EVALUATION OF BILGE WATER-INDUCED PLANT GENOTOXICITY USING RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD)

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ABSTRACT

Indiscriminate disposal of bilge water into water bodies has been identified as a source of environmental pollution. There is currently no study on the genotoxicity evaluation of bilge water using molecular maker. Physicochemical analysis of the wastewater showed that it was slightly acidic with a pH of 6.09 and contained nitrates, phosphates, chlorides, iron and nickel at amounts higher than the national (NESREA) and international (USEPA) maximum limits allowed for effluent discharge. The Random Amplified Polymorphic DNA (RAPD) technique was employed to evaluate the level of DNA alteration in *Allium cepa* root tips exposed to different concentrations of bilge water (1, 5, 10, 25 and 50%). Compared to the control, DNA polymorphism which was reflected by changes in the RAPD profiles as variation in band intensity, disappearance of bands and appearance of new bands, was induced by the different concentrations of bilge water on the genome of *Allium cepa* root cells. The genetic distances shown on the dendrogram revealed that the genotoxicity of the wastewater was concentration-dependent. This study has shown that polymorphism detected by RAPD can be considered as a powerful molecular marker assay for the detection of the genotoxic effect of bilge water.

Keywords: Bilge water, RAPD, DNA damage, *Allium cepa*, Ships

INTRODUCTION

The maritime industry is international in nature and is acknowledged to be a very dynamic component in the socio-economic development of any given maritime nation. It is estimated that the world seaborne trade in 2013 amounted to 9.35 billion tons of cargo (Werschkun, 2014). Associated with phenomenal growth and development in the shipping industry worldwide, there is significant increase in the amount of oil used, most of which leak into the water through bilge pumping. Depending on the design and function of the ship, the bilge water so formed is a mixture of a variety of substances which get accumulated in the bilge wells (Karakulski, *et al*., 1998).

Bilge water containing oily wastes is normally treated by regular operation of oily water separators, filtration and other related shipboard systems (Han *et al*., 2014). As a result of the high cost of treating bilge water by physical processes or by transportation to inland storage and disposal stations, bilge water treatment is an operational problem. Consequently, bilge water is illegally discharged into the surrounding water bodies from ships at various locations; accounting for about 10% of the total oils entering the sea ecosystem (Sivaraman *et al*., 2011).

Cargo throughput in Nigeria's ports in the first quarter of 2014 stood at 19,659,946 million metric tonnes, an increase of 14 per cent over 17,245,923 metric tonnes achieved in 2013. A total of 1,327 ocean-going vessels with a total Gross Registered Tonnage (GRT) of 33,940,386 called at Nigerian Ports compared with 1,172 vessels with a GRT of 28,830,386 in 2013 (NPA, 2014). The International Convention for the Prevention of Pollution from Ships and Act to Prevent Pollution from Ships (MARPOL) have mandated all ships to discharge bilge water containing less than 15 ppm of oil at sea, a regulation that has often been disregarded (OECD, 2003). The regulatory bodies charged with the responsibility for the management of the marine environment are yet to enforce full implementation of important international agreements and programmes, including the London Protocol, to protect the marine environment (IMO/NIMASA, 2013).

The RAPD technique is a selective and sensitive assay for DNA analysis in eco-genotoxicology. A recent advance in molecular biology, the RAPD is used to evaluate the variation at the DNA level. It

is a reliable and reproducible assay and has the potential to detect a wide range of DNA damage as well as mutations caused by heavy metal stress and therefore, it can be applied to study genotoxicity (Atienzar and Jha, 2006). The RAPD technique is suitable for the analysis of DNA extracted from any organism because of its rapidity, applicability to any organism and its ability to detect a wide range of DNA damages and mutation. Previous studies have shown that changes in DNA band patterns observed reflects DNA alteration in genome from single base changes (point mutation) to complex chromosomal rearrangements, and that DNA fingerprinting offers a useful biomarker assay in assessment of genotoxicity (Baeshin *et al*., 2009; Quari, 2010; Ozakca and Silah, 2013; Hassan and Yassein, 2014). To the best of our knowledge, there is no documented information on the phytogenotoxicity evaluation of bilge water using molecular markers. Consequently, the RAPD assay was employed in this study to detect possible DNA damage in *Allium cepa* root cells exposed to bilge water to ascertain the toxicological implications of the wastewater on plant and animal life.

MATERIALS AND METHODS

Collection of Water Samples and Determination of Physicochemical Parameters

The bilge water was collected in April, 2014 from "M/T" Azuryth, an oceangoing vessel used to transport crude oil. It was berthed on high sea in the Lagos port complex (port of Lagos) located at the Apapa area of Lagos. The wastewater, together with control (tap water) were analysed for a number of standard physicochemical properties, including sulphates, phosphates, carbonates, nitrates and chloride according to methods described by APHA (2005). Ten metals namely: lead, copper, cadmium, chromium, iron, zinc, aluminum, cobalt, nickel and manganese were analysed in the water samples according to standard analytical methods (USEPA, 1996; APHA, 2005) using an Atomic Absorption Spectrophotometer (AAS) (PerkinElmer A Analyst 100).

