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Bioaccumulation and Toxicity of Copper and Lead in *Chlorella vulgaris*

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Abstract
This article presents an advance to the knowledge in the solution of heavy metal environmental pollution using living microalgae cells. The study aims to evaluate the toxic effects of copper and lead on the growth of *Chlorella vulgaris* (Beijerinck Novakova, 1890) and to assess its bioaccumulation capacity as a function of the metals concentration and the time of contact with the metals.
Growth inhibition was greater with Cu than Pb. For both metals, the removal efficiency was very fast and decreased with the increase in metal concentration, and the removal efficiency increased with the exposure time. Living algae removed up to 98.7 % of Pb when exposed to low concentrations after 1 hour and exposure and up to 81.97% and 92.53% of Cu after 10 min and 12 h of exposure, respectively. The final metal concentrations in the supernatant and in the microalgae were significant and negatively correlated, which demonstrated the efficiency of *C. vulgaris* in removing metals from wastewaters. Microalgae reduced Cu and Pb from ppm to ppb concentrations in a very short time, so it is recommended for larger scale treatments.

Keywords: Bioremediation; bioaccumulation; *Chlorella vulgaris*; heavy metal removal; water pollution control.

1. **Introduction**

The limited availability of freshwater in many parts of the world and the increasing pollution of these resources demands studies addressing the removal of pollutants. The discharge of heavy metals by metal processing industries is known to have adverse effects on the environment. The disposal of effluents with a high content of Cr, Pb, Cu, and other heavy metals is an increasing concern. The toxicity and health hazards associated with heavy metals have been established beyond any doubt (Volesky 2001). Indeed, wastewater treatment is an ecological and economic necessity (Kumar and Goyal 2009). Metal pollution, for example, can have many biological effects on the structure of freshwater planktonic communities. In particular, they can modify their abundance and richness, and reduce the growth rate of microalgae species, with potentially harmful effects on aquatic ecosystems (Gagneten and Paggi 2009, Gutiérrez et al. 2010, Gagneten et al. 2012).
The methods used to remove metal ions from aqueous solutions include physical, chemical, and biological techniques. Many conventional methods can achieve this goal, such as alkaline precipitation, ultrafiltration, ion exchange columns, electrochemical treatment, filtration membrane technologies, adsorption by activated carbon, and evaporation. However, some of these methods are extremely expensive and ineffective, especially when the metal ion concentration in aqueous solutions is e.g. 1-100 mg L^{-1} (Wang and Chen 2009). Volesky (2001) summarized the advantages and disadvantages of those conventional technologies for removing heavy metals, while Lesmana et al. (2009) and Hashim et al. (2011) compared the efficiencies of different techniques. The need for effective and economically convenient methods for metal removal has motivated the development of new technologies. In recent years, the application of biotechnology to the control of metal pollution has received much attention and it has gradually become a 'hot topic' due to its potential versatility.
An alternative to more expensive technologies is biosorption, using biological materials, such as bacteria, fungi, yeast, microalgae, and macrophytes. These processes may vary significantly depending on the type of material employed (Lodi et al. 2010). Biosorbents can sequester metals rapidly and efficiently, which makes them suitable for reducing the concentrations of heavy metal ions in solution from ppm to ppb levels (Wang and Chen 2006). At present, dead biomass and agricultural waste are mainly employed as biosorbents, although living organisms are also efficient for removing heavy metals (Regaldo et al. 2009; Bajguz 2011; Piotrowska-Niczyporuk et al. 2012). Indeed, the term bioaccumulation is now restricted to processes that involve living biomass, which depend on rapid passive adsorption by chemical groups found on the surfaces of living organisms, followed by slow active uptake. According to Volesky (2007), biological materials can be used for biosorption or the bioaccumulation of metals. However, living

organisms have demonstrated good potential for sewage water treatment but their use has been limited in many cases, especially with acidic water or water with a high metal content.
The effects of heavy metals on microalgae growth and the microalgae efficiency to accumulate metals have been studied extensively, but little information is available about the relationships between the two processes.
The aim of this study was to analyze on a laboratory scale, *Chlorella vulgaris* Beijerinck efficiency in removing copper and lead, and their toxic effects on the microalgae growth, which results on growth inhibition.

