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Genotoxicity evaluation of a pharmaceutical effluent from Owerri, Nigeria, using the *Allium cepa* assay

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ABSTRACT

There is a growing need to ascertain the mutagenic potentials of complex mixtures such as pharmaceutical industry waste waters. In this study, *Allium cepa* assay was employed to investigate the physico-chemical characteristics and genotoxic potentials of a pharmaceutical industry effluent. The inhibition of mitotic division was employed for *in situ* monitoring of cytotoxicity while chromosomal aberration analysis was used to evaluate genotoxicity. The results obtained show chemical oxygen demand (COD), biological oxygen demand (BOD) and certain metallic components such as Fe, Cu, Mn and Zn to be present in values beyond permissible limits by national and international regulatory bodies. Analysis of variance (ANOVA) data revealed significant ($p<0.05$) dose-dependent differences in the mean root length of *A. cepa* exposed to various concentrations of the industrial effluent. An EC_{50} value of 2.1% was obtained, indicating the high toxicity of the effluent even at very low concentrations. The different types of chromosomal aberrations induced by the pharmaceutical effluent included stickiness, laggards and scattered/disoriented chromosomes; sticky chromosomes being the most frequent. These results draw attention to the dire need to characterize pharmaceutical effluents in order to determine the appropriate treatment options before they are released into the environment.

INTRODUCTION

The global increase in the production and utilization of pharmaceuticals has caused the pharmaceutical industry to become major contributors to pollution, particularly due to the discharge of solid wastes and effluents into the environment. Apart from usage in health maintenance and diagnosis, treatment and prevention of diseases in humans, there is an ever increasing demand for pharmaceutical compounds in animal farming to boost protein availability for an ever increasing human population. Increased animal production requires antibiotics, food additives, hormones, pesticides, etc. (Adeoye et al., 2015). Pharmaceutical industries generally generate a diverse set of waste streams during manufacturing and maintenance operations which include spent fermentation broth, process liquors, solvents, spilled materials and used processing aids (Sumpter et al, 2005). It is very difficult to characterize pharmaceutical waste waters because of non-uniform waste stream, consequent of variation in the medicine being produced during any given processing period (Houk, 1992). However, like other industrial effluents, pharmaceutical waste waters are extremely complex mixtures which may contain chemicals and microorganic chemicals (salts, surfactants such as emulsifiers, detergents and dispersants), ionic metals and their metal complexes, toxic organic chemicals, biocides, unmetabolized drugs etc. (Gadipelly et al., 2014). These components are

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primarily responsible for the toxicity of pharmaceutical effluents.

Pharmaceuticals release a host of xenobiotics into the environment and they are also known to be lipophilic and non-biodegradable in nature, coupled with having biological activities (Velagaleti and Burns, 2006; Cleuvers, 2003; Hernandoa et al. 2006; Lateef et al. 2007). The synergistic and/or antagonistic actions of metals and organic compounds contained in the wastewaters from drug manufacturing industries can induce alterations in enzyme biochemistry, biomolecules and cell membrane as a result of increased cellular formation of oxidative stress via the creation of imbalance reactive oxygen species (ROS) and antioxidant systems (Bakare et al., 2003). Thus, their presence in the environment is of serious concern. Again, while in the environment, most pharmaceutical products accumulate in water bodies, aquatic sediments, soil and biological systems, reaching a biologically active concentration with time (Cleuvers, 2003; Hernandoa et al., 2006). A long term exposure of lower concentrations of complex pharmaceutical mixtures on stream biota may therefore result in acute damages, behavioural changes, reproductive damages, accumulation in tissue and inhibition of cell proliferation (Patneedi and Prasady, 2015). Pharmaceutical effluent discharge into surface water therefore constitutes biohazard to man and other living organisms in the environment because they contain toxic substances that are detrimental to health (Adebisi et al., 2007; Adriano, 2001; Bakare et al., 2003).