Plant Material and Treatment

The purple variety of average-sized onion bulbs, *Allium cepa* L. (about 30 g, 15-22 mm diameter) were purchased from a local market in Benin City, Nigeria (6°15'N, 5°25'E) and sun-dried for two weeks. 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. Thereafter, the bulbs were removed from the distilled water and placed on a blotting paper to remove excess water. Seven onion bulbs were utilized for each water sample and the control (tap water). The base of each of the bulbs was suspended on the water sample inside 100 ml beakers in the dark for 7 days. The bilge water samples were changed daily. At the end of the exposure period, the roots with the best growth were removed with a pair of forceps and utilized for chromosomal DNA extraction and RAPD analysis.

DNA Extraction

After seven days of exposure to test treatments, root tips of approximately 2.5-3.5 cm of the onion bulbs were collected, ground in liquid nitrogen, and the total genomic DNA was extracted by a CTAB method based on that of Padmalatha and Prasad (2006) with minor modifications (Qari, 2010). The DNA purity was determined by measuring the optical density using the nanodrop spectrophotometer at 260/280. The quality of DNA was determined using gel electrophoresis and observing under a UV illuminator.

RAPD Fingerprinting

The conditions of DNA amplification followed the procedure of Williams *et al.* (1990) with some modifications (Qari, 2010). Random Amplified Polymorphic DNA (RAPD) was performed using primers OPT19 (5´GTCCGTATGG3´), OPT17 (5´CCAACGTCGT3´), OPH02 (5´TCGGACGTGA3´), OPT05 (5´GGGTTTGGCA3´) and OPT04 (5´CACAGAGGGA3´) (Operon technologies Inc., Alameda, California, USA) for each amplification. Each reaction (25 µL) consisted of 1 mM of $MgCl_2$, 4 mM each of dATP, dCTP, dGTP, dTTP (Boehringer-Manheim, Germany), 400 nM primer, 1.0 U of Taq DNA polymerase (Appligene-Oncor, France), reaction buffer (1

mM $MgCI_2$, 20 mM Tris HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and 50% glycerol). The reaction mixture was overlaid with a drop of mineral oil and incubated in a thermal cycler (Thermal cycler 480, Perkin Elmer-Cetus, USA) programmed as follows: 48 cycles of 1 mm denaturation at 94°C, 1 mm annealing at 37°C and 1.5 mm extension at 72°C, followed by final extension at 72°C, followed by a cooling at 4°C. Tubes containing all reaction products except template DNA were used as negative control.

PCR reaction products were mixed with one-sixth volume of gel loading buffer (analytical grade water containing 36% glycerol, 0.05% bromophenol, 30 mM EDTA and 0.05% xylene cyanol), and then separated by electrophoresis in a 2.4% agarose gel, using a Tris-borate-EDTA (TBE) system (0.5 × TBE = 45 mM Tris-base, 45 mM boric acid, and 1 mM EDTA). Agarose gel dimensions were 12×6×0.5 cm^3. For comparison, DNA molecular size marker (1 kb) was used for each agarose gel.

Statistical Analysis

The data were expressed as mean ± SEM. The differences between mean values and the controls were statistically investigated using student t-tests. Genomic template stability (%) was calculated as 100 − (100 **a/n**), where **a** was RAPD polymorphic profiles detected in each treated sample while **n** was the total number of bands in the control. Polymorphism observed in RAPD profiles include disappearance of a normal band and appearance of a new band in comparison with control RAPD profiles (Williams *et al.*, 1990; Atienzar *et al.*, 1999). The average was then calculated for each experimental group exposed to different concentrations of each bilge water sample.

RESULTS

Physicochemical Parameters of Bilge Water

The physicochemical characteristics of bilge water used in this study are presented in Table 1. The wastewater was slightly acidic with a pH of 6.09 and composed of a variety of dissolved cations and suspended particles. Some of the parameters notably nitrates, phosphates, chlorides and nickel showed deviation from national (NESREA) and international (USEPA) specifications for maximum limit allowed for effluent discharge into water bodies for all categories of industries. Lead, copper, cadmium and chromium were not detected in the wastewater samples.

Table 1: Physicochemical Properties of Bilge Water

Parameter	Bilge Water	NESREA (2009) Limit	USEPA (2009) Limit
pH	6.09	6-9	6.5.-8.5
Turbidity	12.1	-	-
Ammonia	0.04	1	0.03
Sulphates	43.00	250	250
Nitrates	57.01	10	10
Phosphates	54.04	2	-
Carbonates	188	-	-
Chloride	338.00	250	250
Iron	0.13	-	0.3
Zinc	0.04	-	0.12
Manganese	0.02	0.2	0.05
Aluminum	0.02	-	-
Nickel	0.09	0.05	0.005
Cobalt	0.01	-	-

All values are expressed in mg/L except pH (no units). NESREA = National Environmental Standards and Regulations Enforcement Agency (2009), USEPA = United States Environmental Protection Agency (2009) maximum permissible limits for effluent from wastewater.

