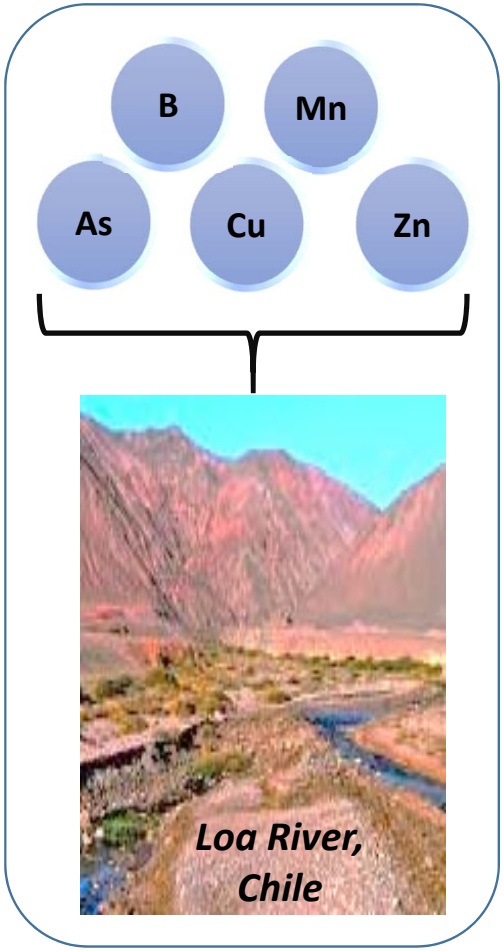
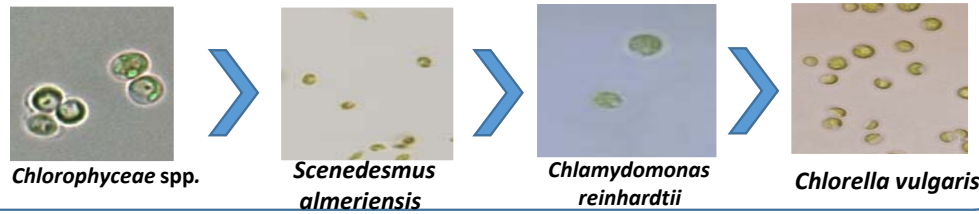
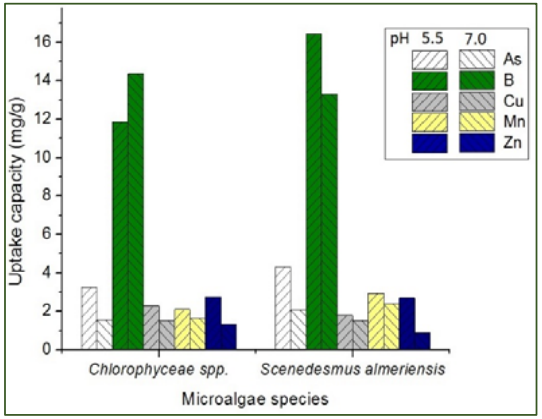
Bioremediation using microalgae biomass



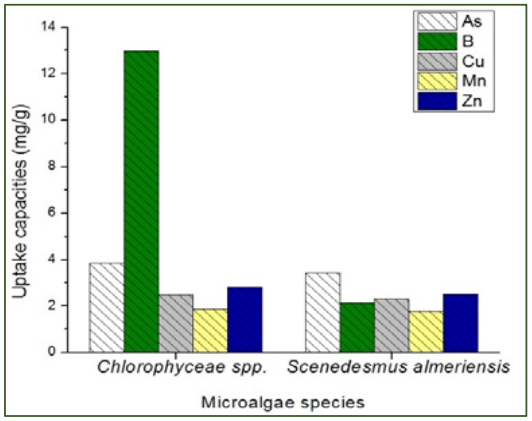
Microalgae tolerance



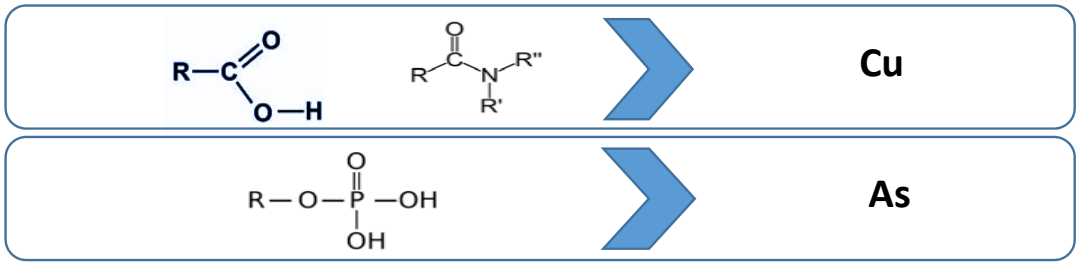
Monometallic Adsorption



Multimetallic Adsorption



FTIR important interactions



Comparative uptake study of arsenic, boron, copper, manganese and zinc from water by different green microalgae

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ABSTRACT

This work represents a comparative uptake study of the toxic elements arsenic, boron, copper, manganese and zinc in monometallic and multimetallic solutions by four green microalgae species (*Chlamydomonas reinhardtii, Chlorella vulgaris*, *Scenedesmus almeriensis* and an indigenous *Chlorophyceae* spp.), evaluating the effect of pH and contact time. Maximum removal efficiencies for each toxic element were 99.4% for Mn (*C. vulgaris*, pH 7.0, 3h), 91.9% for Zn (*Chlorophyceae* spp., pH 5.5, 3h), 88% for Cu (*Chlorophyceae* spp., pH 7.0, 10 min), 40.7% for As (*S. almeriensis,* pH 9.5, 3h) and 38.6% for B (*S. almeriensis*, pH 5.5, 10 min).

B removal efficiencies decreased remarkably in multimetallic solutions (down to 0.2% in *C. reinhardtii*), except for *Chlorophyceae* spp., the only species isolated from a polluted environment. FTIR spectra shown the highest interactions for As (1150-1300 cm^{-1}) and Cu (3300, 1741, 1535, 1350-1400 cm^{-1}). Results confirms microalgae biomass as a potential biosorbent for toxic elements.

HIGHLIGHTS

- *Chlorophyceae* sp. was the most sensible specie, B and Cu interfering its growth.
- Except for *C. vulgaris*, lower toxic elements uptakes were found at pH 7.
- The pH 5.5 enhanced Mn and Zn uptakes in *Chlorophyceae* sp, and *S. almeriensis.*
- Native *Chlorophyceae* sp., shown the best performance in multimetallic studies.
- FTIR spectra of *C. reinhardtii* shown the different binding groups for each element.

Keywords: *adsorption, bioremediation, heavy metals, microalgae, toxic elements*

1. Introduction

Water pollution is considered a top environmental problem that currently affects millions of people in various parts of the world. In this context, heavy metals (HM) entail a severe negative impact on human health and biota due to their toxic nature, recalcitrance and bioaccumulation (Fu and Wang, 2011; Tchounwou et al., 2012). HM are considered stable and non-biodegradable elements that can be bioaccumulated throughout the food chain, thus causing a serious damage to natural ecosystems (Wu et al., 2016).

