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Phyto-genotoxicity Assessment of Sewage Water for Agriculture Applications

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ABSTRACT

The usage of sewage wastewater in agriculture is highlighted by a number of advantages. However, heavy metals and other toxic compounds may exercise harmful effects to soil organisms. The objective of this study was to evaluate phytotoxic potentiality of each of the two stages of the sewage **wastewater** treatment routine, i.e., crude sewage, and secondary effluent. Genotoxicity test was conducted using *Allium cepa* (onion) as test organism. The genotoxicity of sewage effluent was investigated using both morphological and root chromosome assay. Different morphological characters of *Allium* radicals were recorded through 3 days (after 24, 48 and 72 h); these characters included length of radicals, number and appearance. To estimate the mutagenic activity in *Allium cells*, three cytogenetic endpoints were used, namely: 1-mitotic index (MI) 2-nuclear abnormalities (NA) and 3- chromosomal aberrations (CA). Physicochemical analysis revealed the inefficient treatment of sewage where, many elements increased after secondary treatment, and even those elements which decreased did not reach the safe limits for use. Statistical analysis of the genotoxicity test results showed: a significant decrease in root length and mitotic index as well as significant increase in chromosome aberrations. The effect was reduced after secondary treatment. This study proved the occurrence of mutagenic compounds in sewage water after secondary treatment and this requires further treatment to remove them. Moreover, their application to irrigate agricultural land must be stopped because of the mutagenic effects of plants irrigated by such waters.

Keywords: sewage effluents; secondary treatment; *Allium* test, genotoxicity; cytogenetic endpoints.

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INTRODUCTION

In sewage treatment plants (STP), after the sewage had been treated, a sludge rich in organic matter and nutrients is generated as a waste, known as sewage sludge. The composition of this sludge is very variable since it depends on the source of the sewage treatment process and the seasonality [1]. In some countries, there is a preference for the use of sludge in agriculture, since there is considerable land availability and the costs would be relatively low. However, this practice is still incipient, so that the application is made without an adequate management [2]. Sewage water has been used to support agricultural production in many countries over a considerable period of time. It is reported that sludge from sewage plant is recovered to be used to improve the organic content of agricultural soils [3]. Chemical composition of the sludge depends on the origin of the wastewater. Generally it is a compound rich in organic matter and essential nutrients for plants and microorganisms.

Effluent reuse can provide considerable benefit when used under controlled conditions to establish protection of health of both farm workers and consumers of the produce [4]. However, the main limitation observed during the evaluation of possible utilization of sewage sludge in agriculture refers to the presence of metals and other persistent pollutants, which may be toxic to plants, microorganisms, and soil invertebrates [5].

Sewage wastewater also contains a variety of inorganic substances from domestic and industrial sources, including a number of potentially toxic elements such as arsenic, cadmium, chromium, copper, lead, mercury, zinc, etc. Even if toxic materials are not present in concentrations likely to affect humans, they might well be at phytotoxic levels, which would limit their agricultural use. However, from the point of view of health, a very important consideration in agricultural use of wastewater, many of these agents are mutagens, and some are carcinogenic for humans. Thus, scientific studies have detected both toxic and genotoxic effects caused by the liquid effluents dumped into the environment.

Different systems have been used for wastewater treatments. The most appropriate wastewater treatment to be applied before effluent use in agriculture is that which will produce an effluent meeting the recommended microbiological and chemical quality guidelines both at low cost and with minimal operational and maintenance requirements [6]. General terms used to describe different degrees of treatment, in order of increasing treatment level; they are preliminary, primary, secondary, tertiary and/or advanced wastewater treatment. The common onion, *Allium cepa* L. (2n = 16), constitutes a very convenient test system for estimating the harmful effects of chemicals on biological materials. The *A. cepa* assay has been widely used to study the toxicity and genotoxicity of many dangerous contaminants, such as pesticides, dyes, food preservatives and hydrocarbons [7-9]. *Allium* test has been emphasized as a useful, low-cost system. The tests for risks to human health are performed by using various test systems and among these, the *Allium cepa* test. The results of the *A. cepa* test have been considered as an alert for other organisms (i.e. bioindicators). Studies on sensibility and correlation among test systems are fundamental for a more accurate evaluation of environmental risks and for extrapolating the data to other groups of target organisms. A high sensitivity and good correlation with mammal tests and the same sensitivity as test systems of algae and human lymphocytes exist when compared with *Allium cep*a. The aim of this study was to evaluate the genotoxicity of two stages of sewage effluents, namely: crude and secondary treated effluent, by using *in vitro* mutagenicity bioassay on mitotic cells in *Allium cepa* root tips.

MATERIALS AND METHODS

Sewage Effluents

Two different stages of sewage effluent (crude and treated) were collected from El Mansoura station of water sanitation, Egypt. The water samples were regularly collected every two weeks Collection was made by non metallic sampler. The samples were kept in the dark until reaching the laboratory, for chemical analysis and genotoxicity investigations. 1. Crude sewage samples: domestic waste is received through a pipeline in the wastewater treatment plant. The test onions were treated at dilutions of 25% and 50%, 75% and also with an undiluted (100%) sample. 2. The secondary treatment reduces organic matter through the addition of oxygen, which provides anaerobic environment for microorganisms to biologically break down the remaining organic matter.

Test Material

Onion bulbs (*Allium cepa)* were obtained from Agriculture Research Center, Legume and Onion, Research Sections, Giza, Egypt.

Physicochemical analysis of Sewage Effluent

Physicochemical analyses of sewage samples were carried out for the two different stages of wastewater treatment. The parameters analyzed represent the removal efficiency of the nutrients. For each sewage sample collected for the mutagenicity studies, measurements of biochemical oxygen demand (BOD), chemical oxygen demand (COD), water salinity, Electric Conductivity (EC), Hydrogen Ion Concentration (P.H), total hardness, total alkalinity, total nitrogen (TN) and total phosphorus (TP), total chlorides and total sulphates were done. The cations (Na+, K+ and Ca++) were determined in the water samples by using a photometer with propane burner of the type biology (PHF80 U.S.A.). Heavy metals (Cu, Fe, Zn, Pb and Co) were analyzed using atomic absorption spectrophotometer type PERKIN ELMER, 2380.

***Allium* test**

The *Allium* test was carried out as described by Rank et al., [10]. Uniform sized onion bulbs (10-30 g) were selected. The outer loose scales were removed, and scraped that the apices of the root primordium are exposed. Bulbs were submerged in treatment water to about one-quarter the depth of the bulb. The treatment water was changed daily and the bottle tanks should be kept in the dark for the period of the experiment. Tap water was used as control.

After approximately 2-5 days at 20° Ċ. the roots were let to grow to a length of 1-5 cm for cytological analysis. The solutions were changed every day during the experiment. Recovery experiment was carried out by transferring treating bulbs to new bottles filled with tap water for about 72 hours.