Chemical analysis of pharmaceutical effluents does not give information on the toxic effects of the individual components present as mixture of xenobiotics in the effluent nor their potential synergistic and antagonistic interactions in living organisms (Mansour et al., 2012). Experimental toxicity tests are essential for an effective ecotoxicological evaluation. There is therefore a need to evaluate the mutagenic and genotoxic effects of human exposure to pharmaceutical effluents in the environment. A number of bioassays using microbes, plants and animals have been employed to assess the potentials of contaminated water bodies to induce genetic damage (Heath et al., 1984; Vrijheid et. al., 2002). However, pharmaceuticals in industrial waste waters may not be effectively assessed using microbial assays because they may contain antibiotics or bacterial growth inhibitors (Houk, 1992). Hence, chemical characterization along with other test systems, become imperative for effective genotoxicity evaluation. The *Allium* test has been accepted as a standard, easy and sensitive toxicity screening method for complex mixtures in environmental monitoring (Fiskesjö, 1985; Rank and Nielsen, 1993). The assay is also low cost, easy to use and it produces similar results to animal tests because of the similarity in their genetic compositions, hence same response to mutagens (Akinboro et al., 2011).

The rapid strides in industrialization in Nigeria in the last four decades have not been accompanied by the enactment or enforcement of sound environmental waste management policies. The resultant effect is the indiscriminate discharge of untreated industrial effluents into surface water bodies. Of particular interest are the pharmaceutical industries from where large volumes of raw or partially treated waste waters containing complex hazardous substances are continuously discharged into gutters or drains, ending up in streams and rivers. This leads to unprecedented contamination of surface and ground water and the pollution of the ecosystem in general. A number of such industries are located in the Southeast of Nigeria and there is a paucity of data with regards to their potential contribution as environmental hazards (Abu and Mba, 2011) as most published works were carried out in the Western and Northcentral parts of the nation (Bakare et al., 2009; Akintonwa et al., 2009; Anyakora et al., 2011; Lateef et al., 2007; Adeoye et al., 2015; James et al., 2015; Idris et al., 2010). The modified *Allium cepa* assay (Fiskesjö, 1997; Babatunde and Bakare, 2006; Bakare and Wale-Adeyemo, 2004). The *A. cepa* assay was employed in this study, in conjunction with chemical analysis, to evaluate the genotoxicity of a pharmaceutical effluent from Regan Remedies Limited in Owerri metropolis in the Southeast of Nigeria. The results of the study will generate the much needed data that could serve as a scientific basis for regulating the discharge of potentially hazardous substances into the environment.

MATERIALS AND METHODS

Effluent collection and physico-chemical analysis

The raw effluent was obtained from Regan Remedies Limited, a pharmaceutical company located at New Owerri, Imo State, Nigeria (5° 29’ 01” North, 7° 01’ 59” East). The effluent from the industry is discharged into big gutters near the factory, from where it flows into a major water body in Owerri metropolis, the Nworie River. The effluent was collected in two 10 L plastic containers from the point of discharge into the environment. The products of the company include analgesics, multivitamins, antibiotics, antihistamines, sulphonamides and antiemetics. The waste water was filtered upon collection, the pH and other standard physico-chemical parameters such as chemical oxygen demand (COD), total dissolved solids (TDS), biological oxygen demand (BOD), salinity, alkalinity etc. were determined according to standard analytical methods (United States Environmental Protection Agency – USEPA, 1996; American Public Health Association – APHA, 1998). The physico-chemical analyses were carried out at the water chemistry laboratory, Hydrobiology and Fisheries Unit,

Abia State University. The sample was kept in the refrigerator pending use.

Heavy metal analysis

The analyses of some heavy metals such as Zn, Al, Ni, Fe, Mn and Cu were carried out according to standard analytical methods (United States Environmental Protection Agency - USEPA, 1996; American Public Health Association – APHA, 1998). The elements in the digested pharmaceutical effluent were determined using atomic absorption spectrophotometer (AAS). 50 ml of the effluent was measured and weighed in a 100 ml crucible and evaporated to dryness on a hot plate. The crucible containing the residue was transferred to a muffle furnace and incinerated at 550°C for about 4 h. It was cooled in a desiccator and the ashed residue was dissolved with about 5 ml concentrated HCl, diluted with deionized water, filtered and made up to the mark in a 100 ml volumetric flask with deionized water. Heavy metal concentration was analyzed with BUCK 205 atomic absorption spectrophotometer (AAS) using flame atomization. Results are expressed on dry weight basis of each component. The heavy metal analysis was carried out in the Soil Science Laboratory, School of Agriculture and Agriculture Technology, Federal University of Technology, Owerri.

