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Microbiological, physicochemical and genotoxicological assessment of tobacco wastewater

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ABSTRACT

Tobacco wastewater contains many toxic chemical compounds, which may have adverse impact on the environment and human health. To study the toxicity or genotoxic potential of the wastewater, biological tests, such as Ames Salmonella test and SOS Chromotest test were employed. The variability of the results obtained from the entire tests, show a correlation of mutagenic and genotoxic potential of the wastewater. Microbiological and physicochemical analyses were also carried out. The wastewater contained a large number of bacteria: (9.78 X 107 ± 2.00 X 107) and fungi: (3.83 X 104 ± 1.10 X 104). Microorganisms isolated from this study are Staphylococcus cohnii, Anaerococcus hydrogenalis, Propionibacterium acne, Proteus vulgaris, Vibrio vulnificus, Penicillium sp., Aspergillus fumigatus, Aspergillus niger and Rhodotorula glutinis. These microorganisms have been linked with varieties of diseases in living organisms. Physicochemical analysis of the wastewater shows that it contained constituents that can induce mutation in living systems. The synergy of all the tests from this study unequivocally confirms that the tobacco wastewater is highly toxic to living organisms. Drastic measures must be taken by tobacco industries to integrate the knowledge and the control of their wastewaters in infection and environmental pollution regulatory programs.

Keywords: Ames test, Genotoxicity, SOS Chromotest, Tobacco Wastewater and Nigeria

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INTRODUCTION

A quantum jump was registered by Nigerian industries in the past few decades which contributed to high economic growth, but concurrently the growth has also resulted in rigorous environmental pollution. As a result of that, the quality of water is critically affected which is far lower when compared to the international standards. Among all industries, the tobacco industry grades the 18th in chemical waste production [1]. Tobacco wastewater certainly has an impact on both human health and the environment. Apart from that, wastewater from tobacco industry consists of contaminants which slow down the microbial syndicate in biological treatment plants. Flavouring chemicals containing glycogen, alcohol, adsorbable organic halogens (AOX), and pesticides from tobacco leaves and nicotine are known to be sources of these toxic contaminants [2]. More specifically, untreated or allegedly treated tobacco wastewater contains variable amounts of heavy metals such as arsenic (As), lead (Pb), nickel (Ni), cadmium (Cd), copper (Cu), mercury (Hg), zinc (Zn) and chromium (Cr) are known to be constituted in an untreated or allegedly treated tobacco waste water [3; 4], which have the ability to get the germinating crops under irrigation contaminated. These heavy metals have a marked effect on the aquatic flora and fauna. They enter through bio-magnification into the food chain and humans are eventually affected as well. Contamination by heavy metal pollution is the long lasting escalating predicament of oceans, lakes and rivers. The accumulation of heavy metals in fish, oysters, sediments and other aquatic living organisms in the ecosystems have been reported globally [5; 6; 4; 7; 8; 9; 10; 11].

Major tobacco companies have allegedly taken up the environmental impact of tobacco wastewater as part of their assortment for corporate social liability. The environmental qualifications of many tobacco companies have been displayed by describing on their websites their implementation of greener, sustainable and low-impact practices from farm to factory [12; 13; 14]. The development and application of in vitro models of DNA damage and cellular transformation for use in the testing of the effects of wastewater and its toxicants are alleged to be inspected by some of these companies [15]. The tobacco industry's unethical business practices, which have been aimed, inter alia, at deceiving the public about the extent to which tobacco by-products harm people's health, contravene widely accepted ethical considerations.

The screening of water contamination for potentially carcinogenic compounds consequently presents a major concern for humans, animals and microorganisms in the environment. It is tremendously difficult to quantify the risk factors associated with these effluents as they usually occur in concentrations too low for analytical determination, and putative mutagens, with few exceptions, yet undetected [16]. Besides, the effects of mixtures cannot be measured through analytical methods. Therefore, it is highly crucial to investigate tobacco wastewater genotoxicity to ultimately regulate the population exposure using bacterial genotoxicity tests such as Ames test using *Salmonella typhimurium* strain and SOS Chromotest using *Escherichia coli* strains. These assays are often employed to determine toxicity of effluents in which a prior knowledge of toxicants identity and physiochemical properties is not necessary.

MATERIALS AND METHODS

Area of Study

The study was carried out at a Tobacco Company around the Toll gate industrial area which is one of the most rapidly developing and heavily polluted industrial belts of Ibadan. The industrial area is spread over 863.18 hectares of land consisting of about 20 large and medium scale industries like engineering units, steel processing industries, chemical units, paints, pharmaceutical units, textile industries etc. The study area lies between latitude 7°23'47"N longitudes 3°55'0"E. The main water source for the industrial consumption is bore holes. The industrial area utilizes a lot of fresh water per day. However, specific amount of water used was not documented. The effluent discharge, treated and untreated is released into neighbouring environment. This has created health hazards not only for local population but also resulted in disturbances of aquatic life of the Odo-Ona River, flowing near the industrial area.

Sampling of Industrial Waste Water Effluent and Sample Preparation

The industrial waste water effluent samples (number of samples collected, $n = 4$) were collected randomly from all major discharge points of the tobacco company (name withheld for confidentiality). Polythene bottles of 2.5 L and 2.0 L were used to collect the grab water samples The bottles were thoroughly cleaned with hydrochloric acid, washed with tap water to render free of acid, washed with distilled water twice, again rinsed with the water sample to be collected and then filled up the bottle with the sample leaving only a small air gap at the top. The sample bottles were stoppard and sealed with paraffin wax. The samples were refrigerated at 4°C throughout the period of the study.

Physicochemical Analysis

Tests such as pH, temperature, colour and odour were performed in situ. The samples were maintained at 4°C until the bioassays were carried out. Chemical analyses included chemical oxygen demand (COD, mg of O_2/L), biochemical oxygen demand (BOD, mg of O_2/L), total suspended solids (TSS, mg/L), total dissolved solids (TDS, mg/L), total solids (TS, mg/L), nitrate (NO_3, mg/L), sulphate (SO_3, mg/L), ammonia (mg/L), total chlorides (Cl, mg/L) and total hardness (TH, mg/L). The analyses were carried out according to recommended ISO methods [17; 18; 19; 20; 21; 22; 23; 24]. These values are presented as the mean of three individual values measured (Table 1).

Heavy Metal Analysis by AAS Technique

The analysis for the trace metals like chromium (Cr), cadmium (Cd), nickel (Ni), zinc (Zn), copper (Cu), lead (Pb) and iron (Fe) was done by Perkin- Elmer ASS-280 Flame Atomic Absorption Spectrophotometer at the University of Ibadan, Oyo State, Nigeria in accordance with standard analytic methods [25]. The calibration curves were prepared separately for all the metals by running different concentrations of standard solutions. A reagent blank sample was analyzed and subtracted from the samples to correct for reagent impurities and other sources of errors from the environment. Average values of three replicates were taken for each determination.