Macroscopic parameters

Different morphological characters of *Allium* radicals were recorded through 3 days (after 24, 48 and 72 h) these characters included length of radicals, number and appearance.

Genotoxicity test

On the 5th day of treatment, the radical length, number and other morphological characters were recorded in *Allium cepa*. Subsequently some root tips of *Allium*, were chosen randomly from each test solutions, excised and fixed in ethanol: acetic acid (3:1). Radicals (about 1 cm long) were excised, fixed, stained and squashed as described by Kihlman, [11] and Panda and Sahu, [12]. The slides were made permanent by using Canada balsam and microscopically examined to calculate the mitotic index and score the chromosomal aberrations and nuclear (interphase) irregularities.

Statistical analysis

SPSS program [13] was used to determine means and standard deviations from repeated samples (5) of each of the experimental groups. Least Significance Difference (L.S.D.) was used to determine significance (p=0.05) in the data compared to the control.

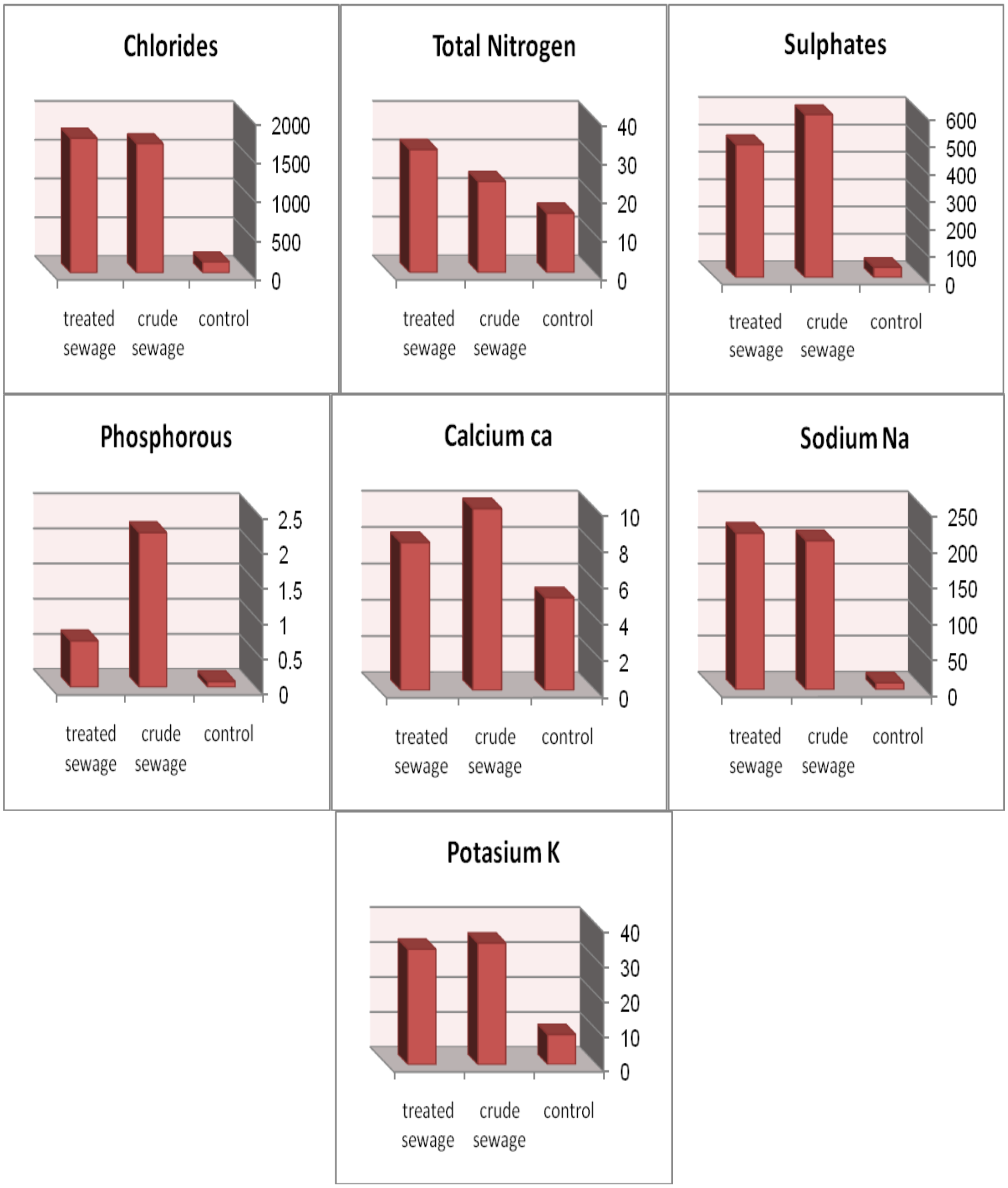
RESULTS

Physicochemical Analysis

Physicochemical analysis of the studied water samples is graphically illustrated in Figs. (1-3). In general, chemical analysis of the studied water samples revealed that, the concentrations of many estimated elements and parameters exceeds the safety limits within crude and treated sewage. The values of some physical and chemical parameters increased as well after the secondary treatment of crude sewage. The data showed that treatment of crude sewage led to increase of concentrations of some elements such as chlorides, total nitrogen, cobalt and iron. pH also increased after treatment. On the other hand, concentrations of some elements decreased after secondary treatment such as sulphate, potassium and cadmium but their concentrations are still not considered within the safety permissible range.

Among the physical parameters, DO (dissolved oxygen) values decreased in both crude and treated sewage. Also, COD (chemical oxygen demand) in crude sewage was very high. The obtained data showed an increase of pH after secondary treatment of sewage. Chlorides were found in unsafe limits in all water samples with higher percentage in treated sewage (Fig.1).

Fig 1: Chemical analysis of sewage effluents showing concentrations of elements (mg/L)



Concerning the concentrations of heavy metals, Fig. 3 showed that there were high levels of potentially toxic metals such as zinc, cobalt, lead, cadmium, copper and others in both sewage water samples. In particular, high concentrations of potentially toxic metals were detected in the treated sewage water. Copper and cadmium were higher in crude sewage whereas treated sewage showed higher cobalt, Zinc and iron. Surprisingly, control samples showed higher concentration of lead compared to the other samples (This may be due to the leakage of lead from lead pipe).

