Phytoremediation of Pb+2, Cd+2 and Cu+2 by an Aquatic Macrophyte *Azolla pinnata* from Industrial Wastewater in Egypt

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ABSTRACT

In this study, *Azolla pinnata* as a free floating aquatic fern was obtained from Agric. Microbial Dept., Soils, Water and Environment Research Institute (SWERI), Agric. Res. Center (ARC), Giza, Egypt to study its accumulation capacity of different concentrations of heavy metals (Pb+2, Cd+2 and Cu+2) from industrial wastewater. As presented in this study, the removal efficiency shown by healthy pre weighed *A. pinnata* was comparatively higher for Pb, Cu and lower for Cd. Overall, *A. pinnata* is efficient in the purifying polluted water from the heavy metals, representing an effective, eco-friendly and low-cost treatment technology. From the results, it can be concluded that *A. pinnata* will serve as the purpose of wastewater treatment in industrial areas which are easily manageable.

Introduction

The pollution in aquatic ecosystems by heavy metals has been attracting considerable public attention over the past few decades (Sood *et al*., 2012). The aquatic ecosystem can be broken down into two basic regions, freshwater (i.e, ponds and rivers) and marine (i.e, oceans and estuaries) (Ramachandra *et al*., 2005). Heavy metals in aquatic ecosystems are considered as serious pollutants due to their environmental persistence, toxicity and ability to be incorporated into food chains (Demirbas *et al*., 2005). Heavy metals are chemical elements with a specific gravity that is at least five times more than the specific gravity of water (Charan *et al*., 2014). The heavy metals enter in tissues through the food chain and accumulate in the body of all living organisms (Doke *et al*., 2012). The metals hazardous to humans include lead, cadmium, mercury, arsenic, copper, zinc and chromium (Abbas *et al*., 2013). Some metals, such as Cu, Co, Fe, Mo, Mn, Ni and Zn are essential mineral nutrients. Others, however, such as Cd and Pb have no known physiological activity (Lasat, 2002). Heavy metals are commonly found in aqueous wastes of many industries, such as paints, pigments, batteries, ceramic glazes, metal products and ammunition production (Mohammad *et al*., 2010).

A high concentration of lead can be found in industrial wastewater, in domestic detergents and other laundry products and in cigarettes (Celebi and Kendir, 2002). The major source of environmental lead is metal smelting (Caussy *et al*., 2003), but agriculture, industry and urban activities are also important sources of Pb pollution (Marchiol *et al*., 2004). Lead is toxic to many vital organs and tissues in the human body including heart, kidneys, reproductive and nervous systems (Waranusantigul *et al*., 2011). Chronic intoxication can lead to encephalopathy mainly in children (Jordao *et al*., 2002).

Cadmium may be released to water by natural weathering processes, by discharge from industrial facilities or sewage treatment plants, atmospheric deposition, by leaching from landfills or soil, or phosphate fertilizers (Morrow, 2001). Cadmium accumulates in the human body affecting negatively several organs: liver, kidney, lung, bones, placenta, brain and the central nervous system (Castro and Méndez, 2008). Cadmium is an element that represents serious environmental hazards because it can be absorbed via the alimentary tract, penetrates through placenta during pregnancy and damages membranes & DNA (Kabata, 2004).

Copper is an essential micronutrient for numerous physiological processes at low concentrations but a toxic metal at high concentrations (Gaetke and Chow, 2003). Excess of Cu may reach living organisms as a result of environmental pollution caused by anthropogenic activities (mining operations, manufacturing industries and agricultural technologies) which can modify the biogeochemical cycles of the metal (Khellaf and Zerdaoui,

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2010). The high toxicity of copper to algae creates a ripple effect throughout the ecosystem and demonstrates that changing one part of an ecosystem will affect the entire ecosystem (Wright and Welbourn, 2002). The term ''phytoremediation'' is a combination of two words: Greek phyto (meaning plant) and Latin remedium (meaning to correct or remove an evil) (Hazrat *et al.*, 2013). While there are numerous descriptions of the term, phytoremediation can be summed up with one clear definition: the use of plants for the removal of pollutants from the environment (Gardea, 2003). Remediation of heavy metals using plants is better in terms of ecology and economy than chemical treatment (Rai, 2011). The ideal type of phytoremediator is a species that creates a large biomass, grows quickly, has an extensive root system and must be easily cultivated and harvested (Clemens *et al.*, 2002). Among these, *Azolla*, a free-floating, fast growing and nitrogen fixing pteridophyte seems to be an excellent candidate for removal and disposal of heavy metals from the polluted aquatic ecosystems (Arora *et al.*, 2006).

The genus *Azolla* was botanically established by Lamarck in 1783 (Kannaiyan and Kumar, 2006). The word *Azolla* has a Greek origin. It results from the agglutination of two words, *azo* and *ollyo*, which means *killed by drought* (Ashton and Walmsley, 1976). *Azolla* has common names of Mosquito fern, Water velvet, Water fern or simply *Azolla*. It has been reported that *Azolla* has a high capacity to accumulate toxic elements such as mercury, cadmium, chromium, copper, nickel and zinc (Rai, 2008) and can be used to remove contaminants from wastewater (Rakhshaee *et al.*, 2006). This fern can also remove nutrients (Forni *et al.*, 2001) and organic substances like sulphonamides (Forni *et al.*, 2002).

A specific feature of this fern is its symbiosis with the cyanobacterium *Anabaena azollae* Strasb. (*Nostocaceae*) which can fixes atmospheric nitrogen. This feature made *Azolla* a very useful plant in food production (e.g. on paddy fields) as a green manure (Artur *et al.*, 2012). *Azolla* is a better macrophyte for aquatic phytoremediation because of its short doubling time (2-3 d), easy harvest, nitrogen fixation ability and tolerance to and accumulation of a wide range of heavy metals (Sood *et al.*, 2012).

Therefore, the aim of this investigation is to: 1) evaluate the effect of industrial wastewater by different concentrations of Pb^{+2}, Cd^{+2} and Cu^{+2} on *A. pinnata* growth, 2) verify the potential of *A. pinnata* as a bioaccumulator of these heavy metals and also recycling of industrial wastewater under greenhouse conditions.

