Cytogeno Toxicity Evaluation of Waste Water Effluent Discharged from Service Station using *Allium Cepa* Bioassay.

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Abstract

Industrial effluents are a main source of toxicants which cause adverse effects on flora, fauna and human health. In order to determine the cytogeno-toxic effects of effluents from service station, Allium cepa bioassay was used. Effluent samples were collected from discharge point and physiochemical characters were evaluated. In growth inhibition assay, root lengths of Allium cepa were measured after 7 days' exposure in effluents (0,1,5,10,25,50,75, and 100% concentration) to determine the EC_{50} (Effective concentration for 50% of growth inhibition). The genotoxicity assay was carried out after 48hrs exposure in effluents (0.01, 0.1, 1, and 10% concentration). Root tip cells were observed under the microscope and the mitotic index (MI), frequency of aberrant cells were calculated. The values of physiochemical characteristics were within the national tolerance limits specified for waste water. There were strong correlations between the concentration and growth inhibition ($R^2 = 0.97$). Effluent had 50% (EC_{50}) of growth inhibitory effects in 80 % of the discharged concentration.

The root growth retardation was significantly concentration dependent ($P < 0.05$). MI values were decreased and total number of aberrated cells were increased with increasing concentration. The dominant aberrations were sticky meta and sticky anaphases. The damages, including bridges, vagrants and c-metaphases and polar slips were also observed in low effluent concentrations. It is concluded that the toxic effect and growth inhibition can be reduced further by diluting the effluent. The proposed A.cepa bioassay is a reliable tool for this study. Genotoxic effects of other industrial effluents can also be monitored by using this assay.

Keywords: *Genotoxicity, Root growth inhibition*

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Introduction

Industrial effluents are a main source of direct and continuous input of pollutants into aquatic ecosystems. Pollution lowers the quality of life in various aspects and also affects the health and life span of humans. Pollutants may be mutagenic or toxic and lead to several human afflictions (Olusegun *et al.*, 2010). Genotoxicity assays are used to evaluate the genotoxic potential of environmental and industrial effluent samples. Higher plants are excellent genetic models to detect environmental mutagens (Rank and Nielson, 1994). *Allium cepa* bioassay is frequently used for environmental monitoring studies of waste, surface water and groundwater quality assessments as it is a low cost, sensitive, short term, effective method and also easy to handle (Grant, 1978).

In Sri Lanka, inland surface waters are being polluted with domestic sewage and industrial effluents. National tolerance limits of Sri Lanka Standards have been established for a set of physio-chemical and microbiological parameters for discharge of industrial effluents. However, no attention has been given for assessing the biological effects of inland surface water and ground water impacted by industrial waste water and effluents (Pathiratne and Kannangara, 2015). Petroleum can harm the environment through air and water pollution. Used motor oil is considered as a dangerous environmental pollutant due to its chemical composition and worldwide dispersion. It contains polycyclic aromatic hydrocarbons (PAH) and high levels of heavy metals which are highly mutagenic and carcinogenic.

The aim of the study was to evaluate the cytogeno toxicity of discharged effluents from service station by determine the root growth inhibition in *Allium cepa* and observe the chromosomal aberrations of the dividing cells. The results of this study are useful for detecting the mutagenic effects by the effluent.

Literature Review

Industrial wastewaters may contain complex chemical mixtures including metallic and organic compounds with potential cytotoxic and genotoxic effects. The evaluation of hazardous wastes and effluents by genotoxicity assays may provide data useful for hazard identification and comparative risk assessment (Claxton, 1998).