Fig 2: Physical parameters of sewage effluents

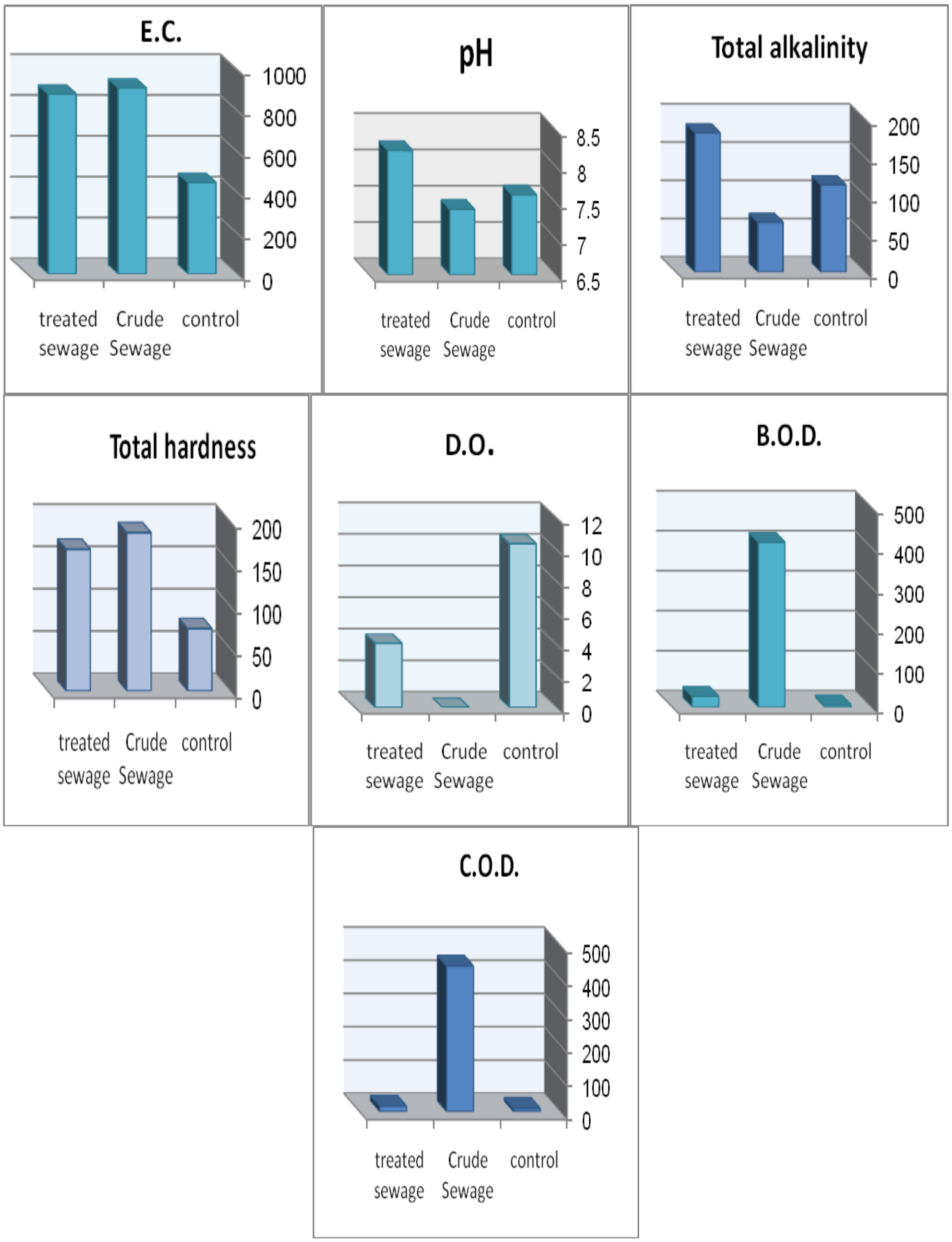
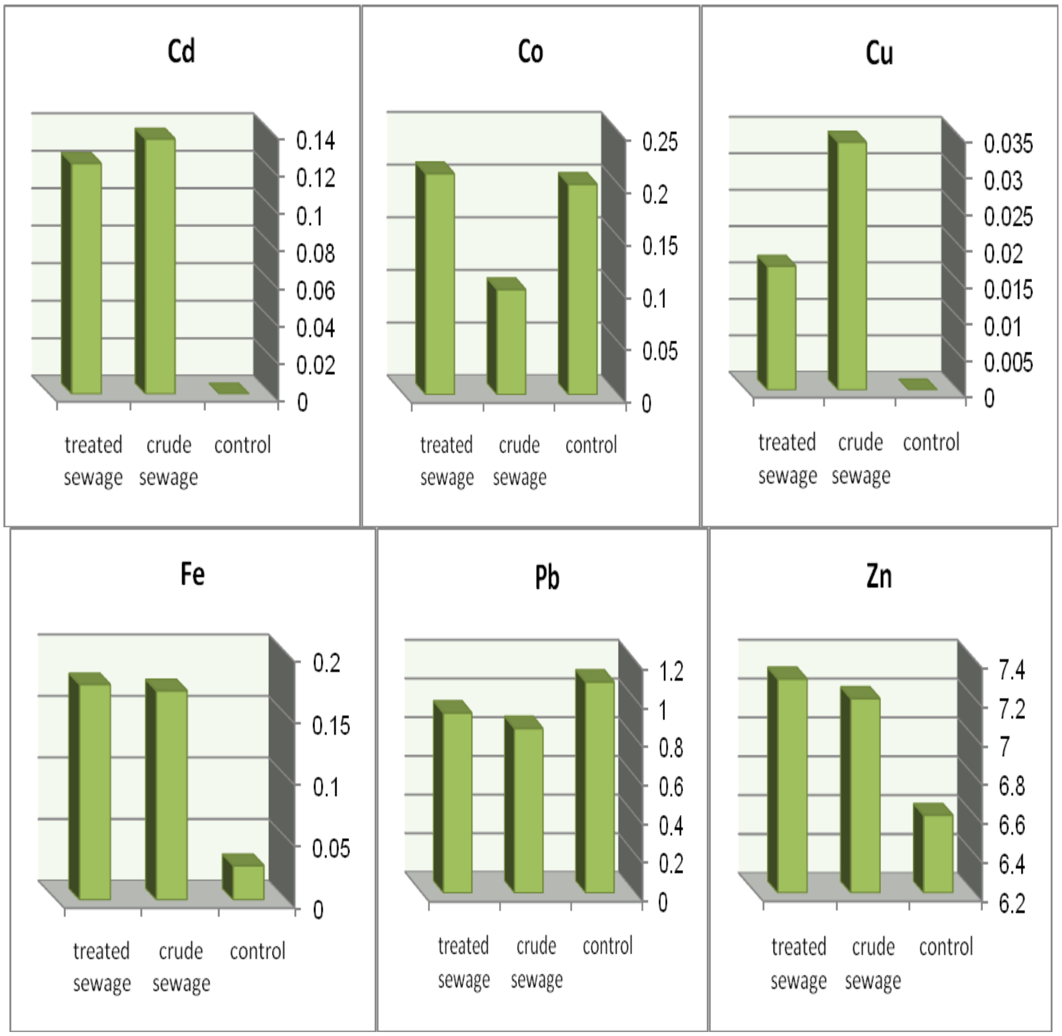


Fig 3: Chemical analysis of sewage effluents showing heavy metal concentrations (mg/l)