RAPD Profiles of Bilge Water

The range of the purity of extracted DNA from treated root tips of onion bulbs was between 1.71 and 2.04. The result of the RAPD profile of nucleotide sequences of the five primers used and the base sequence of the primers tested is presented in Table 2. In all, 150 bands were scored and 27 (18%) were polymorphic.

Table 2: Nucleotide Sequence Showing Total Bands and Percentage Polymorphism in Bilge Water

RAPD Primers	Sequence 5'----3'	G=C Content (%)	Total Bands	Polymorphism (%)
OPT-04	CACAGAGGGA	60	35	14.29
OPH-02	TCGGACGTGA	60	18	0
OPT-05	GGGTTTGGCA	60	20	10
OPT-17	CCAACGTCGT	60	45	20
OPT-19	GTCCGTATGG	60	32	31.43
	Total		**150**	

Table 3: Changes in RAPD Profiles Scored Compared with Control

Treatments (%)	RAPD Band Gain (+)	RAPD Band Loss (-)
1	0	2
5	0	2
10	1	2
25	2	2
50	5	4
Total	8	**12**

Oligonucleotide primer OPH02 showed no polymorphism while OPT04, OPT05, OPT17 and OPT19 showed with OPT19 showing the highest while OPT17 had the highest number of bands. There was an increase in the gain and loss of bands as the concentration of the wastewater increased. For instance, there was an increase in band gain from 2 at 25% to 5 at 50% concentration. Similarly, there was an increase in band loss from 2 at 25% to 4 at 50% bilge water concentration. Altogether, 12 bands were lost while 8 were gained (Table 3). The RAPD profile obtained with the five oligonucleotide primers used produced bands between 120 and 1300 bp in length (Plates 1 -5).

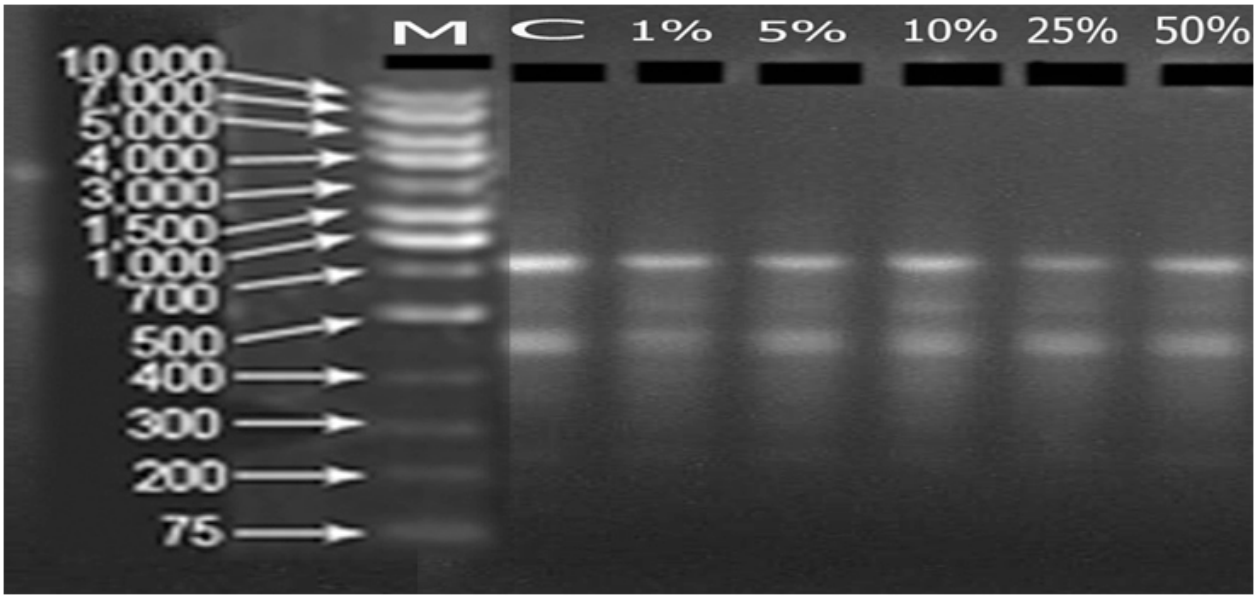

Plate 1: Profiles RAPD of genomic DNA from root cells of *Allium cepa* grown in the bilge water samples using OPH02 (M = DNA ladder, C = control).