Materials and Methods

Propagation of A. pinnata :

A. pinnata used in this study was kindly supplied by Agric. Microbial Dept., Soils, Water and Environ. Res. Inst. (SWERI), Agric. Res. Center (ARC), Giza, Egypt. *A. pinnata* (10 g) were grown in plastic pots (32 cm in diameter and 15 cm depth) containing 1kg soil saturated with 3 liters of tap water. These pots were kept in a greenhouse till *A. pinnata* covered the entire water surface according to El- Shahat (1988). *A. pinnata* was collected and washed gently in running deionized water for several times by using 0.2 meshes screen and then the plants were air dried for 30 min. All experiments were carried out in the greenhouse of Soils, Water and Environ. Res. Inst. (SWERI).

Efficiency of A. pinnata as a bioaccumulator of Pb^{+2}, Cd^{+2} and Cu^{+2} from wastewater:

Collection and analysis of wastewater samples:

Wastewater samples were collected from green area around 6th of October city and brought to the greenhouse in plastic containers for the experimental requirements. The samples were analyzed to determine Pb^{+2}, Cd^{+2} and Cu^{+2} concentrations using Inductively Coupled Plasma Spectrometry (ICP) (Model Ultima 2 JY Plasma- Jobin Yvon) according to Chapman and Pratt (1961). pH values (measured by pH meter), electrical conductivity (EC) (measured by electrical conductivity bridge according to the method of Richards (1954), total nitrogen (determined colorimetrically by using Nessler solution, described by Sauter and Stoub (1990). Soluble phosphorus (determined colorimetrically by using ascorbic acid method described by Watanabe and Olsen (1965), soluble potassium (determined by using inductively coupled plasma optical emission spectroscopy). The Physico-chemical analysis of wastewater samples in the three studied drains is represented in Table (1).

Table 1: Physical and chemical analysis of industrial wastewater samples.

Industrial source	PH	EC ds/m²	mg/L					
			N	P	K	Pb	Cd	Cu
Drain (1)	7.16	2.970	5.380	1.290	14.210	100.207	-	-
Drain (2)	7.26	0.580	1.180	0.220	5.370	-	1.001	-
Drain (3)	7.74	1.670	2.000	0.510	10.500	-	-	98.076

Experimental design and the treatments:

The aquatic plant selected for phytoremediation was *A. pinnata* and wastewater, diluted with deionized water as follows; R1: 25 % wastewater + 75 % deionized water (1:3), R2: 50 % wastewater + 50 % deionized water (1:1), R3: 75 % wastewater + 25 % deionized water (3:1), R4: 100 % wastewater (1:0) and R0: control (aquatic plant with deionized water only) which was necessary to compare the results to study the effects of increasing the concentration of heavy metals on fresh, dry weight (El-Shahat, 1997) and doubling time of *A. pinnata* growth. The accumulation of Pb^{+2}, Cd^{+2} and Cu^{+2} was determined on dry weight basis by using ICP according to Chapman and Pratt (1961).

The wastewater after dilution with deionized water in the proportions as mentioned above was poured into plastic pots (10.0 cm diameter and 7.0 cm in depth). The selected plant, 1 g fresh weight of *A. pinnata* was inoculated in above pots as per the experimental layout and treatments formulated (El- Berashi, 2008). *Azolla* fronds were vegetatively multiplied in 25 sets of plastic pots for each treatment. Three replicates were used for each dilution (one set). The inoculated pots were incubated at a temperature of 33°C ± 2, 14 hr light and 10 hr dark for 25 days under greenhouse conditions during June 2015. Samples of each treatment were taken after zero time, 5, 10, 15, 20 and 25 days of incubation. Pots were kept at a constant volume throughout the experimental periods by frequent irrigation with deionized water or wastewater (ratios for dilution) to compensate water loss by evaporation when it is necessary (El- Berashi, 2008).

The measured parameters:

Fresh and Dry Weight:

Samples of *A. pinnata* fronds were harvested, washed by deionized water and placed under shade between two thick layers of blotting tissue papers for approximately 1 hr. before determining fresh weight. The dry weight of *A. pinnata* was determined by drying fronds in an oven at 70°C to constant weight. Fresh and dry weights of *A. pinnata* were expressed as g/m^2 (El- Berashi, 2008).

Doubling time calculation:

Growth rate of *A. pinnata* in terms of doubling time (D.T.) was calculated by using the following equation according to Aziz and Watanabe (1983):

Doubling time = t/r, whereas:
t   = the duration of *Azolla* growth
r   = [log (wt/wo) / 0.301]
wt = weight of *Azolla* at time t,
wo = weight of *Azolla* at zero time i.e. weight of inoculum.

Determination of heavy metals (Pb^{+2}, Cd^{+2} and Cu^{+2}) bioaccumulation by A. pinnata:

Before digestion to analyze heavy metals, harvested plants were washed with deionized water, air dried, dried at 70°C until constant weight and weighted for the dry weight. The digestion method was applied involving sulfuric acid and perchloric acid as wet digestion procedure according to Chapman and Pratt (1961). 0.1 g dry weight of *A. pinnata* was used for digestion for each sample. Concentrations of Pb^{+2}, Cd^{+2} and Cu^{+2} were determined by using ICP (Chapman and Pratt, 1961). Read of the instrument (mg L^{-1}) multiplied by an inverted extraction ratio (total volume for sample (cm) / sample weight (g)) = mg kg^{-1}.

Statistical analysis:

The data were presented by mean ± standard deviation (n=3). Statistical analysis was carried out as a randomized complete design (Snedecor and Cochran, 1980) using LSD test to compare means of treatments in investigation. Statistical significance was defined as $p < 0.05$.

Results:

Effect of industrial wastewater contaminated with Lead (Pb^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. Pinnata*:

Fresh and dry weights gradually increased with increasing the incubation period from zero time up to 25 days. Fresh and dry weights also increased at treatment (R1) and then gradually decreased from (R2) to (R4) up to 20 days of incubation. After 25 days of incubation, fresh and dry weights gradually decreased from (R1) to (R4) as illustrated in Table (2) and Fig. (1). Maximum fresh and dry weights were observed for *A. pinnata* at (R1) (813.73 ± 23.45 and 61.03 ± 1.76 g/m^2), respectively. These parameters were compared with the control

Table 2: Effect of industrial wastewater contaminated with Lead (Pb^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. pinnata* (Data expressed as mean ± SD).