2. Materials and Methods

2.1. Chemicals

All experiments were conducted using stock analytical grade solutions: $(NO_3)^2$ Cu (Merck 1.19786.0500-NO_3H 0.5 M/L) and $(NO_3)^2$ Pb (Merck 1.19776.0500-NO_3H 0.5 M/L). Both solutions were used at a concentration of 1000 mg L^{-1}.

2.2. Culture conditions

The *Chlorella vulgaris* (CLV2) strain originated from an algae culture collection maintained at the Scientific Research and Superior Education Center of Ensenada, Baja California, México (CICESE). The cultures were grown for 10 days in 2000 mL flasks in sterile conditions using Bold Basal Medium (BBM) (Sager and Granick 1953): $NaNO_3$, 250 mg L^{-1}; $CaCl_2$ $2H_2O$, 25 mg L^{-1}; $MgSO_4$ $7H_20$, 75 mg L^{-1}; K_2HPO_4, 75 mg L^{-1}; KH_2PO_4,175 mg L^{-1}; NaCl, 25 mg L^{-1}; $FeSO_4$ $7H_2O$, 4.98 mg L^{-1}; H_2SO_4, 0.001 mg L^{-1}; H_3BO_3,11.42 mg L^{-1}; EDTA, 50 mg L^{-1}; KOH, 31 mg L^{-1}; $ZnSO_4$ $7H_2O$, 8.82 mg L^{-1}; $MnCl_2$ $4H_2O$, 14.4 mg L^{-1}; MoO_3, 0.71 mg L^{-1}; $CuSO_4$ $5H_2O$, 1.57 mg L^{-1}; and $Co(NO_3)_2$ $6H_2O$, 0.49 mg L^{-1}. The pH of the culture was 6.25. The culture was maintained at a constant temperature (23 ± 1°C), with uniform and continuous aeration and light (0.72×10^{20} photons m^{-2} s^{-1} ± 20%). Continuous stirring of the culture was achieved using a 100 rpm magnetic plate.

2.3. Growth inhibition tests: Cu and Pb toxic effects on microalgae growth

The experimental treatments were prepared according to algal growth inhibition test standards (OECD 1984). Microalgae were harvested during the exponential growth phase (day 7), before being centrifuged twice (10 min at 3500 rpm) and re-suspended in sterile ultrapure water. The concentration of algae in the culture was assessed directly using a Neubauer hemocytometer chamber (1.02×10^6 cells mL^{-1}) and indirectly using a spectrophotometric method (absorbance 1.5 λ at 650 nm, using a Hatch spectrophotometer). The bioassays were conducted in 250 mL flasks containing 100 mL of BBM medium and an initial cell density of 10^4 cells mL^{-1}. Six different Cu or Pb concentrations were added to the algae culture: The Cu concentrations tested were 0.02 [C1], 0.05 [C2], 0.10 [C3], 0.20 [C4], 0.40 [C5], and 0.80 [C6] mg L^{-1}. The Pb concentrations tested were 0.25 [C1], 0.50 [C2], 1.0 [C3], 2.0 [C4], 4.0 [C5], and 8.0 [C6] mg L^{-1}, with their respective controls (without metals); all trials triplicated. The vessels were maintained in an incubation chamber, under controlled conditions, as follows: temperature = 23 ± 1°C, daily shaking, and continuous lighting (0.72×10^{20} photons m^{-2} s^{-1} ± 20%). Growth was monitored on three occasions by cell counting, i.e., three replicates of 100 µL were sampled at 24, 48, and 72 h, and the cells were counted using a 400× Olympus light microscope. Counting at least 25 squares ensured there was an error of <10% (Venrick 1978). The endpoints considered were the effective concentration (EC_{50}) of each metal obtained at 24, 48, and 72 h of the exposure assay. To determine the EC_{50}, the cell density (cells mL^{-1}), was plotted against the metal concentration and analyzed using the Probalg Program. The growth rate (µ) was determined as follows: $\mu = \text{Log2}(N_1/N_0)/t_1 - t_0$ where N_1 = final cell density (cells mL^{-1}), N_0 = initial cell density (cells mL^{-1}), t_1 = final time, and t_0 = initial time (Guillard 1975). The pH values were measured at the beginning and the end of each assay.

