JASEM ISSN 1119-8362

Full-text Available Online at www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *March*, 2011

Vol. 15 (1) 211 - 216

Genotoxicity Screening of Industrial Effluents using Onion bulbs (*Allium cepa* L.)

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ABSTRACT: The potential cytotoxicity and genotoxicity of three industrial wastewaters (brewery, rubber and bottling) in Benin metropolis using the *Allium cepa* test were investigated. A series of five small onion bulbs were cultivated in 0.01, 0.1, 1, and 10% of the wastewaters (v/v) and after 48 hours, one root tip from each bulb was harvested and processed for cytological studies by the aceto-orcein squash technique. At 72h, their cytotoxic effects on the root tips showed strong growth retardation in high concentrations of all the wastewaters. Compared to the control, treatment with the wastewaters resulted in root growth inhibition with EC_{50} values of 35, 50 and 62% for bottling, rubber and brewery effluents respectively, and decrease in mitotic index with increasing concentration for all samples and these were statistically significant ($p<0.05$). Chromosomal aberrations induced in the onion root tip cells were mostly sticky chromosomes and bridges. Chromosomes with disturbed spindles and fragments were also present in appreciable amounts. Based on the EC_{50} values, the bottling wastewater was most toxic, followed by rubber effluent while effluents from the brewery were least toxic. The findings in this study indicate that there are toxic chemicals present in the wastewaters which are responsible for the observed genotoxic effects on the onion root tip cells. The study also reveals that the *Allium* test is a useful and reliable tool for the genotoxicity screening of industrial effluents which could be employed by environmental managers before these effluents are finally discharged into the environment. @JASEM

Industries are undoubtedly indispensable components of a nation's development; however, the impact of industrial wastewaters on aquatic and terrestrial ecosystems has drawn a lot of attention worldwide because of its overwhelming environmental significance. Industrial wastewater originates from the wet nature of most large industries which require large quantities of water for processing and disposal of wastes. Most industries are therefore, located near water sources. Industrial wastewater is not only concentrated but plentiful, so the pollution potential of industrial wastewater is by far greater than that of domestic wastewater. In Nigeria, over 80% of the industries discharge solid wastes, liquid effluents and gaseous emissions directly into the environment without any treatment (Federal Ministry of Water Resources, 1994). Despite existing legislations, of the 200 randomly assessed industries, only 18% perform rudimentary recycling prior to disposal of the wastes (FEPA, 1998).

Several higher plant systems, including bioassays with plant roots have provided cheaper, easier, sensitive, useful, reliable and valuable alternative methods for the determination of the adverse effects of environmental pollutants to the usual assays carried out on experimental animals (Grant, 1978). Most of the studies indicate that there is an excellent correlation between chromosome abnormalities and mutagenic activity found in root-tip systems and those found in mammalian cell systems. Observation of the root tip system of plants therefore constitutes a rapid and sensitive method for environmental monitoring (Majer *et al*., 2005). *Allium cepa* L. has been used as a first-tier assay for the detection of environmental chemicals that may pose genetic hazards from a wide range of pollutants such as sewage effluents (Ukaegbu and Odeigah, 2009), leacheates (Bakare and Wale-Adeyemo, 2004, Chandra *et al.,* 2005) and chemicals (Seetharaman *et al.*, 2004). Cytotoxicity and environmental pollution have been assessed by the *in vivo* onion (*Allium cepa*) root tip cell test which is known to give similar results to *in vitro* animal cytotoxicity tests (Vicentini *et al*., 2001; Teixeira *et al*., 2003). *A. cepa* test has also been used by many authors to evaluate cytotoxicity and genotoxicity of industrial effluents (Rank and Nielson, 1998; Chauhan *et al*., 1999; Grover and Kaur, 1999; El-Shahaby *et al*., 2003, Babatunde and Bakare, 2006; Junior *et al*., 2007; Abdel-Migid *et al*., 2007; Şık *et al.,* 2009; Samuel *et al*., 2010).

