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EFFECT OF CADMIUM AND LEAD ON NITRATE AND PHOSPHATE REMOVAL BY THE DUCKWEED *LEMNA GIBBA*

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Abstract – In the present study, the effect of the heavy metals, such as cadmium and lead on the removal of nitrate (NO_3^-) and orthophosphate (PO_4^{3-}) was assessed using *Lemna gibba*. Duckweed plant was cultured in N and P-rich medium, supplemented with heavy metals. A total of two initials (0.1 and 1 mg/L) concentrations of Cd and Pb were used. Samples were taken every two days to assess plants efficiency in removing both nutrients and heavy metals over Six days. Results showed that in control and in all treatments (Cd and Pb), nitrate and orthophosphate concentrations decreased markedly within the two days of initiating experiments as compared to the initial concentrations (1.76 ±0.01 mg P/L and 850±0.01 mg N/L).The highest phosphate removal efficiencies (percentage removal) were obtained on the fourth day at 1 mg Cd/L and 1 mg Pb/L. Whereas, nitrate removal showed maxima on the sixth day at 1 mg Cd/L and at 0.1 mg Pb/L. As compared to the control, the presence of Cd and Pb at 0.1 mg/L in the culture medium had no effect on phosphate removal, while a Pb concentration of 1 mg/L revealed a better phosphate removal. Cd and Pb at 0.1 mg/L enhanced nitrate removal as compared to control. *Lemna gibba* was able to simultaneously remove Cd, Pb, nitrate and phosphate, major causes of contamination and eutrophication in water bodies.

Keywords: heavy metals, nutrients, phytoremediation, aquatic plant, eutrophication.

Introduction

Heavy metal contamination and eutrophication of aquatic ecosystem are global environmental problems. The problem of water pollution by heavy metals is becoming more and more serious with the increasing industrialization.
Unlike organic substances, heavy metals are essentially non-biodegradable and therefore accumulate in the environment (Ali et al., 2013). Because of their toxicity, cadmium and lead are of prime environmental concern (Scheifler et al., 2002). Over the past five decades, the worldwide release of Cd has reached 22.000 tones (Singh et al., 2003) and a total of 4 million tons of Pb are mined in one year (Dirilgen, 2011). Their bioaccumulation through the food chain can pose risks to human health (Gisbert et al., 2003). The threat of these toxic metals to human and animal health is aggravated by their long-term persistence in the environment (Forstner, 1995). Often present in industrial effluents, cadmium and lead are hazardous to living organisms in the aquatic system (Nanda Kumar et al., 1995).Cadmium occurs in natural and wastewaters, and it originates from many industrial sources such as processing, smelting and mining ores, reclamation of scrap metals, incineration for disposal of waste products, run-off carrying fertilizers and fungicides etc. (Liu et al., 2007). For lead, the most important sources into wastewater include batteries, pigments; paints, petrol, cables, steels, alloys, and plastic industries (Salem et al., 2000).
Also, eutrophication of water bodies is an important global

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environmental problem and it has caused by a surplus of nutrients in the water, especially of phosphorus and nitrogen. Phosphorus (phosphate) has been recognized as one of the most common elements on earth and an essential nutrient to all living organisms (Kamika & Momba, 2015).

Phosphorus exists as phosphates, inorganic and organic forms. The predominant form is orthophosphate which can be used by algae and macrophytes.

In plants, phosphorus plays an important role in photosynthesis, the transfer and storage of energy (ATP), the manufacture of nucleic acids, proteins and carbohydrates (Rychter & Rao, 2005; Kamika & Momba, 2015). However, an excess of phosphorus in the aquatic environment leads to a rapid algal growth, which results in eutrophication (Akpor et al., 2008; Kamika & Momba, 2015).

Similar to phosphorus (phosphate), nitrogen is one of the essential elements for any form of life (Camargo & Alonso, 2006). The most common variety of chemical forms present and used in the aquatic environment are ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) (Rabalais et al., 2009). Excessive concentrations of nitrate in groundwater and surface water can cause environmental and health problems (Akpor et al., 2008). Ammonia that is not ionized can be toxic to marine organisms and aquatic life.

