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ASSESSMENT OF CYTOTOXICITY AND GENOTOXICTY OF TREATED WASTEWATER BY *ALLIUM CEPA* BASED BIOASSAY: A CASE STUDY IN TABUK, KSA

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Abstract:

Water is an elixir for life, yet we are not able to replenish it to nature at the same pace with which we are extracting it. Tabuk, the capital city of the north-west region of KSA produce 110,000 m3/day of wastewater. This wastewater is treated on the outskirts of the city. KSA is considering wastewater as a major water source and aims to achieve its 100% usage by 2025. In Tabuk, most of the treated wastewater is used for groundwater recharge; our aim was to evaluate the water quality by Allium cepa bioassay. This is the first report from the KSA and gives an insight into the cyto-genotoxicity of treated wastewater in the arid environment. In this current study released treated wastewater from Tabuk, wastewater treatment plant was collected from different locations and its physicochemical properties were examined, tap water and distilled water was used as the control. In addition, assessment of cyto-genotoxicity of the said treated wastewater was done by A.cepa bioassay. The water quality parameters such as pH, temperature, conductivity, TDS and Turbidity values shows an increasing trend as we move from S1 to S4. Salinity values were constant and dissolved oxygen value was highest at S1 as compared to all other sites. The heavy metal values of Cr, Mn, Cu, Zn, Cd, and Pb were decreased from S1 to S4. In A. cepa test, macroscopic parameters, root number, and root length were increased from S1 to S4. The microscopic parameter like mitotic index was significantly inhibited at S1 as compare to other sites. Abnormal cell shape, spindle disorientation, chromosomal adherence, fragmentation, chromosomal bridges, and micronuclei were recorded after 5 days. The values concluded that water at S1 and S2 was more harmful compared to S3 and S4. The elemental analysis at S4 and the values of root number, root length, fewer abnormalities in the cell shows possible phytoremediation effect of Phragmitis sp.

Key words: *Cytotoxicity, Genotoxicity, Chromosomal bridges, Phytoremidiation, Aquifer recharge.*

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INTRODUCTION:

We are living in an era where natural resources are depleting and not fit for human consumption due to anthropogenic activities. One such resource is groundwater, water is considered an elixir for life, yet we are not able to replenish it to nature with the same pace with which we are extracting it. Further, it is a scarce resource in the Kingdom of Saudi Arabia (KSA), one of the largest arid countries without permanent rivers or lakes (Aquastat, 2015). Nowadays, partially treated wastewater is used as an artificial method for recharging the groundwater (El-Shaikh and Alhemaidi, 2014; Page et al., 2018). The KSA considers treated wastewater as a major water source, aiming to achieve its 100% use by 2025 (Ouda, 2016). This was aimed to achieve by spending SR 50 billion by the National Water Company (NWC) of KSA on new projects from 2013 to 2017 to improve and increase the Kingdom's water and wastewater infrastructure (Saudi Gazette, 2013). Recently, an announcement was made to add 204 new sewage treatment plants in KSA by 2019 it will treat 2.9 billion m^3 of sewage from the household sector (Saudi Gazette, 2018).

The public water supply system of KSA depends upon groundwater and it is also a critical source for agriculture and industries (Drewas et al., 2012). But as it is often the case with critical resources, groundwater is not always available when and where needed, especially in water-short areas where heavy use has depleted underground reserves. The total actual yearly renewable freshwater resources in Saudi Arabia is estimated to be 2.49 m^3, meanwhile, its yearly freshwater withdrawal is about 22.59 m^3 which is 9.4 times the renewable freshwater resources (Frenken, 2009)

There are additional advantages of artificial groundwater recharge with reclaimed water such as reduction of groundwater levels decline, protection of underground freshwater in coastal aquifers against intrusion from the ocean, and storage of reclaimed water for future reuses (Miller, 2006; Heir, 2016). Water quality standards for indirect potable uses have not been formally adopted in Saudi Arabia (WHO, 2006). However, there is a current effluent guideline standard for unrestricted agricultural reuse enforced by the Ministry of Agriculture and Water (US EPA Guidelines, 2004). It is likely that water quality considerations will be the most stringent for direct injection, as this alternative places treated water directly into the aquifer and has the potential for the shortest residence times in the environmental buffer (Lacy, 2005). Guidelines for aquifer recharge are generally expected to maintain, at a minimum, a drinking water quality standard with additional treatment burdens for multiple barriers with stringent disinfection for pathogen removal. Despite the fact, water treatment in KSA is of the high standard only a small fraction (9%) of the treated water is used for irrigation. The farmers face a lot of problem in using sewage water for irrigation (Saudi Gazette, 2018). Keeping all the above facts, this project was designed to assess the physicochemical properties of the treated wastewater along with heavy metal analysis. Further, cyto-genotoxicity of Treated Wastewater (TWW) was quantified by *A. cepa* based bioassay.

