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Genotoxicity assessment of three industrial effluents using the *Allium cepa* bioassay

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The *Allium cepa* assay was employed, in conjunction with physico-chemical analysis, to investigate the potential cytotoxicity and genotoxicity of three industrial effluents (soap, beverage and paint) from the southeast of Nigeria. For *in situ* monitoring of cytotoxicity level, inhibition of mitotic division was investigated and for genotoxicity evaluation, chromosomal aberration assay was carried out. The results showed certain sample-constituents of the wastewaters (e.g. pH, turbidity) to be at concentrations beyond the maximum permissible limits required by international regulatory authorities. On the basis of the 72 h effective concentration (72 h EC_{50}), the paint effluent was the most toxic while the beverage effluent was the least toxic. The mean root lengths of *A. cepa* exposed to different concentrations of the industrial effluents, when compared to the control, were shown by Analysis of Variance (ANOVA) to be significantly ($p<0.05$) concentration dependent. The three industrial effluents were observed to induce chromosomal aberrations, laggards and sticky chromosomes being the most frequently seen. The findings show that a combination of physico-chemical analysis and genotoxicity assay is effective in assessing industrial effluents for the environmental monitoring of pollutants.

Key words: *Allium cepa*, industrial effluents, soap, beverage, paint, genotoxicity

INTRODUCTION

Waste water and solid discharge from anthropogenic activities has resulted in an alarming rate of pollution of most aquatic and terrestrial environments worldwide. The sources of such wastes include industrial effluents, domestic waste water and agriculture waste water. The rapid strides in the urbanization and industrialization of developing nations have necessitated pollution becoming an inevitable challenge. The problem is further aggravated by wrong handling and indiscriminate disposal of industrial effluents into the various water ways, coupled with the apparent ignorance of urban residents with respect to the dangers associated

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with such acts. Raw water for public water supply has continued to be the recipient of industrial wastes from multiple sources and not much attention has been paid to assessing the biological effects of inland surface waters impacted by such industrial effluent discharge.

Effluents are complex mixtures containing numerous inorganic as well as organic compounds, and most industrial effluents may contain metallic compounds in addition (Nielsen and Rank, 1994). Such pollutants may have direct health implications due to their potential cytotoxic and genotoxic effects (Grover and Kaur, 1999; Lah et al., 2004). The chemicals in effluents can also bioaccumulate (Abdel-Migid et al*.,* 2007) and biomagnify in a food chain (Alimba et al*.,* 2011; Eisen-Cuadra et al., 2013) leading to adverse effects on indigenous biota (Claxton et al*.,* 1998). The complexity of industrial effluents makes it almost impossible to carry out hazard assessment merely on the basis of conventional water chemistry analysis (El-Shahaby et al., 2003). It is therefore imperative to evaluate hazardous wastes and effluents by genotoxicity assays in order to obtain data that can be used for hazard identification and comparative risk assessment (Organisation for Economic Co-operation and Development (OECD), 2015).

Although Nigeria has undergone relatively rapid industrialization over the last four decades, this was not guided by comprehensive environmental awareness, governance, efficient regulatory systems, enforced planning regulations and environmental sound waste management practices. In the southeast of Nigeria, most industries are sited close to residential houses and other urban areas thereby exposing nearby human populations to great health risk from pollution. Of particular interests are the paint, food processing and cosmetic industries, which are the major industries in the region and among others discharge large volumes of raw or partially treated effluents containing hazardous substances continuously into nearby gutters or drains. These end up in streams, rivers and wetlands thereby leading to gross pollution of the ecosystem. There is a general paucity of reports on genotoxicity evaluation of industrial effluents in Nigeria in general (Odeigah, et al., 1997; Bakare et al., 2009; Samuel et al., 2010, Oladele et al., 2013) and little or no reports on the southeast region in particular. A recent study in the region was focused only on the potential impact of effluents on water quality (Oladele et al., 2017).