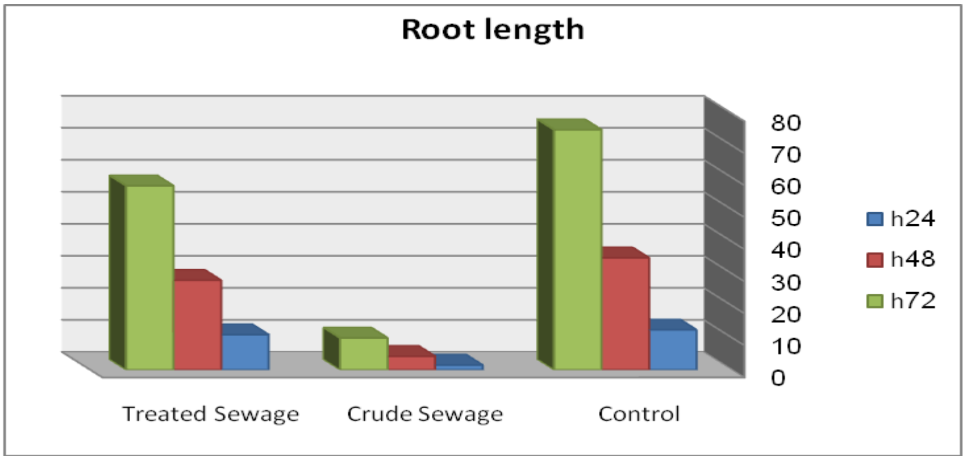


Genotoxicity test

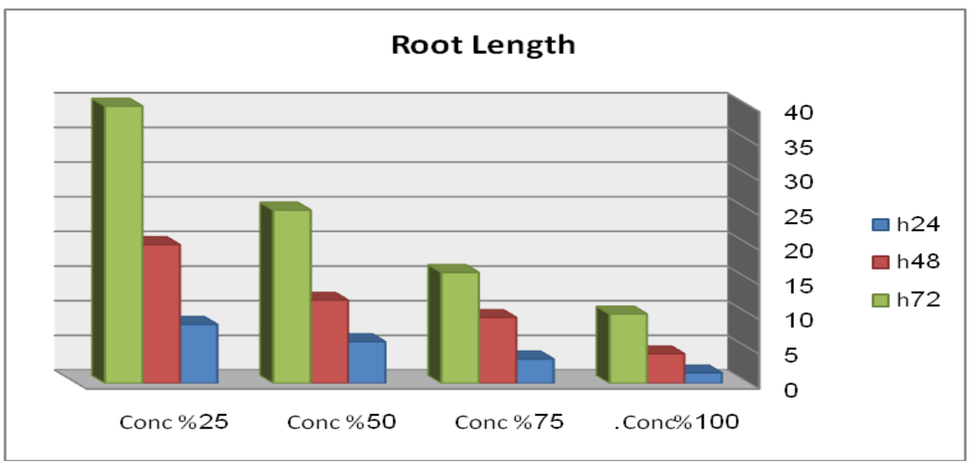
Macroscopic parameters

The length of treated *Allium* roots treated measured each 24 hours through three days. The data are graphically illustrated in Fig.4. After 72 hours, roots reached, 45 mm and 10 mm following treatment with, treated and crude sewage respectively compared to control (75 mm). After 72 hours recovery period, the root lengths reached 40 mm. Root lengths could be ranked as follow: crude sewage < treated sewage < control, as illustrated in plate (1). Root lengths of *Allium* treated with different dilutions of crude sewage are shown in table (4) and graphically illustrated in Fig (4). It is obvious that, the higher concentration of crude sewage led to inhibition in root growth which is presented by decrease in root lengths. Following 72 hours treatment, the root lengths were: 10mm, 16mm, 25mm and 40mm after application with 100%, 75%, 50% and 25% respectively (Plate 2). Another macroscopic parameter which can be used for detecting the toxicity of water was the morphology of roots. Morphological deformations were noticed in roots treated with crude and treated sewage. They mainly were damaged ends and crocket hock shaped ends (Plate 3). Other variations were observed concerning root number, root colors, diameters, viscous roots and precipitations around roots.

Fig.4. Root Lengths of *Allium* following treatment with A) crude and Treated Sewage effluents, B) different Concentrations of Raw Sewage effluents through 3 days period



A



B

Plate (1): Root lengths of *Allium cepa* root after treatment with sewage (crude and treated) after 5 days and recovery after 3 days.

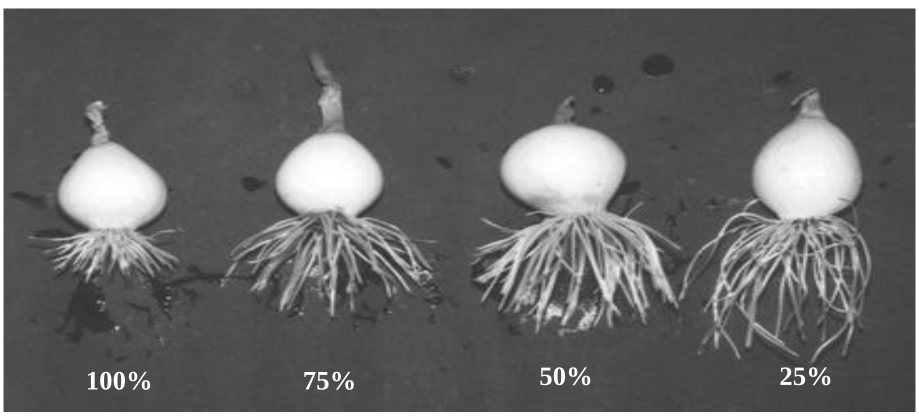


Plate (2) :Root lengths of *Allium cepa* treated with different crude sewage dilutions after 5 days.

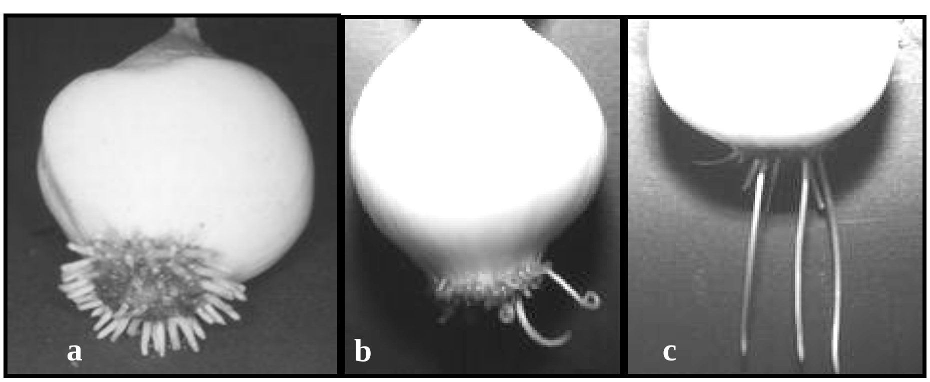


Plate (3): Morphological deformations of *Allium cepa* radicals shape, a: normal, b: crocket hock shaped, c: damaged ends.