MATERIAL AND METHODS:

Site of Work:

Tabuk is one of the largest regions in Saudi Arabia which covers an area of 139,000 km^2 and corresponds to about 7% of the country's total area. In general, the climate of this region is considered as arid (Al-Nafie, 2008). The recent population of Tabuk city is about 520,000 capita with average yearly increasing rate of 3.3% (Statistic yearbook, 2010). The average daily water consumption is 280 liter per capita from the groundwater. In Tabuk city around 110000 m^3/day of wastewater is produced which is treated in a central plant located at the outskirts of the city.

Water sampling:

Water Samples were collected as per American Public Health Association (APHA, 1998) from 4 different locations from the area of Wastewater Treatment Plant (WWTP) and were labeled as Site 1 (S1), outlet of treated water, Site 2 (S2) after 1KM of outlet down the stream, Site 3 (S3) after 1KM of outlet down the stream and Site 4 (S4) after 4 KM of outlet down the stream. Beside these samples Tap water (TW) and Distilled water (DW) was used as Control and negative Control respectively.

Water Quality Parameter:

The TWW, TW and DW were analyzed for a number of standard physico-chemical properties, including pH, salinity, temperature, conductivity, total dissolved solids (TDS), turbidity and dissolved oxygen (DO) as per (APHA, 2005).

Elemental Analysis:

Thirteen metals - Be, Na, Mg, P, K, Ca, Cr, Mn, Fe, Cu, Zn, Cd and Pb were analyzed as per (APHA, 2005). The metal standards were prepared and kept in container pre-cleansed with concentrated nitric acid and distilled water. The analytical determination of trace metal was carried by Inductively Coupled Plasma/Mass Spectrometry (ICP/MS NexION 300D, Perkin Elmer, USA). The following operating conditions of the instruments used in this study. The

RF power was set at 1600W, Nebulizer gas flow was 0.65L/min, Lens voltage 9.55 V, Analog Stage Voltage -1745 V and Pulse stage voltage was 950 V. The absorbance of the standards, treated wastewater and control was taken in triplicate and peak hopping was chosen as scan mode. The dwell time and integration was 40 ms and 1200 ms respectively.

***A. cepa* test:** The *A. cepa* toxicity test was carried out according to the method described by Fiskesjö (1988) with slight modifications for the adoption under Saudi conditions for instance, while Fiskesjö (1988) used the white variety of the onions, locally available varieties of red onions were used and incubation temperature of 25±5ºC instead of 20ºC was maintained. Tap water (containing permissible levels of organic and inorganic substances) was used as control whereas distilled water used as negative control. For the *A. cepa* test, both the macroscopic and microscopic parameters were studied. Among the macroscopic parameters, root numbers and root length was noted down. Mitotic Index (MI), cell, nuclear and chromosomal abnormalities like chromosomal adherence, stickiness, fragmentation, laggards, bridges, and micronuclei were measured as microscopic parameters to ascertain the cytotoxicity and genotoxicity in the *A. cepa*. Photography was done by using Carson HookUpz 2.0 Universal Smartphone Adapter for Microscope using Samsung Galaxy S5 mobile phone.

RESULTS:

Physicochemical properties of water samples:

The water quality parameters of water samples from different sites of treated wastewater along-with tap water and distilled water were recorded in Table 1. The pH of TWW was increased as we move from S1 (6.2) to S4 (7.5) whereas the salinity remained almost constant at all the sites (1.5-1.6 ppm). The temperature of treated water was slightly increased as we move from S1 (26.7 ˚C) to S4 (27.5 ˚C) whereas the temperature of tap water and distilled water was 28.6 ˚C & 26.4 ˚C respectively. Conductivity is greatly influenced by temperature and most fluid increase in conductivity with increase in temperature, this relation was recorded at all sites. TDS analysis conducted at different sites showed an increasing trend from S1 (1800 ppm) to S4 (1995 ppm). Turbidity measures the clarity of the water and is an optical characteristic. In general, water was more turbid at S 4 as compared to control. DO is the non-compound oxygen available in the water body. It is a very good health indicator of a water body. The water samples showed higher DO at the S1 as compare to all other sites. The elemental analysis of water sample showed a decreasing concentration for Be, P, K, Cr, Mn, Cu, Zn, Cd and Pb as we move from S1 to S4 whereas it was increased for Mg, Ca and Fe at same sites (Table 1).