2.4. Cu and Pb removal efficiency test

The microalgae were harvested during the exponential growth phase, centrifuged, and re-suspended in ultrapure sterile distilled water 24 h before starting the tests. The assays were conducted in 100 mL flasks containing 50 mL of ultrapure sterile distilled water with 1.02×10^6 living cells mL^{-1} and three different actual concentrations of Cu (0.45 [C1], 1.30 [C2], and 1.65 [C3] mg L^{-1}) and Pb (1.95 [C1], 2.85 [C2], and 4.83 [C3] mg L^{-1}) and their respective controls (without metals), all triplicated. The vessels were maintained in an incubation chamber at a controlled temperature (23 ± 1°C) with continuous illumination ((0.72×10^{20} photons m^{-2} s^{-1} ± 20%). After 10 min, 30 min, 1 h, 12 h, and 24 h of exposure, the algal cells were harvested by centrifugation at 3500 rpm for 10 min, separating the supernatant from the algal pellet. The total Cu and Pb concentrations in the cells (metals in the algal pellets, i.e., absorbed and adsorbed) were measured by AAS at each time and concentration (see section 2.4.1). All values were the means of three trials with three replicates and were calculated using the following formula, from Perez-Rama et al. (2002): Percentage

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removed (%) = (Amount of metal ion removed: metal in algal pellets) / (Initial amount of metal ion: metal in algal pellets + metal in the supernatant) × 100.

2.4.1. Measurement of the metal concentrations on the supernatant (metal aqueous solutions) and on the algae cells (pellet)

After centrifugation, algae cells (pellet) were digested according to EPA Method 200.3 (US EPA 1991). The supernatant contained the metal ions that remained in the medium after the cells had removed the metal ions. To determine the metal concentration in the supernatant, Cu or Pb solutions were digested according to EPA Method 200.2 (US EPA 1991). The metal concentrations were measured using a Perkin Elmer atomic absorption spectrophotometer (model PE 8000 Analyst), which was equipped with a graphite furnace, using a standard addition technique for calibration. The detection limits for heavy metals were <4 and <3 μg L^{-1} for Pb and Cu, respectively. All glassware and materials were cleaned before metals analysis. Certified analytical grade reagents were used throughout the study. Blanks were run with all experiments. The calibration blank was checked at the beginning and the end of the analysis for each group of samples to ensure that the instrument calibration had not drifted.

2.5. Data analysis

Possible significant differences in the pH were tested by comparing the pH values at the beginning and the end of each assay, using Wilcoxon's matched pairs test.

Possible significant differences in the growth rates of *Chlorella vulgaris* in the control and in the treatments with different concentrations of Cu or Pb were tested using a repeated measures (RM) analysis of variance (ANOVA), followed by Dunnett's test. The same test was used to test for significant differences between the initial Cu or Pb concentrations and the final concentrations, which were measured in the supernatant after different exposure periods. Differences in the percentage (%) of heavy metal removed in the three different concentrations were tested using ANOVA, followed by a Bonferroni post test (Sokal and Rohlf 1969). The normality of the data was confirmed before each test (Kolmogorov-Smirnov test). All statistical analyses were carried out using the package GraphPad InStat (InfoStat 2004). Differences were considered significant if $p < 0.05$. Pearson's correlation coefficients were calculated to test the correlation between the concentrations of Cu and Pb accumulated by *C. vulgaris* and the concentrations of both metals that remained in the supernatants.

3. Results and Discussion

3.1. Cu assays. Growth inhibition tests and Cu removal efficiency

The EC_{50} values in Cu tests were 0.94, 1.00, and 0.45 mg L^{-1} at 24, 48, and 72 h, respectively. There was a decrease in the culture growth rate (μ) as the exposure time and Cu concentration increased. The growth rate was significantly lower and different to the control in C4, C5, and C6 after being exposed for 48 h ($p < 0.01$). After 72 h, there also were significant differences between the control and all the concentrations tested ($p < 0.01$) (Fig 1).

The mean initial and final pH values ranged from 6.20 (± 0.05) to 6.04 (± 0.3), without significant differences at the beginning and the end of each assay ($p > 0.05$).