Technologies based on physicochemical processes, such as chemical precipitation, oxidation, adsorption, ionic exchange, electrochemical treatment and reverse osmosis, are currently available for HM removal in water bodies and wastewaters. Nevertheless, these methods are expensive and inefficient for metal removal from dilute solutions containing less than 100 mg/L of dissolved metal and some of them entail remarkable environmental impacts (Montazer-Rahmati et al., 2011). In this context, bioremediation has emerged as a promising alternative to compete with conventional physical/chemical treatment processes for the removal of toxic minerals and HM. The high affinity and fast kinetics of biological materials make bioremediation cost-effective for the removal of inorganic pollutants at low concentrations (Fu and Wang, 2011). Indeed, algae, bacteria, fungi and plants have been successfully used for HM removal from wastewaters (Akunwa et al., 2014; Konig-Péter et al., 2014).

Among these potential bioadsorbents, microalgae have attracted recent attention based on their large surface area and the presence of multiple binding groups on the microalgal cell surface (Zeraatkar et al., 2016). Cell wall of green microalgae contains heteropolysaccharides composed of carboxylic and sulfate groups for metal sequestration (Kumar et al., 2016). Biosorption and bioaccumulation constitute the most studied mechanisms of microalgae to remove HM from water bodies (Lo et al., 2014; Vijayaraghavan and Balasubramanian, 2015). In this context, multiple microalgae species have been effectively reported as bioremediation agents. In monometallic adsorption studies, *Spirogyra* spp. presented an uptake capacity for Cu (II) of 38.2 mg/g in studies at 100 mg Cu/L (Lee and Chang, 2011), while *Scenedesmus obliquus* supported an uptake of Zn (II) of 22.3 mg/g in studies at 150 mg Zn/L (Monteiro et al., 2011). The Cu (II) sorption capacity of *Chlorella vulgaris* decreased remarkably in the presence of other metals into the

medium, resulting in a loss of 75% and 10% in the Cu uptake efficiency in the presence of Pb (II) and Zn(II), respectively (Rodrigues et al., 2012). Nevertheless, adsorption in multimetallic systems is a very complex process whose mechanisms are not yet fully understood and which requires further studies.

The purpose of this work was to study possible treatments based on microalgae for the highly polluted water of the Loa River, the only watercourse located in the Antofagasta region, Chile. The water of this river contains high concentration of HM, such as copper (Cu), manganese (Mn) and zinc (Zn), but also other toxic elements, including arsenic (As) and boron (B). The origin of this pollution is associated to both, natural causes, and human activity, mainly because the development of industrial mining processes. Water pollution in this area is especially relevant due to the geographic and climatic particularity of the Atacama Desert region. Loa River is the only water supply for indigenous communities and it is an essential resource for industrial and economic activities.

Four microalgae species with promising potential in biosorption (*Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Scenedesmus almeriensis* and an indigenous *Chlorophyceae* spp. isolated from the study area) were tested for the removal of HM, As and B. Adsorption of As and B in microalgae biomass has been scarcely studied and no information about interferences of these elements in HM adsorption has been previously reported. Microalgal viability, growth and uptake capacity tests were conducted for the four microalgae species in monometallic and multimetallic solutions, evaluating the influence of operational factors such as pH and contact time on HM, As and B removal. Finally, FTIR studies were performed to understand the main contact sites between biomass and HM ions and to identify the interferences among the ions in all studies presented.

2. Materials & Methods

2.1. Microalgae cultures and reagents

The microalgae species selected in this study were *Chlamydomonas reinhardtii* 11-32b (SAG Culture Collection of algae), *Chlorella vulgaris* (University of Antofagasta, Chile), *Scenedesmus almeriensis* (University of Almeria, Spain) and a strain isolated from the Loa River in Chile (*Chlorophyceae* spp.). This isolation and purification was carried out by serial dilution and plating. The culture medium used was Bristol medium (UTEX S.A) enriched with a trace metal solution from F/2 of Guillard medium (UTEX media). The pH of the medium was 6.5 ± 0.1. Microalgae biomass was cultivated via inoculation of 50 mL 7-days cultures in exponential growth into 2.1 L gas tight bottles containing 950 mL of fresh culture medium. The bottles were supplied with a constant flow of CO_2 (volume concentration > 99.9%, Abello Linde, Spain) and magnetically agitated (250 rpm) at 23 ± 2ºC for a period of 7 days prior testing. A 12:12 h:h photoperiod using LEDs lamps at 1000 $\mu E/m^2/s$ was used for biomass cultivation, which was conducted under sterile conditions. Optical microscopy analysis was periodically performed in order to check cell culture viability and purity.

The toxic metals and the concentration range of synthetic solutions used in this study were selected based on the composition of the Loa River water. From a recompilation of composition data covering a wide area, representative collecting points were selected and water samples analyzed following the analytical procedure described in section 2.4. Maximum values of toxic elements resulted: 64 mg/L B, 11.7 mg/L As, 1.5 mg/L Cu, 2.1 mg/L Mn, 3.4 mg/L Zn, with pH ranging from 6.85 to 8.49. Initial concentrations of 60

113 mg/L for B, 12 mg/L for As and 3 mg/L for Cu, Mn and Zn were selected, in order to
114 harmonize the HM adsorption study.

115 Stock solutions of As of 600 mg/L and B of 3000 mg/L were prepared using
116 $Na_2HAsO_4 \cdot 7H_2O$ (Sigma Aldrich) and H_3BO_3 (Sigma Aldrich) in ultrapure water. Stock
117 solutions of 150 mg/L of Cu, Mn and Zn were prepared using $CuSO_4 \cdot 5H_2O$, $MnCl_2 \cdot 4H_2O$
118 and $ZnCl_2$ (Sigma Aldrich, Germany) in ultra-pure water. For multimetallic experiments, a
119 synthetic stock solution containing 600 mg/L of As, 3000 mg/L of B and 150 mg/L of Cu,
120 Mn and Zn was prepared. The stock solutions were stored at 4°C and periodically analyzed
121 for quality assurance purposes. Solutions of NaOH (0.1 M) and HCl (0.1 M) were used for
122 pH adjustment. All chemicals employed in this study were analytical grade. All plastic and
123 glass containers were washed in diluted HNO_3 (10% v/v) for 24 h and rinsed 3 times with
124 Milli-Q water (R > 18 MΩ cm) before use.
125

126 ***2.2. Microalgal viability and growth inhibition tests***

127 Viability and growth inhibition tests were carried out to elucidate the influence of metal
128 concentration on the growth of the four microalgae tested. The tests were performed in
129 glass flasks of 120 mL containing culture medium inoculated with a 7-days culture in
130 exponential growth to an initial biomass concentration of 25 mg/L. Monometallic stock
131 solutions were added in order to obtain three different concentrations: As-6, 9 and 12 mg/L;
132 B-60, 120 and 180 mg/L; Cu, Mn and Zn-2, 4 and 6 mg/L in a final volume of 50 mL. The
133 air headspace was filled with CO_2 at 10%. The bottles were incubated under a 12:12 h:h
134 photoperiod at 1000 $\mu E/m^2/s$ at 23 ± 2ºC under magnetic agitation (250 rpm). Control tests
135 without metals were also prepared as above described, adding distilled water to the

cultures. Optical density at 540 nm (OD_{540}), pH and CO_2 headspace concentration were monitored for 7 days.