Table No. 1 Physicochemical properties of water samples collected from different sites of treated wastewater in Tabuk region

S. No.	Parameter	Unit	Tap Water	Distilled Water	Site 1	Site 2	Site 3	Site 4	RCYJ Standard
1	pH	-	7.78	7.22	6.2	6.2	6.5	7.5	6-8.4
2	Salinity	ppm	180.5	16	1.5	1.6	1.6	1.6	-
3	Temperature	˚C	28.6	26.4	26.7	27.0	27.1	27.5	-
4	Conductivity	µS	270	6.84	2.81	3.05	3.04	3.72	-
3	Total Dissolved Solids (TDS)	ppm	395.6	6.25	1800	1940	1939	1995	1750
6	Turbidity	NTU	0.00	0.00	6.00	23	16	26	2
7	Dissolved Oxygen (DO)	mg/L	4	3	7.38	6.54	6.4	7.28	-
8	Beryllium (Be)	ug/L	0.059	0.012	0.059	0.036	0.020	0.016	-
9	Sodium (Na)	ug/L	19597.54	32.459	-	-	-	-	-
10	Magnesium (Mg)	ug/L	10243.49	14.90	26985.79	28422.05	28227.28	28845.50	-
11	Phosphorus (P)	ug/L	15.15	14.90	329.71	326.24	329.13	237.49	30,000
12	Potassoim (K)	ug/L	991.00	57.33	4176.28	3982.16	3895.06	3786.73	-
13	Calcium (Ca)	ug/L	10988.07	52.58	25487.93	26540.27	26641.59	26748.59	-
14	Chromium (Cr)	ug/L	1.41	0.57	5.44	4.51	4.25	4.14	-
15	Manganese (Mn)	ug/L	16.00	2.05	6.14	4.54	3.06	2.60	-
16	Iron (Fe)	ug/L	578.57	46.13	1108.33	1188.77	1244.60	1305.18	-
17	Copper (Cu)	ug/L	2.33	0.80	8.27	8.30	7.44	6.49	-
18	Zinc (Zn)	ug/L	72.10	0.97	31.74	12.09	8.29	7.44	-
19	Cadmium (Cd)	ug/L	0.21	0.03	0.23	0.13	0.08	0.06	-
20	Lead (Pb)	ug/L	0.22	0.06	0.85	0.22	0.15	0.15	-

Different Sites of sample collection: S1: Outlet of Treated water, S2: After 1 KM of outlet down the stream, S3: After 2 KM of outlet down the stream, S4: After 4 KM of outlet down the stream. Beside these samples Tap Water (TW) and Distilled Water (DW) was used as Control. Values are means ± standard errors of atleast 5 replicates; within each column, means followed by the same letter are not significantly different at $p \leq 0.05$ according to DMRT.
RCJY-Royal Commission of Jubail and Yanbu (2010)

A. cepa Test

Macroscopic Parameters of Onion Roots: Root Number and Root Length

Root Number and Root Length: The number and length of roots is a direct method to assess the suitability of water for plant growth. In this case, it was well evident after 1, 3 and 5 days of observation of onion roots grown on water samples from different sites of TWW in the Tabuk region (Figure 1). After 1 day the root numbers in TW were (44.00±4.93) and in S4 (54.66±5.23), similarly, after 3 and 5 days, the root numbers were also increased from S1 (64.33±5.36) to S4 (74.33±6.17). However, in all days of observation root numbers in DW were the least. After 1 day of treatment, the root length did not show much variation (Figure 2) and it was almost same in TW (0.84±0.03) and S4 (0.88±0.03) and the least root length was recorded in S2 and S3 (0.69±0.03). After 5 days the data was significantly different at all sites, minimum root length was recorded in DW (1.94±0.09) and maximum was in S4 (4.91±0.21).

Microscopic Parameters of Onion Roots

Mitotic Index: In Table 2 values of Mitotic Index (MI) were recorded and reflect how cells differ in their capability to divide with respect to treated water collected from different sites. In DW the MI was recorded lowest after 1(5.63±0.47), 3 (6.25±0.36) and 5 (6.49±0.42) days of treatment whereas during the same period it was maximum in S4 (9.39±0.41), (9.63±0.39) and (10.07±0.28) respectively. Further at S1 and S2 MI was low as compared to TW in all days of treatments.

Different Types of Abnormalities: In root tip squash preparation cytological, nuclear and chromosomal changes were observed. Abnormalities of variable natures were recorded in Table 3. Based on the data, cells of S1 showed the highest number of cellular abnormalities (2.50±0.28), nuclear abnormalities (2.05±0.36) and chromosomal abnormalities (2.65±0.31). In the case of TW cellular abnormalities was lowest (0.10±0.06) and no chromosomal abnormalities were recorded whereas nuclear abnormalities were lowest in DW (0.10±0.06). The treated WWTP water samples showed a remarkable effect on the cell shape and size, lobed cell, multilobed cell, foot shape cell, hook shape cell and abnormal cell division was recorded in Figure 3. The nuclear abnormalities such a binucleate cell, micronuclei, spoon shape nucleus in extra-large cell and rectangular shape nucleus was recorded in Figure 4. The chromosomal changes were recorded in the form of abnormal metaphase, improper alignment of the equatorial plate as well as disorientation of chromosomes during anaphase (Figure 5). Further chromosomal adherence at metaphase was observed at Site1 and 2. In addition, we recorded chromosomal Bridges, micronuclei, and abnormalities binucleate cell with micronuclei, spoon shape nucleus in extra-large cell and chromosomal loss at site 1.

