase index (\%)} = \frac{\text{Number of cells in each phase}}{\text{total number of cells in mitosis}} \text{ X } 100$$

Cytogenetic bioassay: At least 50 metaphases were analyzed, from each treatment, using a high power (100X) light microscope. Micronuclei (MN) in Interphase and chromosomal aberrations (CA) in mitosis stages (anaphase-telophase) were quantified. Both % Abc and % Mi were calculated, corresponding the control using tap water. For each treatment concentration, experiments were performed in triplicate.

Statistical analysis

The *A. cepa* CA bioassay data analyzed using the software package SPSS 17.0 after initial natural logarithm (ln) data transformation to obtain a normal distribution of variables and homogenous variances. The frequencies CA and aberrant cells of the samples in each of the five concentrations were subsequently compared by one-way ANOVA followed by the Tukey test for multiple comparisons. Values of $p<0.05$ indicated significance.

Results

Chromosomal abnormalities: Microscopic investigation of *A. cepa* root tip cells displayed that there were numerous observable types of chromosomal anomalies that occur through their mitotic cycle, (see Tables 1 and 2, Figs. 1-4). At the two seasons, cell division (Mitotic index) of treated *A. cepa* was decreased compared to the control group. Furthermore, the data showed that cells displayed high chromosome abnormalities in both seasons. For both seasons, there were statistically significant differences in the means of % Abc and % Mi ($p\leq 0.05$) compared to the control group. There were also statistically significant differences in the means of % Mi and % Abc of *A. cepa* meristem cells that were exposed to concentrations: 100%, 50%, 25% and 12.5%) of the two seasons compared to the control group. The exception noted was the 12.5 dilution of both seasons which showed no significant differences between its mean and the control mean in the case of % Abc and % Mi.

Summer treatment test: In summer time, *A. cepa* that treated with the control water (tap water) displayed 24.6% and 8.4% in their (% Abc) and (% MI) respectively (Table 1, Figs. 1 and 2). However, abnormalities in control were mostly C - mitosis and sticky.

Generally, *A. cepa* that treated with treated wastewater showed a significant decrease in mitotic index compared to the control. The lowest MI was 4.4% at the concentration 100%, whereas, the highest MI was 7.9% at the concentration 12.5%n (Table 1, Figs. 1 and 2). Based on these data, we could also conclude that with few exceptions, this parameter was dose dependent where it decreases as concentration increased. Not surprisingly the highest percentage of aberration recorded by the highest concentration 68.2% at 100%.

Table 1. Percentage of aberrant cells and mitosis in *A. cepa* meristems following different treatment with various concentrations of treated sewage water at Al madenah Al munawarah, Saudi Arabia in summer season. Others are some types of chromosomal aberrations such as chromosome blending, uncoiling, syncytium, divergence, resident, sticking and loop.

Treatment	No. of dividing cell	Chromosome aberrations									No. of cells with aberrant chromosomes aberration	% of cells with aberrant chromosomes aberration	MI
		Polyploidy	C- Mitosis	Bridges	Micronuclei	sticks	Lagging	Tripolar	Irregular prophase	Breaks	Others		
Control	505	5	35	4	7	25	0	2	12	1	33	124	24.6 ± 2.01	8.4 ±2.13
100	264	16	30	10	34	19	2	10	26	7	26	180	68.2 ± 2.96**	4.4 ±1.19**
50	353	12	39	22	28	35	1	4	19	3	32	195	55.2 ± 2.31**	5.9 ±2.33**
25	410	7	39	9	15	33	2	3	20	3	34	165	40.2 ± 2.86*	6.8 ±2.38*
12.5	471	4	35	6	12	30	1	3	14	1	27	133	28.2 ± 2.59	7.9 ±1.78

Significant difference from the control * ($p < 0.05$). **Significant ($p < 0.01$)

Table 2. Percentage of aberrant cells and mitosis in *A. cepa* meristems following different treatment with various concentrations of treated wastewater at Al madenah Al munawarah, Saudi Arabia in winter season. Others are some types of chromosomal aberrations such as chromosome blending, uncoiling, syncytium, divergence, resident, sticking and loop.

Treatment	No. of dividing cell	Chromosome aberrations									No. of cells with aberrant chromosomes aberration	% of cells with aberrant chromosomes aberration	MI
		Polyploidy	C- Mitosis	Bridges	Micronuclei	sticks	Lagging	Tripolar	Irregular prophase	Breaks	Others		
Control	504	5	35	4	7	25	0	2	12	1	33	124	24.6 ± 1.84	8.4±2.13
100	244	15	28	10	31	18	2	10	24	6	24	168	68.9 ± 3.03**	4.1±2.54**
50	330	12	36	21	27	33	1	4	18	3	30	185	56.1 ± 3.11**	5.5±2.38**
25	382	7	37	8	14	31	2	3	19	3	32	156	40.8 ± 2.43*	6.4±2.07*
12.5	437	4	33	6	10	27	1	3	13	1	24	122	28.0 ± 2.14	7.3±2.32

Significant difference from the control * ($p < 0.05$). **Significant ($p < 0.01$)

Regarding to the mutagenicity of *A. cepa* that exposed to treated wastewater in summer season, the percentage of total aberrations and percentage of the observed types of abnormalities were high compared to control (Table 1, Figs. 1 and 2). For the former it was found in contrast with MI parameter where it increased as the concentration increased.