The growth inhibition (G_i) of the microalgae biomass was calculated by comparing optical densities in terms of percentage, as defined by Eq. 1:

$$G_i = \left(\frac{OD_{540\ control} - OD_{540\ sample}}{OD_{540\ control}}\right) 100 \qquad \text{Eq.1}$$

Where $OD_{540\ sample}$ means optical density of the analyzed experiment and $OD_{540\ control}$ optical density of the control test at identical conditions.

2.3. Influence of pH and contact time on the metal uptake efficiency

Metal uptake experiments were carried out in order to study the removal capacity of the four selected microalgae under different conditions of pH and contact time. All tests were performed batchwise using a total volume of 50 mL of metal solution in glass flasks of 120 mL. The initial concentration of biomass (obtained after centrifugation) for all the tests was 1000 mg/L. The uptake of toxic metals by microalgae biomass was calculated in terms of the experimental uptake capacity (q) as follows (Eq. 2):

$$q = \frac{V(C_i - C_f)}{W} \qquad \text{Eq. 2}$$

Where q is the uptake capacity of the biomass at the specific contact time studied (mg toxic element/g of biomass); C_i and C_f are the initial and final metal concentrations, respectively (mg toxic element/ L), V is the volume of suspension (L) and W is amount of dry microalgae (g).

On the other hand, the metal removal efficiency (Y_R) was also evaluated and expressed in percentage as defined by Eq. 3:

$$Y_R = \left(\frac{C_i - C_f}{C_i}\right) 100 \quad \text{Eq. 3}$$

2.3.1. Monometallic adsorption studies

All monometallic adsorption studies were conducted collecting samples at 10 minutes and 3hours. These times were selected in order to compare the initial adsorption on microalgae surface and the adsorption equilibrium value. Equilibrium was considered at 3 h, as reported in previous works (Abdel-Ghani and El-Chaghaby, 2014; Bulgariu and Bulgariu, 2012). In order to study the effect of pH on the removal capacity of the biomass, pH was modified by addition of HCl (0.1 M) or NaOH (0.1 M) to three initial values (5.5, 7.0 and 9.5) prior to metal addition. This pH range was selected based on the viability range of living biomass, since more extreme conditions can significantly affect microalgal metabolism and induce cell damage (Zeraatkar et al., 2016). An aliquot of 1 mL of monometallic stock solution was added into 49 mL of microalgae suspension in order to achieve an initial concentration of As of 12 mg/L, B of 60 mg/L and Cu-Mn-Zn of 3 mg/L. The tests were conducted under magnetic stirring (250 rpm), at 23 ± 2ºC, in the absence of illumination and without CO_2 addition, in order to minimize the microalgae metabolism influence. Samples of 5 mL were taken using sterile syringe at contact times of 10 min. and 3h. Then, the samples were filtered through 0.45 μm-pore diameter membrane filters (Whatman paper), acidified with 30μL of HNO_3 (0.1 M) and stored at 4º C prior analysis of metal concentrations.

2.3.2. Multimetallic adsorption studies

The initial concentrations of the toxic metals studied were those used in the monometallic tests. In order to work on similar conditions as those existing in photobiorreactors for water treatment, the initial pH was adjusted to 7.0 (Posadas et al., 2015; Valdés et al., 2012). The tests were conducted at 23 ± 2ºC under magnetic agitation (250 rpm). During the first 3 h, no illumination nor CO_2 addition were applied, in order to minimize the microalgae metabolism influence. After this contact time, CO_2 was injected in the closed flasks to reach a headspace concentration of 10% under a 12:12 h:h photoperiod at 1000 μE $m^{-2}s^{-1}$. The systems were incubated for 72 h, the typical hydraulic residence time on photobiorreactors used for water treatment (Acién et al., 2012). Samples of 5 mL were taken at 3 h, 24 h and 72 h of contact time. Samples were filtered through 0.45 μm-pore diameter membrane filters (Whatman paper) and acidified with 30 μL of HNO_3 (0.1 M) prior metal quantification. Final Total Suspended Solids (TSS) was determinated for all the tests. Uptake capacities were calculated respect to the average amount of dry microalgae.

The total molar uptake capacity of the microalgae biomass (mg/mmol) was obtained as the sum of the individual molar uptake capacities, calculated by dividing the (q) value between the molecular mass of each element, as defined by Eq. 4:

$$mq = \sum \frac{q_{toxic\ element}}{MW_{toxic\ element}} \quad \text{Eq.4}$$

Where q is the experimental uptake capacity (Eq. 2), and MW is the molecular mass of each toxic element studied.

2.3.3. Fourier transform infrared spectroscopy (FTIR) analysis

Samples of *C. reinhardtii* after adsorption of different elements at pH 7.0 were analyzed by FTIR. In order to analyze the interactions among the HM studied and the interfering effect

of As and B, monometallic (Cu, Mn and Zn) and multimetallic [B+Cu+Mn+Zn], [As+Cu+Mn+Zn] and [B+As+Cu+Mn+Zn] solutions were studied. Concentrations of As of 12 mg/L, B of 60 mg/L and Cu-Mn-Zn of 3 mg/L were used. The tests were conducted at two different contact times: 10 min and 3 h. Biomass was centrifuged at 4500 rpm for 7 min, dried in an oven at 45° C for 48 h and grinded to a fine powder for FTIR determination. Control test was prepared using raw biomass without metallic solution.

2.4. Analytical methods

CO_2 concentration was measured by gas chromatography in a Bruker 430 GC-TCD (Palo Alto, USA), according to (Cantera et al., 2016). The pH was measured using a pH-meter Basic 20+ (Crison, Spain) and cell growth was monitored spectrophotometrically by optical density at 540 nm (OD_{540}) using a Helios-Alpha spectrophotometer (Thermo Scientific, USA). As, B, Cu, Mn and Zn concentration was determined by Inductively coupled plasma optical emission spectrometry (ICP-OES) in octopole reaction System (HP 7500 cc, Agilent, USA). FTIR spectroscopy measurements were performed at 400 and 4000 cm^{-1} in a Model Tensor 27 (Bruker, USA) - with ATR Golden Gate device Tecknokroma model.