Due to several health and environmental hazards caused by phosphates and nitrates, several conventional methods have been developed for the removal of nitrogen and phosphorus These include chemical oxidation, chemical precipitation, ion exchange, etc. (Akpor et al., 2008; Kamika & Momba, 2015). Although these techniques are effective for the removal of nitrates from the contaminated water, their pilot scale operation is expensive and has only limited potential applications (Zaitsez et al., 2008).

Owing to these limitations for the removal of nitrates and phosphates from water and/or wastewater, the most versatile and widely used technology is phytoremediation. Aquatic macrophytes have been widely used to remove nitrogen from both wetlands and wastewaters (Yang et al., 2001; Saha & Jana, 2003; Sooknah & Wilkie, 2004). In synthetic medium containing 100 mg/L of nitrate, water hyacinth reduced the nitrate level to 64%.The efficiency of nitrate removal was further increased to 80% with nitrate concentrations of 200 and 300 mg/L (Ayyasamy et al., 2009). Water hyacinth (*Pontederiaceae*) was more efficient in the removal of nutrients (nitrogen and phosphorus) than Salvinia (*Salviniaceae*) (Petrucio & Esteves, 2000).

Assessment of the contribution of duckweed *Lemna gibba* (*Lemnaceae*) and its associated microorganisms (algae and bacteria forming an attached biofilm) to remove nutrients showed that the biological floating mat complex (plant and microbes) is responsible for removing up to 75% of the nutrients in the wastewater. The macrophyte contributed up to 52% of phosphorus removal by its own growth. The associated organisms and microorganisms removed the rest (Korner & Vermaat, 1998).

No studies have been conducted on the effect of heavy metals on the duckweed ability to remove nitrate and phosphate.

Hence, the purpose of this study was to evaluate the potential of *L.gibba* for the removal of nitrate and phosphate from culture medium under different levels of Cd and Pb stresses and over time. The growth of the plants was also assessed.

Materials and Methods

Plant material and culture conditions

L. gibba (duckweed) was collected from a natural pond in the north of Algeria. The plants were disinfected by immersing the colonies first in 0.5% (v/v) ethanol for few seconds, then in a solution of sodium hypochlorite at 0.5% for 3 min, and finally they were rinsed in sterile distilled water. The stock cultures were maintained at least 4 weeks under axenic conditions in 1L glass beakers containing autoclaved growth medium that consisted of: 118 mg/L $Ca(NO_3)_2{\cdot}4H_2O$; 5.055 mg/L KNO_3; 4.932 mg/L $MgSO_4{\cdot}7H_2O$; 0.68 mg/L KH_2PO_4; 0.1 mg/L Fe-EDTA; 0.286 mg/L H_3BO_3; 0.155 mg/L $MnSO_4{\cdot}7H_2O$; 0.022 mg/L $ZnSO_4$; 0.0079 mg/L $CuSO_4$; 0.00478 mg/L $NiSO_4{\cdot}7H_2O$. The pH was adjusted with the drop-wise addition of NaOH at 0.1 N or HCl at 0.1 N to be between 6 and 6.5. The medium was changed every 7 days. The incubation conditions are as follows: temperature 27±1°C, 54 µmol $m^{-2}s^{-1}$ light intensity, 16h /8h light and dark cycle.

To assess the duckweed ability in metal accumulation, the nutrient medium was supplemented with two nominal concentrations of Cd prepared from $CdSO_4$ (0.1 and 1 mg/L) and Pb prepared from $PbCl_2$ (0.1 and 1 mg/L).

Lemna gibba was cultured in P and N-rich medium. Initial phosphate and nitrate concentrations were 1.76 ± 0.01 mg/L and 850 ± 0.01 mg/L respectively.

The final pH of the solutions was adjusted to 6.0 ± 0.5.

Thirty (30) uniform and healthy colonies (each colony with 2 fronds) of *L. gibba* have been selected as test specimens and inoculated into flasks containing 100 mL of nutrient medium treated with two concentrations of Cd and with two concentrations of Pb (0.1 and 1 mg/L).