Of all the investigations carried out with higher plants that have been recognized as excellent genetic models for the detection of environmental mutagens, the *Allium cepa* assay stands out. It was introduced by Levan (1938) to examine the effect of colchicines on mitotic spindles and has been rated as a standard method in environmental monitoring and toxicity screening of waste water and river water (Fiskesjö, 1985, 1993; Rank and Nielsen, 1993, 1998; Abdel-Migid et al*.,* 2007; Grover and Kaur, 1999; Junior et al*.,* 2007; Vesna et al*.,* 1996).

In this study, the *A. cepa* assay was employed to evaluate the toxicity of waste waters from three industries in Southeast of Nigeria in consonance with chemical analysis. The results of this study will go a long way in providing the much needed data that can be used as a scientific basis for the regulation of the discharge of potentially hazardous substances into the environment.

MATERIALS AND METHODS

Sampling sites and analysis of samples

The soap effluent (Sp) was collected from the PZ Cussons Factory, Aba, Abia State; the beverage effluent (Bv) from Consolidated Breweries, Awomama, Imo State; while the paint effluent (Pt) was from Saclux Paint Industry Ltd., Umuahia, Abia State. The three raw industrial effluents (Sp, Bv and Pt) were collected from the industrial waste water discharge pipes of the respective factories. The effluents from these industries are discharged into nearby municipal rivers and drainages. At the time of collection, the water reaction (pH) and the electrical conductivity (EC) of the samples were determined and the samples were analyzed for other standard physico-chemical parameters such as turbidity, alkalinity, Cl, So_4, Co_3, Na, Ca, No_3, K, organic carbon and inorganic carbon according to standard analytical methods (United States Environmental Protection Agency - USEPA, 1996; American Public Health Association - APHA, 1998). Collection was done in plastic containers and the samples were stored at 4°C (in a refrigerator), pending use.

Biological materials

The common purple onion *A. cepa* L. Stuttgarter Reisen (2n=16, Family Amaryllidaceae) bulbs (2.5 - 2.8 cm diameter) used for the study were commercially procured from Eke Okigwe Market, Abia State, Nigeria. They were sun dried for 2 weeks and the dry bulbs (excluding the rotten ones) were later used for the tests.

The *Allium cepa* assay

The modified assay (Fiskesjö, 1997; Bakare and Wale-Adeyemo, 2004; Babatunde and Bakare, 2006) was carried out using 100-ml beakers. Lead nitrate ($Pb(NO_3)_2$) solution and distilled water were used as positive and negative controls respectively while distilled water was used for the dilution of the industrial effluents. The effluent in each case was equilibrated to room temperature (26±2°C) and diluted with distilled water to produce the series of concentrations investigated. Prior to the test, the outer scales of the bulbs and brownish bottom plates were removed, leaving the ring of root primordial intact. The peeled bulbs were placed into fresh water during the cleaning process so as to protect the primordial from drying. Afterwards, the bulbs were exposed directly to 100, 75, 50 and 25% (v/v, effluent/distilled water) of each of the test liquid. Five onions were used for each concentration of each individual effluent and the control, that is, each concentration was set up in 5 replicates. The base of each onion bulb was suspended on the test liquid in 100-ml beakers in the dark at 27±1°C. The test liquids were changed daily.

Genotoxicity investigation

After 48 h, the root tips of one bulb in each group of the

Table 1. Physico-chemical characteristics of industrial effluents analyzed for genotoxicity.

Parameter	Sp	Bv	Pt	FEPA[a]	USEPA[b]	WHO[c]
pH	13.47	7.50	5.34	6-9	6.5-8.5	6.5-9.5
Electrical conductivity	18.4	25.6	57.6	NS	NS	NS
Turbidity (NTU)	64	49	320	NS	NS	<0.1-5.0
Alkalinity	22500	32	35	250	20	250
Total Hardness	1300	10	40	NS	0-75	100-300
Calcium Hardness	1100	9	26	200	NS	NS
% Nitrogen	0.125	0.118	0.164	NS	NS	NS
NO_3	1.42	0.62	4.91	20	10	50
PO_4	0.68	0.24	2.81	500	5	NS
Na	300	18	50	NS	NS	150
K	750	30	80	250	NS	150
% Total Carbon	0.260	0.408	1.343	NS	NS	NS
% Organic Carbon	0.113	0.064	0.162	2.50	NS	150
% Inorganic Carbon	0.147	0.344	1.181	NS	NS	NS
Moisture content	97.86	99.94	98.43	NS	NS	NS