In a study conducted on the toxicity of wastewaters from three industrial establishments in Benin City, Nigeria, differential lethality was established on *Chironomus travalensis* (*Diptera larvae*) (Olomukoro and Okhumale, 2008). We are not aware of any reports on the genotoxicity of these industrial effluents. This study was therefore undertaken to corroborate or otherwise, the nature and extent of toxicity of the effluents discharged from the same industrial establishments using a higher plant monitoring system, the *Allium cepa* assay.

MATERIALS AND METHODS

Three industrial effluents: a brewery (Br), bottling company (Bt) and rubber processing industry (Rr) were collected from their discharged points within the Benin City metropolis situated between latitudes 6° 06' N, 6° 30' N and longitudes 5° 30' E, 5° 45' E and an area of about 500 square kilometres. Effluents from these industries are discharged into close-by rivers; the brewery (Br) and rubber processing industry (Rr) effluents are discharged into the Ikpoba

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river while the effluent from the bottling company (Bt) are discharged into the Iguosa river.

Onion bulbs *(Allium cepa* L., 2n=16*)* of the purple variety of average size (15-22 mm diameter) were purchased locally in Benin City, Edo State, Nigeria. They were sun-dried for six weeks and the dried roots present at the base of the onion bulbs were carefully shaved off with a sharp razor blade to expose the fresh meristematic tissues. The bulbs were then placed in freshly prepared distilled water to protect the primordial cells from drying up. To account for a number of bulbs in the population that would be naturally slow or poor growing, seven replicate bulbs were used for each test sample and control (tap water) and the best five bulbs were chosen for examination (Rank and Nielsen, 1993). The bulbs were removed from the distilled water and placed on a blotting paper to remove excess water. For root growth inhibition evaluation, the bulbs were exposed directly in 0, 1, 5, 10, 25, 50, 75 and 100% (v/v, effluent/tap water) of each of the test samples. Seven onion bulbs were utilized for each concentration of each wastewater and the control. The base of each of the bulbs was suspended on the effluents inside 100ml beakers in the dark for 72 h. Test effluents were changed daily. At the end of the exposure period, the roots of five onion bulbs with the best growth at each concentration were removed with a forceps and their lengths measured (in cm) with a metre rule. The effect of each sample on the morphology of growing roots was also examined. For the evaluation of induction of chromosomal aberration, five onion bulbs were suspended on 10, 1, 0.1, and 0.01% concentrations (v/v) of each of the effluents and the control for 48 h, at the end of which root tips from these bulbs were cut and fixed in ethanol:glacial acetic acid (3:1, v/v). These were hydrolyzed in 1N HCl at 60° C for five minutes after which they were washed in distilled water. Two root tips were then squashed on each slide, stained with aceto-carmine for 10 min and cover slips carefully lowered on it to exclude air bubbles. The cover slips were sealed on the slides with clear fingernail polish as suggested by Grant (1982). This is to prevent drying out of the preparation by the heat of the microscope (Sharma, 1983). Six slides were prepared for each concentration and the control out of which five were analyzed at ×1000 magnification for induction of chromosomal aberrations. The mitotic index was calculated as the number of dividing cells per 1000 observed cells (Fiskesjo, 1985, 1997). The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of each effluent (Bakare *et al*., 2000).

The effluents were analyzed for a number of standard physico-chemical properties, including dissolved oxygen (DO), total dissolved solids (TDS), biochemical oxygen demand (BOD_5), chlorides, nitrates, and phosphates, according to methods described by APHA (1998). Nine metals (including seven heavy metals) namely: lead, cadmium, copper, iron, zinc, nickel, and manganese were analyzed in the effluent samples according to standard analytical methods (USEPA, 1996; APHA, 1998). Briefly, 100 ml of the effluents were digested by heating with concentrated HNO_3, and the volume reduced to 3-5 ml. This volume was made up to 10 ml with 0.1 N HNO_3. Concentrations of the metals were estimated by using an Atomic Absorption Spectrophotometer (Perkin Eelmer E. Analyst, 2000, USA).

