

Toxicology and health risk assessment of pharmaceutical effluent using duckweed as a Test organism

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Abstract

The toxic effects and risk assessment of a pharmaceutical effluent collected from a pharmaceutical company in Ilorin, Nigeria have been carried out. In this study, duckweed was used as a test system. Chlorophyll a (chl a), Chlorophyll b (chl b) and Carotenoid (Cx+c) contents were determined. The Total Soluble Protein, peroxidase activity and relative growth rate based on biomass (change in weight of the plants), were investigated as indicators for environmental monitoring. The effluent caused inhibition of relative growth rate based on biomass and the loss of photosynthetic pigment content in duckweed plants following exposure to different concentrations of the effluent (0%, 5%, 10%, 20%, 50% and 100%). Peroxidase activity and total heavy metals absorbed from the effluent by the plant markedly increased as the concentration of the effluent increased, while the total soluble protein decreased as the concentration of the effluent increased. Obtained data demonstrate the relevance of duckweed as useful indicators for monitoring toxic effect of pharmaceutical effluent.

Keywords: Duckweed, biomass, Peroxidase activity (POD), Effluent.

Introduction

Industrial waste is the waste produced as a result of industrial activities which include any material that is rendered useless during a manufacturing process such as that of factories, mills and mines. Toxic wastes, chemical wastes and industrial wastes are designations of industrial wastes and

sewage treatment plants which can treat some industrial plants (Maczulak et al., 2010).

Contaminated air, soil and water by effluents from industries are associated with disease burden and these are the reasons for the current short life expectancy when compared with developed nations (WHO, 2002; 2003). These chemicals in effluents are poisonous to man and aquatic life resulting in food contamination (Novick, 1999, WHO, 2003).

A growing concern in many countries is environmental pollution from municipal and industry wastewaters. Due to the enormous number of potentially polluting substances contained in such waters, a chemical-specific approach is insufficient to provide the information about water quality. Therefore, it is essential to use biological test systems with living cells or organisms that give a global response to the pool of micro pollutants present in the sample. Owing to their settled life style, plants are constantly exposed to pollution. In addition, plants are the major energy source for nearly all higher eukaryotes and as such play an active role in transferring contaminants to higher trophic levels (Wang et al., 1995). Among the developmental parameters, the most commonly assessed in ecotoxicological test systems, are growth parameters. The measurement of biochemical responses to chemical contaminants may serve to improve the assessment of biologically significant exposures to toxic chemicals and enhance the ability to assess the risk of effects on the health and survival of toxicant exposed populations. Biomarkers such as pigment content (chlorophyll and carotenoids) and enzyme activities (like peroxidase) are commonly used as parameters for toxicity tests (Mohan et al., 1999). Duckweed is used in water quality studies to monitor heavy metals and other aquatic pollutants, because duckweed, like other water plants, may selectively accumulate certain chemicals. The plants possess physiological properties (rapid growth between pH 5 and 9, and vegetative propagation), which make them an ideal test system.

Industrial pollution has been, and continues to be, a major factor causing the degradation of the environment around us and affecting the water we use, the air we breathe and the soil we live on. Among the various industries, pharmaceutical companies are one of the largest and most important industries in the world. The effluents released from these industries produce a high degree of organic pollution in both aquatic and terrestrial ecosystems

reported in the literature. Hence, this work is desirable. This work is aimed at determining the physico-chemical parameters and the presence of Nitrate, Phosphate, Sulphate and heavy metals in the pharmaceutical effluent and at assessing the effect of pharmaceutical effluent of various concentration (0% 5%, 25%, 50% and 100%) on duckweed using effect on growth, pigment content, total soluble protein, peroxidase Activity (POD) and heavy metal absorbed.

Materials and Methods

Sample Collection

The effluent was taken from the company reservoir where it was kept to be released into the environment at SAM Pharmaceutical Company located in Ilorin in a sterile 5 litres plastic container carried in an ice-chest stored in a refrigerator before analysis. Prior to discharge, the effluent was treated mechanically (a sieve and a sedimentation tank).

Plant Material and Experimental Design

Duckweed was collected from Botanical Garden, Faculty of Biological Science, University of Ilorin. The plants were then planted in a bowl with synthetic fresh water (BG11) and given one week from the time planted to acclimatize to its new environment. Several healthy colonies of the plant with 7-8 fronds (from stock cultures) were transferred to the original (100% effluent) and to different concentration of the effluent (5, 10, 20, and 50%) with BG11 (synthetic fresh water).

Determination of Heavy Metals in the Raw Effluent

For the analysis of Fe, Cr, Zn, and Pb the effluent sample (50 ml) was taken in glass beaker, and 10 ml of concentrated HNO_3 was added to it and heated on a hot plate for slow boiling until the volume was reduced to 15 ml, 10 ml of 1M HNO_3 was added while the boiling continued until the volume was reduced to 10 ml and a clear solution was obtained, it was transferred by filtration through filter paper (Whatmann 41.), into a volumetric flask and volume of filtrate was made up to 50 ml mark by adding deionised water.

The digested sample was kept in a poly propylene bottle at room temperature till it was analysed using AAS (Hussein et. al., 2002).