L. gibba cultured in the growth medium without heavy metals served as controls. All experiments lasted 6 days and were performed in triplicate. The frond number measurements have been monitored on days 0, 2, 4 and 6.

The relative growth (RG%) was determined using Eq.(1) (Megateli et al., 2009):

$$RG\% = \frac{Nt-N0}{N0} \times 100 \quad (1)$$

Where N_t is the average number of fronds at time t, N_0 is the average number of fronds at the beginning of the experiments ($N_0 = 60$).

The growth inhibition rates noted Inh% were calculated as follows (Megateli et al., 2009):

$$Inh\% = \frac{\Delta Nt-\Delta N}{\Delta Nt} \times 100 \quad (2)$$

whereN_t is the average variation of the number of colonies in the control, N is the average variation of the number of colonies in presence of metal

Analysis of heavy metals in plant samples

The metals analyses were carried out by means of atomic absorption spectrophotometry (AAS) using an optical emission spectrophotometer Perkin Elmer (Optima 700 DV). Plants samples were dried during48 h at 80°C. After the drying, the fronds were grounded and digested with (HNO_3/ $HClO_4$) 3/1(v/v). Afterward, each sample was dried and then redissolved in deionized water.

Analysis of phosphate and nitrate in the culture medium

The phosphate and nitrate concentrations of the growth medium were analyzed, using the ascorbic acid and salicylate methods, respectively, as described in standard methods (APHA, 2001) performed by a spectrophotometer HACH (DR/2000).

Statistical analyses of data

Standard deviations were performed using the included statistical functions with Microsoft Excel Office 2003.

Three replicates of all the samples were run to ensure precision of the determinations. Data was subjected to analysis of variance (ANOVA) by SigmaPlot.11. P values less than 0.05 was considered to be significant.

Results

Phosphate removal

The change of orthophosphate concentration in culture medium at different initial Cd and Pb concentrations and over time are shown in Fig. 1.

The orthophosphate concentration in all treatments (Cd and Pb) decreased markedly within 2 days of initiating the experiments and remained almost unchanged after that except in the solution treated with 1 mg/L Pb, in which a significant decrease of phosphate concentration occurred also from day 2 to day 4. A similar tendency was observed in control treatment. Phosphate removal (%) was in the ranges of 75.50-97.53% in all experiments. The removal efficiencies showed maxima on day 4. The greatest values were 90.32% and 97.53% obtained at 1mg/L of Cd and Pb respectively. They were about 86.15% and 88.44% at 0.1 mg/L of Cd and Pb respectively.

Furthermore, Pb at a concentration of 1 mg/L Pb appeared to be able to enhance phosphate removal on day 4 as compared to its respective control (75.75%). No significant differences were noted between the removal efficiencies in the controls and those obtained with all treatments of Cd and at 0.1 mg/L Pb.