Test organism

The common purple onion *A. cepa* L. Stuttgarter Reisen (2n=16, Family Amaryllidaceae) bulbs (2.5-2.8 cm diameter) used for the study were commercially procured from Eke Okigwe market, Abia State, Nigeria. They were sun dried for 2 weeks and the dry bulbs (excluding the rotten ones) were later used for the tests (Babatunde and Bakare, 2006).

The *Allium cepa* assay procedure

The modified assay (Fiskesjö, 1997; Bakare and Wale-Adeyemo, 2004; Babatunde and Bakare, 2006) was carried out using 100 ml beakers and distilled water was used as a negative control and for the dilution of the industrial effluent. The effluent was equilibrated to room temperature (26±2°C) and diluted to produce the series of concentrations investigated (OECD, 2015). Prior to the test, the outer scales of the bulbs and brownish bottom plates were removed, leaving the ring of root primordial intact. The peeled bulbs were placed into fresh water during the cleaning process so as to protect the primordial from drying. Afterwards, the bulbs were exposed directly to 0.5, 1.0, 5.0 and 10% (v/v, effluent/distilled water) of the test liquid. Five onions were used for each concentration of the effluent and the control. The base of each onion bulb was suspended on the test liquid in 100 ml beakers in the dark at 27±1°C. The test liquids were changed daily.

Genotoxicity investigation

After 48 h (Babatunde and Bakare, 2006), the root tips of one bulb in each group of the experimental organisms were fixed separately in ethanol:glacial acetic acid (3:1, v/v) and were used for the chromosomal analysis. The root tips (for each effluent concentration and the control) were hydrolyzed in 1N HCl at 60°C for 5 min and rinsed in distilled water. Two root tips were placed on each slide and stained in aceto-carmine for 20 min (after squashing). Excess stain was removed with filter paper and cover slip was carefully lowered to prevent air bubbles being trapped under. The edges of the cover slip in each case were sealed with clear nail polish as suggested by Grant (1982) to prevent drying out of the preparation by the heat of the microscope (Sharma, 1983). Five slides were prepared for each effluent concentration and for the control. The prepared slides were coded and examined for chromosomal aberrations at high magnification (X1000). The mitotic index (MI) was calculated as percentage of the number of dividing cells per 500 observed cells (Fiskesjö, 1985, 1997) that is, 100 cells were examined per slide per concentration, including the control. The mitotic inhibition was estimated as the percentage of the difference between the mitotic indices of the control and the group, divided by the mitotic index of the control. The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of the effluent (Bakare et al., 2000).

Root growth inhibition test

On the third day (that is, after 72 h) the measurements of each root of the bundle of roots for the remaining four onion bulbs per concentration and the control were taken (Babatunde and Bakare, 2006). The percentage of reduction in root growth in relation to the control and EC_{50} value (concentration of a toxicant that gives half-maximal response after a specified exposure time) was determined from the values thus obtained (Samuel et al., 2010).

Statistical analysis

Analysis of variance (ANOVA) and Pearson correlation analysis were carried out to test for significant

Table 1. Physico-chemical characteristics of the pharmaceutical effluent analyzed for genotoxicity.

Parameters*	Pharmaceutical effluent	NESREA[a]	FEPA[b]	USEPA[c]
pH	6.5	6.0-9.0	6-9	5-9
Colour	Dark brown	NS	NS	NS
COD[d]	201.75	90	50	NS
TDS[e]	82.01	NS	2000	NS
BOD[f]	66.60	50	50	NS
Alkalinity	109.0	150	250	20
Salinity	82.0	NS	NS	NS
Sulphates	96.80	250	NS	750
Phosphate	17.00	2.0	5.0	NS
Ammonia	98.20	10	0.01	0.02
Zn	7.5	2.0	5.0	NS
Ni	0.005	0.05	0.05	NS
Mn	0.15	0.02	0.05	0.05
Al	1.00	NS	NS	NS
Fe	40.84	NS	0.3	0.3
Cu	4.5	0.5	0.01	1.3

*All values are in mg l^{-1} except pH with no units and salinity (ppt.); NS-Not stated (that is, no guideline established); [a]National Environmental Standards and Regulation Enforcement Agency (2009); Permissible limits for effluent discharge into surface water; [b]Federal Environmental Protection Agency (2001); [c]United States Environmental Protection Agency (2006) Standards for effluent discharge regulation; [c]COD, Chemical oxygen demand; [d]TDS, Total dissolved solid; [e]BOD, Biochemical oxygen demand.

relationship (positive or negative) between the root length and effluent concentrations. The analysis was performed using the IBM SPSS ® 21.0 statistical package. The EC_{50} was determined from a plot of root length as a percentage of control against the sample concentrations by using Microsoft Excel computer program. The results were expressed with 95% confidence limits that is 0.05 probability level.