Values are in mg l^{-1} except turbidity and pH with no units; NS-Not stated (that is, no guideline established). [a]Federal Environmental Protection Agency (1991). Permissible limits for effluent discharge into surface water. [b]United States Environmental Protection Agency (1999) National recommendation water quality criteria-correction; [c]World Health Organisation (1996) Guideline for drinking water quality recommendation.

experimental organisms were fixed separately in ethanol:glacial acetic acid (3:1, v/v) and were used for the chromosomal analysis. The root tips (for each effluent concentration and the control) were hydrolyzed in 1N HCl at 60ºC for 5 min and rinsed in distilled water. Two root tips were placed on each slide and stained in aceto-carmine for 20 min (after squashing). Excess stain was removed with filter paper and cover slip was carefully lowered to prevent air bubbles being trapped under. The edges of the cover slip in each case were sealed with clear nail polish as suggested by Grant (1982) to prevent drying out of the preparation by the heat of the microscope (Sharma, 1983). Five slides were prepared for each effluent concentration and for the control. The prepared slides were coded and examined for chromosomal aberrations at high magnification (X1000). The mitotic index (MI) was calculated as percentage of the number of dividing cells per 1000 (400 cells per slide per concentration and control) observed cells in each case (Fiskesjö, 1985, 1997) and the mitotic inhibition was estimated as the percentage of the difference between the mitotic indices of the control and the group divided by the mitotic index of the control. 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).

Root growth inhibition test

The effects of the industrial effluents on the morphology of growing roots of *A. cepa* were examined. The growth inhibition assay was performed as a 72 h semi-static exposure test (Bakare et al*.,* 2009), that is, the test organisms were exposed for 72 h to the different concentrations (100, 75, 50 and 25%) of the industrial effluents. At the end of exposure, the length of the root bundle was measured for the remaining four bulbs at each concentration for each industrial effluent and the control. Growth inhibition was estimated as EC_{50} (concentration of a toxicant that gives half-maximal response).

Statistical analysis

Pearson correlation analysis was carried out to test for significant relationship (positive or negative) between the average root lengths and effluent concentrations. One-Way Analysis of Variance (ANOVA) and Student Newman Keul's (SNK) post-hoc tests were used to test for significant differences in the mean root lengths of *A. cepa* exposed to different concentrations of the soap, beverage and paint effluents. The analysis was performed using the IBM SPSS ® 22.0 statistical package. The results were expressed with 95% confidence limits, that is, 0.05 probability level. The EC_{50} was determined from a plot of root length as a percentage of control against the sample concentrations by using Microsoft Excell computer program.

RESULTS AND DISCUSSION

Physico-chemical characteristics

The physical and chemical properties of the effluents from the soap industry (Sp), beverage industry (Bv) and paint industry (Pt) at their discharge points are shown in Table 1. The results indicate the presence of certain sample-constituents at concentrations beyond the maximum permissible limits required by international regulatory authorities (Federal Environmental Protection Agency (FEPA), United States Environmental Protection Agency (USEPA), World Health Organisation (WHO)). The pH of the soap industry effluent was extremely alkaline (13.47) while that of the paint industry was acidic (5.34) and that of the beverage industry was close to

Table 2. Mean (±SD) root length of *A. cepa* exposed to different concentrations of the three industrial effluents.