Biomass Productivity and Relative Water Content

Duckweed plants were weighed immediately to determine fresh weight (FW) and oven-dried at 65°C for 48 hours at which time constant dry weights were obtained. Dry to fresh weight ratio (DW/FW) was determined according to calculation: dry weight (g)/fresh weight (g). The relative water contents (RWC) was also calculated as described by Chen et al., (2009).

$$RGP = \frac{\text{dry weight (g)}}{\text{fresh weight (g)}} \times 100 \dots\dots\dots (i)$$

$$RWC(\%) = \frac{(FW-DW)}{FW} \times 100 \dots\dots\dots (ii)$$

Biochemical Assays

Determination of Pigment Content

Chlorophyll a (chl a), b (chl b) and carotenoid contents were determined according to the method described by Lichtenthaler et al., (1983). Accurate weight of 0.5g of fresh plant leaf sample was measured and homogenised in tissue homogeniser with 10 ml of extractant solvent (80% Acetone). The homogenised sample was centrifuged for 10,000rpm for 15min at 4°C. The supernatant was separated and 0.5 ml of it was mixed with 4.5 ml of the Acetone. The photosynthetic pigment contents were calculated according to Lichtenthaler (1987). The solution was used to determine Chlorophyll-a, Chlorophyll-b and carotenoids content by measuring the absorbance at wave length of 663nm, 645nm, and 470nm respectively with spectrophotometer. The pigment was calculated from the formula:

$$\text{Ch-a} = 12.25A_{663.2} - 2.79A_{646.8} \dots\dots\dots (iii)$$

$$\text{Ch-b} = 21.5A_{646.8} - 5.1A_{663.2} \dots\dots\dots (iv)$$

$$C_{x+c} = (1000A_{470} - 1.82C_a - 85.02C_b)/198 \dots\dots\dots (v)$$

Total Soluble Protein

Total soluble protein was determined by Bradford assay (Bradford, 1976). Total Soluble Proteins was extracted by weighing 0.5g of fresh plant material (leaves). It was grounded in a pre-chilled mortar by adding 5ml

extraction buffer with pH 7.2. The buffer that was used is phosphate buffer saline (PBS) containing 10g Na_2HPO_4 , 2g KH_2PO_4 , 2.7g KCl and 1.37g NaCl dissolved in distilled water and made up to a volume of 1000 ml. The pH 7.2 of PBS was adjusted with HCl and then autoclaved (Sambrook and Russell, 2001). The grounded leaf material was centrifuged at 12000rpm for 5 min. Supernatant was preserved in a separate centrifuge tube for the determination of soluble proteins while the pellet was discarded.

$$\text{Total Soluble Protein} = \frac{\text{Slope} \times \text{absorbance of supernatant}}{\text{ml of extract used}} \dots\dots\dots(\text{vi})$$

Peroxidase Activity (POD)

Peroxidase activity (POD) was determined after homogenising the plant tissue in potassium phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) buffer (pH 7.0) including 0.1M ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 25000 rpm for 30 minutes at 4°C while supernatant was used for enzyme activity. The reaction mixture was prepared by adding 0.1ml 2-Methoxyphenol, 0.1ml H_2O_2 and 2ml phosphate in 0.1ml enzyme extract. The change in absorbance at 470 nm of the reaction mixture was observed every 30 seconds up to 4 minutes (Chance et al., 1955). The following calculation was made to ascertain peroxidase activity in a sample:

$$\text{Change in } A_{510} = A_f - A_i \dots\dots\dots(\text{vii})$$

Where; A_i = initial absorbance reading

A_f = final absorbance reading

Determination of Heavy Metals in the Plant after 8 Days

0.25 g of the powdered plant sample was digested in 6.5ml of acid solution (HNO_3 , HCl in ratio of 5:2). The resulting solution was heated until a white fume appeared. The clear solution was diluted up to 50ml with distilled water and filtered with Wattman filter paper no.1. The digested sample was kept in a poly propylene bottle at room temperature for the determination of heavy metals using AAS (Hameed et al., 2008).

Results and Discussion

Table 1: Concentration of heavy metals, BOD and COD in the pharmaceutical effluent

	PARAMETERS						
	pH	Fe (mg/l)	Cr (mg/l)	Zn (mg/l)	Pb (mg/l)	BOD (mg/l)	COD (mg/l)
Total amount	5.1	37.860	0.206	3.649	0.040	22.4±0.058	229.6±0.1

Heavy metal analysis obtained by AAS (Table 1 and 2) shows relatively low concentrations of measured metals in the pharmaceutical effluent. However, duckweed ability to accumulate heavy metals has been reported to make it a potential candidate for phytoremediation (Axtell et al., 2003). It has been shown that exposure of plants to toxic metal concentrations generally causes the fast inhibition of cell elongation and expansion (Poschenrieder et al., 2004; Srivastava et al. 2006). Horvat et al. (2007) exposed duckweed to electroplating wastewater rich in Fe and especially Zn and reported that the plant accumulated the metals in great quantities followed by Pb and Cr. However, toxicity of metals in duckweed tissues was observed to decrease in the order of Zn>Fe>Cr>Pb (Axtell et al. 2003). Although the interactions in our multi-metallic samples considerably limit possibility of explaining: the results for single metals, the growth reduction observed in our study could be also due to the suppression of the elongation growth rate of cells exerted by Fe, Zn and Cr.

Table 2: Concentration of heavy metals absorbed by the plant from the pharmaceutical effluent.