Mitotic index (MI)

The values of M.I. were used as a parameter of relative mitotic inhibition and hence cytotoxicity. The percentage of mitotic indices of root meristems are shown in table (1) and illustrated graphically in Fig. (5). All treatments caused a significant decrease in the %M.I. compared to control (17.6% ± 1.02). Mitotic inhibition was more pronounced in root cells treated with crude sewage, presented by less M. I. value (9.5% ± 0.65*). On the other hand, inhibition in cell division in *Allium* was exhibited also by different dilutions of crude sewage (100%). The percentage of M.I. decreases significantly by increasing the concentration of crude sewage. M.I. values are (15.2% ± 0.27*), (12.6% ± 0.42*) and (10.6% ± 0.65*) after treatment with 25%, 50%, 75 % of crude sewage respectively. It is worthy to mention that, after recovery period for 72 hours, the mitotic index increased up to 15.6% ± 0.96*.

Nuclear Abnormalities (NA)

The frequencies of abnormalities in *Allium cepa* root cells that appeared in interphase were shown in table (1). Nuclear abnormalities included micronuclei, fragmented nucleus and binucleated cells as shown in plate (4). Percentage of total nuclear abnormalities increased significantly only in case of crude sewage treatment (177 ± 1.0) and after the recovery period (2.4 ± 0.5) compared to control (0.72 ± 0.26). Non significant increase in total abnormalities was observed in case of secondary treated sewage treatments (1.4 ± 0.63 and 1.52 ± 0.75). Lower concentrations of crude sewage (25% and 50 %) induced low percentage of nuclear abnormalities (0.48 ± 0.5 and 0.57 ± 0.7) respectively, which is statistically insignificant, whereas high concentration 75% led to significant increase in total percentage. A higher frequency of micronuclei (1 %) observed in root cells treated with crude sewage which also caused a higher percentage of binucleuated cells (2.2%). Fragmented nucleoli appeared only after recovery period (0.4%).

Chromosomal Aberrations (CA)

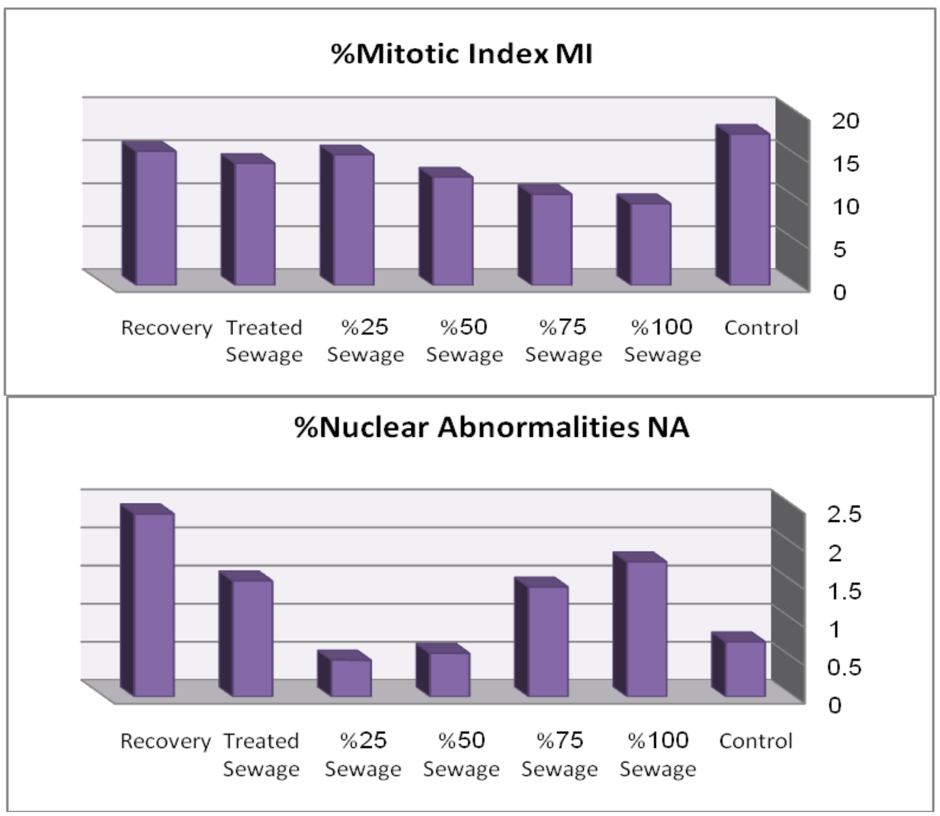
Chromosomal aberrations (CA) in mitotic stages of *Allium cepa* roots following all treatments are summarized in table (1) and shown in plates 5-8. In general, a significant increase in total percentage of chromosomal aberrations was observed compared to control.

A higher percentage was caused by crude sewage (73.2 ± 15.75) compared to control (16.52 ± 7.54). A clear non significant increase in the total percentage of chromosomal aberrations resulted after recovery period (17.48 ± 4.67). The scored aberrations included: stickiness, bridges micronucleus, banded chromosomes, laggard, chromosome breaks, and disturbed metaphase e.g., disorientation, ring chromosome, vagrant chromosome (Plate 6). Stickiness was the main frequent aberrations, observed in prophase and metaphase cells after all treatments.

Treatment		MI%	NA%	%CA
Control		17.6±1.02	0.72±0.26	**16.52±7.54**
100%	crude Sewage	9.5±0.65*	1.77±1.0*	**73.2±15.75***
75%		10.6± 0.65*	1.44±1.07*	**54.64±13.75***
50%		12.6± 0.42*	0.57±0.7	**42.96±6.8***
25%		15.2±0.27*	0.48±0.5	**31.62±7.03***
Treated Sewage		14.2±0.83*	1.52±0.75	**43.06±12.21***
Recovery		15.6±0.96*	2.4±0.5*	**17.48±4.67**

Table 1: Percentage of Mitotic index (MI); total Nuclear Abnormalities (NA) and Total Chromosome Abnormalities (CA) in *Allium cepa* root tips following treatments of: different concentrations of crude sewage; treated sewage; and after Recovery**. (mean ± S.D. * significant at p=0.05).**

Fig.5: percentage of Mitotic index (MI); total nuclear abnormalities (NA); total chromosome aberrations (CA) in *Allium cepa* cells exposed to different treatments of sewage water compared to control