RESULTS AND DISCUSSION

The results of the heavy metals and physico-chemical analysis of the pharmaceutical effluent are presented in Table 1. The pH value of 6.5 obtained was within the normal range but the odour of the wastewater was offensive, probably due to the organic components of the effluent. The values of BOD and COD obtained (66.60 and 201.75 respectively) were higher than the acceptable national and international limits fixed by the different regulatory bodies (NESREA, FEPA and USEPA) for industrial effluents to be discharged into surface water bodies. A high BOD value of an industrial effluent implies high biologically degradable organic load while a high COD on the other hand suggests that the effluent is composed of a mixture of substances at different stages of decomposition. A high value of BOD leads to accelerated bacteria growth in the water bodies receiving industrial effluents and this could in turn result in the consumption of the oxygen in the water bodies. Moreover, high COD and BOD values are associated with modification of metal toxicity and bioavailability, even when such metals are present at very low concentrations (Hamelink et al., 1994). BOD and COD values are thus very important pollution indices of industrial effluents and the reduction of BOD is the main focus of effluent treatment plants (Telka et al., 2012). The values obtained for phosphates and ammonia (17.0 and 98.20 respectively) was also much higher than permissible limits for the discharge of industrial waste waters. Elevated values of phosphates and ammonia become important issues in view of the fact that they can serve as an alternative available source of energy for microorganisms when BOD is depleted, causing an unpredictable swing in biological activity and oxygen demand (especially upsetting the balance of nitrification-denitrification). This is because during waste water treatment, excess air will be required to provide a “safety stock” of BOD and this will increase the energy cost (Sotirakou et al., 1999). High values of phosphates and nitrates therefore become significant organic contaminants in assessing the pollution status of pharmaceutical effluents.

The values of Fe, Cu, Mn and Zn were very high when compared to acceptable limits for effluent discharge into surface waters (National Environmental Standards and Regulation Enforcement Agency - NESREA, 2009; United States Environmental Protection Agency –

Table 2. Inhibitory effects on *A. cepa* root length exposed to various concentration of the pharmaceutical effluent.

Concentration of pharmaceutical effluent (%)	Mean root length ± SD (cm)	Root growth in % of control (cm)	95% confidence limit
0	3.5 ± 1.03	0	0.32
0.5	0.84 ± 0.23	24.0	0.58
1.0	0.39 ± 0.08	11.14	0.71
5.0	0.26 ± 0.05	7.43	0.63
10	0.13 ± 0.05	3.71	0.49
EC_{50}	2.1%		

USEPA, 2006) while the value obtained for Ni was below detectable limit. Previous studies have reported high concentrations of heavy metals and organic compounds in pharmaceutical effluents, even though these have been at varying concentrations beyond the standard acceptable limits (Adeoye et al., 2015; Cleuvers, 2003; Larsson et al., 2007; Hernandoa et al., 2006; Saukpal and Naikwads, 2012). High levels of Fe are known to promote the growth of iron bacteria, which derive energy from the oxidation of ferrous iron to ferric iron on exposure to air (World Health Organization - WHO, 2017). On the other hand, a high value of Cu is known to cause stomach and intestinal distress, liver, kidney damage and anaemia in humans, the adverse effects of Mn in humans include neurobehavioural and psychological symptoms while the presence of Zn may increase the acidity of water (United States Environmental Protection Agency - USEPA, 2003). Some fish can also accumulate Zn in their bodies when living in Zn-contaminated waterways and this can biomagnify up the food chain (Wuana and Okieimen, 2011). The presence of these metals in high values in pharmaceuticals is thus an indication of the potential capacity of the pharmaceutical effluents to constitute a health hazard in the environment.