Microbiological Analysis

The spread plate method was employed for total colony count of bacteria using nutrient agar (bacteria) and potato dextrose agar (fungi) after serial dilution of the sample. The total colony count was determined as described by Nwachukwu [26]. Identification was done on the basis of Bergey's Manual of Determinative Bacteriology using various staining and biochemical tests such as Gram staining, citrate test, catalase test, sulphide, indole and motility test, lactose utilization, starch hydrolysis, methyl red and Voges Proskauer test, and carbohydrate fermentation.

Microscopic examination of fungi growth was achieved by observing the colonial morphology: colour of colony, texture, shape and surface appearance; cultural characteristics: asexual and sexual reproductive structures like sporangia, conidial head, arthrospores, septate or non-septate vegetative mycelia, [27; 28]. The needle mount method as described by Wemedo *et al*. [29] was used for microscopic examinations of the fungi. All identifications of pure isolates were made on the basis of their cultural and morphological characteristics [30; 28; 27]. GIDEON is an online application used for the identification and characterization of the microbes isolated in this experiment.

Genotoxological Testing

Test bacterial strains

The lyophilized genetically engineered strains, *Escherichia coli PQ37* and Salmonella typhimurium TA98/TA 100 were obtained from EBPI kits in Canada and stored at -80ºC and thawed before the assay

Standard Mutagens

Sodium azide (NaN_3, 0.5 µg/100 µl) [CAS no. 26628-22-8] – for use with TA 100 and 2 Nitrofluorene (2- NF, 20 µg/100µl) [CAS no. 607-57-8] – for use with TA 98

The Ames Test

The fluctuation test was performed as described by Legault *et al.,* [31] and conducted without metabolic activation. The lyophilized bacteria was rehydrated and pre-incubated the evening before the assay. This was done by aseptically transferring the nutrient broth (bottle G) into the vial of lyophilized bacteria while mixed thoroughly. The vial was then corked with rubber stopper and incubated at 37^0C overnight (16 to 18 hours). The bacteria were examined for growth indicated by the existence of turbidity. Twenty millilitres (20 ml) of aqueous solution sample was filter-sterilized using the 0.22 µm sterile filter supplied in the kit. The Ames test reaction mixture was prepared aseptically by measuring and mixing 43.24 ml from bottle A + 9.50 ml from bottle B + 4.76 ml from bottle C + 2.38 ml from bottle D + 0.12 ml from bottle E into the reaction mixture container supplied with the kit. 2.5ml of reaction mixture was aseptically dispensed from the tube to each sterile tube containing 17.5ml of the tobacco wastewater and mixed thoroughly. The concentrations of the wastewater used for the assay are 5%, 10% and 1%. The total volume for the tube was 20 ml. Five micolitres (5 µl) of *S. Typhimurium* (TA 100 or TA 98) suspension from the culture grown overnight was withdrawn, added to each of the sample test tubes and mixed thoroughly, with the exception of the reaction blank. The contents of each test tube were poured into a sterile multichannel pipette reagent boat while 200µl aliquots of the mixture were dispensed into each well of a 96-well micro-titration plate using a multichannel pipette. The micro-titration plate was covered with a lid and sealed in airtight plastic bag (s) to prevent evaporation. Please note that it is recommended to store the plate containing Sodium Azide in separate bag as it is commonly found to contaminate surrounding plates, resulting in erroneous results. The plates in the airtight bags were incubated at 37^0C for five days.

SOS Chromotest

The SOS Chromotest was performed without metabolic activation as described by Quillardet and Hofnung, [32] with modifications. About ten (10) to twelve (12) ml of LB growth medium was transferred to dried lyophilized bacteria in a bottle, mixed well by inverting and incubated at 37°C for 12 hours. For further studies, the bacterial suspension of optical density greater than 0.05 was used. The standard solution used was 4-Nitroquinoline oxide dissolved in 1ml of DMSO at a concentration of 10mg/ml (10000µg). 100µl of this standard solution was added to 900µl DMSO. The above step was repeated two more times to get a working standard of 10µg/ml. Then six two-fold dilutions were prepared with saline 10% DMSO.Dispensed 10µl of properly diluted 4NQO control solutions into wells of Column 1. 100µl of overnight bacterial suspension was added into each well of the microplate except in Machine blank well. The remaining wells were used to dispense the 14 two-fold serial dilutions of the tobacco wastewater. (The layout shown in Fig.5 is used for standard testing).

The micro plate was incubated at 37°C for 2 hours. During the 2 hour incubation, genotoxic materials interacted with DNA of the SOS Chromo test Bacteria and induced the De Novo synthesis of the β-Galactosidase. At the last stage of SOS-Chromo test, relative amount of the enzyme produced as a result of this interaction was measured by addition of a blue chromogenic substrate. The Bacteria was tested for ATP activity-viability, using alkaline phosphatase. The Blue coloured chromogen yielded a clearly visible blue colour suitable for Quantitative (by Photometer) Evaluation of Test Result. Blue chromogen was transferred to the dry alkaline phosphatase substrate and mixed well. 100µL of the chromogenic mix was added into each well of the plate and incubated at 37°C, for 60 to 90 minutes until a green colour developed. 50µL of the stop solution was added to each well of the plate to stop the reaction. The absorbance was read at 615nm to measure the genotoxicity activity. To determine viability/cytoxicity of bacteria, the absorbance was read at 405nm.

The Optical densities of the wells were measured in the appropriate wave length for chromogen. At 615nm, only absorption of the blue colour was read and there was no interference of the alkaline phosphatase yellow substrate on blue results.

	1	2	3	4	5	6	7	8
	Standard 4-NQO (µg/mL)	**Tested Material**				**Standard 4-NQO (µg/mL)**		**Tested Material**
A	10	Undiluted	1:128			10	undiluted	1:128
B	5	1:2	1:256			5	1:2	1:256
C	2.5	1:4	1:512			2.5	1:4	1:512
D	1.25	1:8	1:1024			1.25	1:8	1:1024
E	0.625	1:16	1:2048			0.625	1:16	1:2048
F	0.313	1:32	1:4096			0.313	1:32	1:4096
G	Diluents	1:64	1:8192			Diluents	1:64	1:8192
H	Machine blank	Diluents	Diluents			Blank	Diluents	Diluents

Figure 1. Experimental Design of Microplate for testing the wastewater at various concentrations

	Positive Control	**B**	**P/S**	**A+C**
	Test Substance	B	T/S	A+C
	Solvent	B	S	A+C
	Blank	M	S	A+C

B- BACTERIAL SUSPENSION. M- MEDIUM. P/S- POSITIVE SUBSTANCE. S- DMSO.

T/S- TEST SUBSTANCE: GENOTOXIN /CARCINOGEN DILUTIONS.

A+C- THE CHROMOGENIC SUBSTRATE +ALKALINE PHOSPHATASE MIX.