Genotoxicity of environmental contaminants is of great concern, due to the capability of genetic damage to cause health problems. Many of the metals in use motor oil are harmful to human health and living organisms. These metals originate from the fuel and from motor wear. Used oil contains high concentrations of lead, zinc, calcium, barium, and magnesium along with lower concentrations of iron, sodium, copper, aluminum, chromium, manganese, potassium, nickel, tin,

silicon, boron, and molybdenum. However, while the experiments clearly show that PAHs in used motor oil interacted with DNA, most adducts would be repaired, and few would result in genetic lesions. Similarly, DNA adductions were formed when human skin cultures were exposed to used oils (Rafaelvazquez-duhalt, 1989). It is important to conduct mutagenicity or genotoxicity assays in addition to the analysis of conventional water quality parameters to efficiently assess the presence of mutagens in water (Pathiratne & Kannangara, 2015).

Even the epidemiological approaches used to detect the toxic chemicals, they have limitations to detect genotoxic and carcinogenic chemicals. Wide variety of organisms ranging from viruses, bacteria, plants and insects to human cell cultures and intact mammals were employed to evaluate the mutagenicity of environmental chemicals. In order to identify the harmful effects of substances in different concentrations and time of exposure, a variety of tests have been employed, such as cytogenetic tests etc. These tests are commonly used for biomonitoring the extent of pollution and to evaluate the effects of toxic and mutagenic substances in the natural environment (Matsumoto *et al.*, 2006).

Allium/Vicia root chromosomal aberration assay has also been adopted by the International Program on Plant Bioassays (IPPB) for the evaluation of the environmental pollutants. This assay has also been used to monitor the antigenotoxic nature of various plants and plant products (Ma, 1999).

Several higher plant systems, including bioassays with plant roots have provided cheaper, easier, sensitive, useful, reliable and valuable alternative methods for the determination of the adverse effects of environmental pollutants to the usual assays carried out on experimental animals (Grant, 1978). Different parameters of *Allium cepa* such as root shape, growth, mitotic index and chromosomal aberrations can be used to estimate the cytotoxicity, genotoxicity and mutagenicity of environmental pollutant (Amin, 2002). Among these assays, *Allium cepa* L. chromosomal aberration assay have been proven to be effective, sensitive, less costly and used for testing the potential mutagens in both mitotic and meiotic cells. The cytotoxic level of a test chemical compounds can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of cytotoxicity in studies of environmental biomonitoring (Smaka-Kincl, 1996).

The cytotoxic level can be determined by the decreased rate of mitotic index. A mitotic index decreases below 22% of negative control causes lethal effects on test organism while a decrease below 50% has sub lethal effects and is called cytotoxic limit value. Several investigators have used MI as an endpoint for the evaluation of genotoxicity or antigenotoxicity of different chemical treatments. Chromosomal aberrations are characterized by change in either total number of chromosomes or in chromosomal structure which occur as a result of the exposure of chemical treatment. Chromosomal aberrations were grouped into 2 types, clastogenic and physiological

aberrations. Clastogenic aberrations include chromatin Bridge, chromosomal break and ring chromosome whereas physiological aberrations include c-mitosis, vagrant, stickiness, delayed anaphase and laggard (Sharma, 2013). Cytogenotoxcity assay using *A. cepa* have been reported including exposure to a number of pollutants like heavy metals (Pathiratne, 2015), pesticides (Turkoglu, 2012) and complex mixtures containing industrial and municipal wastes (Samuel, *et.al.*,2010)

Materials and Methods

Physiochemical Characterization

Effluent sample was collected from discharge point using grab sampling method. Glass vessels were used for the collection. Physiochemical characters including temperature, pH, EC, BOD_5, NO_3^- and PO_4^{3-} levels were evaluated and compared with national standard limits for discharge of effluents.

Two types of assays including growth inhibition and cytogenotoxicity assay were conducted after the completion of the physiochemical evaluation. Growth inhibition assay was performed to determine EC_{50} and cytogenotoxicity assay was performed to find the aberrations.

Growth inhibition assay: In growth inhibition assay, root lengths of *Allium cepa* were measured with meter rule after 7 days' exposure in different concentration of effluent (0,1,5,10,25,50,75, and 100%) to determine the EC_{50}.