Period (days) / Treatments	F.wt. (g/m^2)						D.wt. (g/m^2)						D.t. (days)					
	Zero-time	5	10	15	20	25	Zero-time	5	10	15	20	25	Zero-time	5	10	15	20	25
R0	128.21	324.80 ± 7.89	388.00 ± 9.37	524.80 ± 15.47	716.27 ± 21.06	1126.53 ± 27.18	9.62	24.36 ± 0.59	29.10 ± 0.70	39.36 ± 1.16	53.72 ± 1.58	84.49 ± 2.04	0.00	3.73 ± 0.10	6.25 ± 0.14	7.39 ± 0.17	8.06 ± 0.13	7.96 ± 0.09
R1	128.21	358.93 ± 8.36	452.93 ± 12.16	658.13 ± 19.34	813.73 ± 23.45	918.00 ± 25.15	9.62	26.92 ± 0.63	33.97 ± 0.91	49.36 ± 1.45	61.03 ± 1.76	68.85 ± 1.89	0.00	3.36 ± 0.08	5.49 ± 0.12	6.36 ± 0.11	7.49 ± 0.11	8.80 ± 0.13
R2	128.21	341.87 ± 8.69	417.07 ± 11.72	605.07 ± 17.27	685.47 ± 19.85	762.40 ± 23.73	9.62	25.64 ± 0.65	31.28 ± 0.88	45.38 ± 1.29	51.41 ± 1.49	57.18 ± 1.78	0.00	3.52 ± 0.09	5.88 ± 0.14	6.70 ± 0.12	8.26 ± 0.14	9.73 ± 0.16
R3	128.21	337.59 ± 7.60	408.00 ± 10.40	561.88 ± 15.53	654.67 ± 18.74	731.94 ± 23.92	9.62	25.32 ± 0.57	30.60 ± 0.78	42.14 ± 1.16	49.10 ± 1.40	54.90 ± 1.80	0.00	3.57 ± 0.08	5.99 ± 0.13	7.04 ± 0.13	8.51 ± 0.15	9.96 ± 0.18
R4	128.21	333.33 ± 7.76	398.27 ± 9.50	512.80 ± 16.07	635.87 ± 18.10	711.07 ± 22.36	9.62	25.00 ± 0.58	29.87 ± 0.71	38.46 ± 1.21	47.69 ± 1.36	53.33 ± 1.67	0.00	3.62 ± 0.09	6.10 ± 0.13	7.50 ± 0.17	8.66 ± 0.16	10.12 ± 0.19
LSD at 0.05	-	14.690	19.450	42.421	36.983	44.613	-	1.054	1.456	2.774	2.292	3.347	-	0.161	0.238	0.257	0.249	0.274

R0: Control; R1 (1:3): 25% wastewater + 75% deionized water; R2 (1:1): 50% wastewater + 50% deionized water; R3 (3:1): 75% wastewater + 25% deionized water; R4 (1:0): 100% wastewater.
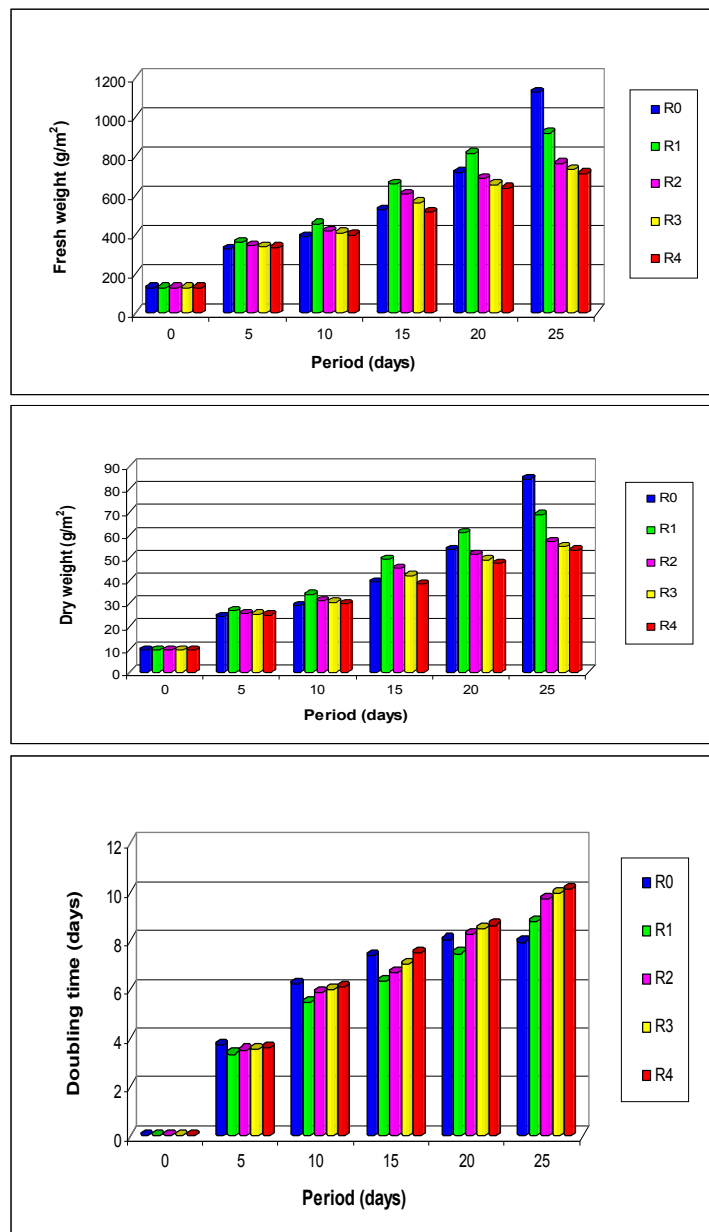

Fig. 1: Effect of industrial wastewater contaminated with Lead (Pb^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. pinnata*.

(R0) (716.27 ± 21.06 and 53.72 ± 1.58 g/m^2) for fresh and dry weight, respectively after 20 days of incubation. The values of fresh and dry weights were significantly different at treatment (R1) compared to the control (R0) after 20 days of incubation.

Doubling time generally decreased at treatment (R1) and then gradually increased from (R2) to (R4) up to 20 days of incubation. After 25 days of incubation, the doubling time gradually increased from (R1) to (R4) as illustrated in Table (2) and Fig. (1). The lowest value of the doubing time was obtained at treatment (R1) (7.49 ± 0.11 days) and this value decreased than that of the control (R0) (8.06 ± 0.13 days) after 20 days of incubation.

