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EVALUATION OF CYTOTOXICITY AND GENOTOXICITY OF TREATED WASTEWATER USING *ALLIUM CEPA* ASSAY IN ALMADINAH ALMONAWRAH, SAUDI ARABIA

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Abstract

The aim of this study was to evaluate the cytotoxicity and genotoxicity of treated wastewater that is discharged in the downstream Alhamed valley, in Almadinah Almonawrah city, Saudi Arabia. Treated wastewater samples were collected from the outlet of sewage treatment plant (STP) and three downstream locations, approximately (10, 20, and 30 km) of the plant. The cytotoxicity and genotoxicity of the samples were assessed using *Allium cepa* mitotic index (MI) and chromosome aberrations (CA) assays respectively. Onion bulbs were exposed to different concentrations (100, 50, 25, and 12.5%) of treated wastewater for 12 hours. Bulbs exposed to tap water were used as negative controls. Chromosome aberrations (CA) were induced by treated wastewater in a dose dependent manner. In addition, CA were significantly higher at the outlet of STP than the downstream locations. Moreover, the frequency of CA decreased as the distance from STP increased. With respect to cytotoxicity, treated wastewater from all locations induced significant decreases in MI and significant increases in the frequency of aberrant cells except the 30 km sampling point (concentration 12.5%). In conclusion, treated wastewater seems to possess genotoxic and cytotoxic compounds that get eliminated by movement of the water away from STP.

Key words: Cytotoxicity, Genotoxicity, *Allium cepa*, Chromosome aberrations, Saudi Arabia.

Introduction

Treated wastewater is considered a valuable water resource for Almadinah Almunawarah, in addition to groundwater, surface runoff and desalinated water (Gutub, 2013). As result of the increase in Almadinah Almunawarah population, treated wastewater is expected to increase in the coming years. Effective treatments of wastewater will be critical to save ground and desalinated water (Kajenthira *et al.,* 2012). Sewer collection system is providing about 45% of the city needs of water. By 2030, this percentage is thought to reach 100% (Saudi Gazette, 2010). Wastewater in Almadinah Almunawarah is treated by the tertiary treatment plant which is located in Al-Khulail area in the northern side of the city. Treated wastewater is then discharged into Alhamed valley to be used by industrial and agriculture sectors (Miller, 2006). The treated water is used mainly by farmers for agriculture, watering green areas and trees in public parks and streets in the city (Gutub, 2013; Shraim *et al.*, 2017). In addition, wastewater can be a valuable source for ecosystem services (Hussain *et al.*, 2002). Furthermore, wastewater can be used in many different helpful ways including recharge of groundwater. However, soils, crops and human health can easily be affected negatively by the reuse of inefficient treated wastewater (Martínez *et al.,* 2013; Jaishankar *et al.,* 2014).

Variety of pollutants coming from domestic and industrial sources can be found in the treated wastewater including organic/inorganic compounds, and heavy metals (Akpor *et al.,* 2014). Water treatment may not be sufficient to degrade these compounds (Rank & Nielsen, 1998; Hussain *et al.,* 2002). The capacity of sewage treatment plant in Almadinah Almunawarah is 300,000 m3 day−1 (Shraim *et al.,* 2017). The plant receives domestic sewage, medical and partially-treated industrial wastewater. Wastewater is supposed to be treated in the plant to reach an international standards quality. Nevertheless, it was documented that the treatment plant does not have an efficient protocol (Shraim *et al.,* 2017). Therefore, in the current study, the genotoxicty and cytotoxicity of treated wastewater from 4 locations downstream of Almadinah Almunawarah treatment plant were investigated using *Allium cepa* assay. This assay has many advantages that make it suitable for such investigation. The *Allium cepa* chromosomes are large and few (2n = 16) and thus, chromosomal damage and cell division disruption can be easily examined (Leme & Marin-Morales, 2008; Khalil *et al.,* 2009; Alotaibi & Barnawi, 2018). Furthermore, using the assay has been shown to be valuable for examining the genotoxicity that can be caused by serious environmental pollutants (Nunes *et al.,* 2011; Roa *et al.,* 2012). The study findings will provide important information about the hazardous contaminants present in the treated wastewater in Almadinah city.