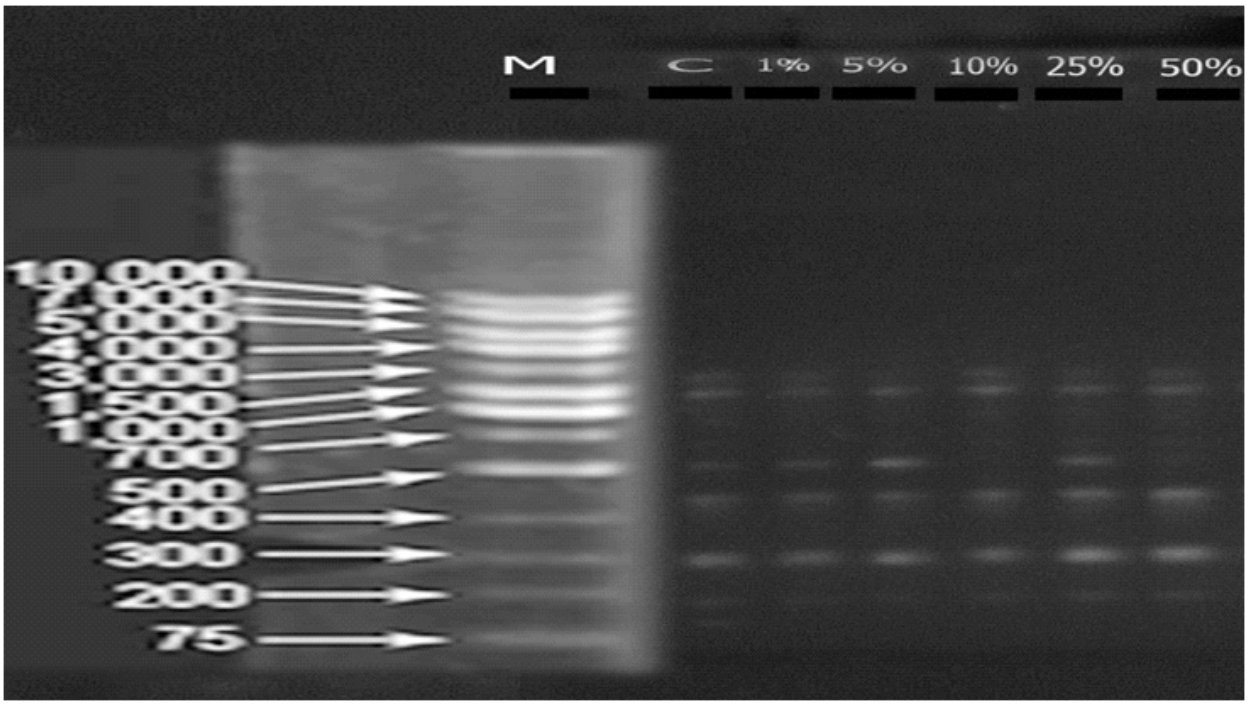

Plate 2: Profiles RAPD of genomic DNA from root cells of *Allium cepa* grown in the bilge water samples using OPT04 (M = DNA ladder, C = control).