A. *pinnata* as a bioaccumulator of Lead (Pb^{+2}) resulted from industrial wastewater (g/m^2):

Results in Table (3) and Fig. (2) showed that, Pb^{+2}accumulation by *A. pinnata* gradually decreased from (R1) to (R4) during all the tested incubation periods from zero time up to 25 days. Hence, the greatest accumulation of Pb^{+2} was observed at treatment (R1) after 25 days of incubation periods (918.26 ± 24.54 g/m^2). The values of Pb^{+2}accumulation were highly significantly different at all treatments compared to the control (R0) during all the tested incubation periods from zero time up to 25 days.

Table 3: *A. pinnata* as a bioaccumulator of Lead (Pb^{+2}) resulted from industrial wastewater (g/m^2) (Data expressed as mean ± SD).

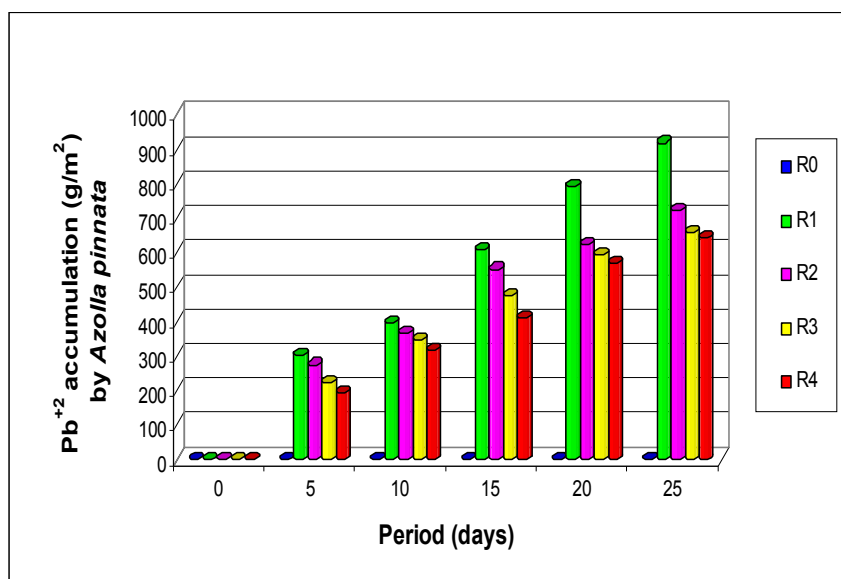| Period (days) Treatments	Zero-time	5	10	15	20	25
			Pb^{+2} accumulation (g/m^2)			
R0	0.00	0.00	0.00	0.00	0.00	0.00
R1	0.00	302.78 ± 6.83	396.76 ± 11.43	608.79 ± 16.21	793.36 ± 21.21	918.26 ± 24.54
R2	0.00	274.48 ± 5.58	365.32 ± 9.29	551.74 ± 15.88	625.16 ± 15.31	722.49 ± 19.78
R3	0.00	223.62 ± 5.28	347.89 ± 8.27	476.00 ± 11.60	593.18 ± 16.20	659.58± 21.83
R4	0.00	191.91 ± 4.88	315.75 ± 6.87	411.70 ± 11.99	568.69 ± 14.98	642.77± 18.58
LSD at 0.05	-	9.254	14.835	22.915	27.841	34.663

Fig. 2: *A. pinnata* as a bioaccumulator of Lead (Pb^{+2}) resulted from industrial wastewater (g/m^2).

Effect of industrial wastewater contaminated with Cadmium (Cd^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. Pinnata*:

Fresh and dry weights gradually increased with increasing the incubation period from zero time up to 25 days. Fresh and dry weights also increased at treatment (R1) and then gradually decreased from (R2) to (R4) up to 20 days of incubation. After 25 days of incubation, fresh and dry weights gradually decreased from (R1) to (R4) as illustrated in Table (4) and Fig. (3). *A. pinnata* couldn't tolerate high concentrations of Cd^{+2}, *A. pinnata* also appeared to be sensitive where fresh and dry weights started to inhibit and this led to its death at treatment (R4) after 20 days of incubation period, respectively. Maximum fresh and dry weights were observed for *A. pinnata* at treatment (R1) (772.67 ± 23.44 and 57.95 ± 1.76 g/m^2), respectively. These parameters were compared with the control (R0) (716.27 ± 21.06 and 53.72 ± 1.58 g/m^2) for fresh and dry weight, respectively

Table 4: Effect of industrial wastewater contaminated with Cadmium (Cd^{+2}) on fresh, dry weight (g/m²) and doubling time (days) of *A. pinnata* (Data expressed as mean ± SD).