Materials and Methods

Samples collection: Treated wastewater samples were collected from Alhamed valley, northern side of AlMadinah Almunawarah, Kingdom of Saudi Arabia. Samples were collected from four different locations as indicated in Fig. 1. These include the outlet after chlorination (tertiary treated), and three locations downstream of the treatment plant of approximately 10 kilometers apart from each other. Stream wastewater is ended at short distance after location 4. Treated wastewater samples were collected in June 2019 in pre-cleaned (by soaking overnight in 10% nitric acid and rinsing with distilled water) glasswares. The collected samples were transported to the laboratory and the pH of the samples was adjusted to 7.2–7.6. Samples were then filtered using vacuum filter funnel (porosity 25–50 µm, Aldrich) and used directly in the *Allium cepa* assay.

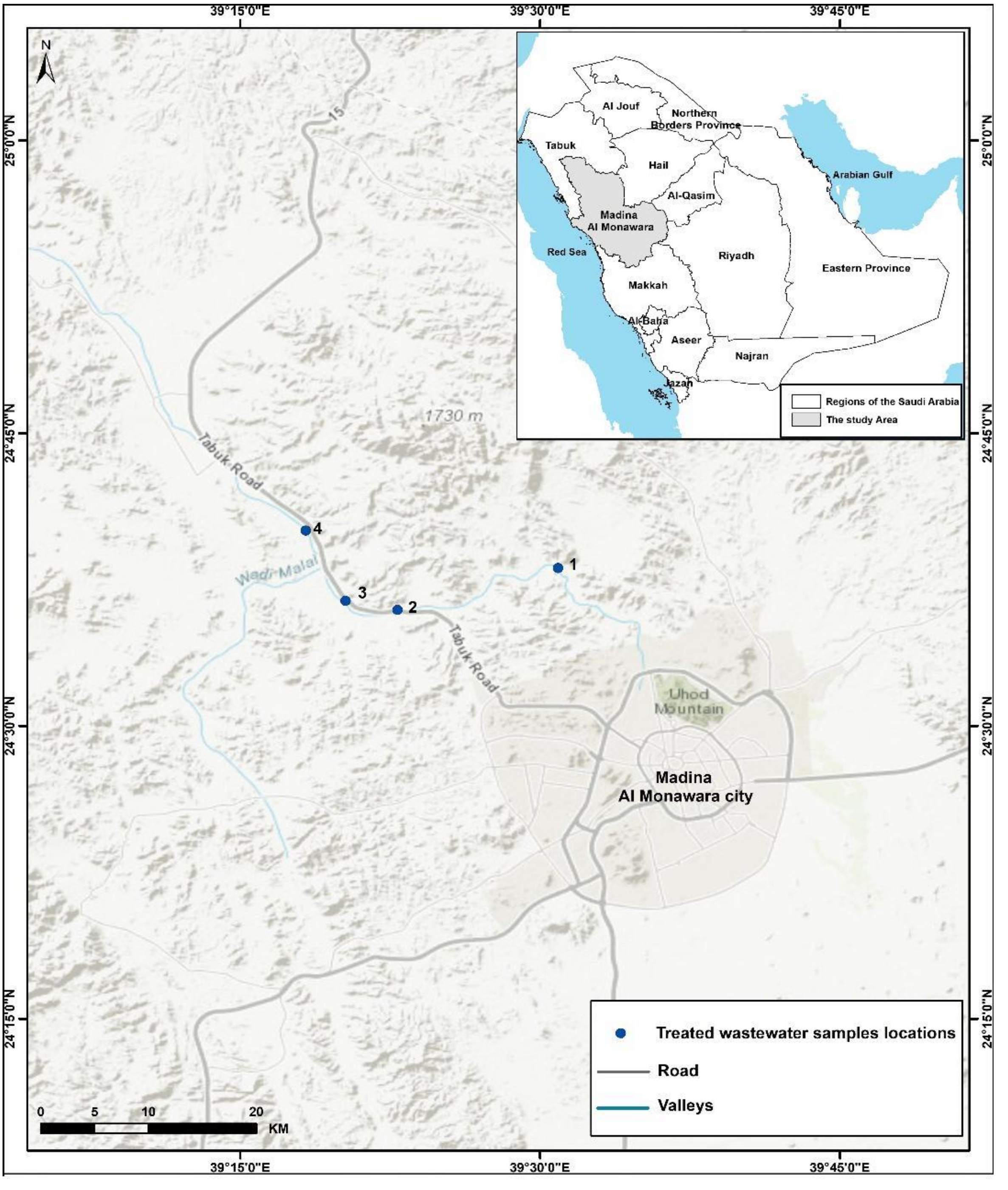

Fig. 1. Location map of sampling in Alhamed valley, northern side of Almadinah Almunawarah, Kingdom of Saudi Arabia. location 1= 24°38′02.9″N39°30′52.6″E , location 2= 24°35'57.3"N 39°22'52.5"E, location 3=24°36'23.4"N 39°20'17.4"E, and location 4= 24°39'54.6"N 39°18'21.2"E.