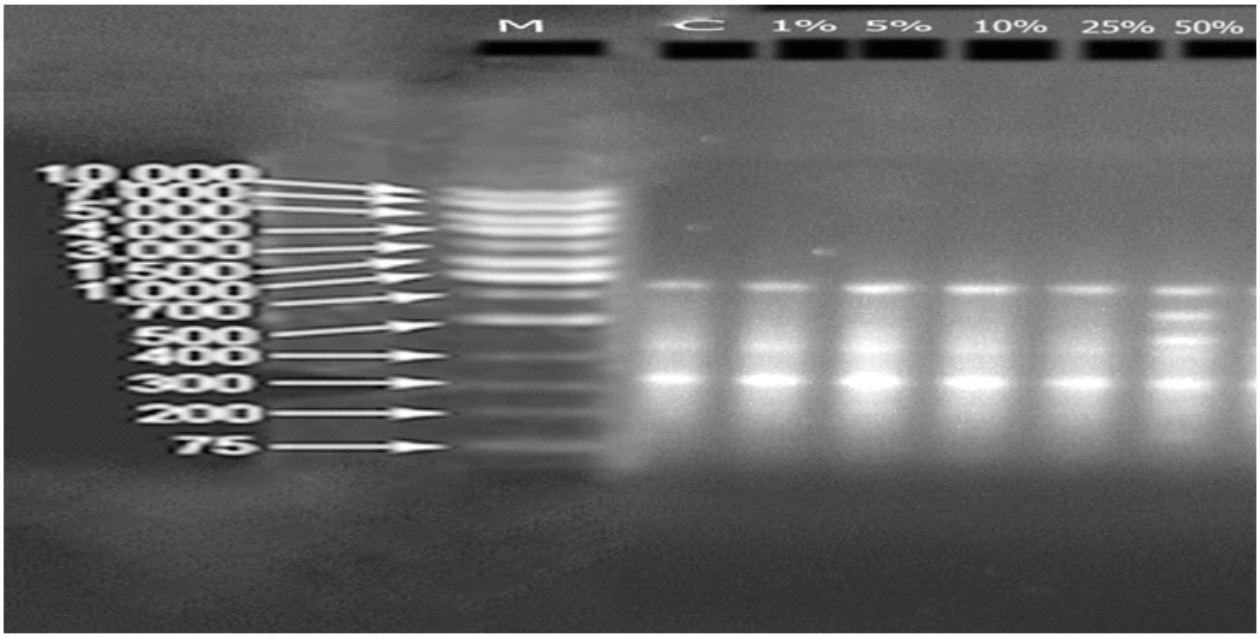

Plate 3: Profiles RAPD of genomic DNA from root cells of *Allium cepa* grown in the bilge water samples using OPT05 (M = DNA ladder, C = control).

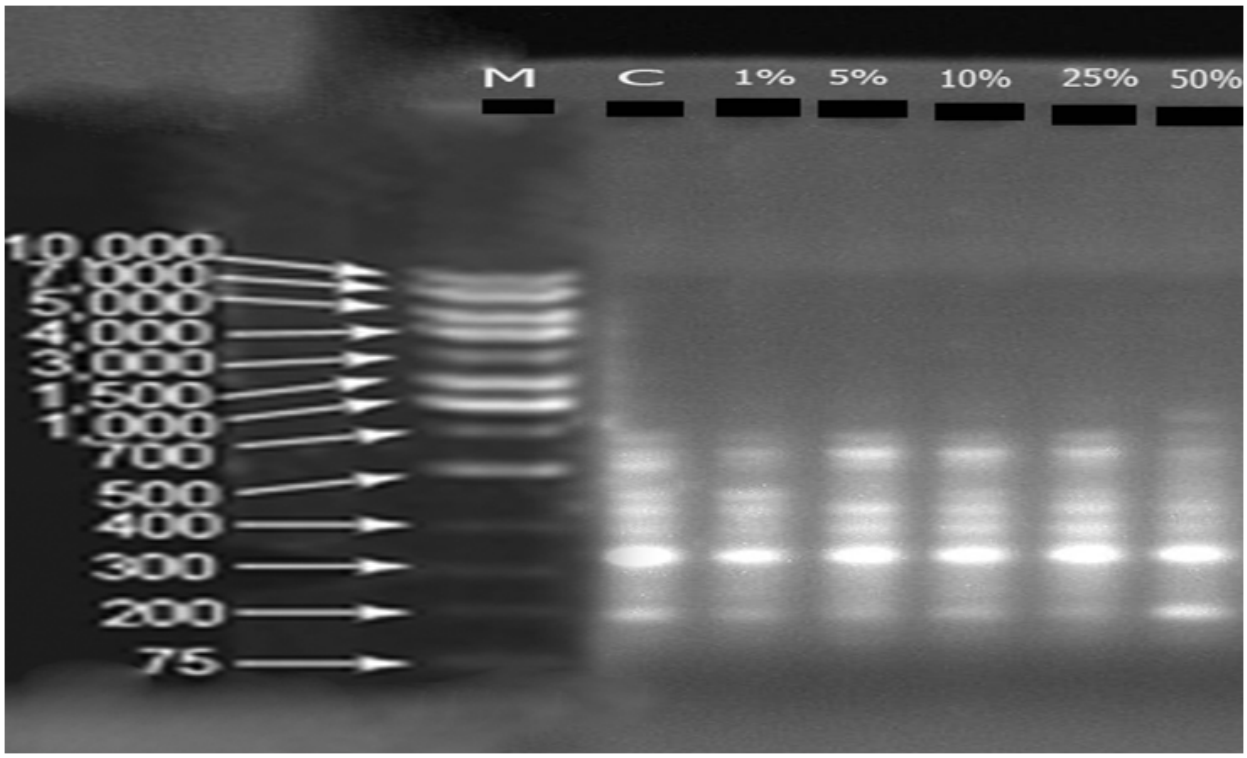

Plate 4: Profiles RAPD of genomic DNA from root cells of *Allium cepa* grown in the bilge water samples using OPT17 (M = DNA ladder, C = control).