Parameters	Metals Concentration absorbed				
	Control	5 %	25 %	50 %	100 %
Fe (mg/l)	2.980	3.030	3.690	6.120	8.490
Cr (mg/l)	0.072	0.094	0.103	0.128	0.146
Zn (mg/l)	0.930	0.996	1.035	1.153	3.920
Pb (mg/l)	0.00	0.00	0.00	0.00	0.00

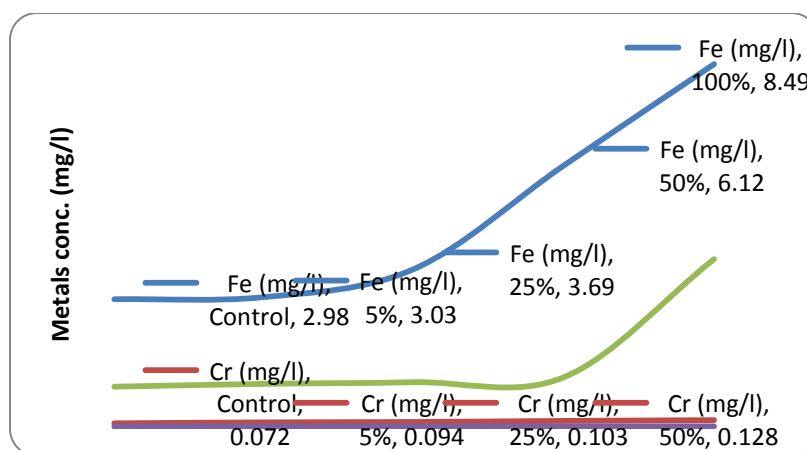


Fig.1: Trends in concentration of heavy metals absorbed at different % dilution of pharmaceutical effluent (mg/l).

Accumulation of the heavy metals content in the plant was dependent on the concentration present in the pharmaceutical effluent. The Fe accumulation level was found to be higher in the plant in all the concentrations of the effluent because it has the highest value present in the effluent (Table 2).

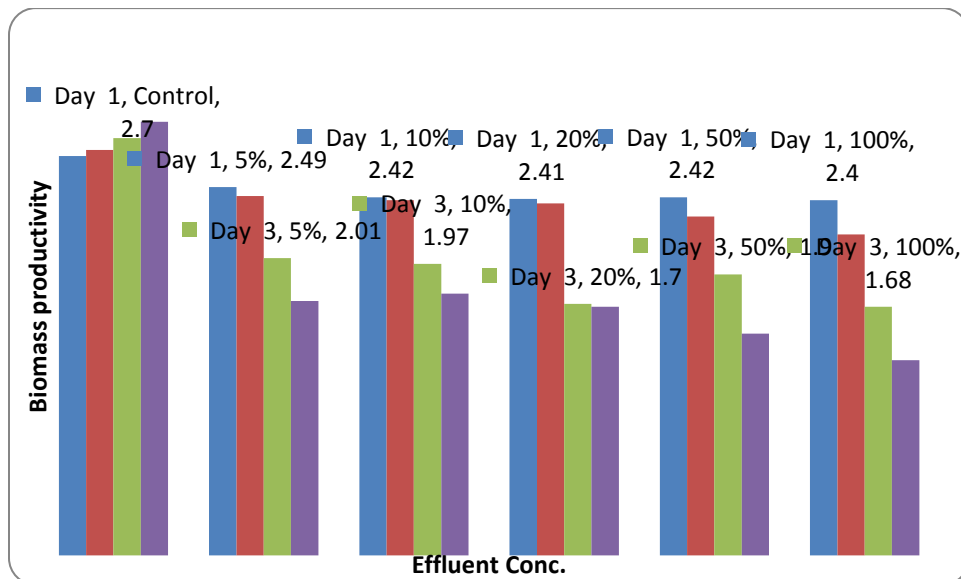


Fig.2: Change in weight of the plant in duckweed exposed for 1-4 days to pharmaceutical effluent.

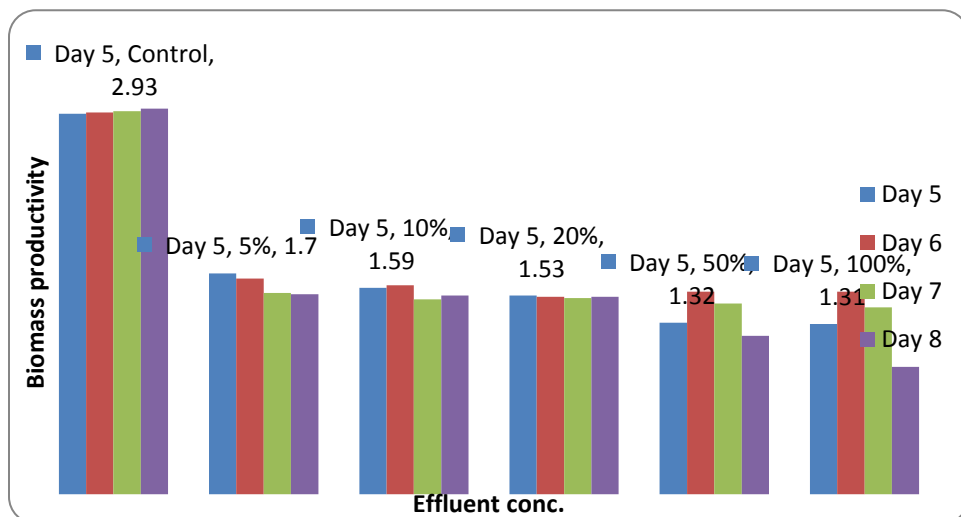


Fig.3: Change in weight of the plant in duckweed exposed for 5-8 days to pharmaceutical effluent.