***Allium cepa* root tips assay:** Common onion (*Allium cepa* L.) bulbs (~5 cm in diameter) were obtained from the local market. The out surfaces of the onions were carefully cleaned to remove any dry roots. Bulbs of *Allium cepa* were placed in beakers containing tab water. The basal ends were dipped in the water. Tab water was replaced every day. Onion bulbs were replaced in different concentrations of treated wastewater when their tips were around 1.5 cm. Treated wastewater concentrations were 100%, 50%, 25%, 12.5%. After 8 h in the treated wastewater, for each concentration, 2 -3 onion bulbs were placed in distilled water for 24 h. Then as described in (Alotaibi & Barnawi, 2018); onion tips were treated with 0.05% colchicine solution for 2.5 h in a dark place. Tips were placed in Carnoy's fixative solution (3:1, absolute alcohol: acetic acid) for 24 hours. 70% ethyl alcohol was used to store selected fixed tips in the refrigerator. Feulgen squash technique was used to assess mitotic study. After washing tips with distilled water, 1 N HCl was used to hydrolyze the tips at 60ºC for 10 minutes. Then, tips were washed with distilled water and then leuco-basic fuchsin stain was used to stain the tips for at least 15 minutes at 25ºC in the dark. Treatments were done independently and in triplicates.

Mitotic index: Light microscope was used to calculate Mitotic index. Three slides were examined for each treatment concentration (2,000 cells for each slide). All stages in the cell cycle (interphase–telophase) were examined. Consequently, cell cytotoxicity was evaluated using the following formulae for mitotic index (Seth *et al.*, 2008).

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

Micronuclei and chromosomal aberration assay: High power (100X) light microscope was used to analyze at least 50 metaphases (per each treatment) for the presence of chromosomal aberrations (CA) and micronuclei (MN) in all mitotic stages (anaphase - telophase). Experiments were done in triplicates.

Statistical analysis: Data was expressed as mean ± SEM using Graph Pad Prism software (version 5) and ANOVA followed by Tukey post-hoc test. $p<0.05$ was considered significant.

Results

Chromosomal abnormalities and Mitotic Index: Microscopic examination of the squashes of *Allium cepa* root tip meristem cells showed several types of chromosomal abnormalities that occurred through their mitotic cycle (Table 1). These include polyploidy, bridges, breaks, micronuclei, lagging, and others (Table 1). The data showed that at the four locations, the number of cells that have chromosome aberrations increased as indicated by % of aberrant cells (Table 1, Figs. 2 and 3). The effects in the four locations cases were concentration-dependent (i.e. damage trend was 100>50>25> 12.5, Table 1). In addition, data of Table 1 and Figs. 2 and 3 showed that mitotic index of *A. cepa* root tip cells were reduced by treatment with wastewater collected from the four locations (p<0.05). Moreover, there were statistically significant differences in the means of % MI and % aberrant cells between the collected samples from the four locations (p<0.05). The exception noted was the 12.5 dilution of location 4 which showed no significant differences between its mean and the negative control mean in the case of % Abc. In general, the magnitude of induced genotoxicity and cytotoxicity decreased as we go downstream of the wastewater treatment plant (location 1 to location 4).

Discussion

Treated wastewater can be considered as a valuable source for Almadinah. It can be used in many ways including irrigation and industrial use. More importantly, treated wastewater can be used for drinking (Gutub, 2013). Various pollutants can be found in the treated wastewater that produced in Almadinah. The fraction of these pollutants might be partially degraded during treatment (Rank & Nielsen, 1998; Hussain *et al.*, 2002). Thus, treated wastewater will carry part of these compounds. The most serious of the pollutant compounds that can be exist in the treated wastewater are heavy metals (Akpor *et al.*, 2014). Human activities may lead to accumulation of heavy metals in treated wastewater. However, heavy metals are non-biodegradable compounds; therefore they can be found in the water for a long time (Jern, 2006). Heavy metals can negatively affect living organisms' lives. For instance, seed germination and plant growth can be decreased (Gardea-Torresdey *et al.*, 2005). Moreover, animals can be clearly affected by heavy metals. This affect can be seen as organ damage, reduction of growth, and cancer which may lead to death (Rehman *et al.*, 2018). Moreover, heavy metals can lead to many chromosomal aberrations such as chromosome break, chromosome loss, c-mitosis, chromosome bridge and polyploidy (Aasha *et al.*, 2019).

