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Original Research

Evaluation of mutagenic activity of an Iraqi wastewater treatment plant effluent with the *Salmonella* fluctuation test

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ABSTRACT:

The purpose of wastewater treatment is to eliminate or reduce water contaminants that impose environmental threats if they are discharged into the environment without the appropriate treatment. The aim of this study was to assess the mutagenicity effects of Al-Dewanyia wastewater treatment plant effluents by the *Salmonella* fluctuation test using the test strains TA98 and TA100 without metabolic activation. Four sizes, 1, 5, 10, and 15 mL, of the effluent sample were used with each bacterial test strain. The results showed that the effluent induced an increase in the number of revertants colonies in the both test strains in a significant dose-dependent manner; $P<0.05$. In the bacterial test strain TA98 the increase in the number of revertants colonies was significant, the mutation rate was more than two regardless of the sample size. In contrast to the bacterial test strain TA98, the induced mutagenic activity in the test strain TA100 was insignificant and the mutation ratio was less than two for all the sample' sizes. The effluents contain high levels of direct-acting frameshift-type mutagens but relatively low levels of direct-acting base pair-type mutagens. The differences in the mutagenicity levels may be due to the various induced genotoxicity types. The study demonstrates that the effluents pose ecological hazards, and thus they should be properly treated to reduce and/or eliminate the potentially genotoxic compounds before being discharged into the receiving environments.

Keywords:

Mutagenicity, *Salmonella* fluctuation test, TA98, TA100, Frameshift, Base-pair.

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INTRODUCTION

Many developing countries are still lacking the required infrastructure for water as well as wastewater treatment, and the negative impact of such lack on the environment is obvious. Several factors are behind the serious lack in operations of Iraqi's wastewater treatment plants, including the absence of permanent programs for environment protection by the government, lack of well-trained engineers and skilled operators, improper design, expenses shortage, and low public awareness about the impacts of direct wastewater discharge into the environment (Alyaseri, 2016). Wastewater is discharged from many sources, including domestic homes, industries, commercial, and agricultural sectors. Since water is a versatile and convenient solvent for several compounds, it is widely used as a transporter of waste materials away from the site of production. The transported waste materials are often toxic to several organisms and cause serious damages to the receiving environments such as rivers, lakes, or streams (White and Rasmussen, 1998). Chemical compounds, including pharmaceutical, household chemicals, and biogenic hormones are commonly released into the environment, and some of these chemicals often pass treatment processes of wastewater (Kolpin *et al.,* 2002). Pharmaceutical compounds, including anti-inflammatory drugs, antibiotics, β-blockers, and X-ray contrast media are widely used and discharged into the environment by pharmaceutical industry effluents and hospitals' wastewater (Jiang and Zhou, 2013). Several toxicity studies have demonstrated that hospital wastewaters might have a genotoxic potential to organisms (Giuliani *et al.,* 1996; Jolibois *et al.,* 2003). Environmental mutagens in wastewater include petroleum-derived hydrocarbons, solvents, certain pesticides, dyes production byproducts, and water chlorination byproducts. Mutagens presence in water is considered a potential hazard to organisms, including human because many of mutagens are carcinogens. Treatment and remediation of some wastes have been shown to reduce or eliminate genotoxicity of toxic compounds. In other cases, the genotoxicity of some waste materials may enhance by the treatment processes (Claxton *et al.,* 1998).

The *Salmonella* fluctuation test has been broadly implemented to detect the mutagenic activity in complex environmental mixtures. The test depends on the detection of histidine-independent revertants in specific strains of *Salmonella* after are being exposed to certain mutagenic compounds. Because of their higher sensitivity to a wide range of mutagens, the most widely used strains for the fluctuation test are TA 98 and TA 100 of *Salmonella typhimurium*. The strains TA 98 and TA 100 have been implemented as the basic strain for the fluctuation test according to DIN38415-4 (1999) and ISO 16240 standards (2002) (Guan *et al.,* 2017). Assessment of water genotoxicity is of an interest because several epidemiologic studies have demonstrated a link between consumption of genotoxic water and increased levels of cancer (Koivusalo *et al.,* 1995). Mutagens have been detected in drinking water and effluents of secondary and tertiary wastewater treatment plants. In Iraq, most of untreated or partially treated wastewaters are discharged directly into water bodies or used for agricultural purposes to irrigate many food crops. The objective of this study is to assess the mutagenicity effect, that is production of frameshift mutation in the test strain TA98 and base-pair substitution in the test strain TA100 of treated effluent collected from Al-Dewaniya wastewater treatment plant by the *Salmonella* fluctuation test.