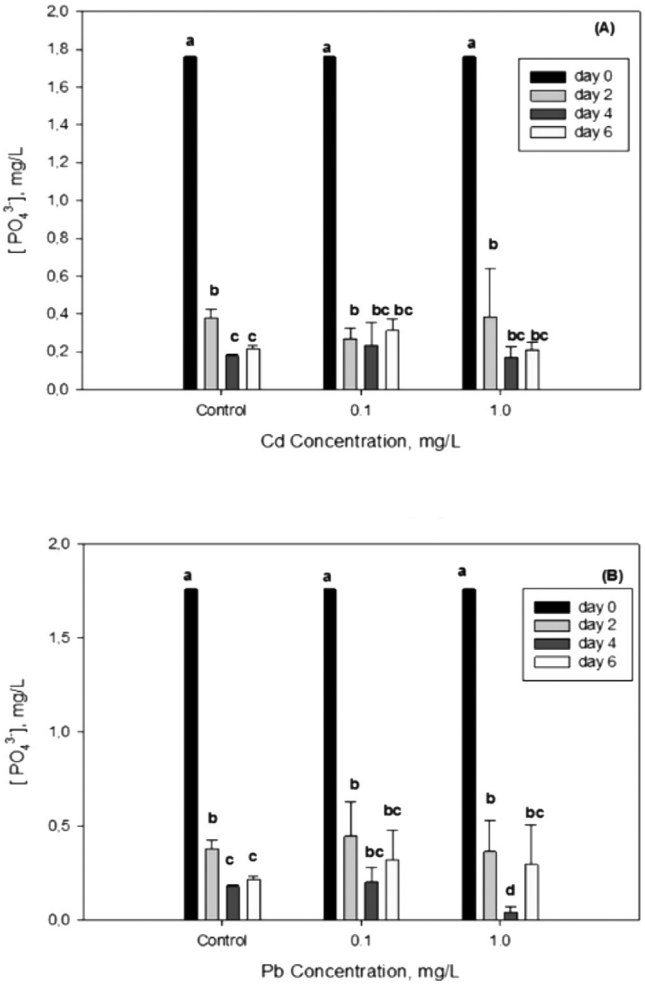

Figure 1. Effect of Cd (A) and Pb (B) on orthophosphate concentration. Values represent mean ± S.D. Different letters indicate significant differences (P< 0.05) according to the ANOVA test.

Nitrate removal

Nitrate concentration in culture medium at different initial Cd and Pb concentrations and over time is illustrated in Fig. 2.
The nitrate concentration in the controls and in all treatments (Cd and Pb) decreased markedly within 2 days of initiating the experiments and remained almost unchanged after that in the controls and in the solution treated with 1 mg/L Pb. Whereas, a significant increase of nitrate concentration is observed from day 2 to day 4 and a decrease once again from day 4 to day 6 at 1 mg/L Cd and 0.1 mg/L Pb treated solutions. Nevertheless, the increase of nitrate concentration between day 2 and day 4 leads to values much lower than the initial concentration and were also below those of their respective controls.
Nitrate removal efficiencies with *Lemna gibba* monitored on days 2, 4 and 6 of the experiments ranged between 48.59% and 78.63% in all experiments. The removal efficiencies showed maxima on day 6. The greatest values of 78.63% and 71.18% were obtained at 1 mg/L Cd and at 0.1 mg/L Pb respectively. Whereas they were about 70% and 57% at 0.1 mg/L of Cd and at 1 mg/L Pb respectively.
As compared to controls, NO_3^- concentration in Cd-treated medium decreased significantly ($p< 0.05$) by 28.92% on day 6 at 0.1 mg/L and by 34.18% and 49.22% on days 2 and 6 respectively at 1 mg/L (Fig.2A). Also, a decrease of nitrate concentration was observed in Pb-treated solution (Fig.2B) with 0.1 mg/L Pb (41.58% on day 2 and 31.55% on day 6).
No significant differences were noted between the removal efficiencies in the controls and those obtained with 1 mg/L Pb.

Growth of *Lemna gibba*

The effect of Cd and Pb on the *L.gibba* growth at different concentrations and exposure times are shown in Fig. 3 and 4. All duckweed samples showed a significant increase in the relative growth on day 6 as compared to relative growth obtained on day 2 ($p<0.05$). On the other hand, treatment with 1 mgCd/L caused a significant decrease in the relative growth of *L. gibba* as compared to the control plants ($p<0.05$) on day 4. Also, a significant decrease in the relative growth was observed for Cd concentration of 1mg/L as compared to that obtained at 0.1 mg/L. However, the plants grown in lower Cd concentration samples (0.1 mg/L Cd) were not different from the control plants ($p \geq 0.05$) on days 2 and 6. Additionally, the tested Pb concentrations induced a significant reduction of the relative growth of the plants on day 6 ($p<0.05$), while this trend was also observed on day 4 in treatments with 1 mg/L Pb. Hence, the highest growth reductions (% inh) were found at 1 mg/L after 4 days of exposure to Cd (41.78 %) and was about 33.81% in the presence of Pb after 6 days.