Yan and Pan (2002) reported lower effective concentrations than the ones determined in the present study. They exposed *Scenedesmus obliquus, Chlorella pyrenoidosa,* and *Closterium lunula* to similar Cu concentrations but at a higher pH (pH = 7) than that tested in this study, which produced EC_{50} values of 50, 68, and 200 μg L^{-1} for the three species, respectively. Wilde et al. (2006) reported that the toxicity of Cu was directly pH-dependent in *Chlorella* spp., where increased pH also increased the toxicity. In the Cu assays in the current study, the mean initial and final pH values ranged from 6.20 (± 0.05) to 6.04 (± 0.39), i.e., there were no significant differences at the start and the end of each assay ($p = 0.578$).

As can be seen in Figure 2, *Chlorella vulgaris* was highly efficient in treatment C1, removing 81.97%, 92.53%, and 95.28% of the available Cu concentration after 10 min, 12 h, and 24 h of exposure, respectively. At 24 h, removed 73.94% and 41.44% in C2 and C3 respectively.

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Figure 1: Growth rate (μ) of *C. vulgaris* after exposure to six different concentrations of Cu (0.02 [C1], 0.05 [C2], 0.10 [C3], 0.20 [C4], 0.40 [C5], 0.80 [C6] mg L^{-1} and the control (without Cu), and Pb (0.25 [C1], 0.50 [C2], 1.0 [C3], 2.0 [C4], 4.0 [C5], 8.0 [C6] mg L^{-1}, and the control (without Pb) for 24 h, 48 h and 72 h, three replicates per treatment. Error bars indicate the (±) standard deviation Asterisks denote significant differences from the control based on ANOVA, RM analysis, and Dunnett's multiple comparisons post test. (*) Significant differences ($p < 0.05$); (**) highly significant differences ($p < 0.01$).

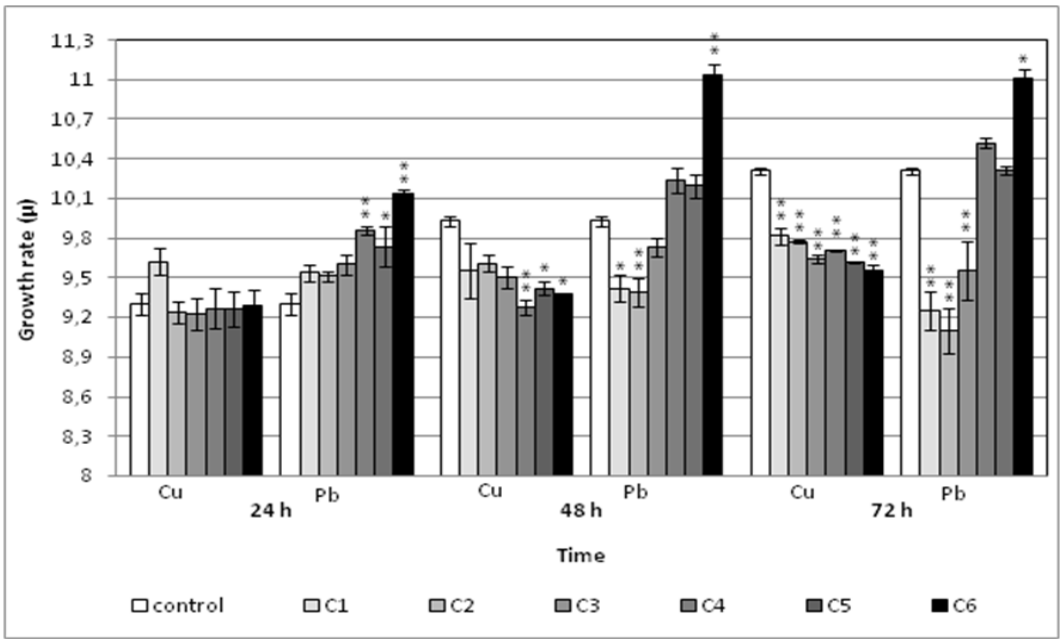

Figure 2: Cu and Pb (%) removal by *C. vulgaris* after 10 min, 30 min, 1 h, 12 h and 24 h exposure to three real concentrations of Cu: 0.45 [C1], 1.30 [C2], and 1.65 [C3] mg L^{-1} and Pb: 1.951 [C1], 2.826 [C2], and 4.83 [C3] mg L^{-1} and the controls (without metals) for 24 h, 48 h and 72 h, three replicates per treatment. Error bars indicate the (±) standard deviation.