Table 2 shows the summary of the growth analysis of *A. cepa* roots exposed to different concentrations of the industrial effluent. The EC_{50} value of 2.1% obtained was very low indicating that the effluent is highly toxic even at low concentrations. This is in agreement with Bakare et al. (2009) who obtained an EC_{50} value of 1.82 in their work with a pharmaceutical effluent from Lagos State, Nigeria. The EC_{50} value is a measure of potency and it is used to determine risk assessment in public health, which in turn is considered essential for making decisions on a scientifically sound basis. Statistical analysis with ANOVA indicated that there was significant ($p<0.05$) difference in the mean root lengths of the test organisms exposed to different concentrations of the pharmaceutical effluent. Generally, root growth inhibition analysis, using Pearson correlation, was observed to be positively correlated to concentration indicating that root growth retardation was significantly ($p<0.05$) concentration-dependent, that is, high growth rate was observed with decreasing effluent concentration. Root growth inhibition is an index for estimating general toxicity and it occurs when roots are exposed to a wrong pH, or to unsolved substances that may prevent nutrition uptake (Fiskesjö, 1993). This results in inhibition of cell division, indicating cytotoxicity. The inhibitory effects can also be on cell extension that is cessation of root elongation which is correlated with the disappearance of mitotic figures. It has been observed that some mechanism (such as microtubule formation, DNA synthesis, nucleoprotein synthesis etc.) associated with cell division is highly sensitive to certain chemicals or metals and is permanently damaged by short exposures (Clarkson, 1965). Thus, the results from EC_{50} estimation and the root growth analysis indicate that the effluent investigated had cytotoxic effects on the roots of *A. cepa*.

The microscopic analysis results are summarized in Table 3. From the data, the estimated MI ranged from 29.2 in the control to 2.4 in the 10% effluent. Mitotic indices of 8.24 (in 2% effluent), 4.04 (in 5% effluent) and 25.8 (in 0.5% effluent), 3.95 (in 5.0 % effluent) had been observed by James et al. (2015) and Bakare et al. (2009) respectively, in their studies on the genotoxicity evaluation of pharmaceutical effluents. The mitotic index (MI) and replication index of root meristem reflects the frequency of cell division and are therefore used as indicators of adequate cell proliferation (Kielkowska, 2017). It is thus a confident endpoint in identifying the presence of cytotoxic pollutants in the environment. The results are in agreement with previous findings and show that pharmaceutical effluents can be highly mito-depressive, the intensity being significantly so ($p<0.05$) with increasing concentrations. One of the causes of mitotic inhibition could be the arrest of cells in the metaphase stage. It could also be as a result of prophase arrest or pre-prophase inhibition or an interference of the cell cycle at anaphase stage (Oloyede et al., 2009). Prophase accumulation is known to be a common deleterious effect of industrial waste water and other complex mixtures on the root tip meristem of plants. This could be consequent of a delay in the breakdown of the nuclear membrane due to 'carry over' inhibitory effects of treatments from interphase stage disturbance or break

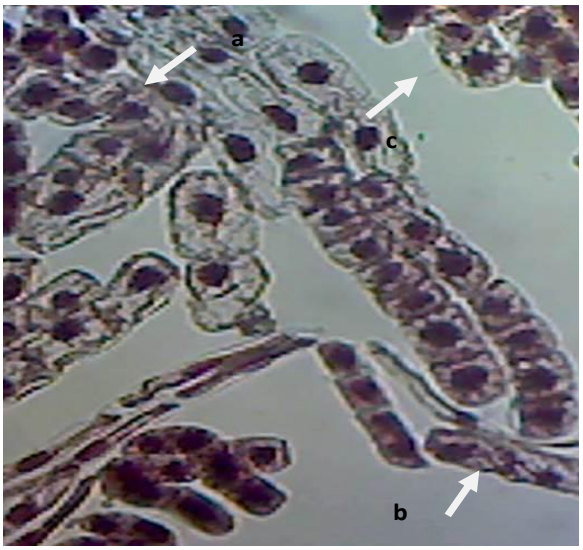

Figure 1. Chromosomal aberrations induced in *Allium cepa* by pharmaceutical effluent. a, Sticky chromosomes; b, laggards; c, scattered chromosomes.