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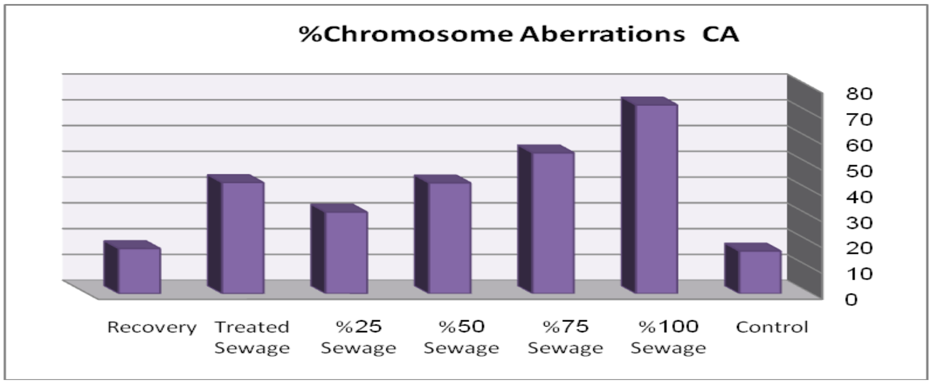

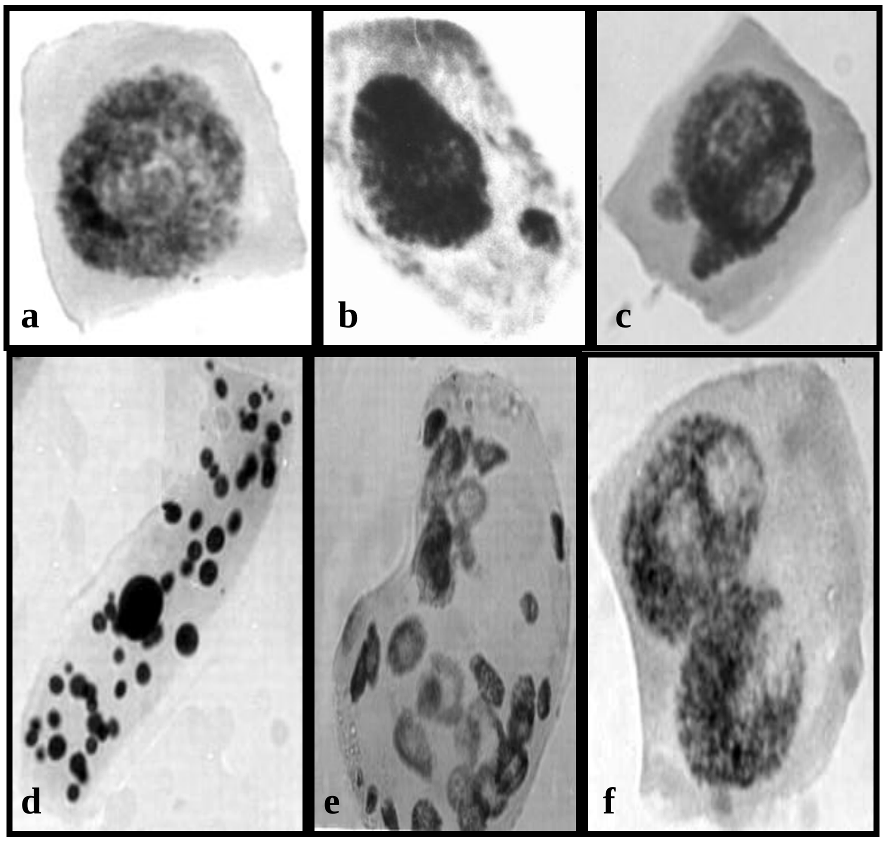

Plate (4) Interphase of *Allium cepa*, a-normal, b-c-micronucleus, d-e- Fragmented nucleus, f-binucleated cell.

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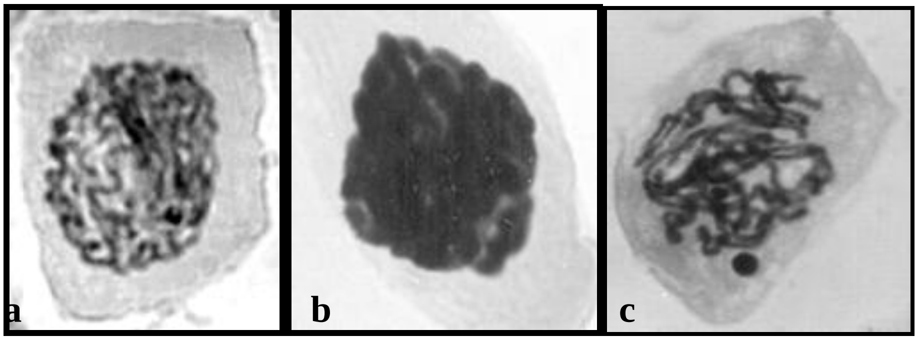

Plate (5): Prophase of *Allium cepa*, a-normal, b- Stickiness, c-micronucleus.