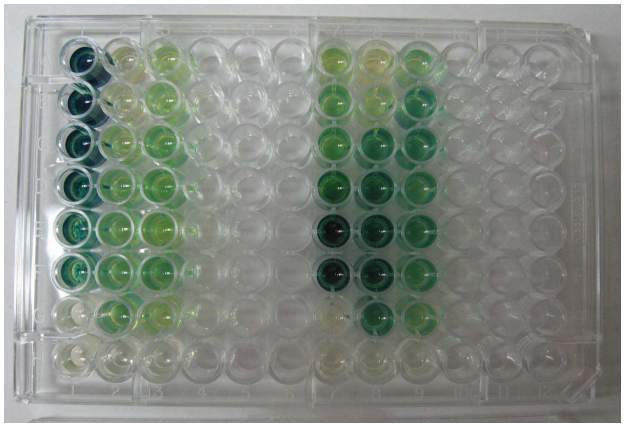

Figure 2. Photograph of Layout for Testing GRC compounds

Statistical Analysis

Analysis of variance of the data was done with SPSS computer program. One-way analysis of variance (ANOVA) and Duncan's mean range test (DMR) were used.

RESULTS

Physicochemical and Heavy Metal Analysis

The physicochemical analyses of the tobacco wastewater are shown in Table 1. The pH was 4.20 with an offensive odour emitted. The wastewater pollution is appalling as the COD (361.02 mg/l), BOD (115.34 mg/l), TDS (988.12 mg/l), TSS (788.12 mg/l) and TSOLIDS (1,776.54) are ridiculously higher than the WHO standard [33]. The nutrients examined in this study were nitrogen (nitrates), sulphur (sulphate) and phosphorus (phosphate) and ammonia. The values obtained are 42.52 mg/l, 560.12 mg/l, 560.16 mg/l and 21.41 mg/l respectively (Table 1).

The heavy metals analysis showed the presence of Zn, Cd, Cu, Co, Fe, and Ni. The mean concentration value of Zn (3.050 mg/l), Cd (0.050 mg/l), Cu (0.244 mg/l), Co (0.099 mg/l), Fe (2.159 mg/l) and Ni (0.127 mg/l) were obtained and presented in Table 2. However, Chromium (Cr) and Lead (Pb) were below detection limits. All the values obtained are high.

Table 1: Physicochemical properties of Tobacco wastewater

S/NO	PARAMETERS	RESULTS (mg/l)	WHO STANDARD
1	C.O.D.	361.02	20.00
2	B.O.D.	115.34	0.00
3	T.D.S.	988.12	500.00
4	T.S.S.	788.12	10.00
5	T.SOLIDS	1,776.54	500.00
6	Total Hardness	1,300.00	200.00
7	Total Chlorides	340.00	250.00
8	Ammonia	21.41	<1.0
9	Nitrate	42.62	<10.00
10	P^H	4.20	6-5-8.5
11	Sulphate	560.16	500

Table 2. Heavy Metal Analysis Results

S/NO	Element of Choice	Reading of Elements by AAS (mg/l)	WHO STANDARD
1	Zinc (Zn)	3.050	<1.0
2	Cadmium (Cd)	0.050	0.01
3	Chromium (Cr)	ND[a]	0.05
4	Copper (Cu)	0.244	0.10
5	Lead (Pb)	ND[a]	0.05
6	Cobalt (Co)	0.099	0.05
7	Iron (Fe)	2.159	1.00
8	Nickel (Ni)	0.127	0.05

[a]*Not detected.*

Microbial Analysis

The wastewater contained a large number of bacteria: $9.78 \times 10^7 \pm 2.00 \times 10^7$ and fungi: $3.83 \times 10^4 \pm 1.10 \times 10^4$ (Table 3). Bacteria isolated from the effluent are *Staphylococcus cohnii*, *Anaerococcus hydrogenalis*, *Propionibacterium acne*, *Proteus vulgaris* and *Vibrio vulnificus* (Table 4). Fungi isolated from this study (Table 5, Fig. 3-6) include *Penicillium sp.*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Rhodotorula glutinis.*

Table 3. Average total bacteria and fungi count

Total viable bacteria count (CFU g^{-1})	Total viable fungi count (CFU g^{-1})
$9.78 \times 10^7 \pm 2.00 \times 10^7$	$3.83 \times 10^4 \pm 1.10 \times 10^4$

± represents standard deviation.

Total count

The quantitative idea about the presence of microorganisms such as bacteria, yeast and mold in a sample was recorded using total viable count (TVC) which represents the number of colony forming units (CFU) per g (or per ml) of the sample.

Bacterial Analysis

Table 9*:* Identification Matrix for Bacteria Isolates

s/n	Gram Pos(+)/ Neg.(-)	Coccus (C)/Bacillus (B)	Growth on MacConkey agar	Growth on ordinary blood agar	Oxidase	Catalase	Glucose fermenter	Beta hemolysis	Motile	Indole	Methyl Red	Voges Paskauer	Citrate	Gelatin Hydrolysis	Starch Hydrolysis	Urea Hydrolysis	Hydrogen Silfide	Maltose	D-Mannitol	D-Sorbitol	D-Dulcitol	Gas from glucose	Lactose	Sucrose	Organism
1	+	C	_	+	-	+	+	-	-	+	+	-	-	+	-	-	N/A	+	-	N/A	N/A	-	-	-	*Staphylococcus cohnii*
2	+	C	N/A	+	+	+	+	-	-	+	+	-	+	-	-	-	N/A	-	+	N/A	N/A	-	-	-	*Anetococcus hydrogenalis*
3	+	B	-	+	-	+	+	+	-	+	+	-	+	+	-	-	N/A	-	+	N/A	N/A	-	-	-	*Propionibacteriuum acnes*
4	-	B	N/A	+	-	+	+	+	-	+	+	-	+	+	-	-	N/A	+	-	-	N/A	-	-	-	*Proteus Vulgaris*
5	-	B	+	+	-	+	+	-	-	-	+	-	-	-	+	-	+	+	+	N/A	+	-	-	-	*Salmonella gallinarum*
6	-	B	-	+	+	+	+	+	+	+	_	_	+	_	_	N/A	+	+	N/A	N/A	-	+	+	-	*Vibrio vulnificus*

**(+): Positive (-): Negative*

Fungi in Tobacco Wastewater

Table 5. Identification Matrix for Fungi Isolates

Organism	*Macroscopic*	*Microscopic*
Penicillium sp	The colonies were slightly raised but the growth was rapid, filamentous and cotton in texture. The colonies were initially white and later became bluish green and then gray green with time. The reverse of the plate was pale yellow.	Has a septate hyaline with branched condiophores. Metulae, phialides and conidia were observed. The metulae carry the flask-shaped phialides. The phialides form brush-like clusters which were referred to as penicillin which carries numerous conidia.
Aspergillus fumigates	Colonies on SDA at 25°C are smoky gray-green with a slight yellow reverse. Rapid growth. Texture was woolly.	Hyphae are septate and hyaline. Conidial are strongly columnar. Conidiophores are smooth-walled and about 300 µm long. Conidia are smooth, subglobose, 2-3.5 µm in diameter.
Aspergillus niger	Colonies on SDA at 25°C were initially white, quickly became black with conidial production. Growth produced radial fissures in the agar.	Hyphae are septate and hyaline. Conidial heads are radiate initially before splitting into columns at maturity. Conidia are brown to black, very rough, globose, and measure 4-5 µm in diameter.
Rhodotorula glutins	Colonies on SDA at 25°C are smooth, glistening or dull. They displayed a rapid growth, sometimes roughened, soft and mucoid in shape. They are pink in colour.	Following 72 hours incubation at 25°C, only globose yeast cells are produced (2.5-10 µm in diameter).