3. Results and discussion

3.1. Microalgal viability and growth inhibition tests

The results evidenced a clear correlation between the growth of biomass, monitored by the increase in optical density and pH, and the consumption of CO_2 (data not shown). Figure 1 depicts the time course of these parameters in a *Chlamydomonas reinhardtii* culture in the

presence of B and Cu. All control tests (absence of metals) presented a lag phase with values in optical density and pH constant between days 0 and 1. Then, culture OD_{540} increased by a factor of 5 between days 1 and 4, while pH increased from 6.5 to > 10 during the same period. Biomass stopped growing at day 4 and a reduction by 10% in the optical density of the cultures was recorded between days 4 and 7. Likewise, a slight decrease in CO_2 concentration was recorded during the first 24 hours of incubation followed by a complete consumption in the following three days.

The results showed that all microalgae species studied were viable regardless of the metal tested, although microalgae metabolisms were partially inhibited in the presence of high concentrations of B, Cu. Tolerance to toxicants was species specific, and microalgae growth was always more affected by the presence of boron likely due to its higher concentration.

Surprisingly, the indigenous *Chlorophyceae* spp., was the most sensitive species, with a severe growth inhibition even at 60 mg/L of B (G_i: 36%). *Scenedesmus almeriensis* growth was also strongly affected by B. Indeed, culture OD_{540} only achieved 56% of the control optical density at 60 mg/L of B and the 20% at higher concentrations. *C. reinhardtii* presented a low inhibition at 60 mg/L of B, but achieved a Gi of 60 % at 120 mg/L and no growth was recorded at 180 mg/L of B. *Chlorella vulgaris* was the more resistant species with no inhibition even at 120 mg/L of B, although B concentrations of 180 mg/L completely inhibited *C. vulgaris* growth.

The most sensitive specie to Cu was *Chlorophyceae* spp., whose metabolism was severely affected at all the concentrations studied. A significant lag phase was presented at 2 mg/L of Cu (Gi: 70% at day 4), although a similar *Chlorophyceae* spp., growth was recorded by

day 7. The time course of pH and CO_2 concentration correlated to biomass growth, confirmed the complete inhibition of *Chlorophyceae* spp., at the highest Cu concentrations. *S. almeriensis* growth was reduced by 10% at 2 and 4 mg/L of Cu, but was severely inhibited at 6 mg/L of Cu (Gi: 80% at day 7). *C. vulgaris* resulted more resistant to inhibition by Cu with Gi: 20% at day 4 and 60% at day 7 at 6 mg/L. Similar Cu tolerance limits were reported in growth inhibition studies at 7 days contact time, with sub-lethal Cu dosages of 1.59 mg/L for *Chlorella sorokiniana* and of 3.18 mg/L for *Scenedesmus acuminatus* (Hamed et al., 2017). These results confirmed the high inhibitory effect of Cu, attributed in previously published works to the influence of this toxic metal in the microalgae metabolism, cell wall structure and cell division (Torres et al., 2017). Only *C. reinhardtii* was tolerant to all the Cu concentrations tested.

On the other hand, the growth of the four microalgae species in the presence of As, Mn and Zn was not affected in the range of concentrations tested, as confirmed by the similar time course of optical density, pH and CO_2 concentration to that of the control. Only a slight lag phase during the growth of *Chlorophyceae* spp., and in the evolution of the pH of its cultivation medium was observed at the highest concentration of As (12 mg/L). Thus, culture OD_{540} by day 4 at 12 mg As/L was 20% lower than that recorded in the control tests. Nevertheless, the concentration of *Chlorophyceae* spp., at 12 mg As/L was similar to that of control by day 7. Surprisingly, although no significant variation in the time course of the optical density or CO_2 concentration compared to the control tests was observed at the 3 Zn concentrations tested, a slight lag phase in the time course of the pH was recorded.

The species in study shown high Zn tolerance compared with previously published results. Therefore, *Nannochloropsis salina* growth was reduced by 50% at 2.64 mg/L Zn

concentration (Dong et al., 2014). Differences in sensitivity could be related with the specie but also with the very low initial biomass concentration of 0.05 mg/L used in that work (Torres et al., 2017; Nalimova et al., 2005).

3.2. Monometallic biosorption studies

The results of the uptake capacity of microalgae at 3 hours at pH 7 showed remarkable differences among species for the metals tested (Figure 2). The As and B removal efficiencies were low, with values ranging from 10.5% (As) and 21.4% (B) for *C. vulgaris* to 20.1% (As) and 25.6% (B) for *C. reinhardtii*. Overall, the uptake capacities were much lower for As (1.31 – 2.56 mg/g) than for B (12.40 -14.84 mg/g), which might be explained by the higher initial concentration of B. Higher removal efficiencies were obtained for Cu, Mn and Zn than for As and B. *C. vulgaris* supported the highest Y_R of 87.9% for Cu, 99.4% for Mn and 88.8% for Zn. Uptake capacities for these toxic metals ranged from 1.51 to 2.65 mg Cu/g, 1.65 to 3.17 mg Mn/g and 0.88 to 2.68 mg Zn/g. Despite the high Y_R found in these tests, the uptake capacities of Cu, Mn and Zn were remarkably lower than those reported in literature likely due to the low metal concentrations used in this research (Mehta and Gaur, 2001; Zeraatkar et al., 2016).

3.2.1 Effect of the pH

The influence of the pH on the uptake capacity (Figure 2) was metal specific. Arsenic adsorption decreased at pH 7.0, with Y_R increasing from 20.1% at pH 7.0 to 33.6% at pH 5.5 and 38.6% at pH 9.5 in *C. reinhardtii* and from 10.5% at pH 7.0 to 32.4% and 29.0% at

pH 5.5 and 9.5, respectively, in *C. vulgaris.* This low adsorption at pH 7 was likely mediated by the pKa (6.8) of As (V) species (Álvarez-Benedí et al., 2005). The highest As removal efficiency was achieved at pH 9.5 with *S. almeriensis* (41.7%).

No clear pH effect was found in the boron adsorption tests. The Y_R for B ranged from 20.4% in *Chlorophyceae* spp., to 33.5% in *C. reinhardtii*, both results at pH 5.5. A negligible pH effect was also observed for Cu biosorption, with lowest removal efficiencies at pH 7 and similar results at pH 5.5 and pH 9.5. Nevertheless, the highest Cu Y_R (87.9%) was found at a neutral pH in *C. vulgaris*. The effect of pH on Cu sorption has been reported in literature with different results depending on the microalgae species and conditions. Optimal uptake capacities were obtained at the range of pH 4-5 for *Chlorella vulgaris* working with 100 mg/L Cu concentrations (Al-Rub et al., 2006) and at pH 6 for *Chlamydomonas reinhardtii* (Flouty and Estephane, 2012).