MATERIALS AND METHODS

Study area and sampling

Wastewater effluent samples were collected from the entering wastewater stream of Al-Dewanyia wastewater treatment plant with GPS coordinates 32.028412, 44.972159–prior to discharge into Al-

Table 1. Concentration of contaminants measured for the plant effluent and compared with the Iraqi standards of effluents. The values represent the mean of the sample triplicates

S. No	Parameters	Mean ± Standard deviation	Iraqi standards
1	BOD5	83.75 ± 7.3	40
2	COD	55.42 ±6.5	100
3	TSS	53.50 ± 6.3	30-60
4	NO_3	19.25 ± 3.2	50
5	PO_4^{-3}	4.53 ± 1.7	3.0
6	NH_3	19.20 ±3.7	10
7	Cl^-	1879 ± 13.6	600
8	H_2S	4.77 ± 1.4	3.0

Dewanyia river, a branch of Euphrates river. The plant is located in the southern part of Al-Dewaniya city, the center of Al-Qadisiya province, and it is the main wastewater treatment facility in the city. The plant receives municipal, industrial and commercial wastewaters from most parts of the city. Wastewaters into the plant are treated through primary and secondary treatment processes. The primary stage consists of a rack screen and grift champers for grift sedimentation. The secondary treatment process is an activated sludge for biological degradation of organic pollutants. Prior to discharge into the river, effluents from the secondary treatment process flow through a chlorine chamber for disinfection. The plant does not contain a dechlorination unit for chlorine removal from the effluents. Triplicates wastewater effluents were collected in 0.5 liter polyethylene containers. The samples were collected from the center of the flow channel at about 40-60% of the water depth to ensure that the water is well mixed. After samples collection, containers were labeled, capped, transported to Al-Qasim Green University, Department of Environmental Health's laboratory in an ice chest, and refrigerated at 4ºC until conducting the analysis.

Analysis of effluent parameters

The physical and chemical parameters of water were conducted to assess the factors that are considered as indicators of plant efficiency in wastewater treatment and effluents pollutions. The parameters measured for the plant effluent were BOD5, COD, TSS, NO_3, PO_4, NH_3, Cl^-, SO_4, and H_2S; their values were 83.75±7.3, 55.42±6.5, 53.50±6.3, 19.25±3.2, 4.53±1.7, 19.20±3.7, 1879±13.6, 4.77±1.4, respectively Table 1. All the values exceeded the Iraqi parameters of effluents.

Mutagenicity fluctuation test

The mutagenicity test kit, including the test strains, *Salmonella typhimurium* histidine auxotrophs TA98 and TA100, reagents, standard mutagens, ultrapure water, and membrane filters were purchased from the Environmental Biodetection Products Inc. (EBPI, Mississauga, Ontario, Canada). Falcon 96-well microplates were purchased from the Ward's Natural Science (Rochester, NY. USA). The *Salmonella* fluctuation test was performed as outlined in the Environmental Biodetection Products, Inc. instructions (2008) in accordance with the procedure described by (Legault *et al.,* 1994). Cell suspensions with approximately 10^8 cell/mL of TA98 and TA 100 were prepared and incubated overnight at 30ºC for bacterial activation. Effluent samples were filtered by a vacuum-filtration through 0.22 µm membranes filters immediately prior conducting the test. The fluctuation test was conducted without metabolic activation (absence of S9 mix). The principle of the *Salmonella* fluctuation test is that test chemicals and the amino acid auxotroph bacteria (*Salmonella typhimurium* TA98 and TA100) are mixed in a liquid media that contains a small amount of histidine and dispensed into micro-plate wells. During the residual growth, a reverse mutation on the marker gene may develop, a mutant clone arises, and afterward produces enough amount of acid due to sugar utilization that changes the assay mixture containing a pH indicator (Sui *et al.,* 2009).