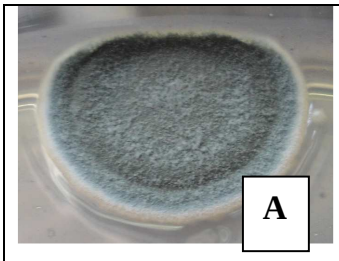

Figure 3. *Penicillum* sp

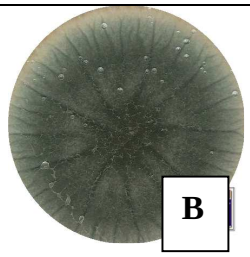

Figure 4. *A. fumigates*

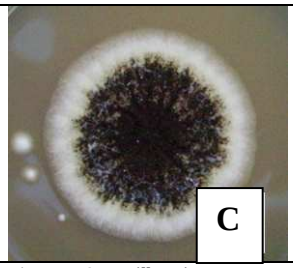

Figure 5. *Aspergillus niger*

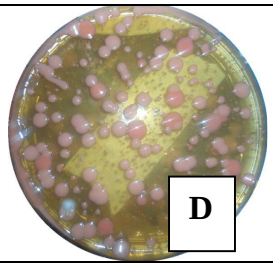

Figure 6. *Rhodotorula glutins*

Table 6. Positively Scored Wells

Plate number	Plate Description	Bacteria Strain	Number of Positively Scored Wells	
			Day 3	Day 4
1	Background	TA 100	9	10
2	Background	TA 100	12	15
3	Background	TA 98	0	4
4	Background	TA 98	2	19
5	Positive Control: NaN3	TA 100	29	80
6	Positive Control: 2-NF	TA 98	13	87
7	Test Sample- 10%	TA 100	21	96
8	Test Sample- 10%	TA 98	2	65
9	Test Sample- 5%	TA 100	0	12
10	Test Sample- 5%	TA 98	0	7
11	Test Sample- 1%	TA 100	8	29
12	Test Sample- 1%	TA 98	1	84

***Legend:**

95 % significance
99 % significance
99.9 % significance
Possibly cytotoxic (less than average of backgrounds)
Value is out of range
Value has decreased over time (wells miscounted)

Genotoxicological Analysis

Ames Test

In the Ames test, the number of positively scored wells are 96 (TA 100) and 65 (TA98) at 10% while at 1%, the positively scored wells are 29 (TA 100) and 84 (TA 98) as shown in Table 6. This implies that at 10% and 1% concentration, the wastewater is 99.9% mutagenic (Table 6, figure. 13-14), while at 5%, it is possibly cytotoxic to

the bacteria, as the number of positively scored well is 12 (TA 100) and 7 (TA 98). The number of positively scored wells in the positive controls NAN3 (TA 100) and NaF (TA 98) are 80 and 87 respectively (Table 11, figure 13-14).

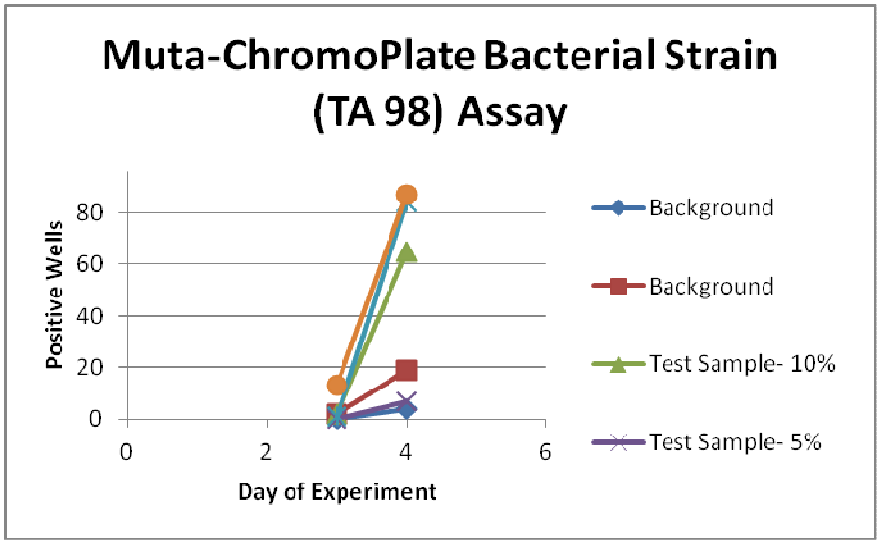

Figure 7. The Number of Positive Wells Scored in a 96-well Microplate using the TA 98 strain

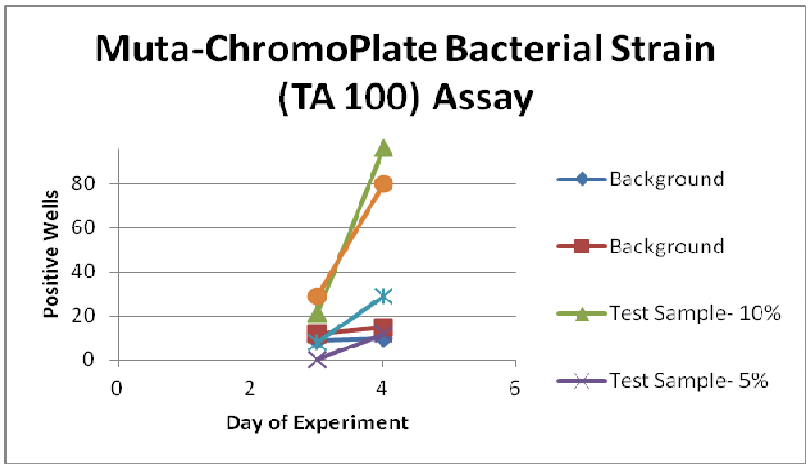

Figure 8. The Number of Positive Wells Scored in a 96-well Microplate using the TA 100 strain SOS Chromo-Test™

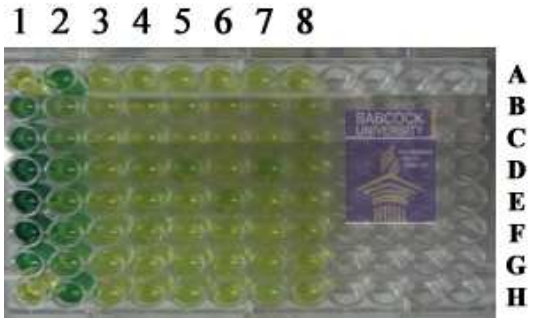

Figure 9. Photograph of Microplate Showing Test Result and Labelling of Microplate