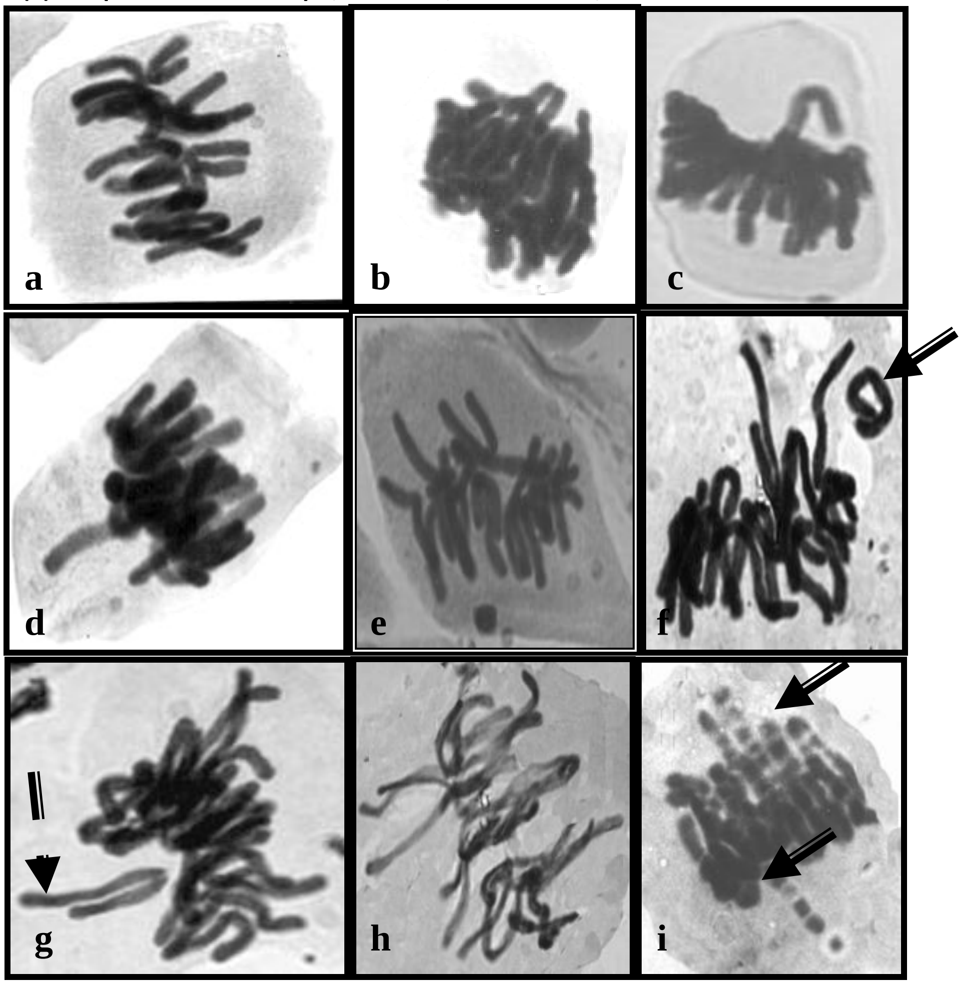

Plate (6): Metaphase cells of *Allium cepa*, a-normal, b-stickiness, c-d-mis-orientation, e -micronucleus, f-ring chromosome, g- vagrant, h-splitting metaphase, i-banded chromosomes.

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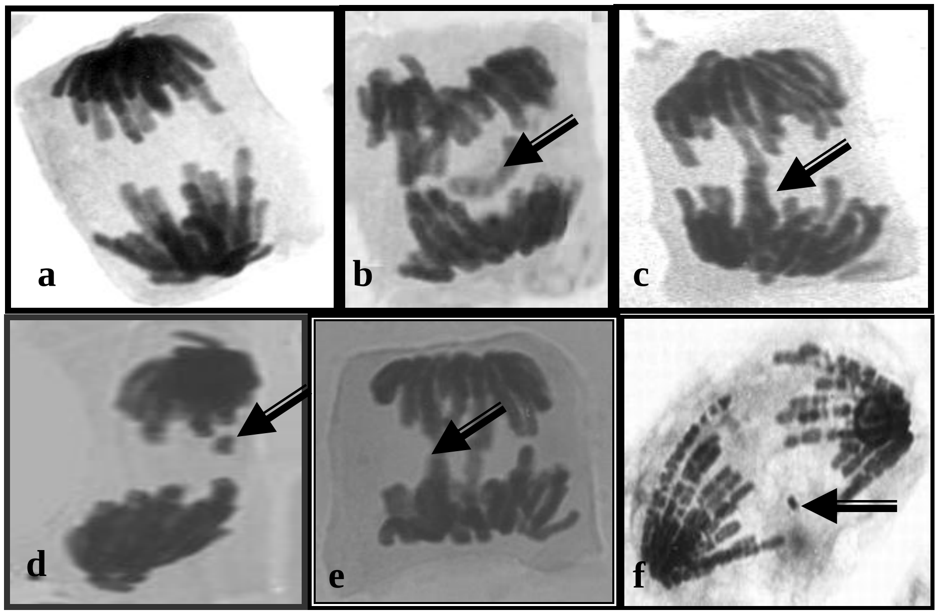

Plate (7): Anaphase of *Allium cepa*, a-normal, b-laggard, c-bridge, d-break, e-dibridges, f-banded chromosome.

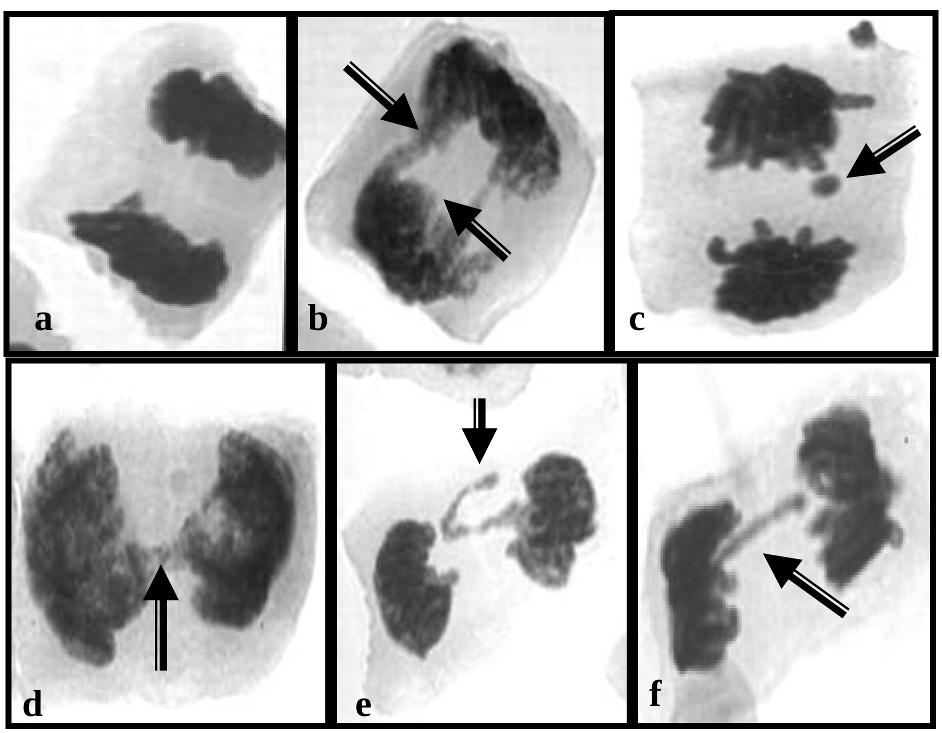

Plate (8): Telophase of *Allium cepa*, a-normal, b-dibridge, c-micronucleus, d- chromatin bridge, e-f-broken bridges.

DISCUSSION

In countries where there is planned reuse for sewage effluents in agriculture, assessment and maintenance of the quality of sewage water is very important. So, extensive monitoring programs need to be carried out, in which hundreds of specific substances are tested for known pollutants, substance-specific risk assessment (with respect to toxicity or genotoxicity) can be achieved, based on the properties of the individual compounds [14].

Water quality testing determines whether water is safe for drinking or irrigation. Typically, tests for water quality identify several indicators that can be used to determine quality of water such as alkalinity, dissolved oxygen, nitrates, pH and inorganic pollutants [14]. Many of these substances are found naturally in water but the actions of man can increase the amounts to where it is not possible to use the water safely. The results of physicochemical and heavy metal analysis of sewage wastewater have been presented in figs.1-3. Chemical analysis of the studied sewage both crude and secondary treated showed the presence of trace elements revealed that, the concentrations of many estimated elements and parameters exceeds the safety limits within crude and treated sewage. According to the literature, these elements tend to induce genotoxic and/or mutagenic effects on plants [15]. The values of some physical and chemical parameters increased as well after the secondary treatment of crude sewage. Although, concentrations of some elements decreased after secondary treatment such as sulphate, potassium and cadmium but their concentrations are still not considered within the safety permissible range. This suggests that the treatment process may be not efficient enough.