| Period (days) / Treatments	F.wt. (g/m²)						D.wt. (g/m²)						D.t. (days)					
	Zero-time	5	10	15	20	25	Zero-time	5	10	15	20	25	Zero-time	5	10	15	20	25
R0	128.21	324.80 ± 7.89	388.00 ± 9.37	524.80 ± 15.47	716.27 ± 21.06	1126.53 ± 27.18	9.62	24.36 ± 0.59	29.10 ± 0.70	39.36 ± 1.16	53.72 ± 1.58	84.49 ± 2.04	0.00	3.73 ± 0.10	6.25 ± 0.14	7.39 ± 0.17	8.06 ± 0.13	7.96 ± 0.09
R1	128.21	353.87 ± 8.17	425.60 ± 11.80	582.93 ± 17.08	772.67 ± 23.44	1027.33 ± 25.91	9.62	26.54 ± 0.61	31.92 ± 0.88	43.72 ± 1.28	57.95 ± 1.76	77.05 ± 1.95	0.00	3.42 ± 0.09	5.78 ± 0.14	6.85 ± 0.13	7.72 ± 0.13	8.33 ± 0.10
R2	128.21	271.73 ± 6.79	355.60 ± 8.39	528.27 ± 15.28	702.53 ± 19.35	875.20 ± 24.24	9.62	20.38 ± 0.51	26.67 ± 0.63	39.62 ± 1.15	52.69 ± 1.45	65.64 ± 1.82	0.00	4.63 ± 0.15	6.80 ± 0.14	7.35 ± 0.15	8.16 ± 0.12	9.03 ± 0.13
R3	128.21	242.18 ± 5.70	283.78 ± 7.57	376.13 ± 9.26	417.00 ± 12.02	454.57 ± 12.73	9.62	18.16 ± 0.42	21.28 ± 0.57	28.21 ± 0.69	31.28 ± 0.90	34.09 ± 0.96	0.00	5.43 ± 0.23	8.70 ± 0.32	9.68 ± 0.22	11.76 ± 0.28	13.66 ± 0.31
R4	128.21	215.33 ± 5.19	268.40 ± 6.03	290.53 ± 6.30	-	-	9.62	16.15 ± 0.39	20.13 ± 0.45	21.79 ± 0.47	-	-	0.00	6.67 ± 0.31	9.35 ± 0.27	12.71 ± 0.32	-	-
LSD at 0.05	-	12.461	16.100	24.275	31.635	37.810	-	0.931	1.204	1.821	2.370	2.743	-	0.354	0.392	0.378	0.291	0.294
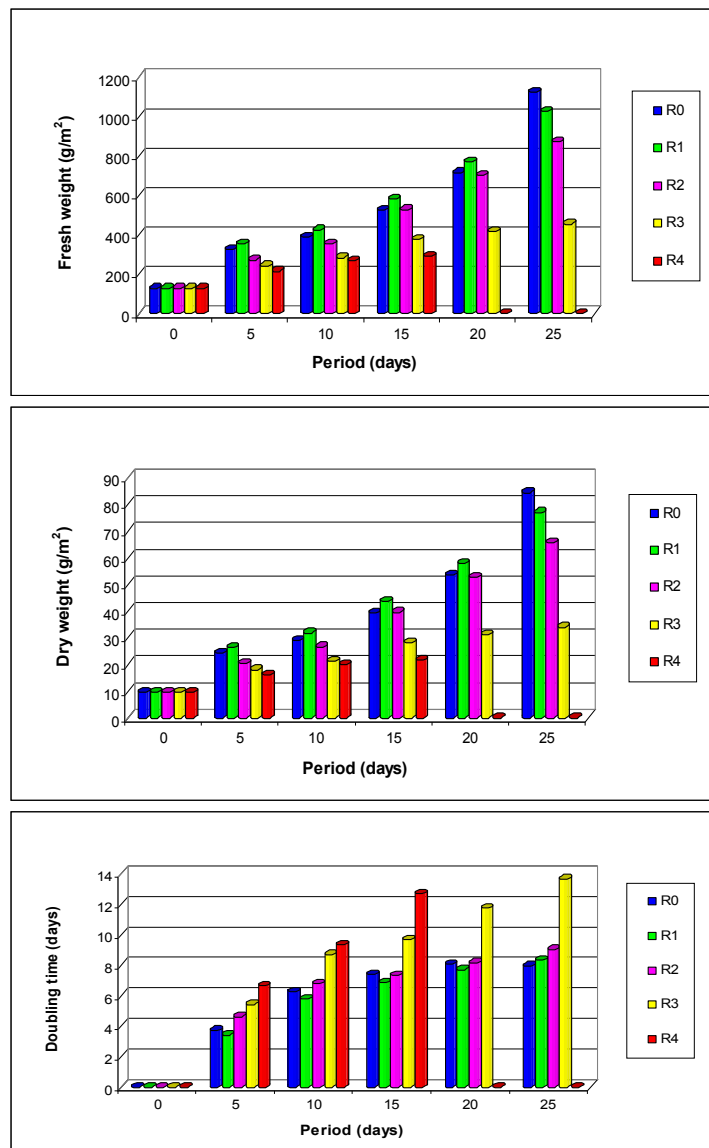
Fig. 3: Effect of industrial wastewater contaminated with Cadmium (Cd^{+2}) on fresh, dry weight (g/m²) and doubling time (days) of *A. pinnata*.

after 20 days of incubation. There was significant difference between the values of fresh and dry weights at treatment (R1) and control (R0) from zero time up to 20 days of incubation.

Doubling time generally decreased at treatment (R1) and then gradually increased from (R2) to (R4) up to 20 days of incubation. After 25 days of incubation, the doubling time gradually increased from (R1) to (R4) as illustrated in Table (4) and Fig. (3). The lowest value of the doubing time was obtained at treatment (R1) (7.72 ± 0.13 days) and this value decreased than that of the control (R0) (8.06 ± 0.13 days) after 20 days of incubation.

A. Pinnata as a bioaccumulator of Cadmium (Cd^{+2}) resulted from industrial wastewater (g/m^2):

Results recorded in Table (5) and Fig. (4) showed that, Cd^{+2} accumulation by *A. pinnata* gradually decreased from (R1) to (R4) during all the tested incubation period from zero time up to 25 days. Hence, the greatest accumulation of Cd^{+2} was observed at treatment (R1) after 25 days of incubation periods (104.56 ± 3.56 g/m^2). The values of Cd^{+2} accumulation were highly significantly different at all treatments compared to the control (R0) during all the tested incubation periods from zero time up to 25 days.

Table 5: *A. pinnata* as a bioaccumulator of Cadmium (Cd^{+2}) resulted from industrial wastewater (g/m^2) (Data expressed as mean ± SD).

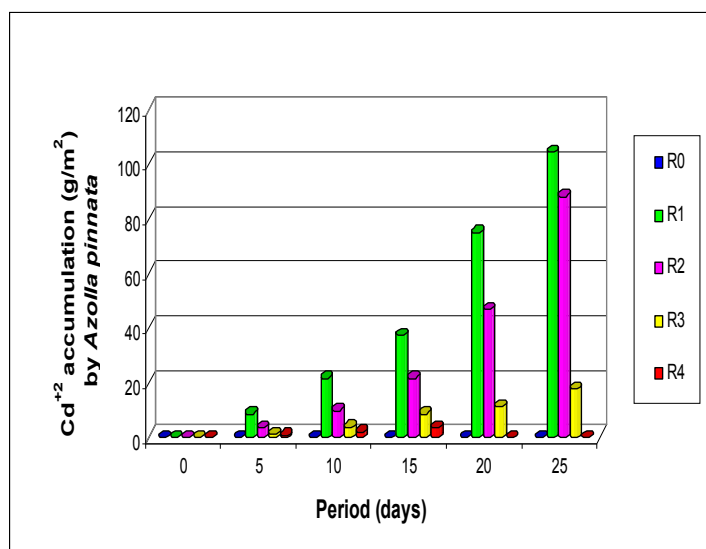| Period (days) Treatments	Cd^{+2} accumulation (g/m^2)					
	Zero-time	5	10	15	20	25
R0	0.00	0.00	0.00	0.00	0.00	0.00
R1	0.00	8.63 ± 0.31	21.70 ± 0.70	37.49 ± 0.67	75.13 ± 2.21	104.56 ± 3.56
R2	0.00	3.48 ± 0.08	9.89 ± 0.37	21.72 ± 0.89	46.91 ± 1.72	88.29 ± 2.34
R3	0.00	1.18 ± 0.00	3.79 ± 0.12	8.60 ± 0.34	11.37 ± 0.42	17.64 ± 0.61
R4	0.00	0.73 ± 0.00	1.98 ± 0.01	3.57 ± 0.10	-	-
LSD at 0.05	-	0.261	0.651	1.635	2.307	3.506

Fig. 4: *A. pinnata* as a bioaccumulator of Cadmium (Cd^{+2}) resulted from industrial wastewater (g/m^2).