Soap effluent (Sp)		Beverage effluent (Bv)		Paint effluent (Pt)	
Concentration (%)	**Mean root length (cm)**	**Concentration (%)**	**Mean root length (cm)**	**Concentration (%)**	**Mean root length (cm)**
Control (0)	3.86 ± 1.05	Control (0)	3.86 ± 1.05	Control (0)	3.86 ±1.05
25	2.31 ± 1.09	25	3.20 ± 0.13	25	2.40 ± 0.12
50	2.19 ± 0.82	50	3.04 ± 0.08	50	2.25 ± 0.09
75	1.73 ± 0.79	75	2.07 ± 0.11	75	1.19 ± 0.08
100	1.15 ± 0.08	100	1.45 ± 0.09	100	1.02 ± 0.05
EC_{50}	58	EC_{50}	84	EC_{50}	52

neutral (7.50). The Sp, Bv and Pt effluents were characterized by considerable pollutants of suspended matter and dissolved matter, thus having high values of turbidity, that is, 64, 49 and 320 respectively, exceeding the maximum permissible limits required by the World Health Organization (WHO). A turbidity value < 5 NTU is considered acceptable as higher values indicate the presence of particulates which can protect bacteria from disinfection thereby stimulating their growth. The total hardness of the soap effluent (Sp) was very high (1300), likewise the Na and K levels. Although elevated levels of total hardness has not been shown to have any adverse health effects, high levels of Na and K have been implicated in different human diseases. Excessive Na in water has been associated with hypertension, although this is yet to be firmly established, while high levels of K is known to cause health defects in susceptible individuals (e.g. kidney dysfunction, heart disease, coronary artery disease, hypertension etc.) (WHO, 1996). However, the analyzed constituents do not by any means represent all or even most of the chemicals that could have been included in the test-samples. The heavy metals and metalloids components of the industrial effluents were not investigated. It is however well known that an effluent is a complex mixture of organic and inorganic chemicals and of many unidentified toxicants known as non-conventional pollutants (NCPs) which, either singly or synergistically, may pose risks of an unknown magnitude to humans.

Macroscopic analysis

The summary of the results of root growth analysis of *A. cepa* exposed to different concentrations of the industrial effluents investigated are presented in Table 2. The estimated EC_{50} values of *A. cepa* exposed to soap, beverage and paint effluents were 58, 84 and 52% respectively. The EC_{50} is used as a measure of potency. The results therefore indicate that the paint effluent is the most toxic while the beverage effluent is the least toxic. Statistical analysis (with analysis of variance - ANOVA), showed that there was a significant ($p<0.05$) difference in the mean root lengths of the test organism exposed to different concentrations of the soap, beverage and paint effluents. Generally, root growth inhibition analysis, using Pearson correlation, was observed to be positively correlated to concentration in all cases. Further Post Hoc analysis using Student Newman Keul's (SNK) test confirmed that root growth retardation was significantly ($p<0.05$) concentration-dependent, that is, high growth rate was observed with decreasing effluent concentration in most cases and vice versa.

Root growth inhibition occurs as a result of the inhibition of cell division (indicating toxicity) and it is thus an index for estimating general toxicity. It occurs when roots are exposed to a wrong pH, or to unsolved substances that may prevent nutrition uptake (Fiskesjö, 1993). The pH value of 5.34 obtained for the paint effluent is quite acidic for a living system and this could be responsible for the high toxicity of the effluent. The inhibitory effects can also be on cell extension, that is, cessation of root elongation which is correlated with the disappearance of mitotic figures. It has been observed that some mechanism associated with cell division is highly sensitive to certain chemicals or metals (such as those found in industrial effluents) and is permanently damaged by short exposures (Clarkson, 1965). Thus, the root growth analysis results indicate that all the effluents investigated had cytotoxic effects on the roots of the test organisms although statistical analysis of the mean root growth of *A. cepa* exposed to the different effluents did not show any significant ($p<0.05$) difference.

Microscopic effects

The cytological analyses of *A. cepa* roots exposed to different concentrations of the industrial effluents examined are presented in Table 3a-c. When compared to the negative control value of 40.3%, there was a statistically significant ($p<0.05$) decrease in mitotic index (MI) with increasing effluent concentration in the onions grown with the three effluents investigated. The

Table 3. Cytological effects of the three industrial effluents on *A. cepa* root cells.