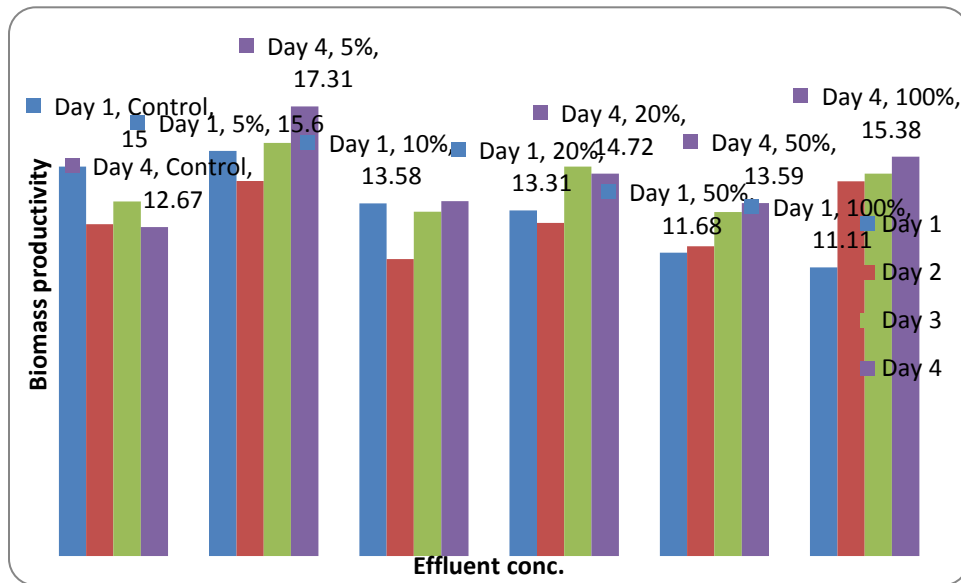


Fig. 4: Relative growth rate based on Biomass in duckweed exposed for 1- 4 days to pharmaceutical effluent.

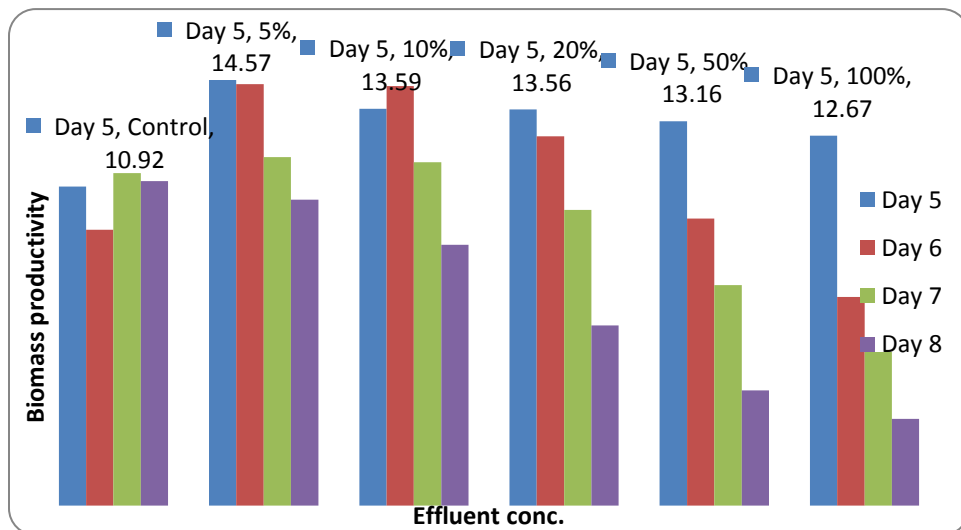


Fig. 5: Relative growth rate based on Biomass in duckweed exposed for 5 - 8 days to pharmaceutical effluent.

Fig 2 and 3 show the relative growth rate based on biomass (Fresh weight) in duckweed exposed for 8 days to pharmaceutical effluent samples collected from SAM Company. It is probably due to the fact that frond count is irrelevant to frond size or biomass. It has frequently been observed that under toxic stress, small buds may protrude and be counted as individual fronds (Mohan et al., 1999; Naumann et al. 2007). However, in the present study final biomass proved to be sensitive parameters. Mackenzie et al. (2003) also found that, besides frond area, growth rate based on biomass is the most sensitive endpoint for detecting chronic toxicity (8 days) in the effluent.

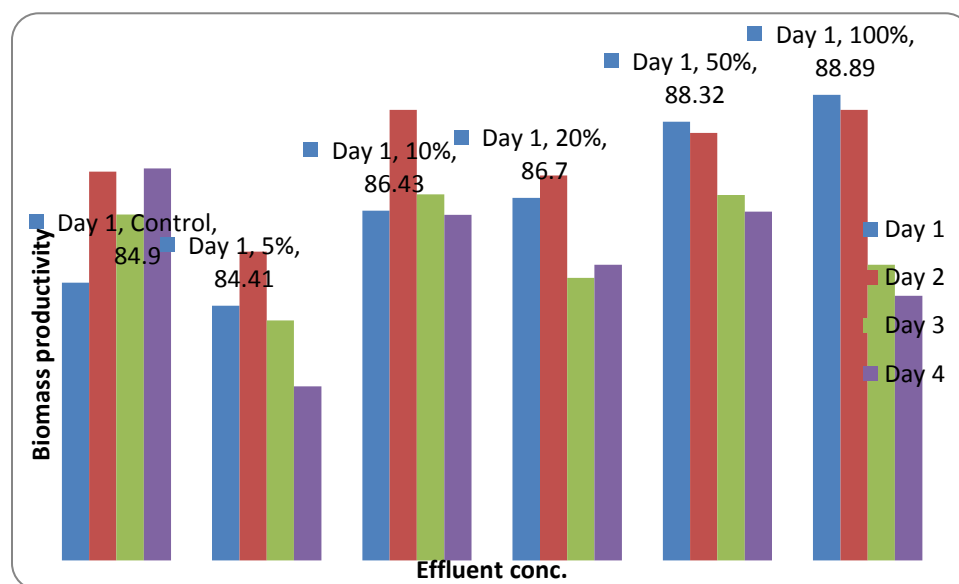


Fig. 6: Relative water contents rate based on Biomass in duckweed exposed for 1 - 4 days to pharmaceutical effluent.