Effect of industrial wastewater contaminated with Copper (Cu^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. Pinnata*:

Fresh and dry weights gradually increased with increasing the incubation period from zero time up to 25 days. Fresh and dry weights also gradually decreased from (R1) to (R4) up to 25 days of incubation as illustrated in Table (6) and Fig. (5). Maximum fresh and dry weights were observed for *A. pinnata* at treatment (R1) (755.60 ± 23.65 and 56.67 ± 1.77 g/m^2), respectively. These parameters were compared with the control (R0) (1126.53 ± 27.18 and 84.49 ± 2.04 g/m^2) for fresh and dry weight, respectively after 25 days of incubation. There was non significant difference between all the values of fresh, dry weights at all treatments and control during all the tested incubation periods from zero time up to 25 days.

Doubling time generally increased from (R1) to (R4) up to 25 days of incubation as illustrated in Table (6) and Fig. (5). The lowest value of the doubing time was obtained at treatment (R1) (9.77 ± 0.17 days) and this value increased than that of the control (R0) (7.96 ± 0.09 days) after 25 days of incubation. *A. pinnata* couldn't

tolerate high concentrations of Cu^{+2} where *A. pinnata* was very sensitive to these high concentrations and this led to its death at treatment (R4) after 10 days of incubation period. After 20 days of incubation, treatment (R3) exhibited the death of *A. pinnata* as well.

Table 6: Effect of industrial wastewater contaminated with Copper (Cu^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. pinnata* (Data expressed as mean ± SD).

| Period (days) / Treatments	F.wt. (g/m²)						D.wt. (g/m²)						D.t. (days)					
	Zero-time	5	10	15	20	25	Zero-time	5	10	15	20	25	Zero-time	5	10	15	20	25
R0	128.21	324.80 ± 7.89	388.00 ± 9.37	524.80 ± 15.47	716.27 ± 21.06	1126.53 ± 27.18	9.62	24.36 ± 0.59	29.10 ± 0.70	39.36 ± 1.16	53.72 ± 1.58	84.49 ± 2.04	0.00	3.73 ± 0.10	6.25 ± 0.14	7.39 ± 0.17	8.06 ± 0.13	7.96 ± 0.09
R1	128.21	243.07 ± 5.47	365.87 ± 9.63	471.73 ± 8.56	553.87 ± 15.80	755.60 ± 23.65	9.62	18.23 ± 0.41	27.44 ± 0.72	35.38 ± 0.64	41.54 ± 1.19	56.67 ± 1.77	0.00	5.43 ± 0.21	6.62 ± 0.16	7.98 ± 0.13	9.48 ± 0.18	9.77 ± 0.17
R2	128.21	196.53 ± 4.68	221.73 ± 6.34	270.13 ± 5.32	405.07 ± 10.87	531.64 ± 14.75	9.62	14.74 ± 0.35	16.63 ± 0.48	20.26 ± 0.40	30.38 ± 0.82	39.87 ± 1.11	0.00	8.06 ± 0.36	12.66 ± 0.64	13.89 ± 0.42	12.05 ± 0.30	12.20 ± 0.24
R3	128.21	188.40 ± 4.98	191.47 ± 4.58	193.15 ± 4.59	-	-	9.62	14.13 ± 0.37	14.36 ± 0.34	14.49 ± 0.35	-	-	0.00	8.93 ± 0.54	17.24 ± 0.77	25.42 ± 1.49	-	-
R4	128.21	174.40 ± 3.32	-	-	-	-	9.62	13.08 ± 0.25	-	-	-	-	0.00	11.36 ± 0.63	-	-	-	-
LSD at 0.05	-	9.957	12.650	15.480	23.170	31.680	-	0.746	1.270	1.676	1.740	2.380	-	0.758	0.831	0.636	0.301	0.249
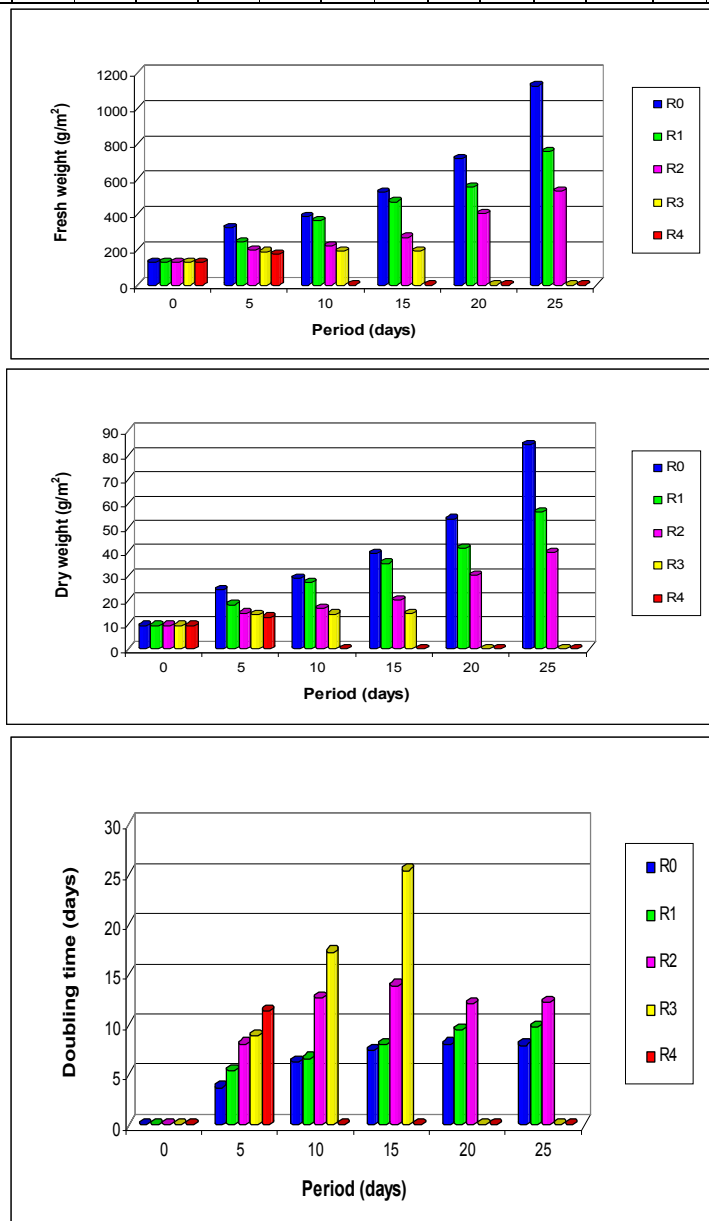
Fig. 5: Effect of industrial wastewater contaminated with Copper (Cu^{+2}) on fresh, dry weight (g/m^2) and doubling time (days) of *A. pinnata*.