Conc. (%)	No of dividing cells	Mitotic index (MI)	Mitotic inhibition (%)	Stickiness	Laggards	Bridges	Fragment	% Freq. of aberrant cells (±SD)
Soap effluent								
Control (0)	403	40.3	0.0	0	0	0	0	0.00 ± 0.00
25	320	32.0	20.60	0	1	2	2	1.56 ± 0.10
50	238*	23.8	40.94	1	0	1	1	1.26 ± 0.60
75	173*	17.3	57.07	0	1	0	0	0.58 ± 0.05
100	104*	10.4	74.19	1	2	0	0	2.88 ± 0.90
Beverage effluent								
Control (0)	403	40.3	0.0	0	0	0	0	0.00 ± 0.00
25	296	29.6	26.55	0	1	1	1	1.01 ± 0.75
50	245	24.5	39.21	1	0	1	0	0.82 ± 0.45
75	206*	20.6	48.88	2	0	0	0	0.97 ± 0.05
100	185*	18.5	54.09	2	1	0	0	1.62 ± 0.82
Paint effluent								
Control (0)	403	40.3	0.0	0	0	0	0	0.00 ± 0.00
25	197*	19.7	51.12	1	3	1	1	3.05 ± 0.86
50	143*	14.3	64.52	0	1	1	0	1.40 ± 0.06
75	96*	9.6	76.18	2	1	0	0	3.13 ± 0.90
100	72*	7.2	82.13	4	6	1	0	15.28 ± 1.25

* Values are significantly different from control at $p<0.05$ (ANOVA).

mitotic index, which is estimated as the ratio of number of cells in mitosis and the total number of cells, is an indirect measure of cell proliferation and it is considered to reliably identify the presence of cytotoxic pollutants in the environment (Chandral and Kulshreshtha, 2004). The mitotic index thus gives an insight into the inhibition of cell division and it is observed microscopically by counting the number of cells in metaphase, which is an index of the meristematic cell.

Dose-dependent inhibition of the mitotic indices in the test organisms could be due to intracellular stress, including DNA damage, preventing cells from entering mitosis. It could also be due to a negative interference of the active substance contained in the effluents tested with DNA synthesis, microtubule formation, impaired nucleoprotein synthesis and reduced level of ATP to provide energy for spindle elongation, microtubule dynamics and chromosomal movements (Majewska et al., 2003; Türkoğlu, 2012). A mitotic index (MI) decrease below 22% of control causes lethal effects on test organism while values below 50% is sublethal and is called cytotoxic limit value (Sharma, 1983). Thus, the mitotic indices show that the effects of the effluents were sublethal at 25 and 50% concentrations in the soap and beverage industrial wastewaters but lethal at higher concentrations (75 and 100%). However, the effects were lethal at all concentrations of the paint effluent.

The microscopic analysis of the cells of *A. cepa* exposed to the test liquid showed that chromosomal aberrations were induced in the root tip cells of *A. cepa* exposed to the different industrial effluents at different concentrations and no aberration was observed in the control group. Most aberrations were observed in *A. cepa* cells exposed to the paint effluent (Pt) indicating that it is the most toxic of the effluents investigated (Figure 1a-c). Previous studies have strongly suggested that the polycationic character of polymeric paint components may underlie their cellular cytotoxicity (Roberts et al., 1996; Hoet et al., 2000). The variation of chromosomal aberrations with the different test liquids was however not dose-dependent. This is in agreement with Odeigah et al. (1997) but in contrast to the observations by Qian (2004) who reported that chromosome aberration increased with increasing effluent concentration. A possible explanation for the former is that with increasing concentration, and consequently increasing toxicity, there was an inhibitory effect on cell division. This might result in prophase arrest with the attendant decline in the observation of chromosome aberration (Odeigah et al., 1997).