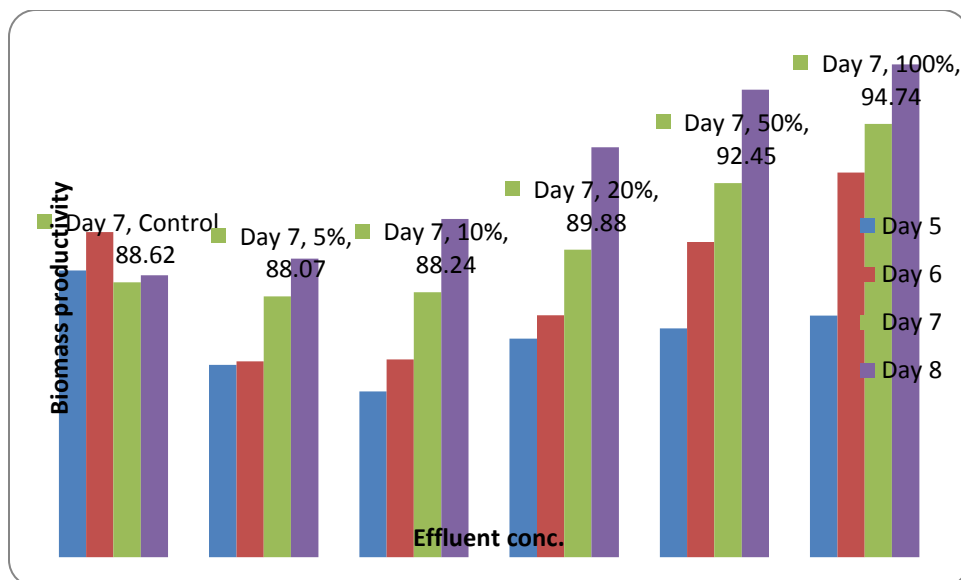


Fig. 7: Relative water contents rate based on Biomass in duckweed exposed for 5 - 8 days to pharmaceutical effluent.

Dry to fresh weight ratio (Dry weight/Fresh weight) measured at the end of the 8 day experiment has shown inverse relation when compared with Relative Growth Rate (Fig. 2 and 3); the parameter significantly increased in all the setup but most in the 5% concentration of the effluent (36.62% increase in comparison to control at day 4). Significant increase of DW/FW as well as inhibition of Relative Growth Rate observed in the experiments suggests bioaccumulation of pollutants, possibly heavy metals, present in the effluent. However, it has been shown that accumulation of heavy metals disturbs the plant water status which eventually results in growth reduction (Perfus-Barbeoch et al. 2002; Poschenrieder et al., 2004). It has been reported that to keep the water balance between cells and external medium, plants are shown to accumulate a wide variety of organic solutes like carbohydrates and quaternary ammonium compounds (Matysik et al. 2002)

Relative water content (RWC) change has been suggested as an indicator of phytotoxicity after heavy metal stress (Zn, Fe, Cr and Pb) in the pharmaceutical effluent (Su et al. 2005). Relative water content in leaves

was slightly higher in the last day of the experiment. It is most likely that lead, Fe, Zn and Cr treatment induced stomata closure triggered over the course of the experiment due to the atmospheric carbon fixing activities that were compromised as a consequence (Brunet et al. 2008). The present study strongly suggests that Pb, Fe, Zn and Cr toxicity in situ leads to triggers of some of the key enzymes of the antioxidant defense system in duckweed plants.

Table 3: Quantification of Chlorophyll a (Chl a) ($\mu\text{g/ml}$) at 663nm in various effluent concentrations with respect to different days.

Conc. (%)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control	3.366 $\pm$ 0.001	3.997 $\pm$ 0.001	5.054 $\pm$ 0.001	6.367 $\pm$ 0.001	6.934 $\pm$ 0.001	7.816 $\pm$ 0.001	8.219 $\pm$ 0.001	9.557 $\pm$ 0.001
5	2.276 $\pm$ 0.001	2.099 $\pm$ 0.001	1.767 $\pm$ 0.001	1.322 $\pm$ 0.001	1.311 $\pm$ 0.001	1.291 $\pm$ 0.001	1.272 $\pm$ 0.001	1.216 $\pm$ 0.001
10	1.985 $\pm$ 0.001	1.610 $\pm$ 0	1.212 $\pm$ 0.001	0.989 $\pm$ 0.001	0.942 $\pm$ 0.001	0.898 $\pm$ 0.001	0.846 $\pm$ 0	0.801 $\pm$ 0.001
20	1.121 $\pm$ 0.001	0.988 $\pm$ 0.001	0.926 $\pm$ 0.001	0.718 $\pm$ 0.001	0.687 $\pm$ 0.001	0.668 $\pm$ 0.001	0.623 $\pm$ 0.001	0.568 $\pm$ 0.001
50	0.909 $\pm$ 0.001	0.772 $\pm$ 0.001	0.778 $\pm$ 0.001	0.690 $\pm$ 0.001	0.647 $\pm$ 0.001	0.646 $\pm$ 0.001	0.517 $\pm$ 0.001	0.507 $\pm$ 0.001
100	0.809 $\pm$ 0.001	0.768 $\pm$ 0.001	0.764 $\pm$ 0.001	0.596 $\pm$ 0.001	0.558 $\pm$ 0.001	0.489 $\pm$ 0.001	0.403 $\pm$ 0.001	0.324 $\pm$ 0.001

Table 4: Quantification of Chlorophyll b (Chl b)($\mu\text{g/ml}$) at 645nm in various effluent concentrations with respect to different days.