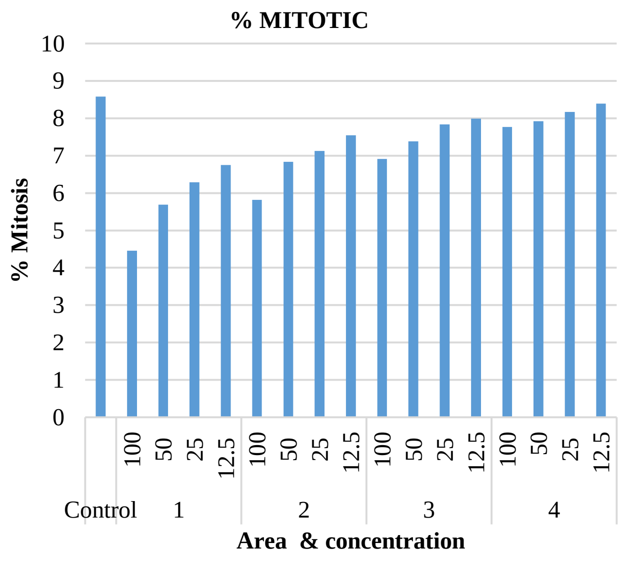

Fig. 2. Percentage of mitosis in *A. cepa* meristems following different treatment with various concentrations of treated wastewater at four different locations (1- 4) in Alhamed valley in Almadenah Almunawarah, Saudi Arabia.

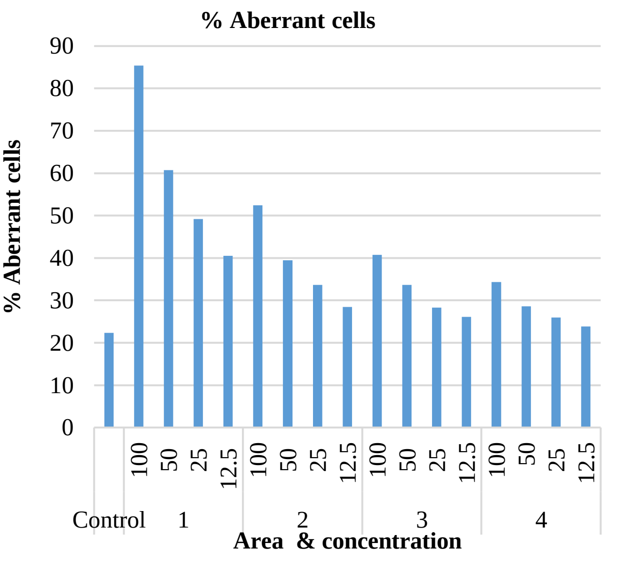

Fig. 3. Percentage of aberrant cells in *A. cepa* meristems following different treatment with various concentrations of treated wastewater at four locations (1- 4) Alhamed valley in Almadenah Almunawarah, Saudi Arabia.

Table 1. Percentage of aberrant cells and mitosis in *A. cepa* meristems following different treatment with various concentrations of treated wastewater at four different locations (1- 4) in Alhamed valley in Almadenah Almunawarah, Saudi Arabia. Values are: Mean ± S.E.M.