Each concentration was set-up in 7 replicates. The test solutions were replaced every 24 hours with fresh solutions. At the termination of exposure, two onions (out of five) with the poorest growth was discarded and the length of the root bundle was measured for the rest five onions. Four longest roots in each bulb were measured. The effective concentration of a chemical producing 50% of Growth inhibition was considered as EC50.

Cytogenotoxicity assay: The genotoxicity assay was carried out after 48hours exposure in effluents (0.01, 0.1, 1, and 10% concentration). Root tips were cut and fixed in ethanol and glacial acetic acid (3:1, v/v). Then the root tips were hydrolyzed in 1N HCl at 60°C for five minutes and washed in distilled water. Finally, they were stained with aceto-carmine for 10 min, squashed on slides and covered with cover slips to exclude air bubbles. Slides were prepared for each concentration and observed under the light microscope with the assistance of an oil immersion lens (x1000 magnification).

Cytogenotoxicity were estimated by observing cytological parameters such as the mitotic index (MI) and number of chromosome abnormalities, including chromosome breaks, stickiness, and polar deviations. The mitotic index was calculated as the number of dividing cells per 1000 observed cells

(Fiskesjo, 1985). The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total dividing cells scored at each concentration of each effluent.

Data Analysis

The means, with 95% confidence limits and the standard deviation for results of the root inhibition and chromosome aberrations at each concentration of the wastewaters were calculated. Data were expressed as Mean ± Standard Deviation of Mean (SD). Differences between the control and different concentrations of the waste waters were analyzed in one-way ANOVA and Tukey multiple range test were used to compare the means, with significance level at $p \leq 0.05$. All the statistical analyses were carried out using SPSS 24.0 statistical package

Results and Discussion

Physiochemical Parameter of Waste Water

The values of physiochemical characteristics were within the national tolerance limits specified for waste water.

Table 1. Physiochemical parameters of waste water effluent

Physiochemical properties	Test value
Temperature	29°C
pH	7.97
EC	1802µS/cm
BOD	20mg/l
NO_3^-	4.95mg/l
PO_4^{3-}	0.75mg/l

Though all the studied parameters were within limit, relatively high levels of pH was observed for effluent which was near to the upper limit (Table1). This may due to discharge of phosphate salts and detergents used in the factory, and relatively higher water hardness of used water (pH 6.4- 8.8, hardness 119-1424mg/l). The high level of total hardness of the effluents can contribute significantly to the hardness of the natural water sources and alkalinity can be attributed to low levels of dissolved oxygen (Olusegun *et.al.*, 2011).

Since all studied physical and chemical parameters of effluent were within the limit, these parameters will not contribute to any impact on environment, and observation of any genotoxic effects by these effluences may be due to other causes such as toxins, PAHs in used motor oil. These compounds are mainly metabolized at the intestinal wall and in the liver, producing free aromatic amines that are potentially carcinogenic and mutagenic

Root Growth Inhibition Analysis

Table 2. Root growth inhibition (%) in the different concentration of effluent

Concentration	Root growth inhibition (%)
1	0.42
5	4.15
10	14.39
25	22.23
50	35.99
75	44.79
100	59.95

Root growth inhibition (%) was calculated as follows:

$$= \frac{\text{mean root growth of control} - \text{mean root growth of specific concentration}}{\text{mean root growth of control}} * 100$$

The results show that root growth inhibition was increased with the increasing concentration of effluents collected from service station (Table 2).