The maximum uptake capacities of Mn and Zn were obtained at pH 5.5 for all microalgae tested except *C. vulgaris. C. reinhardtii* and *S. almeriensis* supported the highest Mn removal efficiencies at pH 5.5 (Y_R of 92.8% and 92.1%, respectively). The effect of pH on Zn adsorption was severe in *S. almeriensis* and *Chlorophyceae* spp., Y_R decreasing from 91.9% and 89.7% at pH 5.5 to 30.0% and 29.0% at pH 9.5, respectively. Maximum removal efficiencies were also found at pH 6 by Yang et al. (Yang et al., 2015) for Mn (II) in *Chlorella minutissima* UTEX2341, and by Monteiro et al. (Monteiro et al., 2009) for Zn in *Scenedesmus obliquus.*

3.2.2 Influence of the contact time

316 The concentration of all the target metals decreased significantly within the first 10 minutes
317 of contact time, which is typically attributed to the binding of the metal to the cell wall
318 surface (Markou et al., 2015; Winters et al., 2017). Figure 3 shows the difference between
319 uptake capacities (q) at 3 h and 10 min of contact time. Thus, the maximum As uptake
320 capacity was reached after 10 minutes and decreased in most of the tests after 3 h of
321 contact, which suggested the exudation of this toxic element by the biomass (Wang et al.,
322 2015). This reduction in As removal was remarkable at pH 7.0, with Y_R decreasing by a
323 factor of 2 for all the microalgae species studied. However, no decrease in the As uptake
324 capacity with time was observed at pH 9.5. In fact, the Y_R of As even increased in *S.*
325 *almeriensis* from 25.8% at 10 minutes to a maximum value of 41.7% at 3 h.
326 Boron uptake also decreased with the time course of the adsorption experiment. The
327 removal efficiency of B in *S. almeriensis* always decreased with time, this decrease being
328 more remarkable at pH 5.5 (from 38.6% at 10 minutes to 28.3% at 3 h.). No correlation
329 between pH and the decrease in Boron Y_R with time was observed, the highest reductions
330 being recorded at pH 7 (from 36.6% to 21.4%) in *C. vulgaris* and at pH 9. 5 in *C.*
331 *reinhardtii* (from 38.2% to 24.3%). The only test resulting in a remarkable increase of B
332 uptake with time was that of *Chlorophyceae* spp., at pH 5.5. In this context, previous
333 studies using the fungi *Aspergillus versicolor* as biosorbent reported the same trend in B
334 biosorption yields with the time course, with Y_R decreasing from 41.4% at 15 minutes to
335 20% - 25% after 1h - 4h of contact time (Laçin et al., 2015).
336
337 On the other hand, the uptake capacities of Cu, Mn and Zn at pH 5.5 increased with contact
338 time for all species studied. This enhancement in the biosorption performance was
339 especially relevant for S. *almeriensis*. Indeed, this microalga supported increases of in the

340 removal efficiency of Mn and Zn from 50.0% to 92.1%, and from 61.6% to 89.7% when
341 the contact time was increased from 10 min to 3 h. Similarly, *C. reinhardtii* supported
342 increases in Y_R from 55.1% and 44.7% (at 10 min) to 92.8% and 87.0% (at 3 h) for Mn and
343 Zn, respectively. The removal efficiency of Cu and Mn in *C. vulgaris* always increased
344 with the time course regardless of the pH. For instance, the Y_R of this microalga at pH 7 for
345 Cu increased from 55.4% at 10 minutes to 87.9% at 3 h. Finally, the removal efficiency of
346 Zn increased from 36.0% at 10 minutes to 84.2% at 3h at pH 7 in *C. reinhardtii*.
347
348 ***3.3. Multimetallic adsorption studies***
349 No inhibition was detected from initial and final values of biomass concentrations, with
350 microalgae biomass growing in all the control and multimetallic experiments. The
351 measured growth resulted lower than 15% (w/w) in all the tests (data not shown).
352 The uptake capacities recorded in the multimetallic biosorption studies are shown together
353 with monometallic results (3 h, pH 7) for a comparative reason, in Figure 4. The lowest Y_R
354 after 3 hours were found for B, which suggests that the presence of other metals interfered
355 on B adsorption in all microalgae tested (except for *Chlorophyceae* spp.), in spite of the
356 high concentrations of this element in the multimetallic solution. B removal efficiencies
357 decreased from the 21.4% in monometallic solutions to 6.6% in *C. vulgaris*, from 25.6% to
358 0.2% in *C. reinhardtii* and from 22.9% to 3.6% in *S. almeriensis*. Similarly, the Mn
359 adsorption was also impacted by the presence of other metals species in *C. vulgaris* and *C.*
360 *reinhardtii*. Thus, Mn removal decreased from 99.4 and 88.8% Y_R values in monometallic
361 solutions to ≈ 60% at 3 h in multimetallic solutions. On the contrary, the As uptake capacity
362 increased by a factor of 3 in *C. vulgaris*, 2.5 in *Chlorophyceae* spp., and 1.7 in *S.*

363 *almeriensis* in multimetallic compared to monometallic solutions. The highest removal
364 efficiencies in multimetallic systems were obtained for Cu and Zn, with Y_R higher than
365 78% for all microalgae tested. Zn removal accounted for 96% in *C. reinhardtii* in
366 multimetallic solution. In this context, the uptake capacities of heavier metals such as As,
367 Cu and Zn in multimetallic media increased at the expenses of a reduction in the adsorption
368 of lighter elements like B and, to a lesser extent, Mn. The same tendency had been reported
369 in multimetallic solutions in *C. reinhardtii* and *Chlorella pyrenoidosa,* whose uptake
370 capacities of Cu and Zn were twice higher than that of Mn (Zhao et al., 2013).
371 Nevertheless, these results suggested that the recorded interference among metals cannot be
372 attributed to a competition by binding sites. The total molar uptake capacities, and
373 therefore the number of occupied sites in *S. almeriensis*, *C. vulgaris* and *C. reinhardtii,* was
374 lower in multimetallic solutions than the molar uptake capacities of B in monometallic
375 solutions (Figure 5). The same trend was observed in terms of mass uptake capacities.
376 However, this phenomenon was not presented in *Chlorophyceae* spp., showing even an
377 increase on its uptake capacities of As, Cu, Zn and Mn while maintaining its B adsorption
378 potential. The only species coming from an environment rich in B, *Chlorophyceae* spp.,
379 supported the best removal efficiencies within the first 3 h, with Y_R of 30.9% for As, 22%
380 for B, 85.2% for Cu, 66.1% for Mn and 95.9% for Zn. On the contrary, the minimum
381 removal efficiencies were recorded in *C. reinhardtii*, with Y_R of 18.8% for As, 0.2% for B,
382 78.1% for Cu and 56.7% for Mn, but 88.3% for Zn.