Schematic Layout of the filling of the each well of the Microplate:
Column 1A - 1F : 6 two-fold dilutions of 4NQO
Column 2A - 2F: 6 two-fold dilutions of 2AA
Column 1G, 2G, 4G, 6G, 8G : Diluent Well (DMSO)
Column 1H, 2H, 4H, 6H, 8H: Machine Blank well (Bacterial suspension)
Column 3A-3H and 4A-4F: 14 two-fold dilutions of Test Compound #1
Column 5A-5H and 6A-6F: 14 two-fold dilutions of Test Compound #2
Column 7A-7H and 8A-8F: 14 two-fold dilutions of Test Compound #3

Visual Analysis of the Results

Table 7. Standard Visual Concentration Gradient Grid of positive 4NQO control

S/No		Grid	Genotoxic Potential
1	-		No
2	+		Poor
3	++		Moderate
4	+++		Good
5	++++		Massive

Table 8. Test Material (Wastewater) Results in correlation to Standard Grid

S/No	Concentration	Genotoxic Potential	
1	10	+	
2	5	+	
3	2.5	+++	
4	1.25	++++	
5	0.625	++++	

Instrumental Analysis of the Results
Analysis of 4NQO
The Optical Density of the wells was measured in the appropriate wavelength for chromogen. At 615nm, only absorption of the blue colour was read and there was no interference of the alkaline phosphatase yellow substrate on blue results. The standard graph was plotted with absorbance values on Y-axis and 4NQO concentration values on X-axis as in graph shown in Fig.10. Table 9 shows the absorbance value for different concentrations of 4NQO.

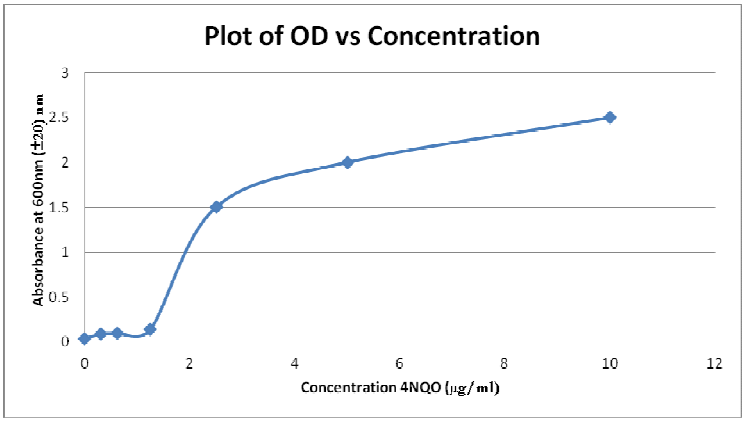

Figure 10. 4NQO- Standard plot of OD vs. Concentration

Table 9. Absorbance value for different concentrations of 4NQO

Sr. No	Concentration (µg/ml)	Absorbance (at 615nm)
1	10	2.5
2	5	2.0
3	2.5	1.50
4	1.25	0.14
5	0.625	0.10
6	0.3125	0.09
7	0	0.039

The SOSIP can be calculated when concentration is expressed in nanomoles. So the values of concentration were converted from microgram to nanomole using the following conversion:

Nanomole Conversion = [Concentration x [Volume Used (µl) / MW] of 4NQO in µg]

Molecular weight (MW) of 4NQO = 190.16g/mol.

Here Volume used is = 10µl (i.e. divided 10 by MW).

Table 10. 4NQO- Concentration in nanomoles

Sr. No	Concentration (µg/ml)	Absorbance (at 615nm)
1	0.525	2.5
2	0.262	2.0
3	0.131	1.50
4	0.065	0.14
5	0.0032	0.10
6	0.0164	0.09
7	0	0.039

Using the absorbance values from Table 10, graph in Fig.11 was plotted by taking Nanomole concentration on X-axis and absorbance on Y-axis. The straight regression line was plotted using the values and the slope of this line was found out. The slope value was used to calculate the SOSIP.

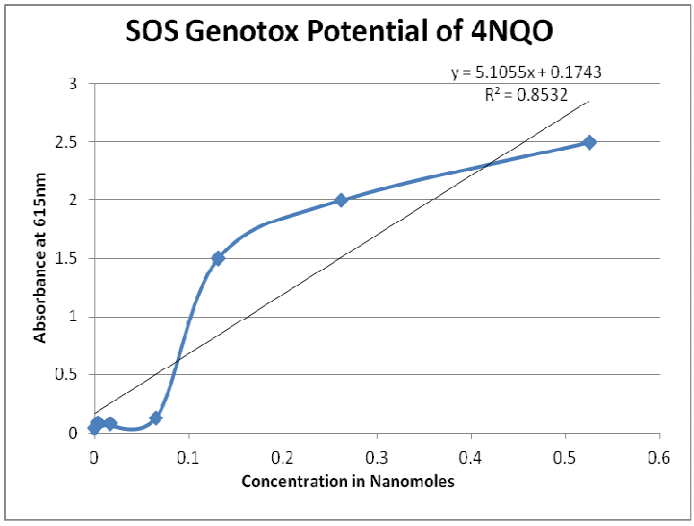

Figure 11: SOS Genotox Potential of 4NQO

The Straight Line Equation: y = mx + c, Where 'm' is the slope.

y = 5.1259x + 0.1273

SOSIP = 10 X (Slope of Linear Regression of the plot of Nanomoles Concentration vs Absorbance)

From the equation, Slope = 5.1055

SOSIP of 4NQO = 10 X 5.125 = 51.05

Since SOSIP of 4NQO > 1.5, we can conclude that 4NQO has significant genotoxic potential in the SOS-ChromoTest™

Analysis of Wastewater

Nicotine constitute 0-6-3.0% of the dry weight of tobacco (Kaiserman and Rickert, 1992**),** its Systematic (IUPAC) name is 3-[(2S)-1-methylpyrrolidin-2-yl] pyridine and its molecular weight is 162.12g/mol. Since nicotine is the active ingredient, it is used for the SOSIP analysis.

Table 11. Absorbance value for different Concentrations of Nicotine (Tobacco Wastewater)

Sr. No	Concentration (nm)	Absorbance (at 615nm)
1	10	3.5
2	5	2.6
3	2.5	1.7
4	1.25	0.15
5	0.625	0.1
6	0.3125	0.06
7	0	0.042