A. pinnata as a bioaccumulator of Copper (Cu^{+2}) resulted from industrial wastewater (g/m^2):

Results obtained in Table (7) and Fig. (6) showed a simultaneous decrease in the levels of Cu^{+2} accumulation by *A. pinnata* from (R1) to (R4). The highest value of Cu^{+2} accumulation was observed at treatment (R1) (642.37 ± 17.81 g/m^2) after 25 days of incubation. The values of Cu^{+2} accumulation were highly significantly different at all treatments compared to the control (R0) during all the tested incubation periods from zero time up to 25 days.

Table 7: *A. pinnata* as a bioaccumulator of Copper (Cu^{+2}) resulted from industrial wastewater (g/m^2) (Data expressed as mean ± SD).

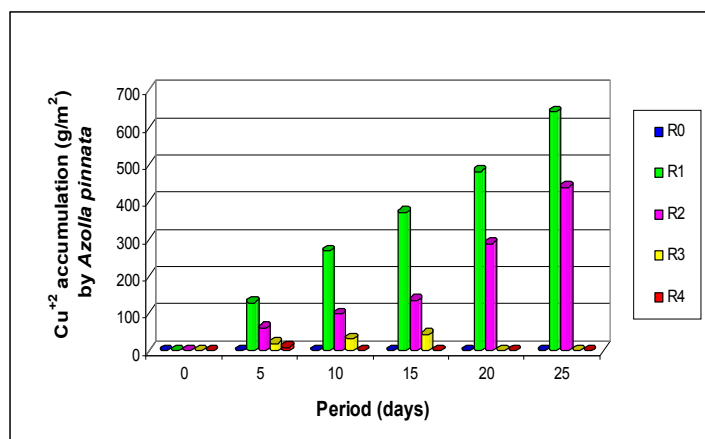| Period (days) \ Treatments	Cu^{+2} accumulation (g/m^2)					
	Zero-time	5	10	15	20	25
R0	0.00	0.00	0.00	0.00	0.00	0.00
R1	0.00	129.67 ± 3.17	268.51 ± 5.20	372.76 ± 9.53	483.10 ± 13.04	642.37 ± 17.81
R2	0.00	61.44 ± 1.76	98.79 ± 2.70	135.61 ± 4.00	288.17 ± 6.32	440.57 ± 11.17
R3	0.00	19.57 ± 0.78	31.92 ± 1.14	44.18 ± 1.76	-	-
R4	0.00	9.58 ± 0.17	-	-	-	-
LSD at 0.05	-	3.030	4.860	8.531	7.953	17.019

Fig 6: *A. pinnata* as a bioaccumulator of Copper (Cu^{+2}) resulted from industrial wastewater (g/m^2).

Discussion

Different dilutions of wastewater by Pb^{+2}, Cd^{+2} and Cu^{+2} (R0, R1, R2, R3 and R4) were used to verify the tolerability of the tested *A. pinnata* to high concentrations of these metals and its potential as a bioaccumulator. Fresh and dry weights gradually increased with increasing the incubation period from zero time up to 25 days. The recorded results are in the same line with those of El- Araby *et al.* (1999); they found that *A. pinnata* recorded its maximum growth with increasing the incubation period up to 25 days. Some non-essential elements (e.g., As, Cd, Hg and Pb) are extremely toxic to biota even at very low concentrations (MoEF, 2011). Lead is a common heavy metal found in industrial effluents, particularly in developing countries, where legislative measures of control are either lacking or are not being strictly enforced (Thayaparan *et al.*, 2013). Consequently removal of Pb (II) from industrial wastewater is important for protection and restoration of waterways.

Fresh and dry weights increased at treatment (R1) and then gradually decreased from (R2) to (R4) up to 20 days of incubation. After 25 days of incubation, fresh and dry weights gradually decreased from (R1) to (R4). *A. pinnata* appeared to accumulate high amounts of Pb^{+2} and consequently became more tolerance to these high concentrations. These results were harmony with Monica *et al.* (2014) who showed that, *A. microphylla* was grown in different concentrations of Pb(NO$_3$)$_2$ and exhibited inhibition of both plant and root growth. After 8th days of lead treatment, the reduction of plant growth was 35% and 43% respectively at 90 ppm and 100 ppm, when compared to the control. However, Pb treatment at 100 mg/l concentration showed toxicity symptoms like chlorosis and drying of edges in plants. Similar response to lead treatment was previously noticed in various plants (Brunet *et al.*, 2008).

Zayed *et al.* (1998) stated that, at high Pb concentrations, *Azolla* growth was reduced after 8th day. The potential of a plant for phytoremediation process is often judged by its bioconcentration factor. The BCF values

over 1000 are considered as evidence of a useful plant for phytoremediation. In this study we found that, BCF of *A. microphylla* for Pb increased significantly with increasing Pb concentration in the growth medium and the highest BCF was observed at 100 mg/l. The values of Pb^{+2} accumulation by *A. pinnata* were highly significantly different at all treatments compared to the control (R0) during all the tested incubation periods from zero time up to 25 days. Lead and cadmium are considered the most toxic heavy metals and have been recognized for their harmful influence on environment where their accumulation via food chain having a serious threat to human health, animals and plants (Nagajyoti *et al.*, 2010). Among the different heavy metals, Cd is one of the most toxic and because of its high availability in soil and water; it can easily enter into the food chain (Singh *et al.*, 2010). *A. pinnata* couldn't tolerate high concentrations of Cd^{+2}, *A. pinnata* also appeared to be sensitive where fresh and dry weight started to inhibit and this led to its death at treatment (R4) after 20 days of incubation period, respectively. Decreased plant growth might be associated with the inhibition of mitotic index noticed with Pb and Cd heavy metal treatment (Vecchia *et al.*, 2005). The values of Cd^{+2} accumulation by *A. pinnata* were highly significantly different at all treatments compared to the control (R0) during all the tested incubation periods from zero time up to 25 days.