The means, with 95% confidence limits and the standard errors for results of the root inhibition and chromosome aberrations at each concentration of the wastewaters were calculated. Data were expressed as Mean ± Standard Error of Mean (SEM). Differences between the control and different concentrations of the wastewaters were analyzed using the Student's unpaired *t*-test. Values of $p \leq 0.05$ were considered to be statistically significant. All statistical analyses were carried out using SPSS®14.0 statistical package.

RESULTS AND DISCUSSION

The physical and chemical properties of the different wastewaters from a bottling plant (Bt), a rubber processing industry (Rr) and a brewery (Br) at their discharge points are presented in Table 1. The effluents were characterized by considerable pollutants of suspended matter, dissolved matters and high values of turbidity. The pH of effluents of the bottling plant (Bt) was alkaline (10.50) while those of the rubber processing plant (Rr) and brewery (Br) were acidic (4.75 and 5.50 respectively. The effluents were characterized by high mean values of total hardness: 417, 320, and 298 for Bt, Rr, and Br respectively. Mean values of total suspended solids (TSS) were 34, 40, and 48 for Bt, Rr, and Br respectively and these were all above limits set for discharge into surface water. Similarly, total hardness of the effluents (417, 320 and 298) for Bt, Rr, and Br respectively, and turbidity exceeded the maximum permissible limits required by WHO. The BOD value in the effluent of Bt (19.90) was highest followed by Br (15.10) and was least in Rr (12). Lead, manganese, cadmium, copper, iron, zinc, and nickel were detected in all the wastewater samples. The overall average levels of most of the parameters of all the three industrial effluents at their discharge points were much higher than the discharge standards set by regulatory bodies (FEPA 1991, USEPA 1999, WHO 1996). These values were out of range with stipulated standards, indicating lack of wastewater treatment prior to discharge. The low DO values from the effluents compared to

FMENV (1992) standards suggest that these industries were producing a lot of organic substances which are high oxygen-demanding wastes (Emongor *et al* 2005). The relatively high levels of alkalinity could be attributed to low levels of DO. The discharge of phosphate salts and detergents used for washing in the factory is a regular source of phosphate in the wastewaters at the discharge point, and since the levels of total hardness were higher than the 100-150 mg/l values normally classified as slightly hard (Onianwa *et al*., 1999), the effluents could contribute significantly to the hardness of the rivers.

Table 1: Mean values of water quality parameters of industrial effluents in Benin City at discharge point.

Parameter	Bt	Rr	Br	FEPA[a]	USEPA[b]	WHO[c]
Temperature (°C)	30	31	28	<40	NS	NS
pH	10.5	4.75	5.5	6-9	6.5-8.5	6.5-9.5
Turbidity (FTU)	68	98	38	NS	NS	5
Total Hardness	417	320	298	NS	0-75	500
TSS	43	40	48	30	NS	NS
TDS	430	720	1500	2000	500	<1200
BOD_5 @ 20°C	19.90	12	15.10	50	NS	NS
DO	2.13	4.24	2.72	NS	NS	NS
Alkalinity	750	340	230	NS	NS	NS
Total solids	715	1012	1970	NS	NS	NS
Nitrates	50.00	86.50	54	20	10	50
Sulphates	78.00	67.50	18	500	250	500
Phosphates	0.91	1.15	0.28	5	NS	NS
Chlorides	248.50	276.90	335	600	250	250
Calcium	306	220	250	200	NS	NS
Copper	0.02	0.1	0.32	<1	0.009	NS
Manganese	0.1	0.1	0.1	5	0.05	NS
Lead	0.1	0.1	0.1	<1	0.003	0.01
Iron	0.64	0.24	0.40	20	0.30	NS
Cadmium	0.07	0.01	0.03	<1	0.002	0.003
Nickel	0.12	0.1	0.15	<1	0.005	0.02
Zinc	0.06	0.3	2.03	<1	0.12	0.01

*Values are means of 3 replicates ±SEM; *All values are in mg/L except temperature, turbidity, and pH with no unit; BOD: Biochemical oxygen demand; DO: Dissolved oxygen; TDS: Total dissolved solids; TSS: Total suspended solids; NS: Not stated; [a]Federal Environmental Protection Agency (1991) Permissible limits for effluent discharge into surface water; [b]United States Environmental Protection Agency (1999).National recommended water quality criteria – correction; [c]World Health Organisation (1996). Guideline for drinking water quality recommendation*