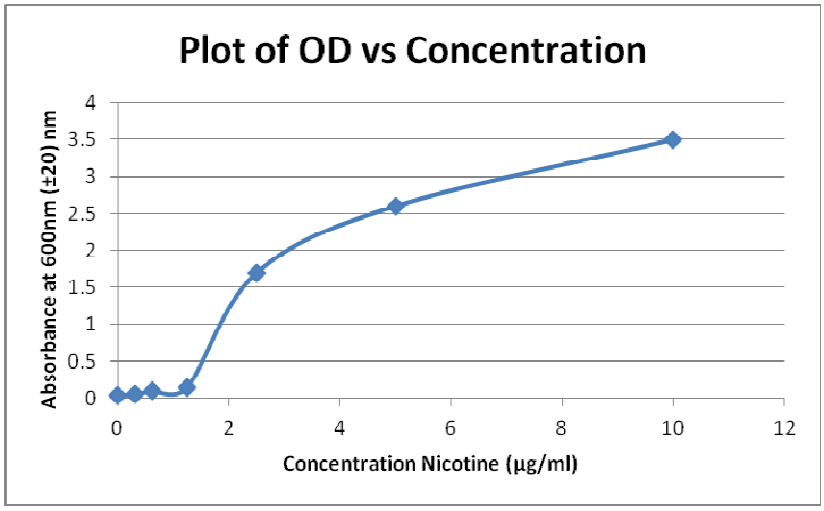

Figure 12. 4NQO- Standard plot of OD vs. Concentration

The SOSIP can be calculated when concentration is expressed in nanomoles. So the values of concentration were converted from microgram to nanomole using the following conversion:

Nanomole Conversion = [Concentration x [Volume Used (µl) / MW] of Nicotine in µg]

Molecular weight (MW) of Nicotine = 162.12 g/mol

Here Volume used is = 10µl (i.e. divided 10 by MW).

Table 12. Nicotine- Concentration in Nanomoles

Sr. No	Concentration (nm)	Absorbance (at 615nm)
1	0.617	3.5
2	0.308	2.6
3	0.154	1.7
4	0.077	0.15
5	0.0385	0.1
6	0.0193	0.06
7	0	0.042

Using the absorbance values from Table 12, Graph in Fig. 13 was plotted by taking Nanomole concentration on X-axis and Absorbance on Y-axis. The straight regression line was plotted using the values and the slope of this line was found out. The slope value was used to calculate the SOSIP.

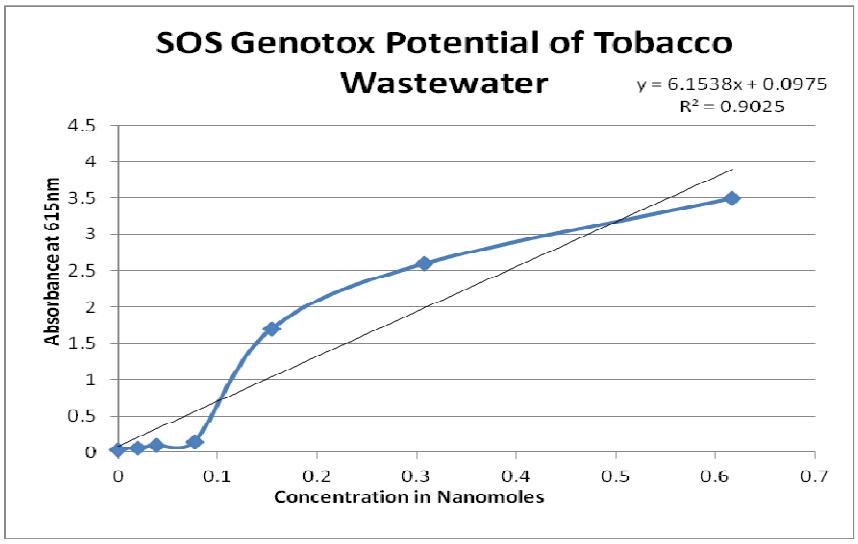

Figure 13. SOS Potential of Tobacco Wastewater (Nicotine)

The Straight Line Equation: y = mx + c, Where 'm' is the slope.

y = 6.1538x + 0.0975

SOSIP = 10 X (Slope of Linear Regression of the plot of Nanomoles Concentration vs Absorbance)

From the equation, Slope = 6.1538

SOSIP of Nicotine= 10 X 6.1538 = 61.54

Since SOSIP of Nicotine > 1.5, hence, the tobacco wastewater has a significant genotoxic potential in the SOS-ChromoTest.

DISCUSSION

An evaluation of the microbiological and genotoxic potential of tobacco wastewater from a tobacco company in Nigeria (name withheld for confidentiality) was performed with Ames *Salmonella* Test, and SOS chromotest, along with the physicochemical and heavy metal analyses. Result of these tests strongly imply that the tested wastewater present a genotoxic effect.

Chemical Analysis

The physicochemical analyses of the wastewater found it to be acidic (4.20). The wastewater pollution is appalling as the COD (361.02 mg/l), BOD (115.34 mg/l), TDS (988.12 mg/l), TSS (788.12 mg/l) and TSOLIDS (1,776.54) are ridiculously higher than the WHO standard (WHO, 1985). The nutrients examined in this study were nitrogen (nitrates), sulphur (sulphate) and phosphorus (phosphate) and ammonia. The values obtained are 42.52 mg/l, 560.12 mg/l, 560.16 mg/l and 21.41 mg/l respectively (Table 1). Plants growing around the river in which the water has been discharged may experience excessive growth due to these nutrients. In the same vein, fish consumed from the river by humans, will definitely have an adverse effect on them.

The heavy metals analysis showed the presence of Zn, Cd, Cu, Co, Fe, and Ni. The mean concentration value of Zn (3.050 mg/l), Cd (0.050 mg/l), Cu (0.244 mg/l), Co (0.099 mg/l), Fe (2.159 mg/l) and Ni (0.127 mg/l) were obtained and presented in Table 2. However, Chromium (Cr) and Lead (Pb) were below detection limits. A critical look at the mean values of the metal content showed that all the studied metals are above the WHO threshold limits (WHO, 1985). These studied elements are individually known to be mutagens and carcinogens. In other words, they are toxicants. It is reasonable to say that the genotoxic effect of the effluent as validated from the various test of this study can be a result of the interactions of these metals which can be more deleterious than the individual effects. These heavy metals have the horrendous ability to induce mutations and cancer in living cells [34]. Research have shown that Fe, Cd, and Cu induced reactive oxygen species (ROS) in eukaryotic systems [35], which can cause DNA, protein and lipid damage (35; 36; 37]. Cu for instance has been reported to be responsible for high incidence of stickiness and c-mitosis in *A.cepa* [38]. According to Banu *et al*., [39], subjection of mice to Zn results in single strand breaks in DNA as measured by the comet assay. Zn has also be shown to cause deceleration of root growth, decrease in micronucleus frequency and delayed cell division while Ni has been reported to produce discerning damage to heterochromatin [40]. This finding is in line with prior reports on genotoxic hazards of industrial wastes [41; 42]

Microbiological Analysis

The wastewater contained a large number of bacteria: ($9.78 \times 10^7 \pm 2.00 \times 10^7$) and fungi: ($3.83 \times 10^4 \pm 1.10 \times 10^4$). This is in accordance with Beattie and Lindow [43] who suggest that tobacco is rich in microorganisms that naturally colonize the tobacco plants (Table 3). Microbes isolated from the effluent (Table 4 and 5) have been linked with varieties of disease in living organisms. *Staphylococcus cohnii* can cause catheter related blood stream infection (44; 45] while *Anaerococcus hydrogenalis* is associated with vaginal infections and ovarian abscesses [46]. *Propionibacterium acne* is responsible for the etiology of acne and post-operative/device related infections such as joint prostheses, shunts and prosthetic heart valves [47]. *Proteus vulgaris* is popularly known as the causative agent for urinary tract and wound infection [48] while *Salmonella gallinarum* causes fowl typhoid [49] and *Vibrio vulnificus* is known to cause disease in those who eat contaminated sea foods and symptoms include vomiting, diarrhoea, abdominal pain and immunocompromised liver disease [50].