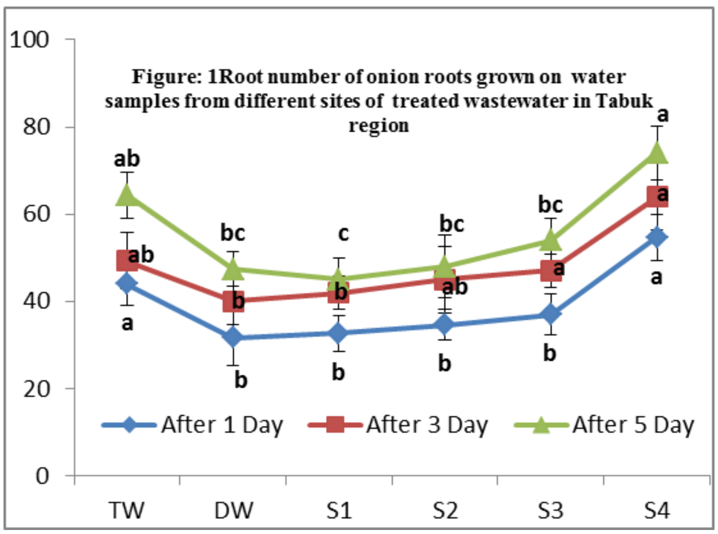


Figure: 1Root number of onion roots grown on water samples from different sites of treated wastewater in Tabuk region

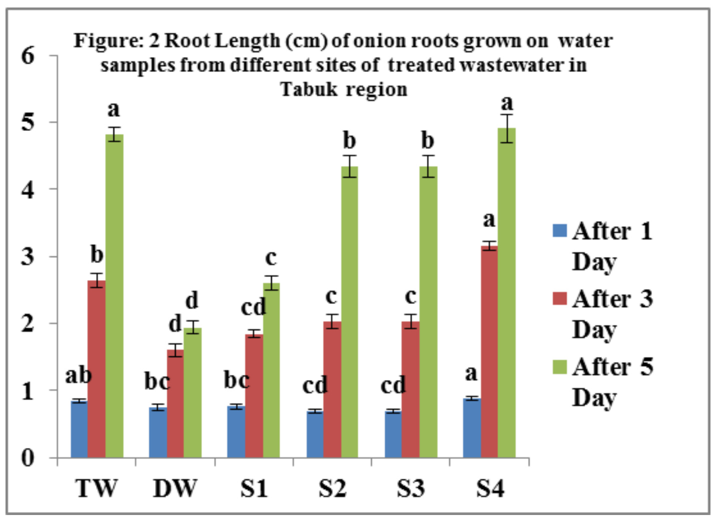


Figure: 2 Root Length (cm) of onion roots grown on water samples from different sites of treated wastewater in Tabuk region

Different Sites of sample collection: S1: Outlet of Treated water, S2: After 1 KM of outlet down the stream, S3: After 2 KM of outlet down the stream, S4: After 4 KM of outlet down the stream. Beside these samples Tap Water (TW) and Distilled Water (DW) was used as Control and Negative control respectively.. Values are means ± standard errors of atleast 5 replicates; within each column, means followed by the same letter are not significantly different at p≤0.05 according to DMRT.

DISCUSSION:

Due to the increasing population and high demands of goods and services large numbers of new cities and industries are mushrooming up in every nook and corner of various countries. Tabuk is one such region, it is considered as one of the fastest growing city in Saudi Arabia, recently the government announced to build an advanced city under NEOM Project in this region to meet the vision 2030 (Saudi Gazette, 2017). It means in the coming years the population of Tabuk will increase at a very fast pace. There is a need to check the available infrastructure of Tabuk, knowing its shortcomings, thereby paving a way to improve the upcoming project. The WWTP of Tabuk is discharging an enormous amount of treated water to replenish the groundwater. This is the first report from the KSA where we investigated the effect of TWW on *A. cepa* bioassay to assess the cytotoxicity and genotoxicity in the arid environment. Further, this report will be helpful to check the suitability of discharged TWW for aquifer recharge. Before performing the bioassay, physicochemical properties of the water was evaluated, parameters such as conductivity, TDS and turbidity showed an increasing trend as we move from S1 to S4 this is in accordance with (Marinelli et al., 2000) which suggest that increase in electrical conductivity of flowing water is due to the presence of dissolved materials. The pH change was not very high and in the purview of RCYJ standard this was in accordance with (Hara and Marin-Morales, 2017), the authors observed no major change in pH of freshwater and treated water. DO is required for the survival of aquatic life and its evaluation reads the biochemistry of streams and lakes. In our study DO was highest at the outlet of WWTP, it was because water was gushing out from the outlet at fast speed and was in direct air contact.