The preparations of assay treatments are shown in Table 2. Four sizes of the effluent sample were used, which were 1, 5, 10 and 15 mL. The micro-plates were

Table 2. Preparations of the assay treatments, Negative Control (NC) and Positive Control (PC). A triplicate was made for each treatment

Item	Standard (mL)	Effluent (mL)	H_2O (mL)	Reaction mixture 2.5 mL	Bacteria 5 µL (10^{-8})
Blank	-	-	17.5	+	-
Negative control TA98	-	-	17.5	+	TA98
Negative control TA100	-	-	17.5	+	TA100
Positive control TA98	0.1 2-NF	-	17.4	+	TA 98
Positive control TA100	0.1 NaN_3	-	17.4	+	TA100
Treatment/TA98	-	15	2.5	+	TA98
	-	10	7.5	+	TA98
	-	5	12.5	+	TA98
	-	1	16.5	+	TA98
Treatment/TA100	-	15	2.5	+	TA100
	-	10	7.5	+	TA100
	-	5	12.5	+	TA100
	-	1	16.5	+	TA100

prepared in triplicates for each sample's size for the both test strains. Sodium azide (NaN_3) and 2-nitrofluorine (2-NF) were used as positive controls without metabolic activation for TA100 and TA98 strains, respectively. Sodium azide and 2-nitrofluorine are chemically known mutagens that induce base pair substitutions and frame shift mutations in several organisms. The positive control for the bacterial strain TA98 was made from 0.1 mL NaN_3, 17.4 mL distilled water, and 5 µL of the bacterial cells suspension 10^8. Similarly, the positive control for the bacterial strain TA100 was made from 0.1 mL 2-NF, 17.4 mL distilled water, and 5 µL of the bacterial cell suspension 10^8 Table 2. Sterile ultrapure water samples without metabolic activation were used as negative controls for the two strains. Microplates containing bacteria, reagent mixtures, and effluent samples were covered, sealed in plastic bags, and incubated at 37°C for 5 days.

Evaluation of mutagenicity

During incubation period, the number of revertants colonies and surviving colonies formed in each plate was subsequently counted. All the turbid, yellow,

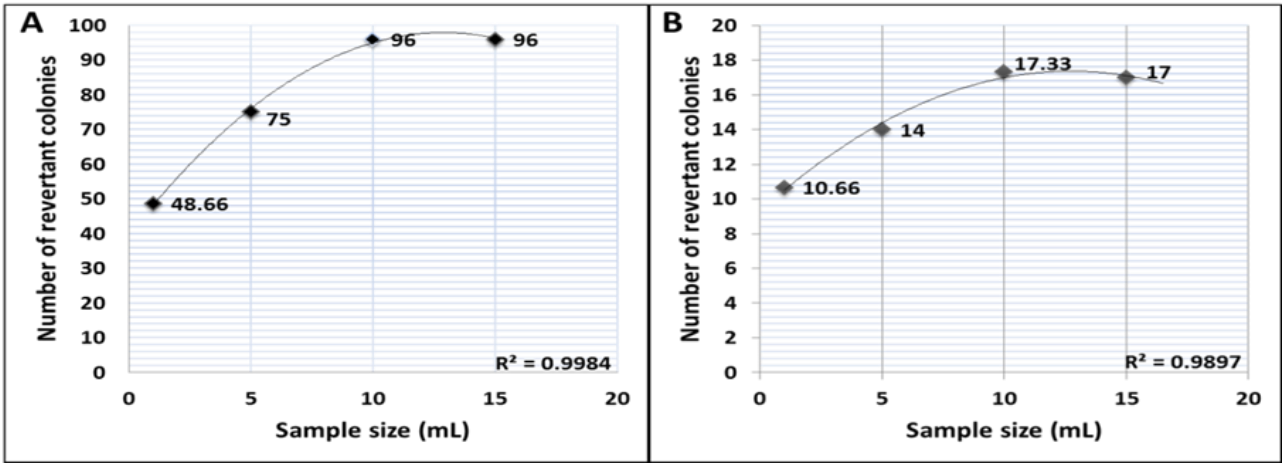

Figure 1. The correlation between the sample size and the number of revertant colonies; A: The correlation between the sample size and the number of revertant colonies for the bacterial test strain TA98; B: The correlation between the sample size and the number of revertant colonies for the bacterial test strain TA100.