Furthermore, cells that treated with wastewater displayed more chromosomes aberrations (CA) and less MI compared to the control group. The effect in both cases was concentration-dependent.

Winter treatment test: In winter, results of cells that exposed to treated wastewater were similar to the summer result. However, some few differences were noticed. Following the same pattern of summer time, MI values were decreased as the concentrations increase. The MI was 7.28 and 4.07 at 12.5% and 100% respectively (Table 2, Figs. 3 and 4).

It is obvious that percentages of chromosomal aberrations in winter were similar to the aberrations in summer time. Highest and lowest CA were 68.9% and 28.0% at 100 % and 12.5 % respectively (Table 2, Figs. 3 and 4). These values were 68.2% and 28.2% of the treatments of summer time respectively (Table 1).

Discussion

Water pollution has crucial effects on land, underground water, biotic and abiotic component of the ecosystem. In addition, it is also causing mutagenic effects on strategic crops, vegetables, animals and human. Therefore, it really has high ability to promote genetic damage that can be inherited and affect coming generations and cause numerous health problems (Batista *et al*., 2016).

Due to desert climate, high temperature and agriculture water loss at Almadenah Almunawarah city, Saudi Arabia. Therefore, treated water is considered as an easy and available source of water in this area. In addition, many farmers might have not the knowledge of the bad effect of treated wastewater. As a result of that, the usage of this treated wastewater will increase. Not surprisingly, crops and vegetables irrigated with polluted water or grown in polluted soil will cause many health problems for human (Wahid *et al.,* 2004) as mental retardation, liver and kidney damage (Matsuno *et al.,* 2004; Uzair *et al.,* 2009).

The low percentage of MI in this study indicates cell cycle disturbances or chromatin dysfunction because of pollutant DNA interactions. Moreover, the presence of heavy metals, trace metals and pesticides are highly expected in the treated wastewater. Consequently, types of genomic damages are expected (Marcano *et al*., 1998; Fatima & Ahmad, 2006).

Overall look on the present data for the treated group, a significant decrease in mitotic index is obvious compared to the control and it is clearly affecting cellular proliferation. The lowest MI was 4.07% at100% concentration. This result proves the cytotoxic of such treated water as it is mostly used for irrigation with this concentration. These findings are consistent with that previously recorded by many authors (Sandra *et al.*, 2010; Maxim *et al.*, 2013; Asya *et al.*, 2015). According to these data we could also conclude that with few exceptions, this parameter was dose dependent where it decreased as concentration increased.

It was documented that higher percentages of aberrations indicated the cytotoxic effects of wastewater on cell division. The mutagenic activity of treated wastewater showed various types of aberrations, these types were polyploidy, micronucleus (Egito *et al.*, 2007). Moreover, treated wastewater caused sticky chromosomes at various stages of mitosis, bridges, c-mitosis (metaphase and anaphase) and disturbed cells. Thus, treated wastewater, lead to a complete destruction of nucleus structure and hydrolyze the nucleic acid material. According to the cytotoxic effects of this water on living cells, heredity material will be also affected.

Aberrant mitotic stages can occur as result of spindle poisoning. Therefore, it leads to chromosome disruptions during mitotic cell division. Toxic metals can induce genotoxicity in two pathways. First of all, it can do cross-linking with DNA and/or proteins. Second, it can generate and reactive oxygen species (Costa *et al.*, 2002; Chandra *et al.* 2005; Fatima & Ahmad, 2006; Glin´ska *et al.*, 2007; Achary *et al.*, 2008; Kaur *et al.*, 2014). It has been found that samples from downstream of a chemical factory displayed stickiness more than the negative control.

Moreover, samples from downstream of an artificial fertilizer plant exhibited anaphase bridges and stickness more than the negative control (Radic´ *et al.*, 2010). In addition, it also found that areas close to a pharmaceutical plant encourage more c-mitosis, anaphase bridges, and stickiness to occur than did the negative control (Radic´ *et al.*, 2010).

Water quality can be clearly affected by climate. Additionally, the effects of environmental pollutants could be clearly examined by biological assays even if the effects of chemicals were not known (Chandra *et al.*, 2005; Barbosa *et al.*, 2010). However, the presence of hazardous environmental pollutants in the water can be observed in the Allium test (Vujosˇevic´ *et al.*, 2008).