Among the physical parameters DO (dissolved oxygen) values decreased in both crude and treated sewage indicating the organic wastes contamination which leads to the deoxygenating of water [16]. Also, COD (chemical oxygen demand) in crude sewage was very high which was found to be correlated with genotoxicity in *Vicia faba* [17]. pH is generally acknowledged to be the principle factor governing concentration of soluble and plant available metals [18]. In this study, an increase of pH after secondary treatment of sewage was recorded. This may indicates the increase in alkaline wastes after treatment process. Total hardness of the sewage was found to be at high values in both crude and treated sewage (186, 66 and 167.26 mg/l respectively). Sulphate and nitrate are among the anions responsible for the hardness [19]. On the other hand, agricultural wastes lead to increase in phosphorus level which is reported in case of crude sewage [20]. Pathogen removal has been considered an objective for reuse of effluents in agriculture [21] and chlorination is a common water disinfectant method which is able to reduce microbial water pollution. However, genotoxic compounds can also be produced if precursors are present in the chlorinated water. Sewage water contains variable levels of organic matter, which may be the main source of potentially toxic and carcinogenic by-products of disinfection with chlorine [22]. Higher concentration of chloride content is known as a cause of toxicity to plants and proved genotoxic potentiality in *A. cepa* and *Tradescantia* assays [23]. In the present study chlorides were found in unsafe limits in all sewage samples with higher percentage in treated sewage (Fig.1). In addition, the heavy metals present in such sewage water either in its treated or crude sewage forms may accumulate in the soil or could be taken up by the roots of the cultivated plant organs to exceed the permissible limits which ultimately may either lead to retardation of plant growth or making its organs becoming unsuitable or hazard for use in feeding animals and /or man.

Parameters such as root shape and growth, frequencies of mitosis and abnormal cell division can be used to estimate the cytotoxicity, genotoxicity and mutagenicity of environmental pollutant [24, 25]. The *Allium* test has many advantages as genotoxicity screening assay, one being that *Allium* root cells posses the mixed function oxidase system which is capable of activating promutagens or genotoxic chemicals. In the *Allium* test, inhibition of rooting and the appearance of stunted roots indicate retardation of growth and cytotoxicity, while root wilting explains toxicity [26]. Nevertheless both growth retardation and root wilting are accompanied by suppression of mitotic activity and occurrence of chromosomal aberrations. The present study provides evidence that sewage effluent inhibited root growth and caused growth retardation. The inhibition of growth may be due to high rate of chemical oxygen demand which affected certain physiological processes leading to the disturbance in the balance between promoter and inhibitors of endogenous growth regulators [27]. Growth inhibition was most marked at 100% concentration. There was also a marked decrease in root length when compared with the control. Sewage water also induced crochet hooks which have been shown by other studies to be useful sign of toxicity [24]. Crochet hooks might be due to presence of heavy metals in sewage water.

Mitotic index is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the mitotic phase of the cell cycle. Hence, the decrease in the mitotic index of *A. cepa* meristematic cells could be interpreted as cellular death [28]. The mitodepressive effect was often used in tracing cytotoxicity [29]. In this study, the cells of *A. cepa* root tips after treatment with sewage effluents showed decrease in mitotic index with increasing concentration. There were significant differences between all treated groups and control group in mitotic index. This may be due to abnormal conditions of the cells induced by the treatments.

Several types of chromosome aberrations were considered in the four phases of cell division (prophase, metaphase, anaphase and telophase) to evaluate chromosomal abnormalities. Chromosome aberrations analysis not only allowed estimation of genotoxic effects, but also enabled evaluation of their clastogenic and aneugenic actions [10]. The abnormalities of chromosomes could be due to the blockage of DNA synthesis or inhibition of spindle formation [28]. The chromosome aberrations observed were chromosome stickiness, bridges, c-mitosis and vagrant chromosomes. These aberrations were due to the effect of sewage on the spindle formation and thus resulted in cell division disturbances. Chromosome bridges indicating the clastogenic effect caused by chromosome breaks, whereas vagrant chromosomes and c-metaphases increase the risk for an euploidy [30]. Some of the physiological aberrations that were most frequent in this study were stickiness (Plate 5b& 6b). They are indicative of a highly toxic usually irreversible effect, leading to cell death [26]. This could be responsible for the completely decayed roots found in 100% concentration.

A remarkable correlation between the frequencies of stickiness and the bridges was observed. This supports the hypothesis that stickiness may result from improper folding of chromosome fibers which makes the chromatids connected by means of subchromatid bridges [31]. Chromosome stickiness is caused probably through immediate reactions with DNA during its inhibition periods, causing DNA-DNA or DNA-protein cross linking [24]. This stickiness may be also interpreted as a result of depolymerization of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fibre units of chromatids and the stripping of the protein covering of DNA in chromosomes [32]. Chromosome bridges may be caused by stickiness of chromosomes which made their separation and free movements complete and thus they remained connected by bridges. A low frequency of c-mitosis (Plate 5g) and vagrant chromosomes was also observed. Their presence may be attributed to the failure of the spindle apparatus to organize and function in a normal way. Similar observations have been made by other workers where c-mitosis was regarded as indicative of a weak toxic effect which may be reversible. However, these changes may induce the formation of polyploid cells when not reversed [26]. Vagrant chromosomes that were not organized to a specific stage of the mitotic division were also observed. This abnormality may be caused by unequal distribution of chromosomes with paired chromatids in which resulted from nondisjunction of chromatids in anaphase. Vagrant chromosomes were weak c-mitotic effect indicating risk of aneuploidy [33].

Besides to the types of chromosome aberrations, the formation of nuclear abnormalities in interphase cells was determined. The percentage of micronucleated cells was obviously higher than control group at all tested concentration. The induction of micronucleus in root meristem cells of *A. cepa* is the manifestation of fragments or vagrant chromosomes [34]. They appear at the end of the cell division as a result of both chromosome breakage and spindle dysfunction. An increase in the frequency of micronucleated cells is considered a result of chromosomal and genomic damage caused by clastogens or spindle poisons [34]. The presence of binucleated cells reveals the inhibition of cell wall formation between cells [33]. Fragmentation was observed in this study in root tips of *Allium cepa* cells after recovery period which may be attributed to dissolution of chromatin [35]**.** Recovery period may be required to capture the micronucleus and may be other abnormalities which were induced around the G1 or S stage of the parental meristematic cells and revealed at the interphase or prophase the daughter cells [36]**.** According to the literature, all aberrations may be caused by the action of some trace elements/metals. Some metals are potentially genotoxic / mutagenic and are strongly related to environmental pollution.

The impacts of using sewage effluent as fertilizer on the environment are difficult to predict. However positive results in *Allium* test should be considered a signal of warning as this may constitute risk to environment and human health. Also, the occurrence of mutagenic compounds in sewage water after secondary treatment was proved and may requires further treatment to remove them. Therefore it is recommended that sewage application to irrigate agricultural land must be stopped because of the mutagenic effects of such waters.

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