Table 3. Mean number of revertant colonies ± standard deviation in negative and positive controls for both bacterial test strains TA98 and TA100

S. No	Item	Test strain	Mean number of revertants colonies ± SD
1	Blank	-	0 ±0.0
2	NC	TA98	24 ±1.5
3	NC	TA100	10 ±2.0
4	PC	TA98	96 ±0.0
5	PC	TA100	96 ±0.0

or partially yellow wells are considered revertants (positive) colonies, and the purple wells are considered surviving (negative) colonies. The mutagenic activity was evaluated based on the mean number of revertants colonies in treatment triplicate plates. The sample was judged to be a positive response when the number of revertants colonies reached twice or more than that of the negative control, or only if the mutation rate MR $\geq$ 2. MR represents the number of mutants colonies on a test plate/number of spontaneous mutants colonies on negative control plate (Guan *et al.,* 2017).

Data analysis

Values were expressed as the mean ± standard deviation. The statistical differences between revertant colonies in treatments versus negative controls were determined using the procedure of results analysis of fluctuation tests developed by (Gilbert, 1980). The correlation between the number of revertant colonies and sample size was performed by Pearson correlation by IBM SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The results from the *Salmonella* fluctuation test are shown in Table 3 and Table 4. The mutagenicity of Al-Dewanyia wastewater treatment plant effluent was investigated with the *Salmonella* fluctuation test using the test strains TA98 and TA100 without metabolic activation. This instant mutagenicity test is a sensitive tool in the analysis of complex environmental mixtures for the detection of contaminants with genotoxic/mutagenic properties. The accuracy of the test was proved; the number of revertant colonies in the blank was zero while the number of revertant colonies in the positive control was 96 for both bacterial test strains Table 3. The number of revertant colonies (yellow, partially yellow, or turbid wells) for the four sample's sizes of both test strains are summarized in Table 4. All the sample sizes induced frameshift (in TA98) and base-pair substitution (in TA100) with different levels of mutagenicity. Heavy metal, polycyclic aromatic hydrocarbons (PAHs), Polychlorinated Biphenyls (PCBs), pesticides, heterocyclic amines, and pesticides have been identified

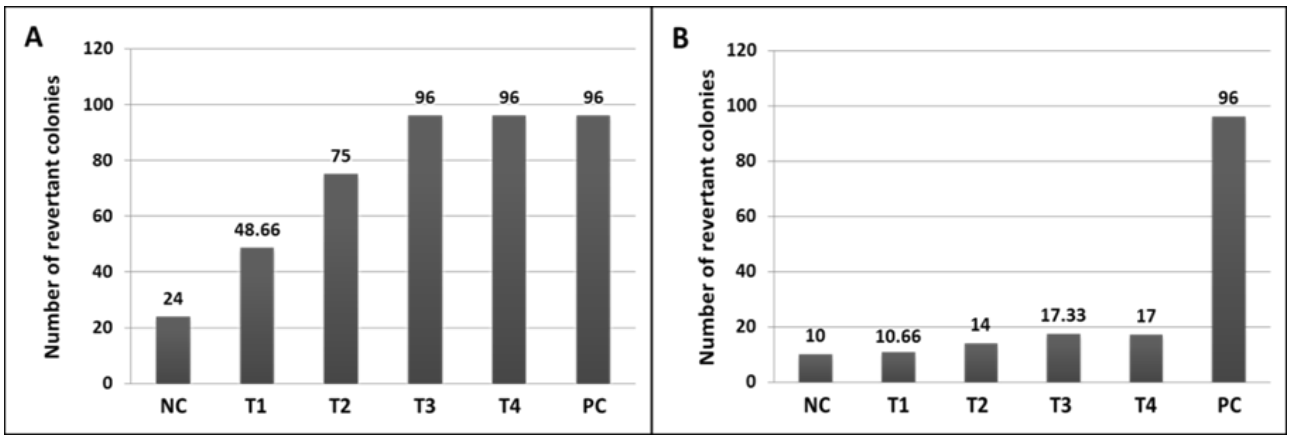

Figure 2. Comparison of the revertants colonies in positive and negative control and treatments. Symbols NC and PC are the negative and positive control, respectively. T_1, T_2, T_3, and T_4 represent treatment with the sample sizes 1, 5, 10, and 15 respectively

Table 4. Mean number of revertant colonies ± standard deviation in the sample triplicates inoculated with TA98 and TA100

S. No	Sample size (mL)	Mean number of revertant colonies in the sample triplicates (TA98) ± SD	Mutagenicity ratio MR	Mean number of revertant colonies in the sample triplicates (TA100) ± SD	Mutagenicity ratio MR
1	1	48.66 ±5.8	2.02	10.66 ±1.5	1.06
2	5	75.00 ±4.5	3.12	14.00 ± 2.6	1.40
3	10	96.00 ±0.0	3.94	17.33 ± 2.0	1.73
4	15	96.00 ±0.0	4.00	17.00 ±1.0	1.70

in wastewaters and exposure to these chemicals induces genotoxicity/mutagenicity in organisms (Filipic, 1995; Ohe *et al.,* 2004).