The trace element analysis was done to evaluate the harmful effects of human activities; most of them are ends up in wastewater and are lethal to both humans as well as aquatic life due to bioaccumulation (Gajalakshmi and Ruben, 2014). The major contributors for trace elements in sewage water are household effluents, drainage water, business discharge (Hospital discharge, car washes, other setups etc.) atmospheric deposition and vehicular emissions such as brake linings, tires, asphalt wear, oil leakage etc. (Baysal et al., 2013) Besides that leather, textile, pigments, dyes, galvanizing, electroplating, metallurgical and paint industries release a substantial amounts of heavy metals in wastewater (Ahluwalia and Goyal, 2007). These elements damage the vital body system such as Pb affects the central nervous system, hemoglobin synthesis and cause kidney damage in humans whereas Cd affects calcium metabolism in animals, Cr is accumulated by aquatic species by passive diffusion. The eco-toxicological studies suggests the most dangerous metals are mercury, lead, cadmium, and chromium (VI) (Malik, 2004)

Table 2: Mitotic Index of onion roots grown on water samples from different sites of treated wastewater in Tabuk region

	TW	DW	S1	S2	S3	S4
After 1 Day	6.12±0.61[b]	5.63±0.47[b]	5.97±0.56[b]	8.19±0.61[a]	8.19±0.61[a]	9.39±0.41[a]
After 3 Day	7.64±0.44[bc]	6.25±0.36[d]	7.13±0.58[cd]	8.73±0.48[ab]	8.73±0.48[ab]	9.63±0.39[a]
After 5 Day	8.08±0.31[bc]	6.49±0.42[d]	7.45±0.35[cd]	8.85±0.44[b]	8.85±0.44[b]	10.07±0.28[a]

Different Sites of sample collection: S1: Outlet of Treated water, S2: After 1 KM of outlet down the stream, S3: After 2 KM of outlet down the stream, S4: After 4 KM of outlet down the stream. Beside these samples Tap Water (TW) and Distilled Water (DW) was used as Control and Negative control respectively. Values are means ± standard errors of atleast 5 replicates; within each column, means followed by the same letter are not significantly different at $p \leq 0.05$ according to DMRT.

Table 3: Different types of abnormalities of onion roots grown on water samples from different sites of treated wastewater in Tabuk region

Abnormalities	TW	DW	S1	S2	S3	S4
Cellular	0.10±0.06[c]	0.20±0.09[b]	2.50±0.28[a]	1.90±0.14[b]	0.50±0.11[c]	0.25±0.09[c]
Nuclear	0.25±0.09[c]	0.10±0.06[c]	2.05±0.36[a]	1.15±0.26[b]	0.65±0.18[bc]	0.30±0.10[c]
Chromosomal	0.00±0.00[d]	0.15±0.42[d]	2.65±0.31[a]	1.30±0.28[b]	0.65±0.19[c]	0.30±0.12[cd]

Different Sites of sample collection: S1: Outlet of Treated water, S2: After 1 KM of outlet down the stream, S3: After 2 KM of outlet down the stream, S4: After 4 KM of outlet down the stream. Beside these samples Tap Water (TW) and Distilled Water (DW) was used as Control and Negative control respectively. Values are means ± standard errors of atleast 5 replicates; within each column, means followed by the same letter are not significantly different at $p \leq 0.05$ according to DMRT.