The table compares mean root length of *A.cepa* exposed to different concentration of effluents from service station. Significant reduction in root lengths were observed in 10% concentration and more than10% concentration effluents from service station compare to 1%, 5% concentration and control($P<0.05$). This analysis revealed that the root growth retardation was significantly concentration dependent (Table3)

Table 3. Mean root length of A.cepa exposed to different concentration of service station effluent

Concentration (%)	Mean Root length(cm) ± SD
Control (0)	11.810 ± 0.6206^{a}
1	11.760 ± 0.5670^{a}
5	11.320 ± 0.3518^{a}
10	10.110 ± 1.3867^{b}
25	9.185 ± 0.6360^{c}
50	7.560 ± 0.7735^{d}
75	6.520 ± 0.8115^{e}
100	4.730 ± 0.5420^{f}

Each value represents the Mean ± SD of replicates (n=5), values represent with the same superscript letter along the column are not significantly different (p>0.05) in one-way ANOVA. SD-standard deviation Mean ± SD with the same superscript letter along the column not significantly different (p>0.05) in one-way ANOVA. SD-standard deviation.

Determination of EC_{50}

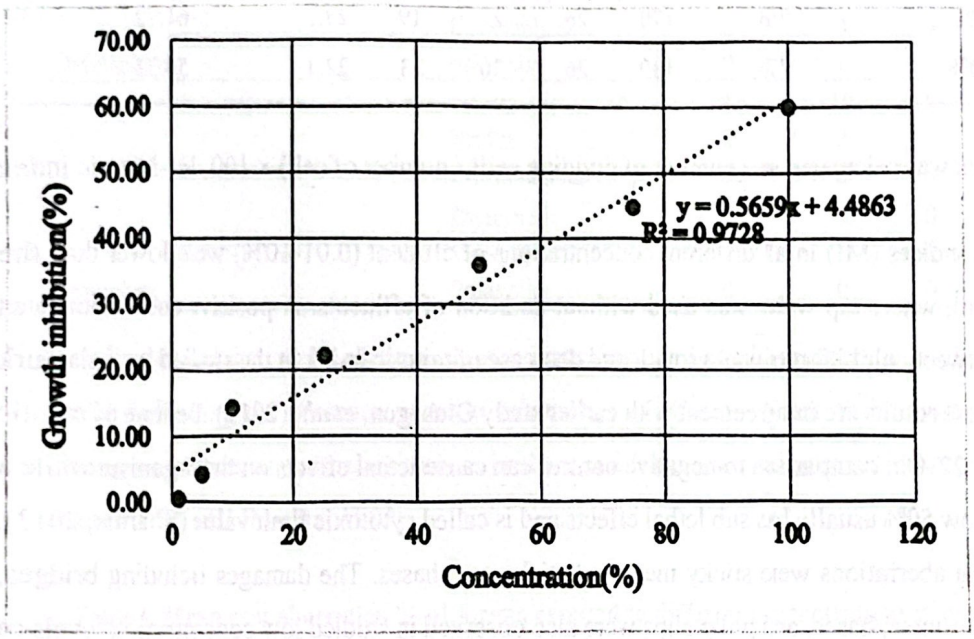

Figure 1. Growth inhibition of *A.cepa* roots exposed to service station effluent

The root growth inhibition in different concentrations of service station effluents are shown in dose-response curves (figure 1). Increasing percentage of growth inhibition of root were observed in dose-response curves with increasing concentrations of effluents. Strong correlation was observed between the concentration and growth inhibition ($R^2 = 0.97$). The estimated EC_{50} (concentration of a chemical producing 50% of the total effect) of *A.cepa* exposed to service station was 80%, and the effluent had 50% (EC_{50})of growth inhibitory effects in 80 % of the discharged concentration level.

Cytogenotoxic Effects

Mitotic indices (MI) were decreased with increasing concentration, at the same time total number of aberrated cells were increased with increasing concentration. The mitotic indices (MI) of *A. cepa*meristematic cells were decreased to 58.33% in the 10% service station effluents compare to control.