Conc. (%)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control	4.069 $\pm$ 0.001	5.271 $\pm$ 0.001	7.577 $\pm$ 0.001	8.536 $\pm$ 0.104	9.703 $\pm$ 0.001	11.159 $\pm$ 0.001	11.837 $\pm$ 0.001	12.434 $\pm$ 0.001
5	3.857 $\pm$ 0.001	3.296 $\pm$ 0	3.270 $\pm$ 0.001	3.169 $\pm$ 0.001	2.521 $\pm$ 0.001	2.106 $\pm$ 0.001	1.687 $\pm$ 0.001	1.345 $\pm$ 0.001
10	1.939 $\pm$ 0.001	1.343 $\pm$ 0.001	1.285 $\pm$ 0.001	1.215 $\pm$ 0.001	1.133 $\pm$ 0.001	1.110 $\pm$ 0.001	1.072 $\pm$ 0.001	0.968 $\pm$ 0.001
20	1.364 $\pm$ 0.001	1.318 $\pm$ 0.001	1.262 $\pm$ 0.001	1.246 $\pm$ 0.001	1.138 $\pm$ 0.001	1.105 $\pm$ 0.001	1.001 $\pm$ 0.001	0.984 $\pm$ 0.001
50	1.232 $\pm$ 0.001	1.151 $\pm$ 0.001	1.059 $\pm$ 0.001	0.933 $\pm$ 0.001	0.829 $\pm$ 0.001	0.727 $\pm$ 0.001	0.643 $\pm$ 0.001	0.438 $\pm$ 0.001
100	1.207 $\pm$ 0.001	1.102 $\pm$ 0.001	0.902 $\pm$ 0.001	0.855 $\pm$ 0.001	0.784 $\pm$ 0.001	0.691 $\pm$ 0.001	0.646 $\pm$ 0.001	0.169 $\pm$ 0.001

Table 5: Quantification of Carotenoid (Cx+c)($\mu\text{g}/\text{ml}$) at 470nm in various effluent concentrations with respect to different days.

Conc. (%)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control	3.050 $\pm$ 0 .001	4.891 $\pm$ 0 .001	6.543 $\pm$ 0.001	10.313 $\pm$ 0.001	10.836 $\pm$ 0 .001	11.119 $\pm$ 0.001	11.604 $\pm$ 0	16.739 $\pm$ 0 .001
5	2.668 $\pm$ 0 .001	2.601 $\pm$ 0 .001	2.115 $\pm$ 0.001	1.725 $\pm$ 0 .001	1.657 $\pm$ 0. 001	1.588 $\pm$ 0.001	1.594 $\pm$ 0 .001	1.517 $\pm$ 0. 001
10	1.939 $\pm$ 0	1.343 $\pm$ 0 .001	1.285 $\pm$ 0	1.215 $\pm$ 0 .001	1.200 $\pm$ 0	1.110 $\pm$ 0.001	1.072 $\pm$ 0 .001	0.968 $\pm$ 0. 001
20	1.485 $\pm$ 0 .001	1.490 $\pm$ 0	1.217 $\pm$ 0	1.148 $\pm$ 0	1.146 $\pm$ 0. 001	1.140 $\pm$ 0.001	1.135 $\pm$ 0 .001	1.087 $\pm$ 0. 001
50	1.483 $\pm$ 0 .001	1.480 $\pm$ 0 .001	1.205 $\pm$ 0.001	1.200 $\pm$ 0	1.120 $\pm$ 0. 001	0.636 $\pm$ 0	0.782 $\pm$ 0	0.559 $\pm$ 0
100	1.418 $\pm$ 0 .001	1.424 $\pm$ 0 .001	1.128 $\pm$ 0	1.107 $\pm$ 0	1.022 $\pm$ 0	0.487 $\pm$ 0	0.531 $\pm$ 0 .001	0.325 $\pm$ 0. 001

The decline in total chlorophyll and carotenoids contents as well as growth inhibition can be regarded as general responses associated with metal toxicity. At the end of the 8 days exposure, duckweed grown on 50% diluted and especially on 100% pharmaceutical effluent showed signs of localized death of cells and tissues (dark brown). Accordingly, a marked decrease in chlorophyll a (Fig.8) and b (Fig.9) contents compared to the control were detected in plants exposed to synthetic fresh water. The carotenoids content was much less affected in comparison with those of chlorophylls (Fig. 10). The carotenoids are involved in the protection of chlorophyll but they also serve as antioxidants to quench or scavenge the free radicals and reduce the damage to cell membrane and DNA. The loss of photosynthetic pigment content has been reported in duckweed plants following exposure to Cu, Pb and Ni (Axtell et al. 2003; Hou et al. 2007; Kanoun-Boule' et al. 2009). Overall, the destruction of photosynthetic pigments by heavy metals could be due to impairment of the electron transport chain and inhibition of important enzymes (Van et al.,1990) associated with chlorophyll

biosynthesis or peroxidation processes in chloroplast membrane lipids by the reactive oxygen species (Sandalio et al. 2001).