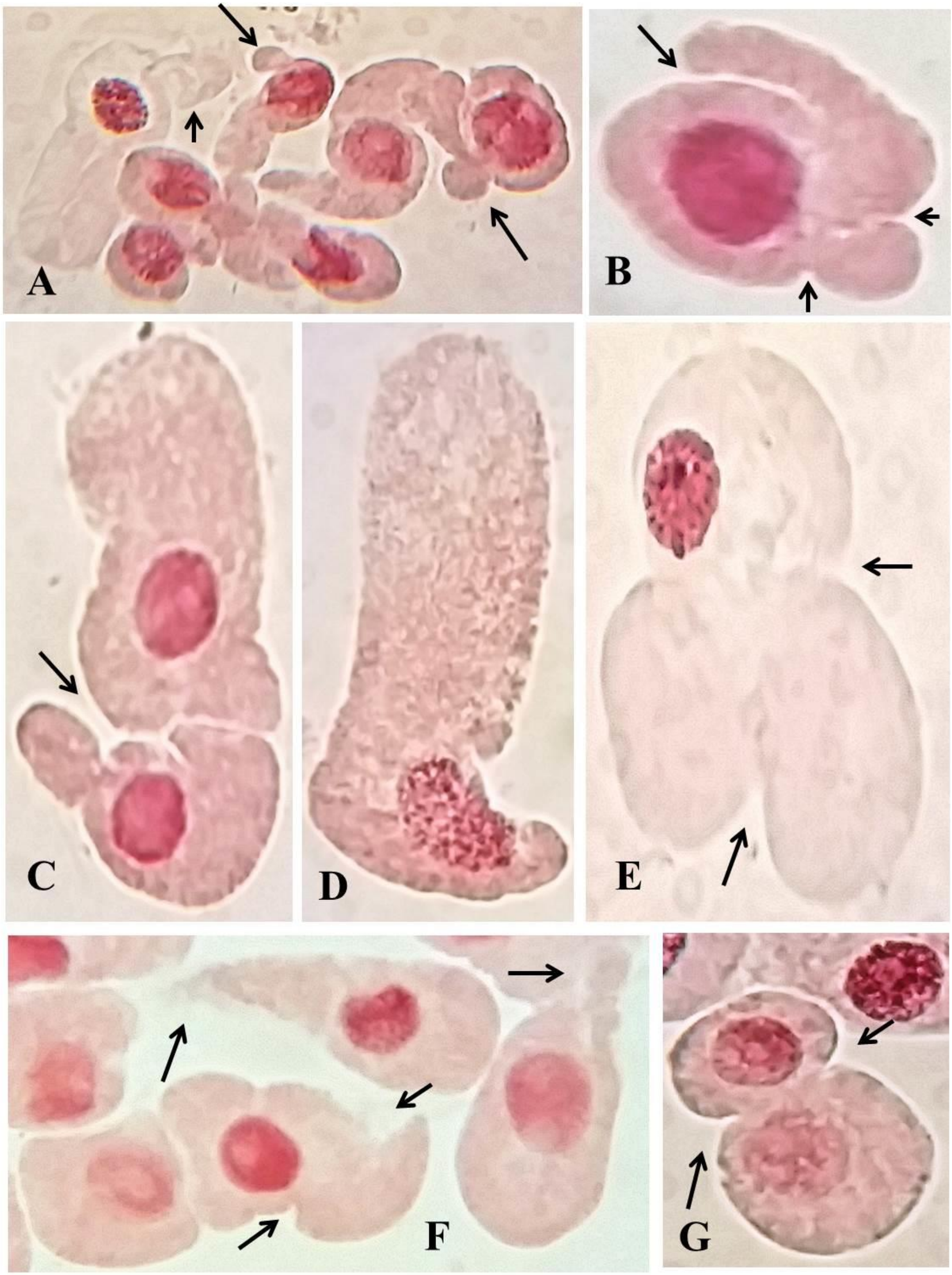
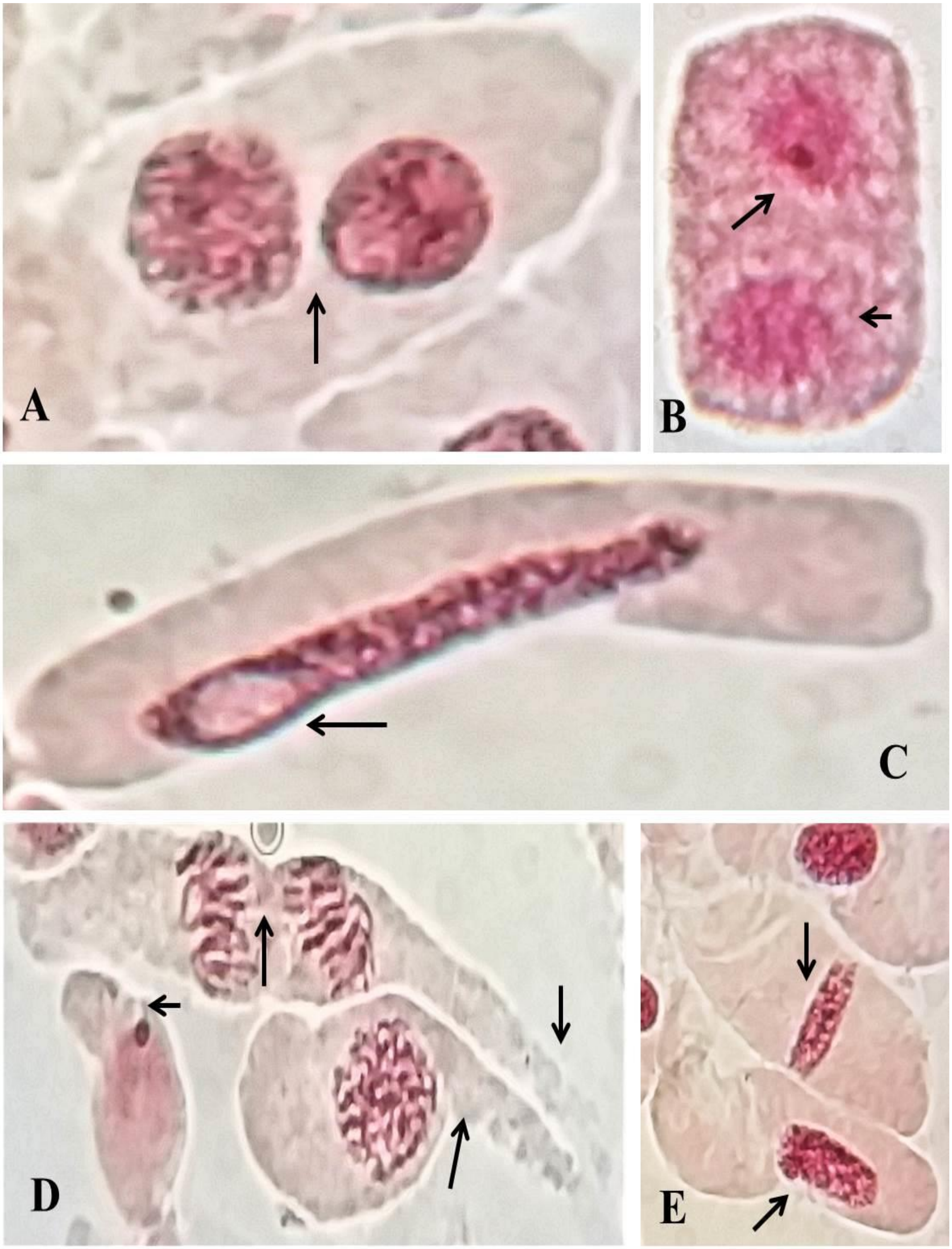