In general, chromosome aberration induction by the industrial effluents indicates their genotoxic effects. The genotoxicity of various types of industrial wastewaters

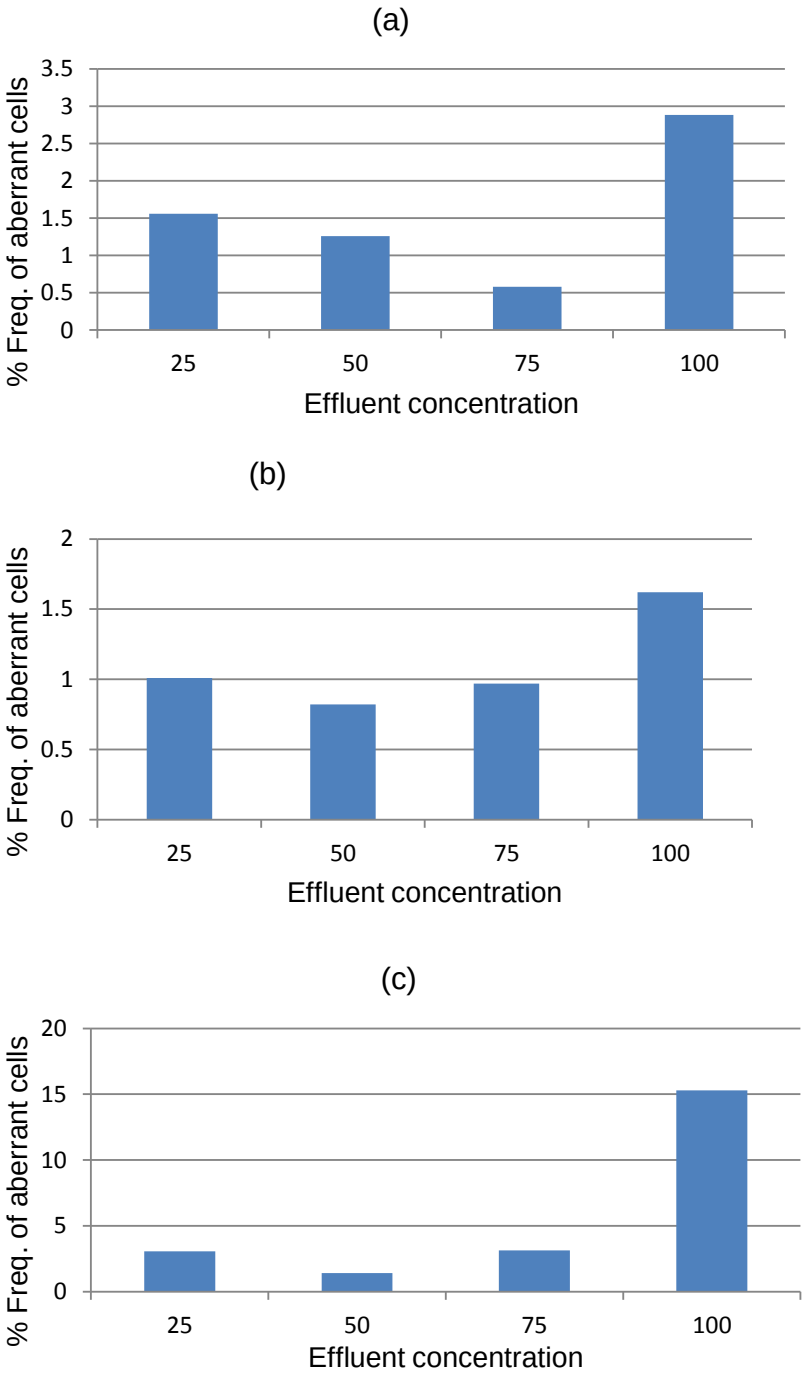

Figure 1. Histogram showing the percentage of chromosomal aberrations at the different concentrations of the three industrial effluents (a) Soap Effluent (b) Beverage Effluent and (c) Paint Effluent.

using the *Allium* test has been severally reported. Odeigah et al. (1997) reported the genotoxicity of oil field wastewater; El-Shaby et al. (2003) reported the genotoxicity of industrial wastewater from the Sandub area in Mansoura district in Egypt, while Babatunde and Bakare (2006) reported the toxicity of wastewaters from Agbara Industrial Estate, Nigeria. Olorunfemi et al. (2015) had also reported the toxicity of process water from Nigeria Agip Oil Company (NAOC) and Kannangara and Pathiratne (2015) reported the genotoxicity of textile