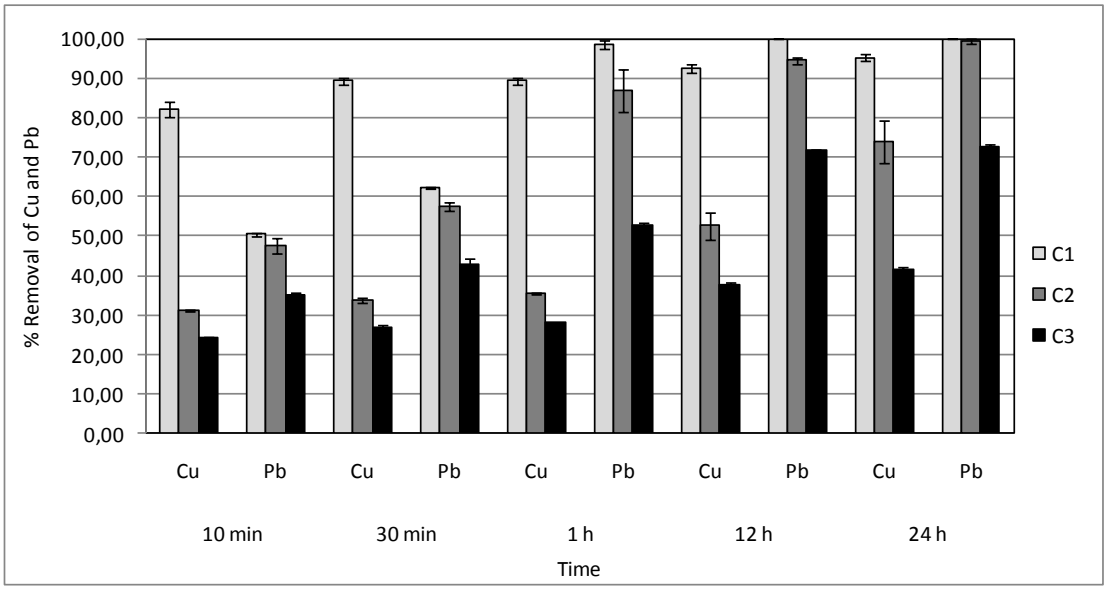

Table 1 shows Cu and Pb concentrations (mg L^{-1}) in the supernatant (metal aqueous solution) after 10 min, 30 min, 1 h, 12 h, and 24 h of microalgae exposure. Although not all the Cu was removed from the metal solution in C1 and C2, there were highly significant differences ($p << 0.01$) after 10 min exposure compared to the beginning of the experiment (0 min) and to all the other times tested. In C3, we found highly significant difference after 30 min of exposure compared to the beginning of the experiment (0 min) and to all the other times tested.

There were significant negative correlations between the final concentration of Cu in algae and that in the supernatant, i.e., r = –0.639, $p = 0.0104$, $r^2 = 0.408$; r = –0.993, $p < 0.0001$, $r^2 = 0.986$; and r = –0.722, $p < 0.0023$, $r^2 = 0.522$ for C1, C2, and C3, respectively.

According to the above results, has been demonstrated that microalgae removed high percentages of Cu after a short (10 min) time of exposure. By contrast, Yan and Pan (2002) reported 6 days of exposure to obtain removal efficiencies of 95%, 79%, and 67% (initial concentration of 0.05 mg L^{-1}) with *C. pyrenoidosa*, *C. lunula*, and *S. obliquus*, respectively.

It is widely known that the pH is a very important factor that affects the adsorption of metal ions because of its effects on metal solubility and the degree of dissociation of functional groups located on sorbent surfaces. According to Vannela and Verma (2006), the metal sorption rate decreases considerably with an increase in alkalinity (pH > 6.0–7.0) because the metal ions precipitate as hydroxides. At pH < 3.0, the hydrogen ions compete with metal ions for the same adsorption sites on the biosorbent. In the Cu assays, the mean pH values were 4.89 (± 0.65) to 4.24 (± 1.07).