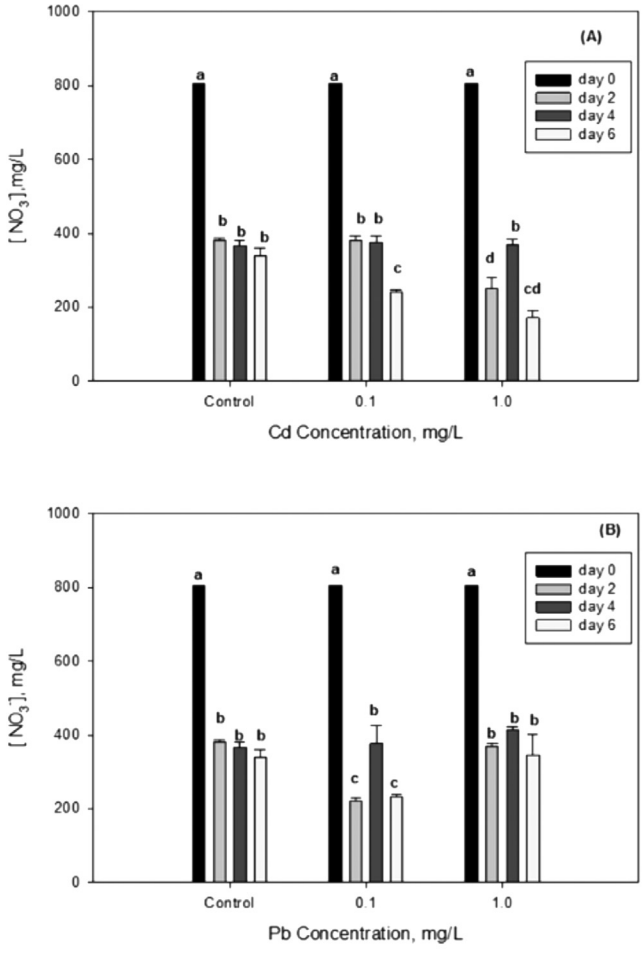

Figure 2. Effect of Cd (A) and Pb (B) on nitrate concentration**.** Values represent mean ± S.D. Different letters indicate significant differences ($P< 0.05$) according to the ANOVA test.

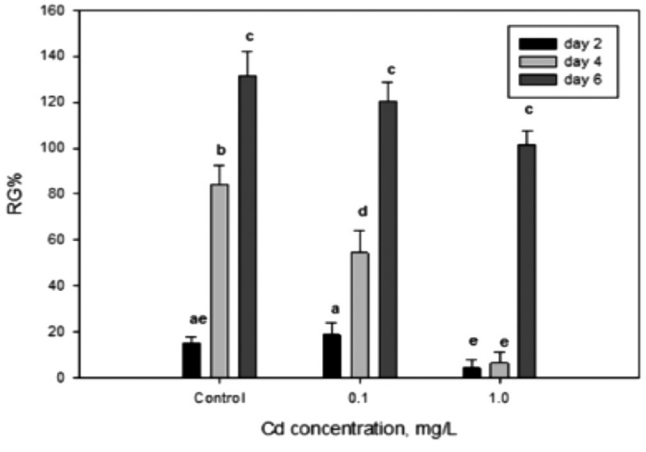

Figure 3. Effect of Cd on growth of *Lemna gibba* at different concentrations and exposure times. Values represent mean ± S.D. Different letters indicate significant differences ($P< 0.05$) according to the ANOVA test.

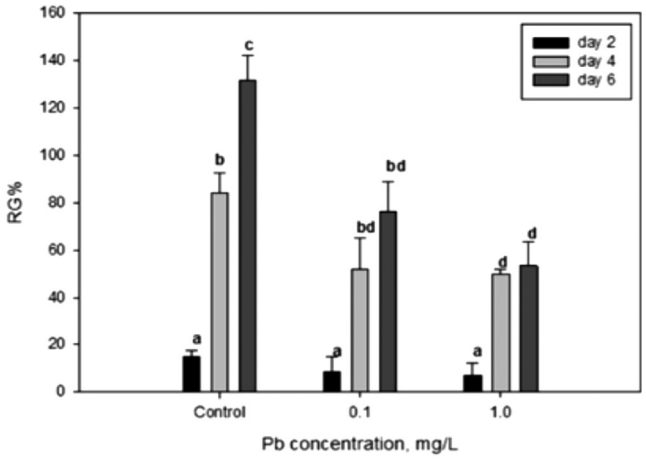

Figure 4. Effect of Pb on growth of *Lemna gibba* at different concentrations and exposure times. Values represent mean± S.D. Different letters indicate significant differences (P< 0.05) according to the ANOVA test.