Figure 3: Cellular Abnormalities. A&C: Lobed Cell; B &E: Multilobed Cell; D: Foot Shape Cell; F: Hook Shape and Pointed End Cell; G: Abnormal Cell Division.



gure 4: Nuclear Abnormalities A & B: Binucleate Cell with micronuclei; C: Spoon Shape Nucleus in Extra Large Cell; D: Abnormal Cell with Tail, Micronuclei & Chromosomal Bridge; E: Rectangular Shape Nucleus.

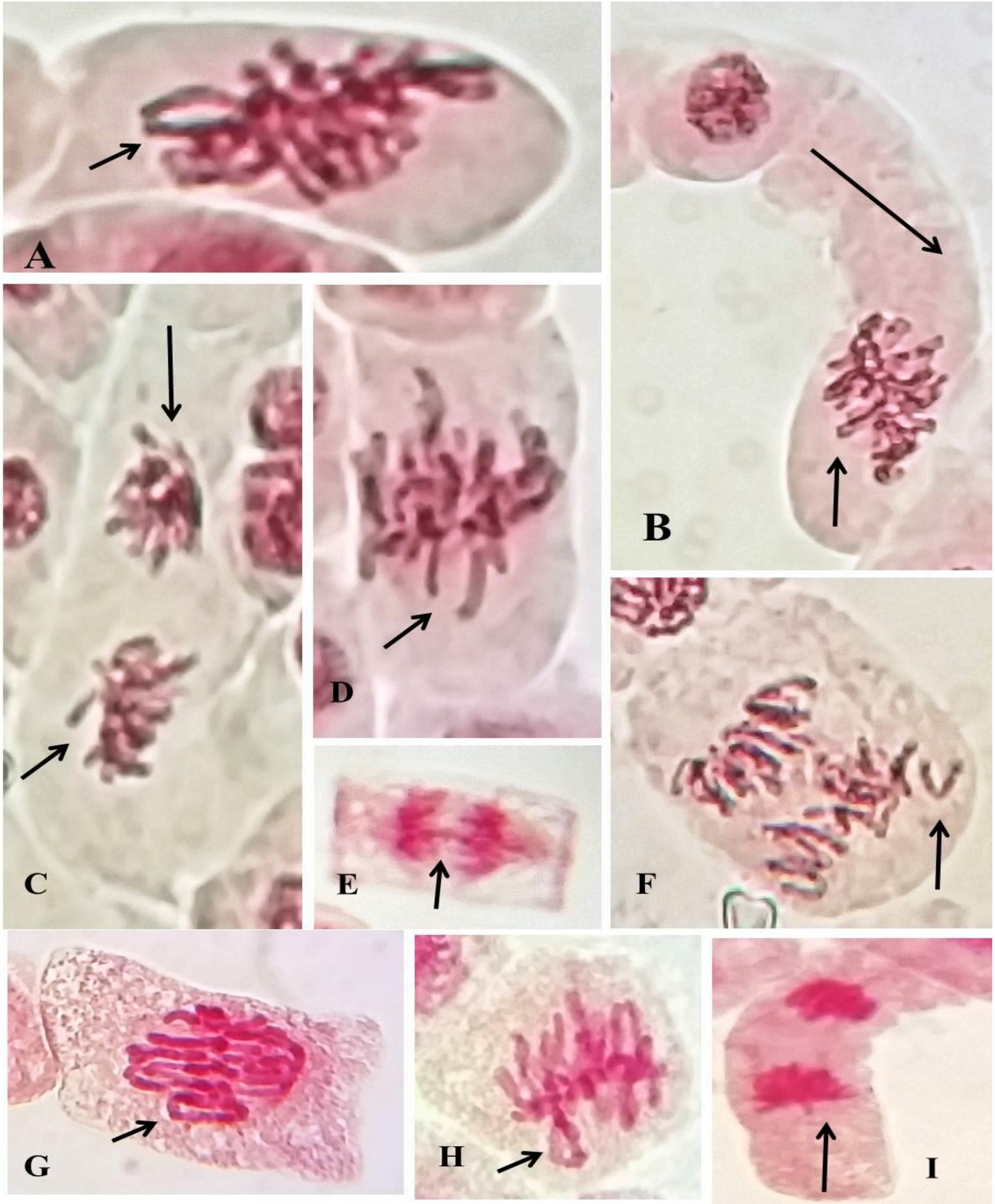


Figure 5: Chromosomal Abnormalities A, G & H: Abnormal Metaphase with chromosomal Loop; B: Abnormal metaphase with improper equatorial plate; C: Abnormal Anaphase disorientation of chromosome movement; D: Abnormal metaphase with non-alignment of metaphase plate; E: Chromosomal Bridge; F: Abnormal anaphase with non-aligned spindle; I: Abnormal anaphase with Improper Spindle Movement

In order to measure onion root growth, root number and length were recorded; it was found that the TWW released at S1 contains substances that affect the growth and development of cells. As the water moves away from the WWTP we found that its toxicity level was decreased and comparatively better growth in cells was observed at S4. The elemental analysis of the treated water sample showed a decreasing concentration for Cr, Mn, Cu, Z, Cd, and Pb as we move from S1 to S4. It suggests the reason for abnormalities and less growth of onion roots at S1 and S2. The abnormalities and root number and growth were less observed at S3 and S4 the possible reason for this might be the phytoremediation effect of *Phragmites sp.* grown at the outlet of the treated wastewater. The phytoremediation effect of *Phragmites* is already reported (Ahmad et al., 2014; Hechmi et al., 2014; Bello et al., 2018) which supported our hypothesis. The concentration of Mg, Ca and Fe at the same sites were increased and it is well-known macro-elements (Tripathy et al., 2014) which supported the growth of the onion roots at S4. This is a new insight into the nature of the arid environment, due to the continuous release of TWW the arid land becomes wetland and *Phragmites sp.*is removing heavy metals from the TWW.

This is first of its kind of work in the KSA where we checked the treated water but similar kind of work in relation to aquatic pollution has been reported (Barbério, 2013; Verma and Srivastava, 2018). In our study, MI was also recorded to measure cytotoxicity along with cell abnormalities. MI was low at S1 and S2 as compare to TW in all days of treatments; this is in accordance with the findings of Olorunfemi et al. (2015) in case of bilge water study and Glínska et al. (2007) report suggesting disturbances in the cell cycle or chromatin