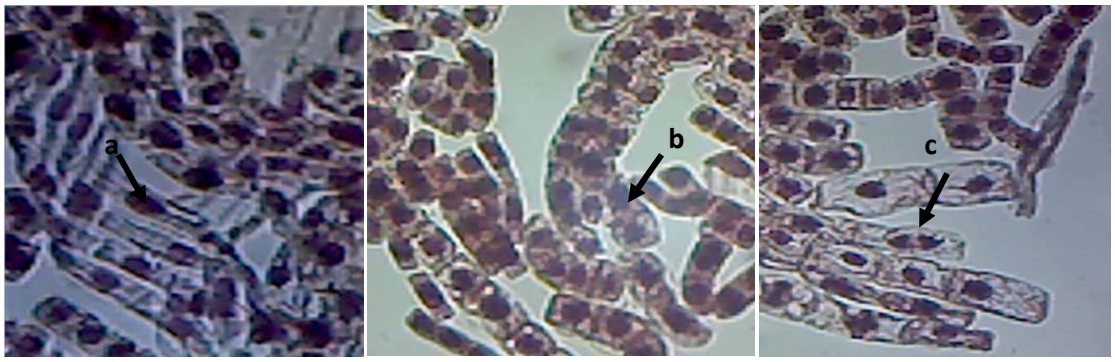

Figure 2. Chromosomal aberrations induced in *Allium cepa* by industrial effluents. (a) sticky chromosomes (b) disoriented chromosomes (c) bridge.

dyeing effluent and leachate from a tannery effluent. Hemachandra and Pathiratne (2017) also observed that raw water induced statistically significant root growth retardation, mitodepression and chromosomal abnormalities in the root system of plants. However, effluents were observed to provoke a relatively cytogenotoxic effects in *A. cepa* but toxicity in most cases was considerably reduced to raw water level with effluent dilution.

The most common cytogenetic aberrations observed were stickiness followed by laggards while other aberrations observed at various frequencies were bridges (Figure 2). Fragments were the least recorded. Chromosomal aberrations indicate the presence of certain cytotoxic or genotoxic substances in the industrial effluents investigated as no aberrations were observed in the controls (0%). The presence of sticky chromosomes indicate a highly toxic, irreversible effect which could lead to cell death while vagrant chromosomes are consequent of weak C mitotic effects indicating a high risk of aneuploidy (Fiskesjö, 1985, 1988). Many of the chromosomal aberrations induced by the action of various types of mutagenic agents might be due to the dysfunction of nuclear spindle (Fiskesjö, 1985). Structural chromosomal aberrations (such as bridges and breaks) are indicators of clastogenic actions; numerical ones (e.g. chromosome losses, delays, adherences, multipolarity and C-metaphases), are usually consequent of abnormal segregation while stickiness (that is, chromosomes without telomeres, fusing with other broken chromosome ends) may be induced by DNA breaks, inhibition of DNA synthesis at S-phase and replication of altered DNA (Metin and Burun, 2010; Glinska et al., 2007). Chromosome stickiness or adhesion as physiological aberration is a type of physical adhesion that involves mainly the proteinaceous matrix of the chromatin material and might be interpreted as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fibre units of chromatids, and a stripping of the protein covering DNA in chromosomes (Mercykutty and Stephen, 1980).

Conclusion

The results from the study show that the industrial wastewaters investigated were all toxic although to different degrees and thus their disposal constitutes serious pollutants to be reckoned with in the environment. It is therefore obvious that the discharge of the effluents into water bodies could lead to pollution of surface water and subsequently impair biolife. The results are also a clear indication of the reliability and high sensitivity of the *A. cepa* assay in the detection of genotoxicity of industrial waste waters because the test system provides information to evaluate action mechanisms of an agent about its effects on the genetic material (clastogenic and/or aneugenic effects). There is also a demonstration that in the *A. cepa* test, there is usually a relationship between growth retardation, mitotic indices and chromosomal damage (genotoxicity) (Olorunfemi et al., 2012). According to Samuel et al. (2010), the onion root growth inhibition test should be integrated in the whole effluent test (WET) program and a specified EC_{50} should be fixed as a condition to be met before the disposal of effluent into the environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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