The mutagenic effects in the bacterial strain TA98 were observed in all the effluent sample sizes, and the mutagenic activity was significant. The maximum mutation rate was 4.0, which was reported at two sizes of the sample; 10 and 15 mL (Figure 2A and Table 4). A significant correlation was observed between the sample size and the number of revertants colonies for the bacterial test strain TA98; $P<0.05$ (Figure 1A and Table 5). Similar to our findings, Guan *et al.* (2017) found the mutagenic activity is a dose-dependent; the mutagenicity ratio increases as the sample size increases. Our results indicated that the effluent contained potential mutagenic compounds, which did not require an activation to exert their mutagenic effects to the strain TA98. Liu *et al.* (2015) reported that the mutagenic effects on the bacterial strain TA98 were significant in sediment extracts, regardless of the presence or absence of metabolic activation. A mutagenicity study by Umbuzeiro *et al.* (2004) showed that a mutagen or mixture of mutagens in surface water and treated industries effluents are the major cause of frameshift mutations in the bacterial strain TA98 regardless of metabolic activation. The effluent sample has a high concentration of chlorine (1897 ppm), which exceeded the world as well as the Iraqi standards of the treated wastewaters (Table 1). This contaminant may be responsible for the mutagenic effects in the test strain. Crebelli *et al.* (2005) reported that the test strain TA98 was highly sensitive to wastewater samples treated with 4mg/l of sodium hypochlorite and peracetic acid, and the number of revertants colonies was more than twice of the negative control value.

On the other hand, the effluent did not cause significant increase in the number of revertant colonies in the bacterial test strain TA100. In all sizes of the sample, the mutation rate was less than two, indicating that the effluent poses low level of mutagenic activity to induce a significant base-pair substitution in the test strain TA100 Table 4 and Table 5. The maximum muta-

Table 5. Pearson correlation analysis between the sample size and number of revertant colonies for the bacterial test strain TA98

Correlations			
		Sample size	Number of revertant colonies
Sample size	Pearson correlation	1	0.921*
	Sig. (1-tailed)		0.039
	N	4	4
Number of revertant colonies	Pearson correlation	0.921*	1
	Sig. (1-tailed)	0.039	
	N	4	4

*. Correlation is significant at the 0.05 level (1-tailed).

Table 6. Pearson correlation analysis between the sample size and number of revertant colonies for the bacterial test strain TA100

Correlations			
		Sample size	**Number of revertant colonies**
Sample size	Pearson correlation	1	0.914*
	Sig. (1-tailed)		0.043
	N	4	4
Number of revetant colonies	Pearson correlation	0.914*	1
	Sig. (1-tailed)	0.043	
	N	4	4

*. Correlation is significant at the 0.05 level (1-tailed).

tion rate was 1.73 when the sample size was 10 mL (Figures 1B and 2B). At the highest sample size, which was 15 mL, the mutation rate was 1.7 Table 4. The correlation analysis showed a significant correlation between the sample size and the number of revertant colonies (Table 6). The variation in the induced mutagenicity levels in the test strains TA98 and TA100 may be caused by the differences in the induced genotoxicity/mutagenicity type (Liu *et al.,* 2015).

CONCLUSION

The fluctuation test indicated that effluents collected from Al-Dewanyia wastewater treatment plant induced genotoxicity in the both test strains; TA98 and TA100. However, the strain TA98 was more sensitive than TA100 to mutagens. The study showed that the effluents induced both frameshift mutation in TA98 and base-pair substitution in TA100, and the dominant mutation was the frameshift. The variations in the mutagenicity levels may be due to the differences in the induced genotoxicity/mutagenicity types. The salmonella fluctuation test is a very sensitive and useful tool for the detection of mutagens in waters; however, this test cannot identify and quantify the contaminants responsible for mutagenicity. Therefore, analytical studies should be undertaken to identify and quantify the compounds that cause the genotoxicity in order to identify the source of the toxicant and consequently take a preemptive and/or restorative actions. Furthermore, such studies assist in locating the proper treatment for wastewater before being discharged into the receiving environment and posing ecological hazards.

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