Table 1: Cu and Pb concentrations (mg L^{-1}) in the supernatant after 10 min, 30 min, 1 h, 12 h, and 24 h of exposure to *C. vulgaris*. ([1])

Copper						
Time	0 min	10 min	30 min	1 h	12 h	24 h
Control	0.000 (± 0.00)	0.004 (± 0.01)	0.019 (± 0.022)	0.002 (± 0.001)	0.009 (± 0.01)	0.009 (± 0.006)
C1 (0.45 mg L^{-1})	0.45 (± 0.013)	0.088 (± 0.023)**	0.050 (± 0.006)**	0.056 (± 0.01)**	0.044 (± 0.012)**	0.026 (±0.011)**
C2 (1.30 mg L^{-1})	1.3 (± 0.020)	1.015 (± 0.024)**	0.971 (± 0.055)**	0.958 (± 0.008)**	0.677 (± 0.039)**	0.367 (± 0.163)**
C3 (1.65 mg L^{-1})	1.65 (± 0.019)	1.546 (± 0.015)	1.378 (± 0.044)**	1.413 (± 0.028)**	1.289 (± 0.083)**	1.072 (± 0.062)**
Lead						
Time	0 min	10 min	30 min	1 h	12 h	24 h
Control	0.000 (± 0.00)	0.000 (± 0.001)	0.007 (± 0.012)	0.003 (± 0.006)	0.000 (± 0.00)	0.000 (± 0.00)
C1 (1.951 mg L^{-1})	1.951 (± 0.022)	1.377 (± 0.007)**	1.126 (± 0.032)**	0.026 (± 0.044)**	0.000 (± 0.00)**	0.000 (± 0.00)**
C2 (2.862 mg L^{-1})	2.862 (± 0.011)	1.682 (± 0.264)**	1.496 (± 0.096)**	0.341 (± 0.308)**	0.151 (± 0.052)**	0.018 (± 0.032)**
C3 (4.83 mg L^{-1})	4.83 (± 0.013)	2.784 (± 0.096)**	2.754 (± 0.183)**	2.075 (± 0.123)**	1.283 (± 0.051)**	1.134 (± 0.06)**

([1]) (±) Standard deviation (three replicates per treatment). Asterisks denote significant differences between the initial concentration and all the other times, tested with ANOVA, RM analysis, and Dunnett's multiple comparisons post test. (*) Significant differences ($p < 0.05$); (**) highly significant differences ($p < 0.01$).

3.2. Pb assays. Growth inhibition tests and Pb removal efficiency

The EC_{50} values in Pb assays were 0.16, 0.49, and 1.85 mg L^{-1} at 24, 48, and 72 h, respectively. Growth inhibition rate was significantly different and lower to the control in the concentrations C1, C2, and C3 after 48 h and 72 h of exposure ($p < 0.01$). By contrast, Pb stimulated the proliferation of cells, showing higher and significantly different growth rate to the control in the higher

concentration C6 (Fig 1). After 24 h, the growth rate of *C. vulgaris* differed significantly to the control in C4, C5, and C6 during this period ($p < 0.05$). The mean initial and final pH values were 6.17 (± 0.1) to 6.94 (± 1.3), without significant differences at the beginning and the end of each assay ($p > 0.05$).

The percentage of removal in C1 was similar to that in C2 ($p > 0.05$), but in C3 was significantly lower to C1 and C2 ($p < 0.001$). *Chlorella vulgaris* removed 50.43% and 47.69% of Pb after 10 min exposure, 98.74% and 86.89% after 1 h exposure for C1 and C2, respectively. After 1 h, almost all the Pb was removed in C1 (98.74%), and the maximum (100 %) was removed after 12 h. In C2, 94.56% after 12 h and 99.31% after 24 h. In C3, the lower values (71.81% and 72.62%) were obtained after 12 and 24 h (Fig 2).

As can be seen in Table 1, there were highly significant differences in the concentration of Pb in the supernatant (metal aqueous solution) and the initial concentration after all times tested ($p<<0.01$).

There were significant negative correlations between the final concentration of Pb in the algae and that in the supernatant, i.e., $r = –0.826$, $p < 0.0001$, $r^2 = 0.683$; $r = –0.880$, $p < 0.0001$, $r^2 = 0.775$; $r = –0.937$, $p < 0.0001$, $r^2 = 0.878$ for C1, C2, and C3, respectively.