The mean root lengths of *A. cepa* grown in the industrial effluents are shown in Table 2. Generally, root growth retardation was observed in all the tested effluents; high growth rate was recorded in onion bulbs exposed to low concentrations and vice versa. In particular, mean root lengths obtained from onion bulbs grown in the wastewaters were in the decreasing order: Bt>Rr>Br at all concentrations, for instance, % root growth of control for the stock sample of Bt was 28.9 while those of Rr and Br were 46.98 and 53.63 respectively. Compared to the onion bulbs grown in the control, the root tips of onion bulbs grown in the industrial effluents were characterized by twists and crotchet roots (roots bent upwards resembling hooks). The induction of root malformations in *A. cepa* has been shown to be useful signs of toxicity in previous studies (Babatunde and Bakare, 2006, Bakare *et al*., 2009, Olorunfemi *et al*., 2011). Results from cytotoxic and genotoxic evaluation of the industrial effluents are indicative of a linear relationship between macroscopic and microscopic parameters for all the wastewaters. There was concentration-dependent decrease in root growth and the order of induction of root growth inhibition based on EC_{50} values was Bt>Rr>Br effluents. These values indicate that the samples were toxic; comparatively, the Bt effluent has the most inhibitory and mitodepressive effects than all the other effluents.

Table 2: Root length of *Allium cepa* after cultivation in different concentrations of industrial effluents.

Conc (%)	Bt			Rr			Br		
	Mean Root length±S.E. (cm)	RG(%) of control	95% CL	Mean Root length±S.E. (cm)	RG(%) of control	95% CL	Mean Root length±S.E. (cm)	RG(%) of control	95% CL
0	3.60±0.20	100	44	3.60±0.20	100	44	3.60±0.20	100	44
1	2.97±0.17	82.20	38	3.18±0.18	88.41	38	3.37±0.18	93.61	38
5	2.91±0.28	80.80	63	2.99±0.26	83.06	41	3.15±0.22	87.52	40
10	2.48±0.27	68.90	66	2.86±0.31	79.44	62	3.14±0.17	87.22	24

25	2.01±0.18	55.80	39	2.69±0.21	74.72	53	3.13±0.15	86.90	33
50	1.87±0.13	51.90	29	2.58±0.22	71.67	41	3.02±0.10	84.55	26
75	1.76±0.12	48.90	31	2.35±0.14	65.28	41	2.87±0.09	79.71	18
100	1.04±0.08	28.90	41	1.69±0.09	46.98	41	2.07±0.10	53.63	20
EC_{50}		35%			50%			62%	

RG (%) of control expressed as % root growth of the control; 95%CL: 95% confidence limit. * $P \leq 0.05$, level of significance of root growth inhibition compared with the untreated control. p values, (> 0.05) level as compared to controls, Values are Mean ± SEM

Results of the microscopic analysis of the treated *Allium cepa* root tips are summarised in Tables 3 and 4. Chromosomal aberrations were induced at all the tested concentrations and were statistically significant (p<0.05). The most frequent aberrations were bridges and sticky chromosomes; of these Bt effluent had the highest number, followed by effluents from Rr. Chromosomes with disturbed spindles and fragments were also present in appreciable amounts. In *A. cepa*, whenever chromosome aberrations occurred, there were almost always certain growth restrictions. Sticky chromosomes represent poisoned chromosomes with sticky surface and probably lead to cell death (Fiskesjo, 1997). Sticky chromosomes at metaphase and anaphase stages were abundant in the *Allium* test indicating that these effluents contain substances that are very toxic. The results of physico-chemical analyses corroborate this assertion. The fact that Bt effluent had the highest number of sticky chromosomes suggests that it is the most toxic of the effluents. Conversely, Br with the least number of these aberrant chromosomes suggests that it is the least toxic. Also frequent were bridges and fragments; such anomalies (i.e. the induction of chromosomal fragments and bridges at anaphase) give an indication of mutagenic events in the cell (Mishra, 1993).