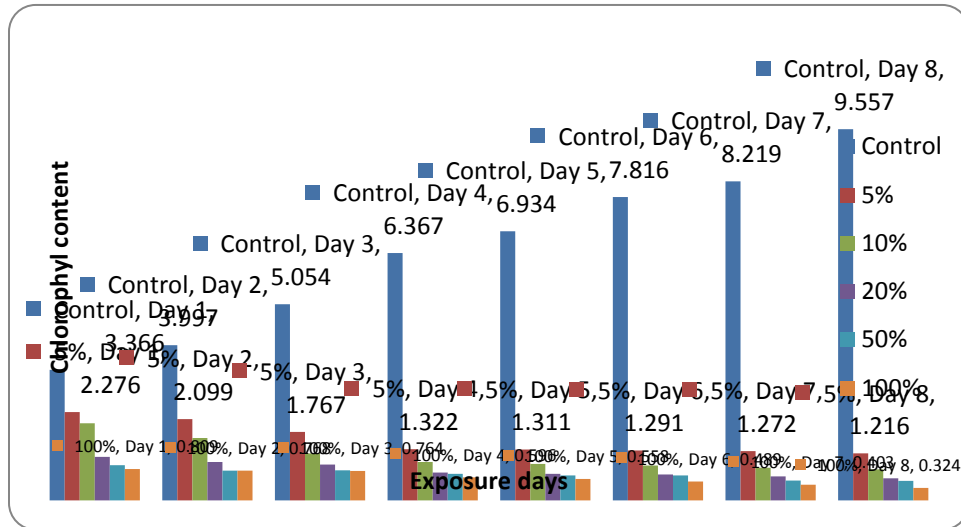


Fig.8: Relative chlorophyll a (chl a) contents in duckweed exposed for 8 days to pharmaceutical effluent.

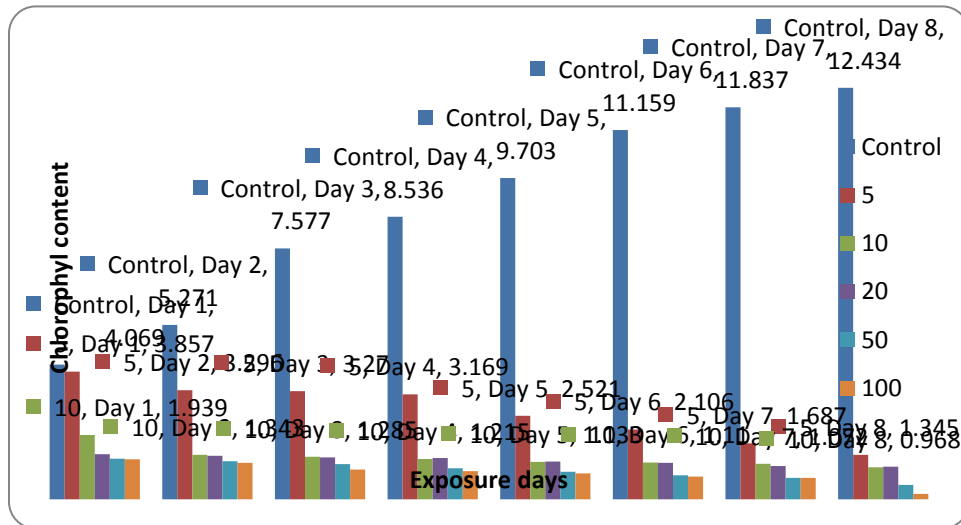


Fig.9: Relative chlorophyll b (chl b) contents in duckweed exposed for 8 days to pharmaceutical effluent.

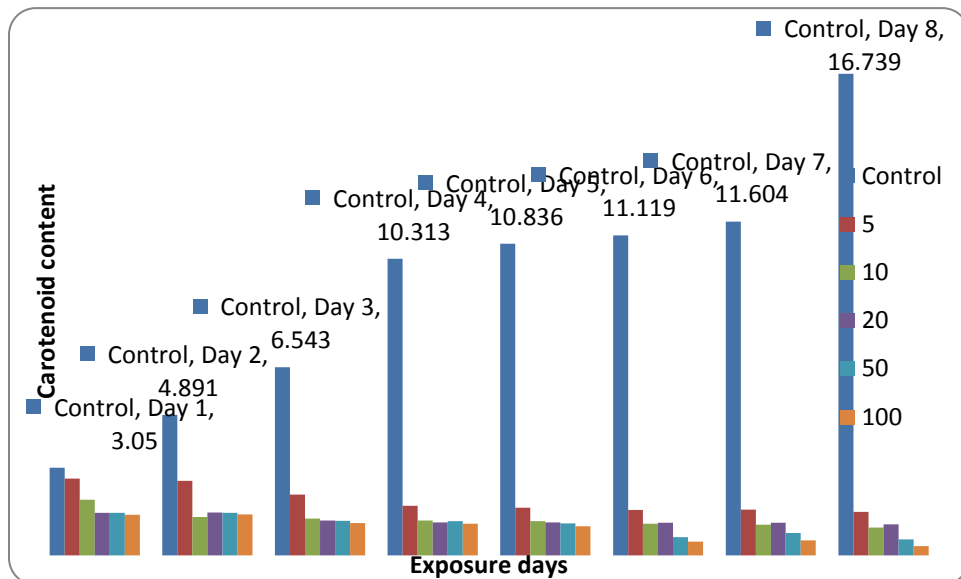


Fig.10: Relative carotenoid (Cx+c) contents in duckweed exposed for 8 days to pharmaceutical effluent.