383

384 On the other hand, a limited impact of the contact time on As and B adsorption in
385 multimetallic solutions was observed regardless of the microalgae (Figure 4). The B uptake

386 capacity of *C. vulgaris* decreased with time down to a removal efficiency of 1% as reported
387 in section 3.2.2 in monometallic solutions. Only a slight increase with time of B removal
388 efficiency in *C. reinhardtii* (Y_R 5.5%) was observed. The Mn uptake capacities of *S.*
389 *almeriensis* and *C. reinhardtii* increased with the contact time in multimetallic solutions,
390 with Y_R increasing from 63.0% and 56.7% at 3 h to 78.0% and 85.6% at 72h, respectively
391 (as recorded in monometallic solutions). The Zn removal efficiencies decreased with time
392 for all the species. This reduction was remarkable in *Chlorophyceae* spp., and *C. vulgaris*
393 and resulted in Y_R values at 72 h of 42.5% and 49.4%, respectively. The Cu uptake capacity
394 of *Chlorophyceae* spp., also decreased with the contact time, with an associated reduction
395 in Cu removal efficiency from 85.2% to 61.8%. Overall, these results did not suggest the
396 occurrence of competition for free sites in the microalgae cell wall since the reduction in
397 the uptake capacity over time for some metals was not balanced by the increase in the
398 adsorption of others (Figure 5).
399

400 ***3.4. Fourier transform infrared spectroscopy (FTIR) analysis***

401 *C. reinhardtii* was selected as a model microalga for the FTIR analysis due to the
402 remarkably different uptake capacities exhibited in monometallic and multimetallic
403 solutions. FTIR spectra of *C. reinhardtii* incubated for 10 min and 3h in different metallic
404 solutions were compared to raw biomass as control (e-supplement Fig.1). The spectrum
405 after 10 min exposure to the multimetallic solution of B, As, Cu, Mn and Zn showed
406 important peak modifications in the ranges 1150-1300 cm^{-1} (related with phosphate ester
407 group P=O), 1350-1400 cm^{-1} (amide III), 1535 cm^{-1} (amide II), 1645 cm^{-1} (amide I) and
408 3300 cm^{-1} (O-H and N-H stretching). Nevertheless, the intensity of these peaks decreased

409 after biomass exposure for 3 h to the multimetallic solution. Interference in the interaction
410 of cations with the carboxyl and amide groups have been previously reported in
411 multimetallic adsorption studies of Ni, Zn and Pb in *C. vulgaris* and *Arthrospira (Spirulina)*
412 *platensis* (Rodrigues et al., 2012), and of Zn in *C. vulgaris* (Ferreira et al., 2011).

413 The spectra of *C. reinhardtii* after a 10 minutes exposure to monometallic solutions showed
414 a remarkable impact of Cu on the amide III and the 1000-1150 cm^{-1} zone, which are related
415 to C-C and C-O stretching mode of the polysaccharides hydroxyl groups. Cu was the only
416 metal affecting the 3300 cm^{-1} zone, probably due to the formation of aqueous complexes.
417 Peaks of 1535 cm^{-1} and 1741 cm^{-1} (C=O group) were also affected by Cu. The impact of Cu
418 was remarkably reduced after 3 h of exposure, although the change in the uptake capacity
419 with contact time was not relevant for this metal (section 3.2.2). Mn and Zn caused a minor
420 modification in the FTIR spectrum compared to the control biomass.
421 The impact of As and B was assessed by adding these elements to a mixture containing Cu,
422 Mn and Zn, showing their respective FTIR spectrum with the control at Figure 6e, 6f.
423 Important modifications in the spectrum of [As-Cu-Mn-Zn] at 1153 cm^{-1} and 1228 cm^{-1}
424 bands were presented. These bands modifications (which increased with the exposure time)
425 suggested that As adsorption was mediated by the presence of the P=O groups in the *C.*
426 *reinhardtii*. On the other hand, the main differences in the FTIR spectrum of the biomass
427 exposed to [B-Cu-Mn-Zn] quaternary mixtures compared to the raw biomass control can be
428 explained by the presence of Cu and Mn in the solution. A negligible effect of boron was
429 observed. These results agree with the low B uptake capacity of *C. reinhardtii* in
430 multimetallic solutions.

431

4. Conclusions

Viability tests demonstrated tolerance of the four microalgae, resulting *Chlorophyceae* spp., the most sensible with inhibition starting from B 60 mg/L and Cu 4 mg/L. Except for *C. vulgaris,* higher uptake capacities were found when shifting pH from neutrality, especially for As, B and Cu. Maximum Mn and Zn uptakes with *Chlorophyceae* spp., and *S. almeriensis* were obtained at pH 5.5. *Chlorophyceae* spp., shown a relevant performance in multimetallic studies, enhancing As and metals removal, and maintaining B uptake, which decreased dramatically in other microalgae. FTIR shown interaction of Cu with carboxyl and amide groups, and As with P=O group.

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565

Figures captions

Figure 1. Time course of the optical density, CO_2 headspace concentration and pH in *Chlamydomonas reinhardtii* cultures (Initial concentration: 0.025 mg/L) in the presence of Boron (A, C, E) and Copper (B, D, F); [–■–]: Control (0 mg/L), [-●-]: (B 60 mg/L, Cu 2 mg/L), [▲]: (B 120 mg/L, Cu 4 mg/L), and [-▼-]: (B 180 mg/L, Cu 6 mg/L).

Figure 2. Influence of the pH on the 3h uptake capacities (mg/g) of 1g/L suspensions of *Chlorophyceae* spp. (CS), *Scenedesmus almeriensis* (SA), *Chlorella vulgaris* (CV) and *Chlamydomonas reinhardtii* (CR) for monometallic solutions with initial concentrations of A) Arsenic 12 mg/L, B) Boron 60 mg/L, C) Copper 3 mg/L, D) Manganese 3 mg/L and E) Zinc 3 mg/L, at pH 5.5 (■), pH 7.0 (▨) and pH 9.5 (▩).

Figure 3. Difference between uptake capacities (q) at 3 hours and 10 min of contact time (mg/g) in 1g/L suspensions of ■: *Chlorophyceae* spp. (CS), ▨: *S. almeriensis* (SA), ▩: *C. vulgaris* (CV), ▤: *C. reinhardtii* (CR), for monometallic solutions with initial concentrations of A) Arsenic 12 mg/L, B) Boron 60 mg/L, C) Copper 3 mg/L, D) Manganese 3 mg/L and E) Zinc 3 mg/L.

Figure 4. Uptake capacities (mg/g) at pH 7 for A) Arsenic, B) Boron, C) Copper, D) Manganese and E) Zinc in 1g/L suspensions of *Chlorophyceae* spp. (CS), *Scenedesmus almeriensis* (SA), *Chlorella vulgaris* (CV), *Chlamydomonas reinhardtii* (CR). Monometallic solution, 3h: (■), Multimetallic solution, 3h: (▨), Multimetallic solution, 24h: (▩) and Multimetallic solution, 72h: (▤).

Figure 5. Comparison of molar uptake capacities (mmol/g biomass) of boron monometallic solution and multimetallic solutions at pH 7 in 1 g/L suspensions of *Chlorophyceae* spp.