Table 3: Cytological effects of effluents of Bt, Rr and Br on cells of *Allium cepa* L.

Conc (%)	**Bt**			**Rr**			**Br**		
	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells.	Mitotic index	% of aberrant cells
0	660	13.20	-	660	13.20	-	660	13.20	-
0.01	430	8.60	4.88	480	9.60	2.29	490	9.80	2.45
0.1	370	7.40	5.14	388	7.76	4.38	410	8.20	3.66
1	311	6.22	7.08	306	6.12	7.51	397	7.94	3.78
10	298	5.96	7.72	300	6.00	7.33	314	6.28	5.41

*5000 cells per conc. of each effluent and the control

Compared to the control value of 13.20, there were lower mitotic indexes with increasing concentrations in all the effluents. In particular, the lowest MI value of 5.96 was recorded for 10% Bt effluent, while MI values of 6.00 and 6.28 were recorded for Rr and Br effluents at the same concentration respectively. Conversely, there was increase in mitotic inhibition values with increasing concentration in all effluents (Table 4). The decline of mitotic index and increase in mitotic inhibition in *A. cepa* with increasing concentrations of wastewaters reflects cytotoxicity. Compared to the control, mitotic index values recorded at various effluent concentrations showed decline in the order Bt>Rr>Br while the mitotic inhibition values were in the increasing order Bt<Rr<Br. These results are in agreement with our earlier studies (Olorunfemi and Ewhre, 2010, Olorunfemi *et al.*, 2011) and studies of other workers (Samuel *et al.*, 2010). Decline of mitotic index below 22% in comparison to negative control can cause lethal effects on the organism (Antonsiewicz, 1990).

Concentration of Effluent	Chromosome aberration per 1000 cells									% of Aberrant cells	Mitotic Index	Mitotic Inhibition
	Bi-nucleated	Laggard	Bridge	Fragment	Stickiness	Micro nucleus	Vagrant	c-mitosis	Disturbed spindle			
Control	-	-	-	-	-	-	-	-	-	-	13.20	-
Bt												
0.01%	3	2	4	2	6	1	1	1	1	4.88	8.60	34.9
0.1%	2	1	4	3	5	-	1	2	1	5.14	7.40	43.9
1%	1	3	4	3	6	1	2	1	-	7.08	6.22	52.9
10%	1	1	6	3	7	1	2	1	1	7.72	5.96	54.9

Rr												
0.01%	1	2	3	1	1	1	-	-	2	2.29	9.60	27.3
0.1%	-	4	4	1	1	1	-	-	6	4.38	7.76	41.2
1%	-	4	3	4	2	5	1	-	3	7.51	6.12	53.6
10%	1	3	5	2	7	1	-	-	3	7.33	6.00	54.6
Br												
0.01	1	3	4	1	1	1	-	-	1	2.45	9.80	25.8
0.1%	1	4	2	2	4	1	-	-	1	3.66	8.20	37.9
1%	3	1	5	1	4	-	-	-	1	3.78	7.94	39.9
10%	2	4	3	2	2	-	-	-	4	5.41	6.28	52.4

Table 4: Mitotic Inhibition of *A. cepa* Root tips grown in wastewaters of Bt, Rr and Br.

Results from this study shows that a strong correlation exist between the cytological values obtained with the differential toxic response of *Chironomus travalensis kieffer* to the industrial effluents earlier investigated (Olomukoro and Okhumale, 2008). In a study on the cytogenotoxicity evaluation of two industrial effluents using *Allium cepa* assay (Samuel *et al*., 2010), the authors suggested that the onion root growth test should be fully integrated into the Whole Effluent Toxicity (WET) programme by giving a particular EC_{50} that an industrial effluent must meet before being allowed to be discharged into the aquatic environment. Results of this study have also shown that *A. cepa* chromosomal assay is a reliable tool for monitoring the genotoxic effects of industrial effluents and wastewaters; it would undoubtedly serve as a useful tool for monitoring the genotoxic effects of industrial effluents and wastewaters before they are discharged into the environment.

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