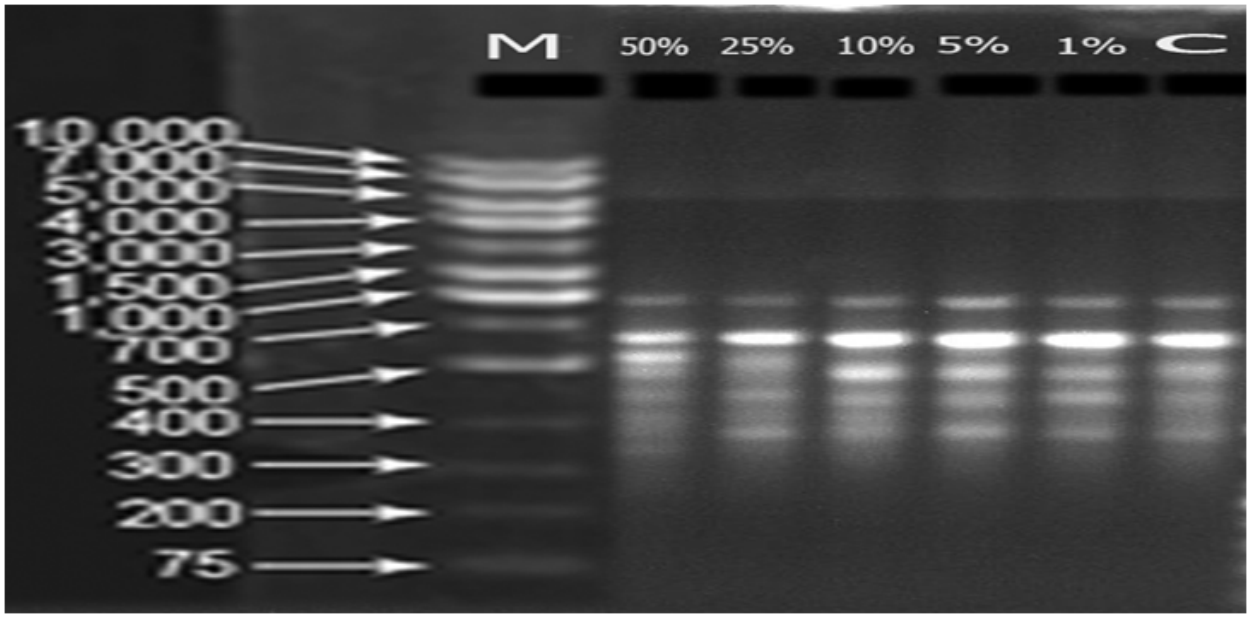

Plate 5: Profiles RAPD of genomic DNA from root cells of *Allium cepa* grown in the bilge water samples using OPT19 (M = DNA ladder, C = control).

Statistical Analysis

The squared Euclidean distance method was used to construct dissimilarity values to estimate the level of DNA polymorphism among control and test samples. The Squared Euclidean distance between the control and the 1%, 5 %, 10 % and 25 % bilge water concentrations was 1, while a Squared Euclidean distance of 8 existed between control and onion bulbs exposed to 1% sample. However, the higher concentrations of the bilge water were separated by a distance of 25 on the rescale (Fig. 1).

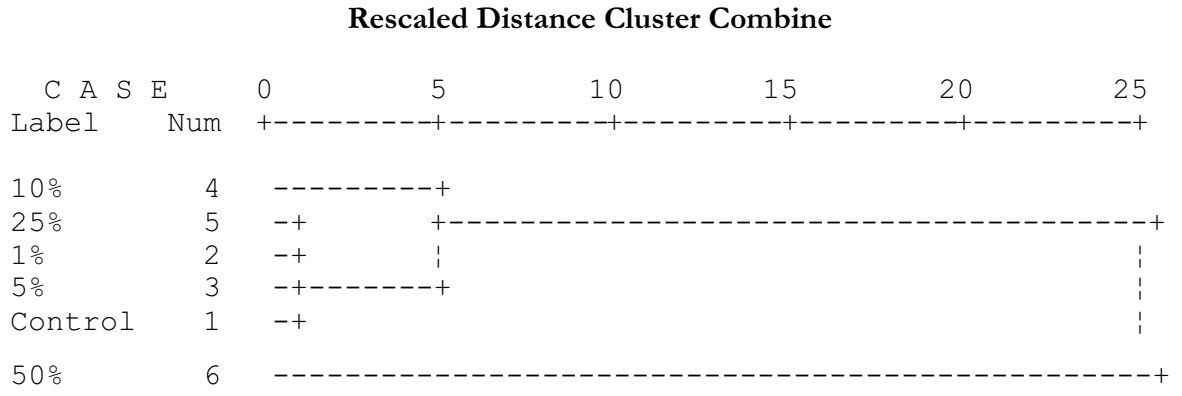

Fig. 1: Dendrogram representing genetic distance among five concentrations of bilge water-treated *Allium cepa* by UPGMA method based on RAPD analysis.

DISCUSSION

In this study, DNA polymorphism was induced by different concentrations of bilge water on *Allium cepa* root cells to detect wastewater-induced genotoxicity and this was reflected by changes in the RAPD profiles as variation in band intensity, disappearance of bands and appearance of new bands in the RAPD profile. Similar results have been obtained with the RAPD fingerprinting of *Allium cepa* DNA damage induced by flusilazole (Ozakca and Silah, 2013), textile azo dyes (Hassan and Yassein, 2014), ballast water (Olorunfemi *et al.*, 2014) and in other plant materials (Enan, 2006; Swaileh *et al.*, 2008; Cenkci *et al.*, 2009).