Finally, based on presented data in this study we could confirm that cytotoxic effect of treated wastewater in Al-Khlail area in the northern side of the city of Al-Madinah Al-Munawarah, and in spite of its treatment is still not suitable for human use or agricultural purposes. Accordingly, we strongly suggest that treated wastewater must be biologically tested after chemical treatment to ensure it is safe for human being usage.

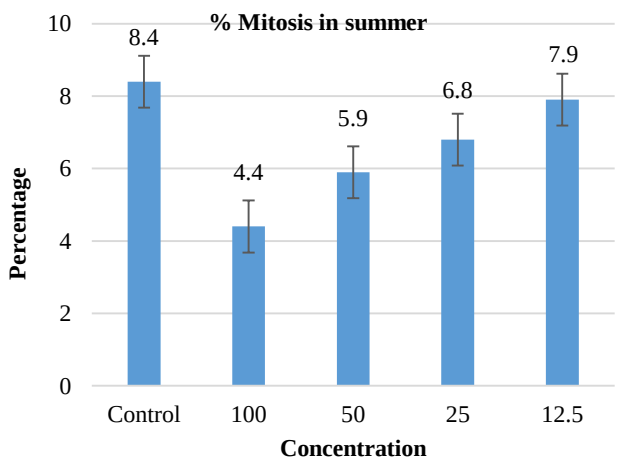

Fig. 1. Percentage of mitosis in *A. cepa* meristems following different treatment with various concentrations of treated sewage water at Al madenah Al munawarah, Saudi Arabia in Summer season.

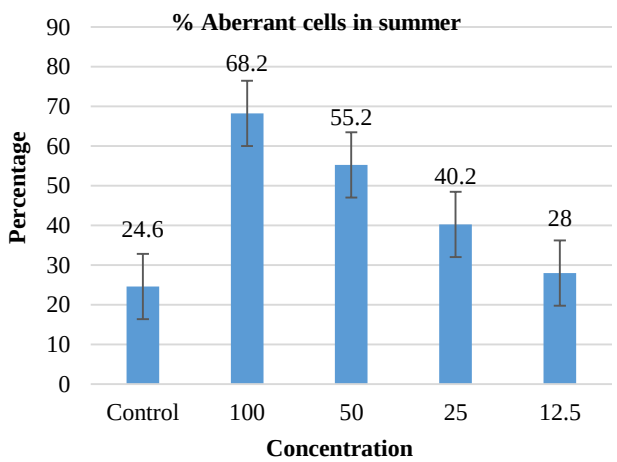

Fig. 2. Percentage of aberrant cells in *A. cepa* meristems following different treatment with various concentrations of treated sewage water at Al madenah Al munawarah, Saudi Arabia in Summer season.

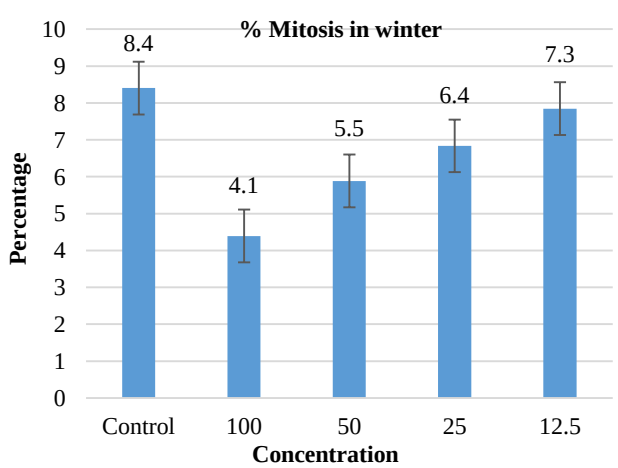

Fig. 3. Percentage of mitosis in *A. cepa* meristems following different treatment with various concentrations of treated sewage water at Al madenah Al munawarah, Saudi Arabia in Winter season.

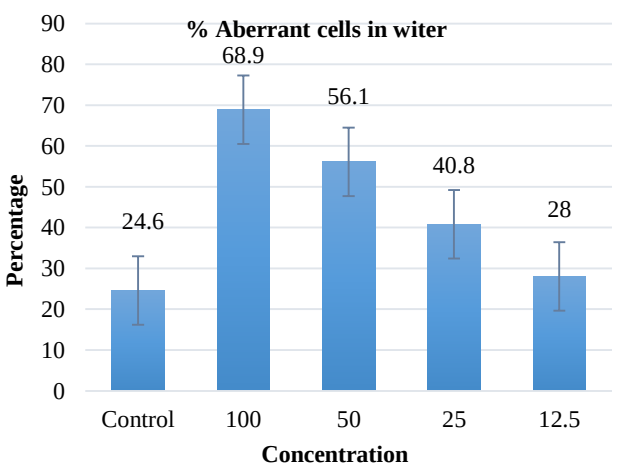

Fig. 4. Percentage of aberrant cells in *A. cepa* meristems following different treatment with various concentrations of treated sewage water at Al madenah Al munawarah, Saudi Arabia in Winter season.

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