Table 3. Cytological effects of the pharmaceutical effluent on *A. cepa* root cells.

Concentration of pharmaceutical effluent (%)	No of dividing cells	Mitotic index (MI)	Mitotic inhibition (%)	Stickiness	Laggards	Scattered/ Disoriented	Percentage frequency of aberrant cells (±SD)
Control (0)	146	29.2	0.0	0	0	0	0.00 ± 0.00
0.5	61*	12.2	58.22	4	2	2	13.11 ± 1.02
1.0	39*	7.8	73.29	4	3	2	20.08 ± 1.55
5.0	21*	4.2	85.62	2	1	1	19.05 ± 2.01
10	12*	2.4	91.78	2	1	0	25.00 ± 1.73

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

down in spindle apparatus (Abu and Duru, 2006). A mitotic index (MI) decrease below 22% of control causes lethal effects on test organism while values below 50% is sub-lethal and is called cytotoxic limit value (Sharma, 1983). The results obtained therefore indicate the high toxicity of the pharmaceutical effluent.

The chromosomal aberrations observed included stickiness, laggards, and scattered / disoriented chromosomes (Figure 1), sticky chromosomes being the most observed. The observed aberrations were not dose-dependent and this is contrary to the findings of Bakare et al. (2009). A possible explanation for this observation could be that with increasing effluent concentration and the attendant cytotoxicity, there is an inhibitory effect on cell division. This must have resulted in prophase arrest with the attendant decline in the observation of chromosome aberration (Odeigah et al., 1997). According to Ping et al. (2012), the occurrence of sticky chromosomes as a physiological aberration is a type of physical adhesion that involves mainly the proteinaceous matrix of the chromatin material. This might be interpreted as a result of depolymerization of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fiber units of chromatids and stripping of the protein covering of DNA in chromosomes (Mercykutty and Stephen, 1980). Sticky chromosomes therefore indicate the presence of a highly toxic substance, inducing irreversible effects in the physical state of the chromatin (Fiskesjö, 1985). The other chromosomal aberrations induced by the mutagenic agents in the pharmaceutical effluent might be due to the dysfunction of nuclear spindle and the observed decrease in the percentage of chromosomal aberrations in the root cells of *A. cepa* exposed to higher concentrations of the pharmaceutical effluent may be as a result of very small fraction of cells undergoing division consequent of mitotic inhibition.

In general, the results obtained from both macro-

scopical (root growth inhibition) and microscopical (chromosomal aberration) parameters show that there is a linear relationship between the two endpoints. They confirm that the test liquid is cytotoxic, mutagenic and genotoxic and also corroborate previous findings, which have established the presence of elevated values of different metallic components from various pharmaceutical effluents. In their study on the genotoxic assessment of a pharmaceutical effluent Bakare et al. (2009) observed high values of Zn, Ni and Mn (1.20, 0.020 and 0.46 respectively) while Abu and Mba, (2011) obtained high values of TDS, Cu, Zn and Pb (2000-42000, 6.0-20.0, 17.3-147.6 and 0.05-2.2 respectively). Anyakora et al. (2011) also recorded high values of Ni and Zn (0.02 and 3.0 respectively) although the concentration of different metals fluctuated with the different batches of the pharmaceutical effluents investigated and this they attributed to particular products being manufactured at each point in time. James et al. (2015) observed sticky chromosomes, bridges and vagrants in the chromosomes treated with the pharmaceutical effluents investigated for genotoxicity. In addition to induction of malformations in the test organisms' (*A. cepa*) roots, they also recorded a decrease in MI with increasing effluent concentration coupled with an abundance of sticky chromosomes with increasing effluent toxicity. It is therefore obvious that the indiscriminate discharge of the untreated pharmaceutical effluents in particular into water bodies could lead to pollution of surface water and subsequently impair biolife. The results obtained in this study also clearly demonstrated the efficiency of the *A. cepa* assay for monitoring the genotoxic effects of pharmaceutical effluents. Samuel et al. (2010) had recommended that the onion root growth should be integrated in the whole effluent test (WET) program and a specified EC_{50} should be fixed as a condition to be met before effluent is discharged into the environment so as to prevent the pollution of both surface water bodies and even ground water since they are all hydrologically connected.

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