(CS), *Scenedesmus almeriensis* (SA), *Chlorella vulgaris* (CV), *Chlamydomonas reinhardtii* (CR), in A) Monometallic boron solution- 3h, B) Multimetallic - 3h, C) Multimetallic - 24h and D) Multimetallic - 72h. For Boron (), Arsenic (), Copper (), Manganese () and Zinc ().

Figure 1.

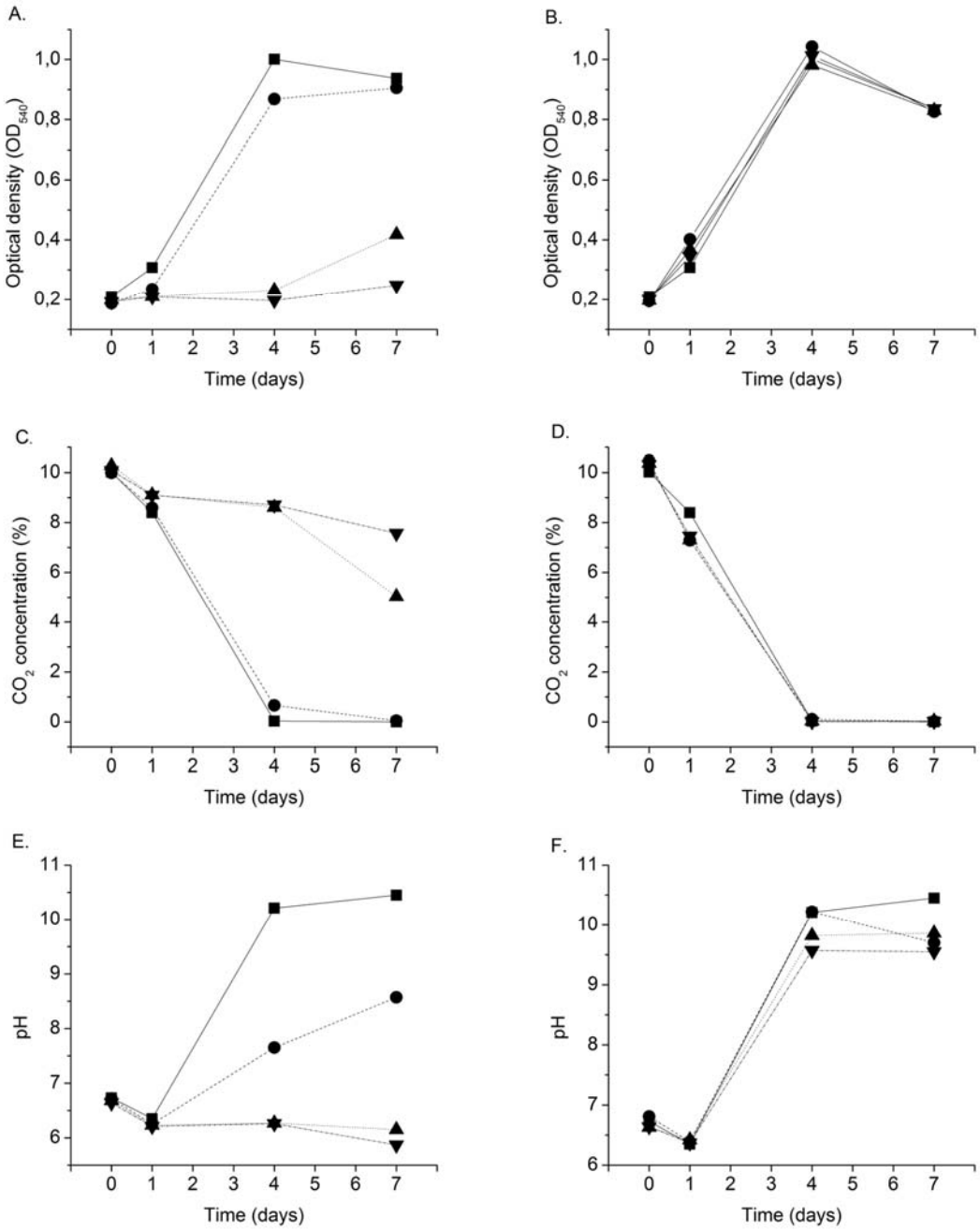

A.
Optical density (OD_{540})
Time (days)
B.
Optical density (OD_{540})
Time (days)
C.
CO_2 concentration (%)
Time (days)
D.
CO_2 concentration (%)
Time (days)
E.
pH
Time (days)
F.
pH
Time (days)

Figure 2.