Cd and Pb accumulation

At Cd concentrations of 0.1 and 1 mg/L, the Cd accumulations were respectively, 0.253 ± 0.087 and 1.161 ± 0.231 on day 2, 0.337 ± 0.045 and 2.321 ± 0.129 on day 4 and 0.6392 ± 0.06 and 2.676 ± 0.0007 mg/g dry weight at the end of the experiment (Fig. 5A).

Results of the ANOVA analysis showed that at 0.1 and 1 mg/L Cd, there was a significant increase of Cd content in the plant on days 2, 4 and 6 at the tested Cd concentrations. Also, a significant increase of Cd content in the aquatic plant is observed as the Cd concentration increased from 0.1 to 1 mg/L. For Pb, the most important accumulations were 3.075 ± 0.364 and 6.284 ± 0.145 mg/g dry weight at 0.1 and 1 mgPb/L on day 6 (Fig. 5B).On days 2 and 4, the Pb amounts accumulated at 0.1 mg/L were not significantly different from that taken-up at 1mg/L ($p<0.05$).

In the present study, *L. gibba* accumulated Cd and Pb to the highest concentrations of 2.676 ± 0.0007 mg/g dry weight and 6.284 ± 0.145mg/g dry weight when exposed to 1 mg/L of Cd and 1 mg/L of Pb respectively.

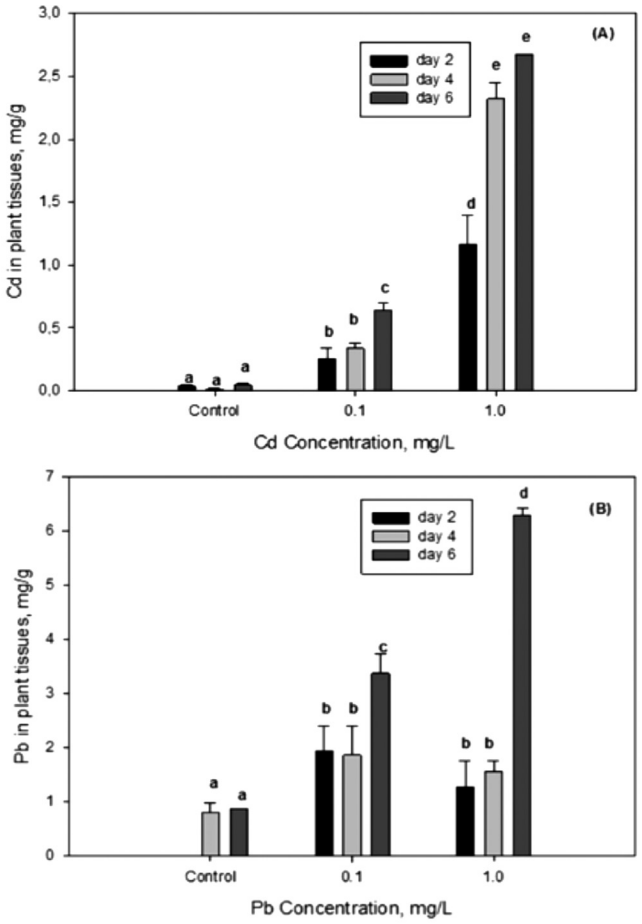

Figure 5. The accumulation of Cd (A) and Pb (B) by *Lemna gibba* at different concentrations and exposure times. Values represent mean ± S.D. Different letters indicate significant differences (P< 0.05) according to the ANOVA test.