Table 4. Mitotic indices of *A.cepa* root cells in different concentrations effluent

Concentration	Interphase	Dividing Cells				MI	MI (% as control)
		Pro	Meta	Ana	Telo		
Control (0%)	616	270	40	37	37	38.4	100
0.01%	710	238	16	22	14	29	75.52
0.10%	730	201	28	30	11	27	70.31
1%	763	170	26	22	19	23.7	61.72
10%	776	139	26	26	33	22.4	58.33

Mitotic index was calculated as: (number of dividing cells / number of cell) × 100, MI-Mitotic index

The Mitotic indices (MI) in all different concentrations of effluent (0.01-10%) were lower than the MI of control, where tap water was used without addition of effluents. A positive correlation was observed between inhibition of root growth and decrease of mitotic index in the studied by Aslanturk (2010). These results are in agreement with earlier study Olusegun, *et al.*, (2011). Decline of mitotic index below 22% in comparison to negative control can cause lethal effects on the organism while a decrease below 50% usually has sub lethal effects and is called cytotoxic limit value (Sharma, 2013). The dominant aberrations were sticky meta and sticky anaphases. The damages including bridges, vagrants and c-metaphases, and polar slips were also observed in studied low concentration levels of effluents (0.01, 0.1, 1, and 10%) (Figure 3) compared to normal cell stages (Figure2)

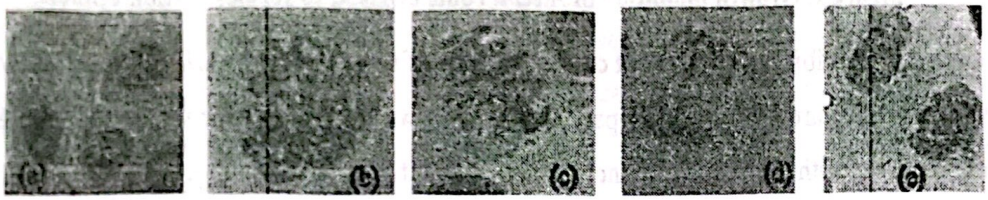

Figure 2. Allium cepa root tip meristematic cells showing normal cell stages (a) interphase; (b) prophase; (c) metaphase; (d) anaphase and (e) telophase.((x1000 magnification)

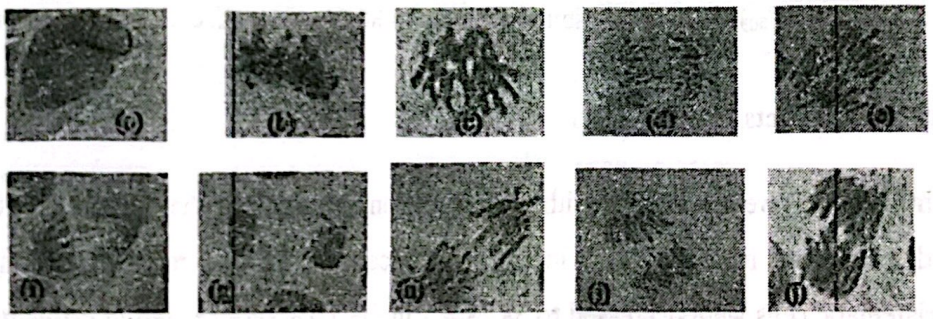

Figure 3. *Allium cepa*root tip, interphase cells with (a) notched nucleus; prophase cell with (b) sticky metaphase; (c) c-metaphase; anaphases with (d,e) Disturbed spindle (f) Polar slip (g) sticky chromosome, (h,i) vagrant (j)chromosomal bridge ((x1000 magnification)

Table 5. Chromosomal aberrations *of A.cepa* root cells in different concentrations of effluents

		Concentration of effluents (%)				
Phase	Aberrations	Control	0.01	0.1	1	10
Prophase	Micro nuclei	0	0	0	0	0
	Disturbed	0	0	0	0	0
	Sticky	5	3	7	15	19
	Chromosomal loss	0	0	0	0	0
	C metaphase	3	0	0	2	2
Metaphase	polar slip	0	1	1	0	1
	Sticky	1	10	5	4	7
	Bridge	2	2	5	2	5
	Chromosomal loss	0	0	0	0	0
	Disturbed	0	0	0	1	1
	Vagrant	1	1	2	1	2
Anaphase	Polar slip	0	0	0	0	1

Results showed that sticky chromosomes at metaphase and anaphase stages were abundant (Table 5) which indicating the presence of toxic substances in effluent. These toxic substances may cause mutagenic effects as anomalies such as bridges and fragments and give an indication of mutagenic events in the cell (Mishra, 1993).