Fungi were also isolated from this study (Table 5, Fig. 3-6). *Penicillium* sp. is of major importance in the natural environment as well as food and drug production. *Aspergillus fumigatus* is the causative agent for aspergillosis [51] while *Aspergillus niger* can also cause otomycosis and lung diseases if the spores are inhaled [52]. Finally, *Rhodotorula glutinis* has been associated with cases of meningitis [53], endocarditis [54], ventriculitis, peritonitis, endophthalmitis [55] central venous catheter-infections, fungemia, and sepsis [56] have so far been reported.

The isolation of these organisms is consequential as this effluent was collected at the point of discharge into a nearby river, which may not only serve as a source of drinking water to the immediate community, but also a food source through fishing.

Genotoxicological Testing Using Bacteria Bioassays

The simultaneous use of Ames' *Salmonella* test and the SOS chromotest allow the preliminary screening of the complex mediums like the tobacco wastewater using mutant strains of *S. typhimurium* (TA98 and TA 100) and *E.coli* (K-12). These two genotoxicity tests are sensitive to different genotoxic effect (substitution mutation, frame-shift mutation or primary DNA damage) and the variability of the obtained results tends to show a correlation of mutagenic and genotoxic potential of the wastewater.

Evaluation of Mutagenicity Using Ames *Salmonella* Test

Mutagenicity of the wastewater was assessed using the Ames test with *Salmonella typhimurium* TA 98 and TA 100 strains. The Muta choromotest kit is 96-well plate and each plate is considered a colony. The assay is dose dependent and the effluent tested positive with both strains which suggests that reverse mutation occurred. This is confirmed as the bacteria in the colony continued to grow, the colour in the well turn from purple (negative) to yellow (positive) [57]. The *Salmonella* tester strains are exposed to increment doses (1%, 5% and 10%) of the wastewater (in the without the S9 metabolic mixture) in which only the newly mutated, histidine-independent cells grow to form colonies. On the last day of the assay, the number of positively scored wells are 96 (TA 100) and 65 (TA98) at 10% while at 1%, the positively scored wells are 29 (TA 100) and 84 (TA 98).

This implies that at 10% and 1% concentration, the wastewater is 99.9% mutagenic (Table 6, figure. 7-8), while at 5%, it is possibly cytotoxic to the bacteria, as the number of positively scored well is 12 (TA 100) and 7 (TA 98). The number of positively scored wells in the positive controls NAN_3 (TA 100) and NaF (TA 98) are 80 and 87 respectively (Table 6, figure 7-8). This suggests that the wastewater is more mutagenic than the positive controls, NAN_3 and NF which are known mutagenic substances [57]. Chemicals, such as ammonia, humectants (acrolein), insecticides, herbicides, fungicides, and flavouring which are found in tobacco are reasonable explanation for the mutagenicity of the wastewater. These chemicals are found to be mutagenic (with and without S9) in the Ames *Salmonella* test and the SOS chromotest [58].

Evaluation of Mutagenicity Using SOS Chromotest

The SOS chromotest results using tester strain *E. coli* PQ37, without metabolic activation as described by Quillardet and Hofnung [32] confirmed that the wastewater is genotoxic. The criterion to consider a sample as positive in the SOS chromotest differs between the authors [59; 58]. A sample is considered a sample as an SOS repair system inducer if the SOSIP value is higher than 1.5, the b-galactosidase activity significantly increases compared to the solvent control, the result is reproducible, and when it is possible there is a dose–effect relationship. 4NQO was used as positive control to compare colour gradation (visual analysis, Table 7-8, Fig. 9) and SSOIP values (instrumental analysis, Table 9) while sterile distilled water was used as negative control.

For the instrumental analysis, the SSOIP value of the positive control, 4NQO is 51.05 (Fig. 11) while the test compound is 61.54 (Fig. 13). Since both values are greater than 1.5, which is the standard, we can say that the wastewater has a significant genotoxic potential. However, it is important to note that 71 is the published value for the 4NQO in the original Quillardet *et al.* [32] chromotest procedure, but the SOSIP may change from time to time due to changing incubation conditions and the age of the bacteria. Therefore, it is better to use the actual value of the known standard. The active ingredient in tobacco is nicotine [60], however there is no found published value for the SOSIP of nicotine. From the study, we can ascertain that the inhibition of cell division by the wastewater, due to its genotoxic potential, causes the expression of SOS function coded by *sfiA* that activates *lacZ* operon which is detected using a spectrophotometer [32]

For visual analysis, the colour density of the positive 4NQO was used (Table 8). At lower concentrations, (1.25 and 0.625), the wastewater is massively genotoxic (++++) while at 2.5, it is moderately genotoxic (+++) while at 5.00 and 10.00 concentrations, it has a poor genotoxic potential (+) (Table 7-8). It is worthy to note that high concentrations do not necessarily induce any positive response due to acutely toxic concentrations in which the cells are killed outright. As the material is diluted out, toxicity is reduced and a positive reaction (deep green colour) may then appear indicating chronic genotoxicity.

The synergy of all the tests from this study unequivocally confirms that the tobacco wastewater is highly toxic to living organisms.

CONCLUSION

In conclusion, the uniformity of the results obtained from the entire test carried out in this study validates the hypothesis that the tobacco wastewater is genotoxic. The simplicity and economical cost of the procedure make genotoxicity bioassays desirable for environmental monitoring and risk assessment. This study also exemplified that genotoxicity bioassays should be a requisite tool in the evaluation of wastewater toxicity before its discharge into the environment. Finally, this study showed the effectiveness of combining microbiological, physicochemical analysis

with cytogenetic methods to better ascertain the toxicity of chemical pollutants and their influence on living organisms.