Discussion

Several researchers have assessed the performance of duckweed in removing nitrogen and phosphorus.

Korner & Vermaat (1998) showed that depending on the initial concentrations, the duckweed covered systems removed 63 - 99% of the initial total phosphorus in three days. They also reported the removal efficiency of 14.0 to 92.2% phosphorus in wastewater system using *L. gibba*. The experimental study of Vermaat & Hanif (1998) for domestic wastewater with Lemnaceae resulted in 77 % removal of total phosphorus.

EL-Kheir et al. (2007) reported that *L. gibba* was found to be very effective in the removal of nutrients, soluble salts, organic matter, heavy metals, and in eliminating suspended solids, algal abundance and faecal coliform densities. Our results confirmed these high removal efficiencies.

In this study, it was found that in the controls and in all treatments (Cd and Pb), orthophosphate and nitrate concentrations decreased markedly within 2 days of initiating the experiments compared to the initial concentrations of 1.76 mg/L and 805 mg/L respectively. According to Ozengin (2007), the maximum removal of the total phosphate by *duckweeds* (*Lemna minor* L.) occurs at 360 minutes, and the maximum removal of the total nitrate was observed at 1440 minutes for industrial and municipal wastewater.

For phosphate concentrations, there is a further decrease from day 2 to day 4 which was significant ($p<0.05$) in the controls and at 1 mg/L Pb solution and insignificant ($p>0.05$) for Cd solutions and at 0.1 mg/L Pb, leading to maximum efficiencies of 89.75% in the control, 86.51% and 90.32% at 0.1 mg/L Cd and 1 mg/L Cd respectively. For Pb solutions at 0.1 mg/L and 1 mg/L maximum efficiencies were about 88.44% and 97.53%. From day 4 to day 6, there was no phosphate concentration decrease.

For nitrate, solutions at 1 mg/L Cd and at 0.1 mg/L Pb, the concentrations increased significantly from day 2 to day 4 and decreased once again from day 4 to day 6. Nevertheless, when nitrate concentrations increased, concentration values were under initial concentrations. In the control solution and at 1 mg/L Pb, little to no decrease of nitrate concentration was observed.

In the present study, the relative growth of *L. gibba* increased over time in both Cd and Pb treated medium particularly leading to higher phosphates and nitrates removal from the medium. These results suggest that *L. gibba* has a strong capability to endure the toxicity of Cd and Pb at appropriate levels. Plants looked green and healthy at the concentrations of the metals to which *L. gibba* was exposed for both toxicants.

In general, the removal efficiencies of phosphate were more than nitrate removal efficiencies. Hence, duckweed plant is not effective for nitrate and nitrogen removal as compared to phosphate removal.

From the results of Selvarani et al.(2015), it was shown that 0.125 mg/L, 0.250 mg/L and 0.500 mg/L of *Glyphosate* resulted in the decline of the phosphate removal from 75% to 37.5% (almost half) in the presence of duckweed whereas the phosphate removal was 75% without adding toxicant.

This may be due to the fact that plant growth is affected by *Glyphosate* toxicant. In another study conducted by Liu et al. (2017), phosphorus and nitrogen removal was inhibited under higher salt stress.

In our study, there was a significant increase of Cd and Pb contents in the plant at the tested Cd and Pb concentrations as compared to the control values which cause no growth or inhibition of the plant growth. Considering all the treatments, Pb-treated plants accumulated larger quantities of this toxicant compared to Cd-treated plants. Nevertheless, phosphate and nitrate removal is not inhibited.

Conclusions

The dramatic growing in the human population and industrialization caused an increase in water resource pollution. This environmental damage has become a global issue due to the public health concerns. The present study suggested that *L. gibba* has a high tolerant ability to Cd and Pb. It grew well and did not show any visual symptoms. Additionally, heavy metals stress did not inhibit nitrate and orthophosphate removal by the aquatic plant. High removal efficiencies were achieved over the exposure period. Phytoremediation using *L. gibba* seems to be a good option for the simultaneous removal of heavy metals and nutrients (nitrate and phosphate).

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