Table 6. Mean root aberration % of A.cepa exposed to different concentrations of industrial effluent

Concentration (%)	Mean Aberrant cells (%) + SD
Control(0)	3.35 + 3.06a
0.01%	6.068 +2.27a
0.1%	6.530 + 6.82a
1%	10.992 + 4.24ab
10%	17.054 + 5.50b

Each value represents the Mean $\pm$ SD of replicates(n=5), values represent with the same superscript letter along the column are not significantly different ($p>0.05$) in one-way ANOVA. SD-standard deviation

The results show that aberrant percentage of cells were significantly higher (Table 6) for the treatment more than 1% concentration of service station waste water effluent ($p<0.05$) than others.

Conclusion

The cytogeno toxic potentials of waste water effluent generated from service station was evaluated by analysing root growth of *Allium cepa* and chromosomal aberration of root cells in different

concentration level. The tested values of physiochemical parameters were within the safe limits which confirm that there were no physio chemical depressions in root growth and expected growth inhibition and aberration may be due to other constituents of effluents. Significant differences were observed in mean root lengths of *A. cepa* exposed to different concentrations of effluents with control, this shows that effluents from have some growth inhibitory effects on the environment. The root growth inhibition is significantly concentration dependent thus root growth was significantly reduced with increasing concentration ($p<0.05$). Morphological changes like brown, thick and short roots were observed with increasing concentrations.

The EC_{50} values of service station was 80% which indicate that 50% and more than 50% of growth inhibitory effects were observed similar to its discharged concentration with direct exposure of roots. Further increase in discharged concentration will lead to more growth inhibition effects. Thus, the results of this study suggest that the toxic effects can be reduced by diluting the effluent from these industries.

Root aberrations were increased with increasing concentration ($p<0.05$); the chromosomal aberration is also concentration dependent. Sticky chromosomes in the metaphase, C-metaphase, disturbed anaphase, anaphase bridge, polar slips and chromosomal losses during anaphases were mostly observed in the dividing cells.

The significant difference was observed between control and 1% concentration of effluent from service station. As this study was undertaken for low concentrations levels (0.01-10%), it is obvious that the discharge (100%) effluents show cytogeno toxicity.

The differences in toxicity between the waste water effluents are probably due to some variation in the chemical composition of the wastewater. The results from this study could constitute an important knowledge of the toxicology of oil products. However, further studies should be carried out to investigate the effects of the whole compliment of the oils and to identify compounds, or interactions of compounds, responsible for the observed toxicity. In this way, service station effluents that exert hazard on the environment may be manufactured. Additionally, when the wastewater effluents are discharged into the land, the lands may become unproductive. The area may not be used for any of the cultivating activities in the nearest future.

The effluents tested in the present project are considered to be bio toxic in long term effect, and many steps of refinement may be needed prior to discharge. Also, comprehensive toxicity and genotoxicity studies should also be conducted on ground water resources around this area. Leaching of the chemicals from the effluents may contaminate the ground water of surrounding environment, so this study can be a basic tool or pre-study for further studies on ground water pollution by these effluents.

Results of this study show that *A.cepa* chromosomal assay is a reliable tool for monitoring the genotoxic effects of industrial effluents before they are discharged into the environment. Furthermore, the toxicology studies using plant and animal test systems are suggested to conduct by using these effluents for concrete conclusions

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