The highest number of disappeared bands was observed at concentrations of 25% and 50% of the bilge water sample. This suggests that the wastewater at these concentrations is capable of inducing DNA damage that will result in band loss. Disappearing bands are likely due to changes in oligonucleotide priming sites, originated from rearrangements and less likely from point mutations and DNA damage in the primer binding sites (Liu *et al.*, 2009). Atienzar *et al.* (2000) reported that mutation can only be responsible for the appearance of new bands if they occur at the same locus in a sufficient number of cells (a minimum of 10% of mutations may be required to get new PCR product visible in agarose gel) to be amplified by PCR. New bands could be attributed to mutation while the bands which disappeared could be attributed to DNA damage.

The cluster analysis method is considered as one of the most effective methods in numerical analysis regarding band scoring and analysis of RAPD fingerprinting. It can be used to calculate the distances between every pair of entity and then summarize the community data sets. In this present study, cluster analysis was done to estimate the level of DNA polymorphism between the control plant and those treated with bilge water. A dendrogram was constructed using distance matrix by using UPGMA method of SPSS software. This result clearly showed that bilge water contains genotoxic substances. Swaileh *et al.* (2008) demonstrated that there is a larger distance between control and raw wastewater as compared to control and treated plants.

Enan (2006) investigated the genotoxic effects of heavy metals (lead, copper, magnesium and cadmium) in kidney-bean (*Phaseolus vulgaris*) seedlings. He reported that DNA damage induced by the heavy metals was reflected by changes in RAPD profiles and the disappearance and appearance of bands of the treated plants. Similar changes have been reported in RAPD banding patterns following treatment with cadmium in *Zea mays* (Shahrtash *et al.*, 2010) and mercury in *Mentha arvenvsis* seedlings (ShaManikandan and Venkatachalam, 2013) compared with normal seedlings.

Bilge water has been implicated in the cytotoxicity and induction of chromosome aberration in *Allium cepa* root tips exposed to the wastewater. Compared to the control, treatment with the

wastewater resulted in significant inhibition of root growth and decrease in mitotic index with increasing concentration. The chromosomal aberrations induced in the onion root tip cells were mostly sticky chromosomes, C-mitosis, vagrants and bridges (Olorunfemi and Duru, 2014).

Results obtained from the physicochemical analysis of the bilge water showed that it was acidic and contained nitrates, phosphates, chlorides and nickel, which were comparatively higher than standard limits for effluent discharge (NESREA, 2009; USEPA, 2009). Iron, zinc, manganese, aluminium and cobalt were also detected in the wastewater. The DNA damage shown by the RAPD profiles are likely to have been caused by these parameters in the wastewater.

CONCLUSION AND RECOMMENDATION

The data presented in this study show that bilge water induced DNA damage in *Allium cepa* root cells suggesting that illegal disposal of the untreated wastewater in the aquatic ecosystem could pose direct or indirect health hazards to the environment and living organisms. The study has shown that polymorphism detected by RAPD can be considered as a powerful molecular marker assay for the detection of the genotoxic effect of bilge water. RAPD analysis can therefore be used for the evaluation of toxic effects on plants caused by bilge water and other wastewaters.

REFERENCES

American Public Health Association APHA 2005. *Standard Methods for the Examination of Water and Wastewater*. 21st ed. American Public Health Association, Washington DC, 120 p.

Atienzar, F.A., Cordi, B. and Evenden, A.J. 1999. Qualitative assessment of genotoxicity using random amplified polymorphic DNA: Comparison of genomic template stability with key fitness parameters in *Daphnia magna* exposed to benzo[a]pyrene. *Environmental Toxicology and Chemistry* 18: 2275-2282.

Atienzar, F.A. and Jha, A.N. 2006. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: a critical review. *Mutation Research* 613(2-3): 76-102.

Atienzar, F.A., Cordi, B., Donkin, M.E., Evenden, A.J., Jha, A.N. and Depledge, M.H. 2000. Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmate*. *Aquatic Toxicology Journal* 50(2): 1-12.

Baeshin, N.A., Sabir, J.S.M. and Qari, S.H. 2009. Cytogenetic and molecular evaluations of genetic effects of leaf extract of *Rhazya stricta* (Decne) on *Allium cepa* root tip meristems. *Egyptian Journal of Genetics and Cytology* 38: 73-83.

Cenkci, S., Yildiz, M., Ciğerci, I.H., Konuk, M. and Bozdağ, A. 2009. Toxic chemicals induced genotoxicity detected by random amplified polymorphic DNA (RAPD) in bean (*Phaseolus vulgaris* L.) seedlings. *Chemosphere* 76(7): 900-906.

Enan, M.R. 2006. Application of random amplified polymorphic DNA (RAPD) to detect the genotoxic effect of heavy metal. *Biotechnology and Applied Biochemistry* 43(3): 147-154.