Chlorophyll and carotenoids are the central part of the energy manifestation of every green plant system. Any significant alteration in their levels is likely to cause a marked effect on the entire metabolism of the plant. The growth reduction recorded in the study may be due in part to the decrease of chlorophyll content produced by heavy metals present in the pharmaceutical effluent. In fact, in duckweed and some other water plant species the degradation of chlorophyll or the inhibition of its biosynthesis, has been proposed as being responsible for photosynthesis and growth reduction caused by Zn, Cr, Fe and Pb (Ku"pper et al. 1996). Decreased plant growth might be associated with the inhibition of mitotic index with Pb, Fe, Zn and Cr present in the effluent (Vecchia et al. 2005).

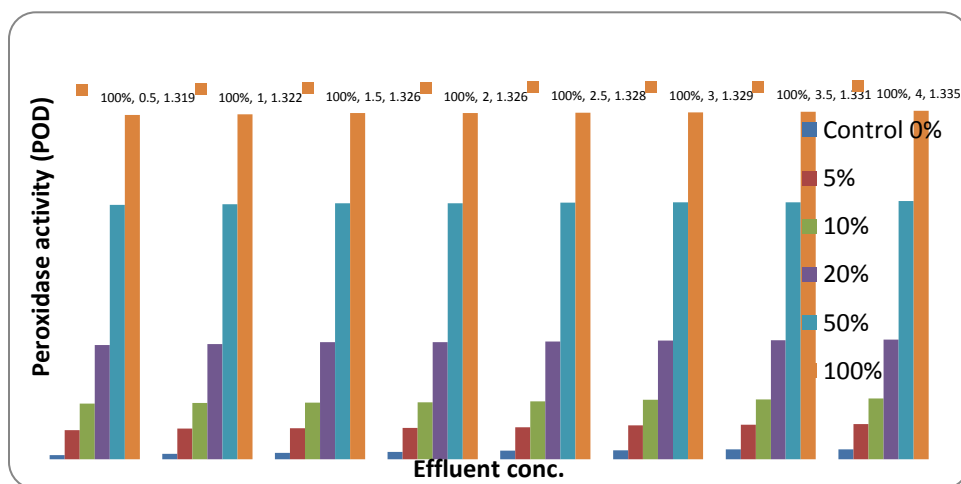


Fig 11: Showing the absorbance of POD contents exposed for 8 days to different concentration of a pharmaceuticals effluent

When the different concentrations of pharmaceutical effluent was tested, almost linear POD increase was observed (Fig. 11). Various pollutants, including heavy metals, are known to induce a change in extractable POD activity (Van et al., 1990; Siesko et al., 1997). There are many studies suggesting an inverse correlation between the peroxidase level and the growth rate (Siegel et al., 1967; Bacon et al. 1997; Lin et al., 2001). Peroxidase plays a key role in the stiffening of the cell wall and in processes associated with plant growth through the formation of phenolic cross-link (Fry 1986).

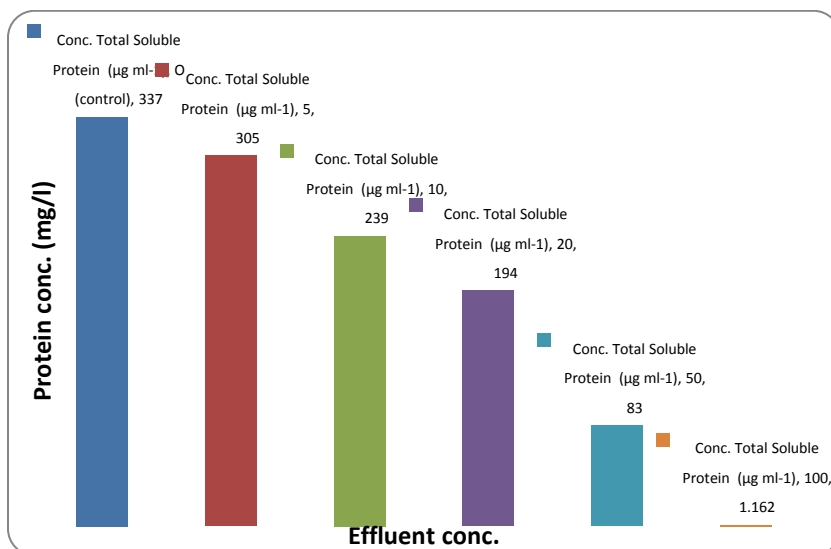


Fig 12: Shows the concentration of total soluble protein contents in duckweed exposed for 8 days to pharmaceutical effluent.

Protein degradation relative rates were measured at the 8 days of the experiment using duckweed plant. However, the information supplied by Figure 12 suggests the onset of senescence (to grow old) in the duckweed by the end of the experiments, as evidenced by the decrease in total chlorophyll and total protein. The result presented in Figure 12 show the relative rate of degradation of the total soluble protein measured in the duckweed.

Conclusions

The results show the suitability of duckweed for pharmaceutical effluent quality assessment as all selected parameters showed consistency with respect to the effluent used in the experiment. The possible reason for such consistency might due to the properties of the plant. Moreover, water and substances to be tested are taken up directly through the leafy fronds (Naumann et al., 2007). The results obtained suggest that phyto- and genotoxicity tests with duckweed should be used in the biomonitoring of municipal, agricultural and industrial effluents because of their simplicity, sensitivity and cost-effectiveness.

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