A. pinnata couldn't tolerate high concentrations of Cu^{+2}, where *A. pinnata* was very sensitive to these high concentrations and this led to its death at treatment (R4) after 10 days of incubation period. After 20 days of incubation, *A. pinnata* also died at treatment (R3). This result was similarly with that recorded by Sheo and Anita (2011) they showed that, *Azolla* changed its colour from yellow to brown indicates the death of the macrophytes. In contrast to this, we can conclude that *A. pinnata* could be a good candidate for the phytoremediation of low concentrations of copper from polluted water. The values of Cu^{+2} accumulation by *A. pinnata* were highly significantly different at all treatments compared to the control (R0) during all the tested incubation periods from zero time up to 25 days. Rauf *et al.* (2009) reported that, copper (Cu^{+2}) is an essential plant micronutrient and often occurs in high concentrations in the aquatic ecosystems.

Copper plays very important role in cell function and energy transfer as well as it is essential in structural stability of chromosomes (Mishra *et al.*, 2008). Although Cu is an essential micronutrient for normal plant metabolism, playing an important role in a large number of metalloenzymes, photosynthesis related plastocyanin and membrane structure, it has been reported to be among the toxic heavy metals (Li and Xiong, 2004). In addition, Cu has spread contamination level due to its use as a mineral pesticide in agriculture (Ahmad *et al.*, 2008). As presented in this study, the removal efficiency shown by healthy pre weighed *A. pinnata* was comparatively higher for Pb, Cu and lower for Cd.

Accumulation of heavy metals in *Azolla* and their mobility from the root to the shoot can be correlated with damage caused by the loss of essential nutrients. The contents of cadmium and copper for the *Azolla's* whole plant grown in the presence of 10 ppm metal were 6,021 and 5,365 ppm, respectively, as detected by atomic absorption of digested plants. Cadmium and copper contents in the roots of these plants were 14,328 and 23,941 ppm, respectively and their contents in the shoot were 4,857 and 2,748 ppm, respectively (Sela *et al.*, 1988).

A. filiculoides L. grown for 3-7 days in nutrient media containing 8-15 ppm of different heavy metals was found to contain about l0000 ppm cadmium, 1990 ppm chromium, 9000 ppm copper, 9000 ppm nickel and 6500 ppm zinc (Sela *et al.*, 1989). The content of heavy metal in the roots was two-to five-folds higher than in the shoots and 98% of the heavy metals were bound to the insoluble fraction of the *Azolla*. The content of heavy metals in dead dried *Azolla* was 3-7 times higher than in the living plants. The accumulation of Cd, Cu, Ni and Zn was several times higher from the solution diluted 1:40 than from the solution diluted 1:8, the content of Cd, Cu, Ni and Zn in *Azolla* was about 1% on a dry weight basis and was found to be 500-1000 times higher than the concentration of these metals in the growth medium except for Cr which was only 100 times more concentrated in the *Azolla* (Sela *et al.*, 1988).

Also, the concentration factors for heavy metal ions recorded here in *Azolla* are far higher than in the other plants, with amounts of Cd, Cu, Ni and Zn in the *Azolla* 500- to 1000-fold higher than in the growth medium. According to Nuzhat *et al.* (2015), results found *A. pinnata* as a hyperaccumulator of Cu, Zn and moderate accumulator of Pb, Cr and Cd. Hyper bioaccumulator factor in *A. pinnata* may be due to highest accumulation capability of heavy metal from a system. The bioabsorption capacity of the plant was decreasing in the order of Zn>Cu>Pb>Cr>Cd (Nuzhat *et al.*, 2015). *A. pinnata* removed 92.7, 83.0, 59.1, 65.1, 95.0, 90.0 and 73.1% of the initial Fe, Zn, Cu, Mn, Co, Cd and Ni, respectively from mixture of wastewater (Elsharawy *et al.*, 2004). In this respect, Arora *et al.* (2004) reported that, *A. pinnata* have been shown to absorb Cr, Pb, Cd, Zn and other heavy metals and showed tolerance when present in low concentrations.

Cohen *et al.* (2002) recorded that, *Azolla* binds heavy metals in a wide range of concentrations with high effectiveness. It was shown that it could be an efficient accumulator of Au (Antunes *et al.*, 2001), As (Zhang *et al.*, 2008), Cd, Cu, Zn, Pb (Rakhshaee *et al.*, 2006), Sr (Cohen *et al.*, 2002) and others (Sood *et al.*, 2011). It can also remove sulpha drugs (Forni *et al.*, 2001). Atomic absorption spectrophotometric analysis have shown initial concentration of these metals in *A. pinnata* as Cu (0.02ppm), Pb (0.085ppm), Cr (0.07ppm), Cd (0.006ppm) and Zn (0.06ppm) and after 10 days period the plant has accumulated Cu (0.90ppm), Pb (0.42ppm), Cr (0.27ppm), Cd (0.042ppm) and Zn (2.1ppm) in the order of Zn>Cu>Pb>Cr>Cd (Nuzhat *et al.*, 2015). Aquatic macrophytes can accumulate significant quantity of heavy metals in their tissue ($10-10^6$) times greater concentration than in

the water (Snežana *et al*., 2005). Albers and Camardese (1993) found that the concentrations of metals in aquatic plants can be more than 100,000 times greater than in the associated water.

According to Nuzhat *et al*. (2015) has revealed the role of free floating macrophyte (*A. pinnata*) in phytoremediation technology where it has an excellent performance in removing the metals and was able to remove huge amount of heavy metals in 10 days of the experimentation period. The results of the present analysis coincide with the previous studies and suggested that *A. pinnata* is an efficient macrophyte and can play an important role in the bioremediation of aquatic ecosystems and wastewater treatment which are under heavy metals stress of anthropogenic pressure.

Conclusion

From the above results, it has been concluded that the *A. pinnata* is a potential candidate for accumulation of heavy metals and recycling of industrial wastewater. Overall, *A. pinnata* representing an effective, eco-friendly and low-cost treatment technology and could be used in heavy metal accumulation from industrial wastewater.

Acknowledgment

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