Han, M., Chen, Y., He, F. and Yu, L. 2014. Treatment of oily wastewater with WPO and CWO. *Journal of Korean Chemical Society* 58(1): 68-71

Hassan, G.M. and Yassein, A.A. 2014. Cytogenotoxicity evaluation of water contaminated with some textile azo dyes using RAPD markers and chromosomal aberrations of onion (*Allium cepa*) root cells. *Egyptian Journal of Genetics and Cytology* 43:39-57

International Maritime Organisation/Nigeria Maritime Administration and Safety (IMO/NIMASA) 2013. *Report of the National Workshop on the London Protocol in Nigeria*, Lagos, 26-28 February 2013, 42 p.

Karakulski, K., Morawski, W.A. and Grzechulska, J. 1998. Purification of bilge water by hybrid ultra-filtration and photo-catalytic processes. *Purification Technology* 25: 163–173.

Liu, W., Yang, Y.S., Zhou, Q., Xie, L., Li, P. and Sun, T. 2007. Impact assessment of cadmium contamination on rice (*Oryza*

sativa L.) seedling at molecular and population levels using multiple biomarkers. *Chemosphere* 67: 1155–1163.

Manikandan, R. and Venkatachalam, P. 2013. Application of random amplified polymorphic DNA (RAPD) analysis to detect the genotoxic effect of mercury (Hg) heavy metal stress on *Mentha arvenvsis* seedlings. *Plant Cell Biotechnology and Molecular Biology* 14(3&4): 84-89.

National Environmental Standards and Regulation Enforcement Agency (NESREA) 2009. Federal Republic of Nigeria Official Gazette, National Environmental (Sanitation and Waste Control). Federal Government of Nigeria Printer, Abuja, Nigeria, FGP 112/102009/L000 (OL54). No. 60 (96); pp. 1057–1102.

Nigerian Ports Authority (NPA) (2014). Nigerian Ports Authority Press Releases, 26/28 Marina, Lagos. Available online: http://www.nigerianports.org/news.aspx?id=375 (accessed on 11 November, 2014).

Olorunfemi, D.I., Okieimen, E.A. and Ovwemuvwose, J. 2014. DNA integrity of onion (*Allium cepa* L.) root cells exposed to ballast water. *Studia Universitatis "Vasile Goldiș", Seria Științele Vieții, (Life Sciences Series)* 24(3): 305-309.

Olorunfemi, D.I. and Duru, L. 2014. Effects of bilge water on mitosis and antioxidant enzyme activities in *Allium cepa* L. Being a poster presentation at the *24th Annual Meeting of the Society of Environmental Toxicology and Chemistry* (SETAC) Europe Congress Centre, Basel MCH Messe Schweiz (Basel) AG, Messeplatz, Basel, Switzerland. 11th – 15th May, 2014

Organisation for Economic Co-operation and Development (OECD) 2003. Cost Savings Stemming from Non-Compliance with International Regulations in the Maritime Sector. Paris, France. http://www.oecd.org/dataoecd/4/26/2496757.pdf. (Accessed 9/12/2014).

Ozakca, D.U. and Silah, H. 2013. Genotoxicity effects of flusilazole on the somatic cells of *Allium cepa*. *Pesticide Biochemistry and Physiology* 107: 38-43.

Padmalatha, K. and Prasad, M.N.V. 2006. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. *African Journal of Biotechnology* 5(3): 230-234.

Qari, S.H.M. 2010. DNA-RAPD fingerprinting and cytogenetic screening of genotoxic and antigenotoxic effects of aqueous extracts of *Costus speciosus* (Koen). *Journal of King Abdulaziz University: Science* 22: 133-152.

Shahrtash, M., Mohsenzadeh, S. and Mohabatkar, H. 2010. Cadmium-induced genotoxicity detected by the random amplification of polymorphism DNA in the maize seedling roots. *Journal of Cell and Molecular Research* 2(1): 42-48

Sivaraman, C., Ganguly, A., Nikolausz, M. and Mutnuri, S. 2011. Isolation of hydrocarbonoclastic bacteria from bilge oil contaminated water. *International Journal of Science and Technology* 8(3): 461-470.

Swaileh, K.M., Hussein, R. and Ezzughayyar, A. 2008. Evaluating wastewater induced plant genotoxicity using random amplified polymorphic DNA. *Journal of Environmental Toxicology* 23(1): 117-122.

United States Environmental Protection Agency (USEPA) 2009. Drinking Water Contaminants. Washington, DC, USA. (Available online: http://water.epa.gov/drink/contaminants/ index.cfm#Lis.)

United States Environmental Protection Agency (USEPA). 1996. *Acid Digestion of Sediments, Sludges and Soil Method* - 3050B. USEPA, Washington, DC.

Werschkun, B., Banerji, S. and Hofer, T. 2014. Emerging risk from ballast water treatment: the run-up to the International Ballast Water Management Convention. *Journal of Chemosphere* 112: 256-266.

Williams, J., Kubelik, A.R. and Livak, K.J. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research* 18: 6531-6535.