Treatment		No. of dividing cell	Chromosome aberrations										No. of cells with aberrant chromosomes aberration	% Aberrant cells	% Mitosis
			Polyploidy	C-Mitosis	Bridges	Micro-nuclei	sticks	Lagging	Tripolar	Irregular Prophase	Breaks	Others*			
Control		515	10	27	4	7	21	4	2	12	1	27	115	22.33	8.58
1	100	267	18	33	20	35	21	9	11	29	8	44	228	85.39 ± 2.64 ***	4.45 ± 2.33 ***
	50	341	12	29	19	28	35	8	10	21	7	38	207	60.70 ± 2.16 **	5.68 ± 2.26 **
	25	378	11	28	16	24	34	6	8	20	5	34	186	49.21 ± 2.68 **	6.30 ± 2.45 **
	12.5	405	11	26	14	21	30	4	6	17	4	31	164	40.49 ± 2.24 **	6.75 ± 2.48**
2	100	349	10	27	17	26	31	7	9	19	6	31	183	52.44 ± 2.43 ***	5. 38 ± 2.51 ***
	50	411	8	26	16	23	27	6	8	17	4	27	162	39.42 ± 2.43 **	6.85 ± 1.87 **
	25	428	6	23	14	22	24	5	6	16	3	25	144	33.64 ± 3.17 *	7.13 ± 2.91 **
	12.5	453	5	21	12	21	21	5	5	14	2	23	129	28.48 ± 2.74 *	7.55 ± 1.87 *
3	100	415	9	27	15	23	29	5	7	19	4	31	169	40.72 ± 3.32 **	6.92 ± 2.87 **
	50	443	7	24	13	20	26	4	6	17	3	29	149	33.63 ± 2.86 *	7.38 ± 3.07 *
	25	470	6	22	11	18	25	3	4	15	2	27	133	28.30 ± 2.68 *	7.83 ± 2.63 *
	12.5	479	6	21	10	17	24	2	3	14	2	26	125	26.10 ± 2.81 *	7.98 ± 3.17 *
4	100	466	9	25	14	22	28	4	5	18	3	32	160	34.33 ± 2.64 **	7.77 ± 2.24 **
	50	475	6	23	12	19	24	3	3	15	2	29	136	28.63 ± 3.08 *	7.92 ± 2.73 **
	25	490	6	21	11	16	23	2	3	15	2	28	127	25.92 ± 2.15 *	8.17 ± 2.69 *
	12.5	503	5	19	10	15	23	2	2	14	2	28	120	23.86 ± 1.75	8.38 ± 1.91

* Significant at ($p<0.05$) as compared to control
** Significant at ($p<0.005$) as compared to control
*** Significant at ($p<0.001$) as compared to control

In this study, genotoxicity effects of treated wastewater on *Allium cepa L* have been evaluated at four locations along Alhamed valley, northern side of Almadinah, Saudi Arabia (Fig. 1). Cells were highly affected at location one. However, cells were less affected at locations two; three and four (Table 1 and Figs. 2 and 3). The lowest MI was 4.45 at 100 concentration on location one. However, the MI was increased gradually to be 8.38 at concentration 12.5 on location four. On the other hand the percentage of chromosomal abnormalities were at the highest value which was 85.39 at concentration 100 of location one. Whereas, the lowest value for chromosomal abnormalities was 23.86 at 12.5 concentration of location four. As indicated by MI and Abc, cells were gradually affected starting from location one and ending at location four. Cell cycle disruption and chromosomal abnormalities are mostly due to pollutants DNA interactions. The results of this study are consistent with previous documented studies by many authors (Radić *et al.,* 2010; Maxim *et al.,* 2013; Dragoeva *et al.,* 2015; Alotaibi & Barnawi, 2018). Moreover, treated waste water can clearly cause different types of mutagenic activities such as polyploidy and micronucleus (Egito *et al.,* 2007). Additionally, sticky chromosomes, bridges and c-mitosis can be seen as result of treating cells with treated wastewater. Pollutants in treated wastewater will surly affect heredity of living organisms (Glinska *et al.*, 2007; Achary *et al.*, 2008; Kaur *et al.*, 2014; Abubacker & Sathya, 2017).

It has been recently documented that contamination of soil by heavy metals which located near wastewater pond depends on various factors. One of these factors is how far the soil from wastewater pond, which has heavy metals (Alaswad, 2020). Moreover, the author found that some heavy metals are able to move in soil than others. Consequently, close soil to wastewater was highly contaminated with heavy metals, whereas far soil was less affected by heavy metals. Our result in this study is consistent with Saleh O. Alaswad, study. Cells exposed to treated wastewater from location one displayed high chromosomal abnormalities. While, cells exposed to treated wastewater from locations two, three and four displayed less chromosomal abnormalities (Table 1, Figs. 2 and 3). However, the lowest chromosomal abnormalities value was found in location four. MI was found significantly lower in location one compared to the control group (see Table 1 and Fig. 2). Additionally, MI was increased gradually in locations two, three and four. However, there was no significant difference between MI at concentration 12.5 in location four and the control group. As mentioned in our study and Saleh O. Alaswad, it is obvious that far place from source of wastewater receives low amount of heavy metals and other pollutants. Accordingly, wastewater from far locations will not lead to high chromosomal abnormalities.

In conclusion, treated wastewater seems to possess genotoxic and cytotoxic compounds that get eliminated by movement of the water away from STP. Thus, heavy metals and other pollutants might be precipitate along the stream of the treated wastewater.

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