dysfunction induced by metal–DNA interactions. The abnormal cell with lobed and multi-lobed shapes was observed at S1 and S2 along with hook and foot shape cell. The nuclear structure was also affected because of the varying concentrations and complex interactions of pesticides and heavy metal compounds. In our results, nuclear abnormalities were recorded at different sites (S1, S2 &S3) as compared to TW and DW. The interphase nuclei showed binucleate cell with micronuclei, spoon shape nucleus in extra-large cell and rectangular shape nucleus. Our results were in accordance with other workers (Fernandes et. al., 2007; Migid et al., 2007; Caritá & Marin-Morales, 2008; Leme et al., 2008; Olorunfemi et al., 2015, Verma et al., 2016; Salles et al., 2016). These authors also observed lobulated nuclei, nuclear bud and polynuclear cells as nuclear abnormalities. The presence of lobulated nuclei and polynuclear cells is an indicator of cell death process (Leme et al., 2008). Micronuclei formation is generally considered as an indicator of genotoxicity (Chandra et al., 2005; Kaur et al., 2014). Changes in the structure of chromosomes and the number of chromosomes are the characteristic features of identifying chromosomal abnormalities. Inceer et al. (2000) reported that chromosome bridges, delays, adherence, and breaks are frequently occurring chromosomal abnormalities. These changes are due to the effects of certain chemicals or physical agents (Russel, 2002). The present study thus substantiates the above facts as we have also recorded scores of chromosomal abnormalities like chromosomal adherence, chromosomal loops, chromosomal bridges, abnormal mitosis and improper alignment of metaphase plate, improper orientation of spindle.

CONCLUSION:

The KSA is considering wastewater as a major water source and aims to achieve its 100% usage by 2025. In Tabuk, most of the treated water is used for groundwater recharge; therefore, it is necessary to assess the water quality of released water. In this first report from the KSA, cyto-genotoxicity of TWW on *A. cepa* bioassay was recorded in an arid environment. The water at S1 and S2 was more harmful to the normal growth of the plant. However, as the water moves downstream its cyto-genotoxicity was decreased a possible reason is the phytoremediation effects of the *Phragmites sp.* This is a preliminary study on WWTP in Tabuk region and requires regular monitor so that it can establish the effectiveness of WWTP in aquifer recharge. Further, the heavy metal analysis in root and shoot of *Phragmites sp.* is also advised.

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