Somewhat lower efficiency was reported by Ferreira et al. (2011), based on dried *C. vulgaris* biomass, where the Pb sorption was mainly affected by the initial metal concentration, with a maximum Pb removal efficiency of 86.5% after 2 h exposure with 0.5 mM = 103.6 mg L^{-1}.

In our survey, using living algae and very low Pb concentrations (1.951–4.83 mg L^{-1}) we obtained higher removal efficiencies.

Arunakumara et al. (2007) reported that low Pb concentrations (0.5–1.0 mg L^{-1}) stimulated the growth of the cyanobacteria *Synechocystis* sp. after 48 h exposure. For the same time period, the Pb concentrations in metal aqueous solution were 0.424, 0.763, and 1.722 mg L^{-1}, after starting with 2, 4, and 6 mg L^{-1}, respectively. By contrast, and similarly to the Cu assays results reported, the current study detected a higher removal efficiency in a lower time (0.026 mg L^{-1} after 1 h and 1.134 mg L^{-1} after 24 h), starting with similar initial Pb concentrations (1.951 and 4.83 mgL^{-1}).

Ferreira et al. (2011) showed that a pH range of 4.0–6.0 was the optimum for sorption of each metal, although Pb precipitated at the higher limit of this range (pH = 6.0). In this study, the mean pH at the start and the end of each assay ranged from 4.89 (± 0.65) to 3.37 (± 0.84) in the Pb assays, which promoted the dissociation of functional groups and the binding of metal ions to cell surfaces, thereby preventing lead precipitation.

3.3. Processes involved in Cu and Pb toxicity

Many processes may affect algal growth, and the effects have been widely studied. For instance, Baron et al. (1995) reported that Cu had a direct impact on photosynthesis by inhibiting photosynthetic electron transport in PSII. Qian et al. (2009) showed that Cu decreased *rbcL* gene transcription, which encodes the large subunit of Rubisco, thereby decreasing the assimilation of CO_2, affecting the activities of enzymes, and inhibiting the mRNA expression of genes encoding related enzymes. According to Nacorda et al. (2007) and Saçan et al. (2007), Pb toxicity can be attributed to its direct interaction with the thylakoid membranes of chloroplasts. These organelles are considered to be the most sensitive to heavy metal exposure in *C. vulgaris* cells.

Several authors have reported different mechanisms of living organisms when exposed to heavy metals, which are associated with their growth and accumulation capacity. In particular, phytohormones and growth regulators have important roles in the regulation of growth and the development of microalgae. They can also modify the bioaccumulation of heavy metals by algae (Piotrowska-Niczyporuk et al. 2012). Recently, Bajguz (2011) studied the effects of brassinolide (phytohormones) on growth, the accumulation of heavy metals by *C. vulgaris*, and the cellular levels of phytochelatins, chlorophyll, monosaccharides, and protein, which confirmed that heavy metals can elicit a variety of adaptive responses.

In this line, Mallick (2004) described mechanisms such as intracellular complexation and detoxification that facilitate the storage of metals, which might also improve the metal removal efficiency. Moreover, several studies have shown that *Chlorella* spp. produce higher levels of phytochelatins, carotenoids, and antioxidant enzymes when exposed to metals. These biomolecules protect the organelles and cell metabolism from adverse heavy metal toxic effects.

Finally, the strong negative correlations between the final metal concentrations in the supernatants and the metal concentrations in the microalgae has demonstrated the efficiency of *C. vulgaris* in removing metals. To the best of our knowledge, this is the first demonstration that *C. vulgaris* can reduce Cu and Pb concentrations from ppm to ppb levels in aqueous solutions after a very short exposure time.

4. Conclusions

The growth rate of *C. vulgaris* was more severely affected by Cu than by Pb.

The metal removal efficiency decreased with increases in the metal concentration and increased with the exposure time.

Microalgae recovered up to 100% of Pb when exposed to low concentrations, 81.97% of Cu after 10 min of exposure and 92.53% after 12 h. Thus, the removal process was rapid, reducing the Cu and Pb concentrations from ppm to ppb levels. The final metal

concentrations in the supernatant and the microalgae were negatively correlated, thereby showing the efficiency of *C. vulgaris* in removing metals from metal aqueous solutions on a laboratory scale. Therefore, this is recommended as an eco-friendly method that should be tested for wastewater treatment on a larger scale.

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