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REFERENCES

[1] Novotny TE, and Zhao F, *Tobacco Control*, **2007**, 8, 75–80.
[2] Sponza DT, *Aquatic Ecosystem Health & Management*, **2001**, 4, 479-492 (14).
[3] Sharma RK., Agrawal M, and Marshall FM, Effects of Waste Water Irrigation on Heavy Metal Accumulation in Soil and Plants, Paper presented at a National Seminar, Bangalore University, Bangalore, **2004**, 7, 8.
[4] Singare PU, Lokhande RS, Naik KU, *Interdisciplinary Environmental Review*, **2010a**, 11(1), 90-107.
[5] Aghor A, *Daily DNA*, **2007**, 27(9), 345-355.
[6] Patil D, A lot's Fishy about Our Creek and Lake Fish, **2009.**
[7] Singare PU, Lokhande RS, Jagtap AG, *International Journal of Global Environmental Issues*, **2011**, 11(01), 28-36
[8] Adams WJ, Kimerle RA, Barnett JW, *Environ Sci Technol*, **1992**, 26 (10), 1864-1875.
[9]] Maher BS, Marazita ML, Moss HB, Vanyukov MM, *J Med Genet*, **1999**, 88, 71–78.
[10] Kennicutt MC, Wade TL, Presley BJ, Requejo AG, Brooks JM, Denoux GJ, *Environ Sci Technol*, **1994**, 28(1): 1-15.
[11] Singare PU, Lokhande RS, Pathak PP, *J Environmental Protection*, **2010b**, 1(1), 121-128.
[12] Imperial Tobacco Group PLC (ITG), Tobacco, **2008**.
[13] Philip Morris International (PMI), *Biologia*, **2008**, 2, 96-100.
[14] British American Tobacco (BAT), **2008**.
[15] British American Tobacco (BAT), Cancer, **2011**.
[16] Jolibois B, Guerbet M, *Ann Occup Hyg*, **2006**, *50*, 2.
[17] ISO 7888, Water Quality: Determination of Electrical Conductivity, **1985**.
[18] ISO 6060, Water Quality: Determination of Chemical Oxygen Demand, **1989**.
[19] ISO 10523, Water Quality: Determination of pH, **1994**.
[20] ISO 11923, Water Quality: Determination of Suspended Solids by Filtration through Glass-fibre Filters, **1997**.
[21] ISO/TR 11905, Water Quality: Determination of Nitrogen — Part 2: Determination of Bound Nitrogen, After Combustion and Oxidation to Nitrogen dioxide, Chemiluminescence Detection, **1997.**
[22] ISO 14911, Water Quality: Determination of Dissolved Li+, Na+, NH4+, K+, Mn2+, Ca2+, Mg2+, Sr2+ and Ba2+ Using Ion Chromatography—Method for Water and Wastewater, **1998**.
[23] ISO 5815, Water Quality: Determination of Biochemical Oxygen Demand After n Days (BOD) — Part 1: Dilution and Seeding Method with Allylthiourea Addition, **2003**.
[24] ISO 6878, Water Quality: Determination of Phosphorus: Ammonium Molybdate Spectrometric Method, **2004**.
[25] American Public Health Association (APHA), 7th Edition, *Standard Method for the Examination of Water and Wastewater*, Washington D.C, **1989**, pp. 1268.
[26] Nwachukwu SCU, *J Environ Biol*, **2000**, 21: 241 - 250.
[27] Winn WC, Allen SD, Janda WM, Koneman EW, Procop GW, Schreckenberger PC, Words GL, *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th Edition, Lippincott Williams and Wilkins, Baltimore, **2006.**
[28] Burnett JH, *Fundamentals of Mycology*, 2nd Edition, Edward Arnold Publishers Ltd., London, **1976**.
[29] Wemedo SA, Obire O, Akani, NP, *Asian J. Biol. Sci*, **2012**, 5, 46-51.
[30] Alexopoulos CJ, Sun SH, *Introductory Mycology*, 2nd Edition, John Willey and Sons Inc., New York, **1962**.
[31] Legault R, Blaise C, Trottier S, White PA, *Environ Tox Water Qual*, **1996**, 11, 151-165.
[32] Quillardet P, De Bellecombe C, Hofnung M, *Mutat Res.*, **1985**, 147, 79-95.
[33] World Health Organization (W.H.O), Guidelines for Drinking Water Quality, Geneva Conference Records 3, **1985**.
[34] Fowler BA, Kahng MW, Smith DR, *Environ Health Perspect*, **1994**, 102 (3), 115-116.
[35] Radetski CM, Ferrari B, Cotelle S, Masfaraud JF, Ferard JF, *Sci Total Environ*, **2004**, 333, 209 - 216.
[36] Glaris D, Evangelou A, *Critical Rev Oncol Hematol*, **2002**, 42 (1), 93 - 94.

[37] Sharma A, Sharma MK., Kumar M, *Chem Biol Interact.*, **2009**, 180, 20-30.
[38] Fiskesjo G, *Environ Toxicol Water Qual.*, **1998**, 9, 235-241.
[39] Banu BS, Devi KD, Maliboob M, and Jamil K, *Drug Chem Toxicol.*, **2001**, 24, 63–73.
[40] Costa M., Salnikow K, Consentino S, Klein Huang X, Zhaung Z, *Environ Health Perspect*, **1994**, 102 (3), 127.
[41] Omura M, Inamasu T, Ishinishi N, *Bull Environ Contam Toxicol*, **1991**,46: 561 - 568.
[42] Bakare AA, Lateef A, Amuda OS, Afolabi RO, *Asian J Microbiol Biotech Environ Sci*, **2003**, 5, 11-17.
[43] Beattie G, Lindow S, Bacterial Colonization of Leaves: A Spectrum of Strategies. *Phytopathology*, **1999**, 89, 353-359.
[44] Fernandes AP, Perl TM, Herwaldt LA, *S Clin Perform Qual Health Care,* **1996**, 4: 107–109.
[45] Szewczyk EM, Różalsk M, Cieślikowski T, Nowak T, **2004**, *Folia Microbiol*, 49, 123–131.
[46] Ezaki T, Kawamura Y, Li N, Li Z, Zhao L, and Shu S, **2001**, *Int. J Syst. Evol Microbiol*, 51, 1521-1528.
[47] Perry A, Lambert P, *Expert Review of Anti-infective Therapy*, **2011**, 9 (12), 1149–56.
[48] Williams GJ, Stickler DJ, *Med Microbiol* 57, **2008**, 1135-40.
[49] Lister SA, Barrow P, *Poultry Diseases*, 6th Edition, Saunders Elsevier, Philadelphia PA, **2008**, pp. 130-133.
[50] Oliver JD, *Epidemiol Infect.*, **2005**, 133 (3), 383–91.
[51] Latge JP, *Clin Microbiol.*, **1999**, 12, 310–350.
[52] Schuster E, Dunn-Coleman N, Frisvad JC, van Dijck PWM, *Applied Microbiology and Biotechnology*, **2002**, 59, 426–435.
[53] Gyaurgieva OH, Bogomolova TS, Gorshkova GI, *J Med Vet Mycol*, **1996**, 34, 357-9.
[54] Naveh Y, Friedman A, Merzbach D, Hashman N, *British Heart Journal.*, **1975**, 37, 101-104.
[55] Pinna A, Carta F, Zanetti S, Sanna S, Sechi LA, *Brit J Ophthalmol*, **2001**, 85, 759.
[56] Alliot C, Desablens B, Garidi R, Tabuteau S, *Clin Oncol-Uk,*. **2000**, 12, 115-117.
[57] Ames B, McCann J, Yamasaki E, *Mutat Res* **1975**, 31, 347-364.
[58] Ruiz MJ, Marzin D, *Mutat. Res*, **1997**, 390, 245–255.
[59] Dayan J, Deguingand S, Truzman C, and Chevron M, *Mutat Res*, **1987**, 187, 55–66.
[60] Colby SM, Drobes DJ, West R, *Nicotine & Tobacco Research*, **2005**, 7, 667-709.