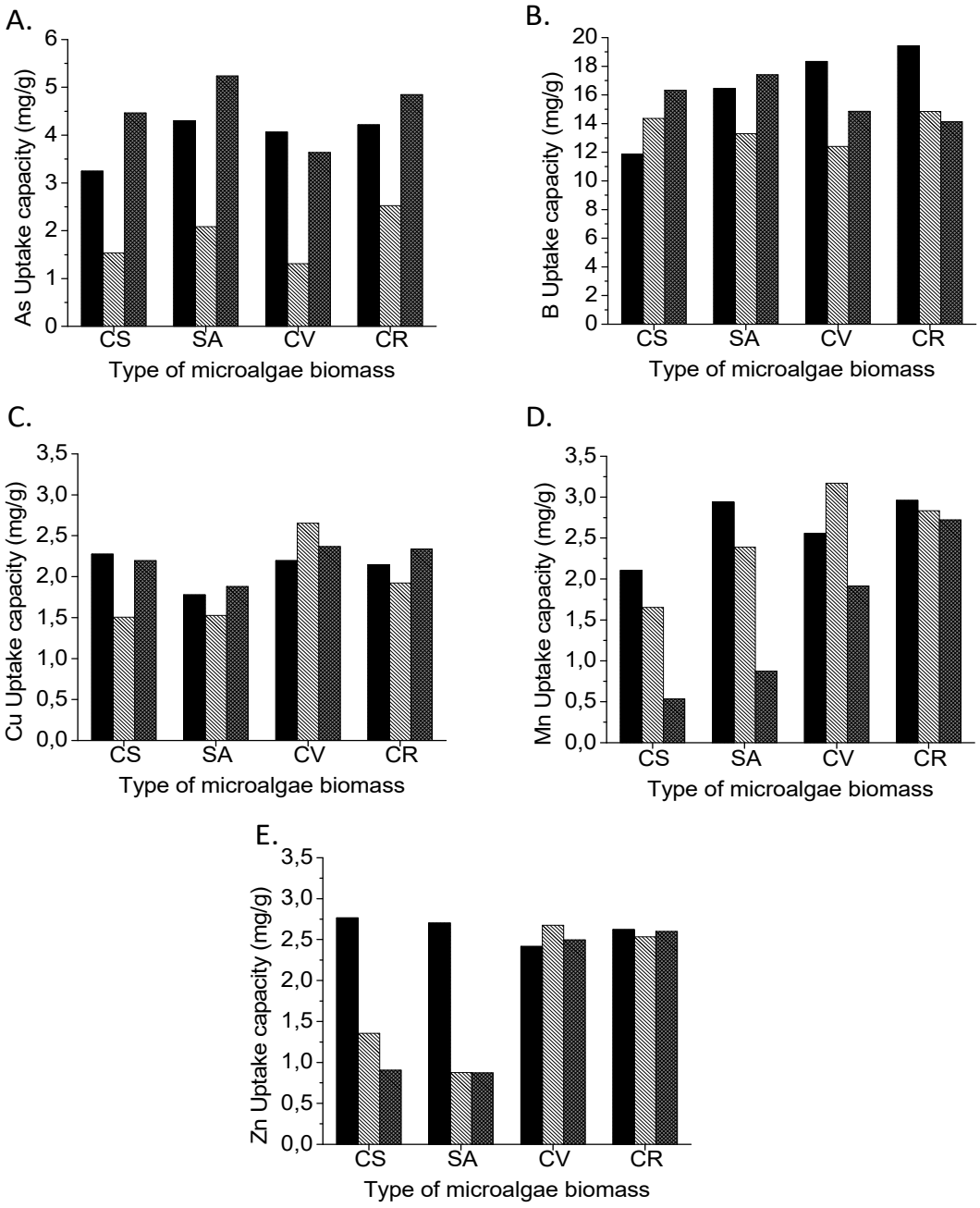

A.
As Uptake capacity (mg/g)
0
1
2
3
4
5
6
CS
SA
CV
CR
Type of microalgae biomass
B.
B Uptake capacity (mg/g)
0
2
4
6
8
10
12
14
16
18
20
CS
SA
CV
CR
Type of microalgae biomass
C.
Cu Uptake capacity (mg/g)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
CS
SA
CV
CR
Type of microalgae biomass
D.
Mn Uptake capacity (mg/g)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
CS
SA
CV
CR
Type of microalgae biomass
E.
Zn Uptake capacity (mg/g)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
CS
SA
CV
CR
Type of microalgae biomass

Figure 3.

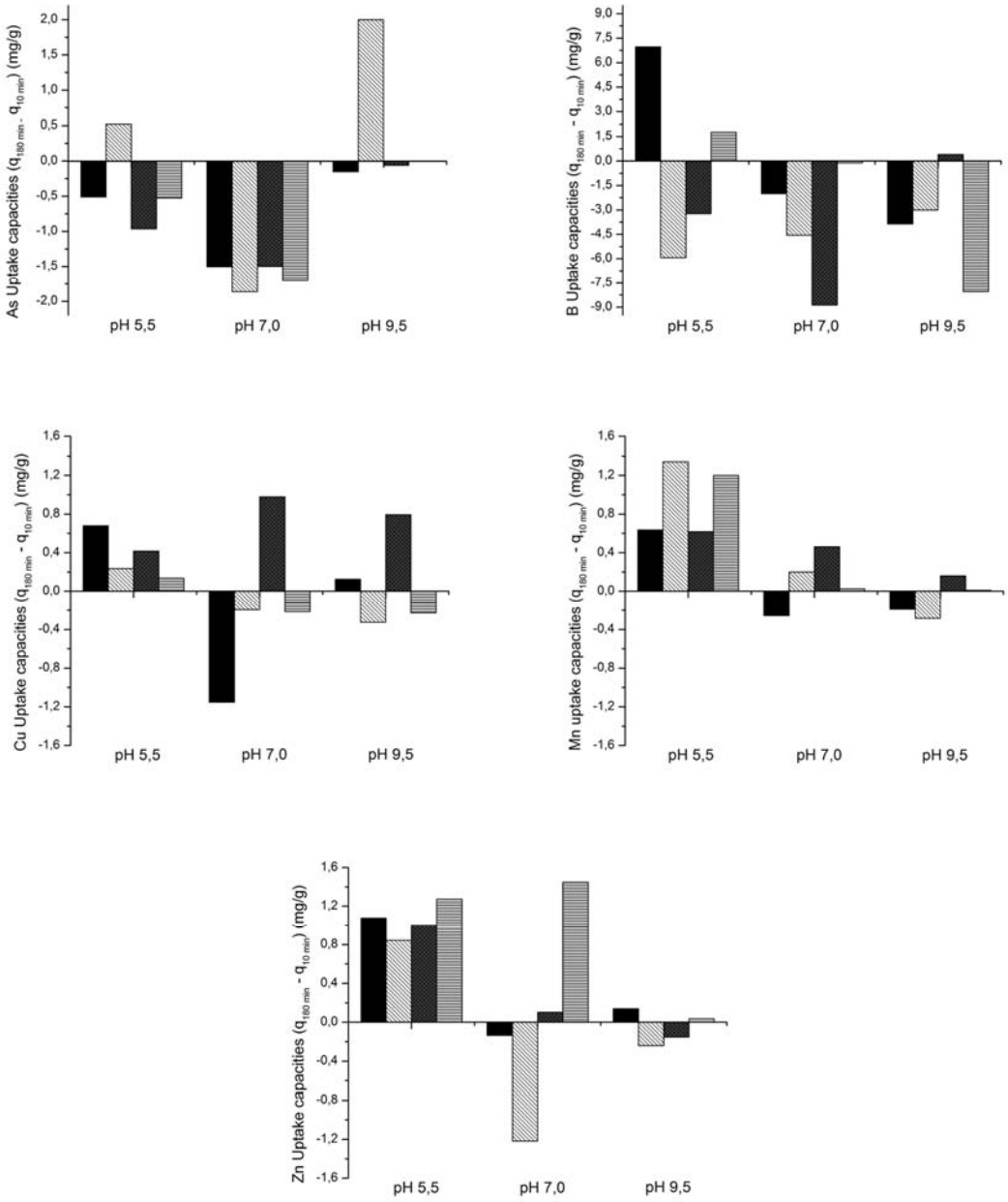

As Uptake capacities ($q_{180\ min}$ - $q_{10\ min}$) (mg/g)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
pH 5,5
pH 7,0
pH 9,5
B Uptake capacities ($q_{180\ min}$ - $q_{10\ min}$) (mg/g)
9,0
7,5
6,0
4,5
3,0
1,5
0,0
-1,5
-3,0
-4,5
-6,0
-7,5
-9,0
pH 5,5
pH 7,0
pH 9,5
Cu Uptake capacities ($q_{180\ min}$ - $q_{10\ min}$) (mg/g)
1,6
1,2
0,8
0,4
0,0
-0,4
-0,8
-1,2
-1,6
pH 5,5
pH 7,0
pH 9,5
Mn uptake capacities ($q_{180\ min}$ - $q_{10\ min}$) (mg/g)
1,6
1,2
0,8
0,4
0,0
-0,4
-0,8
-1,2
-1,6
pH 5,5
pH 7,0
pH 9,5
Zn Uptake capacities ($q_{180\ min}$ - $q_{10\ min}$) (mg/g)
1,6
1,2
0,8
0,4
0,0
-0,4
-0,8
-1,2
-1,6
pH 5,5
pH 7,0
pH 9,5

Figure 4.

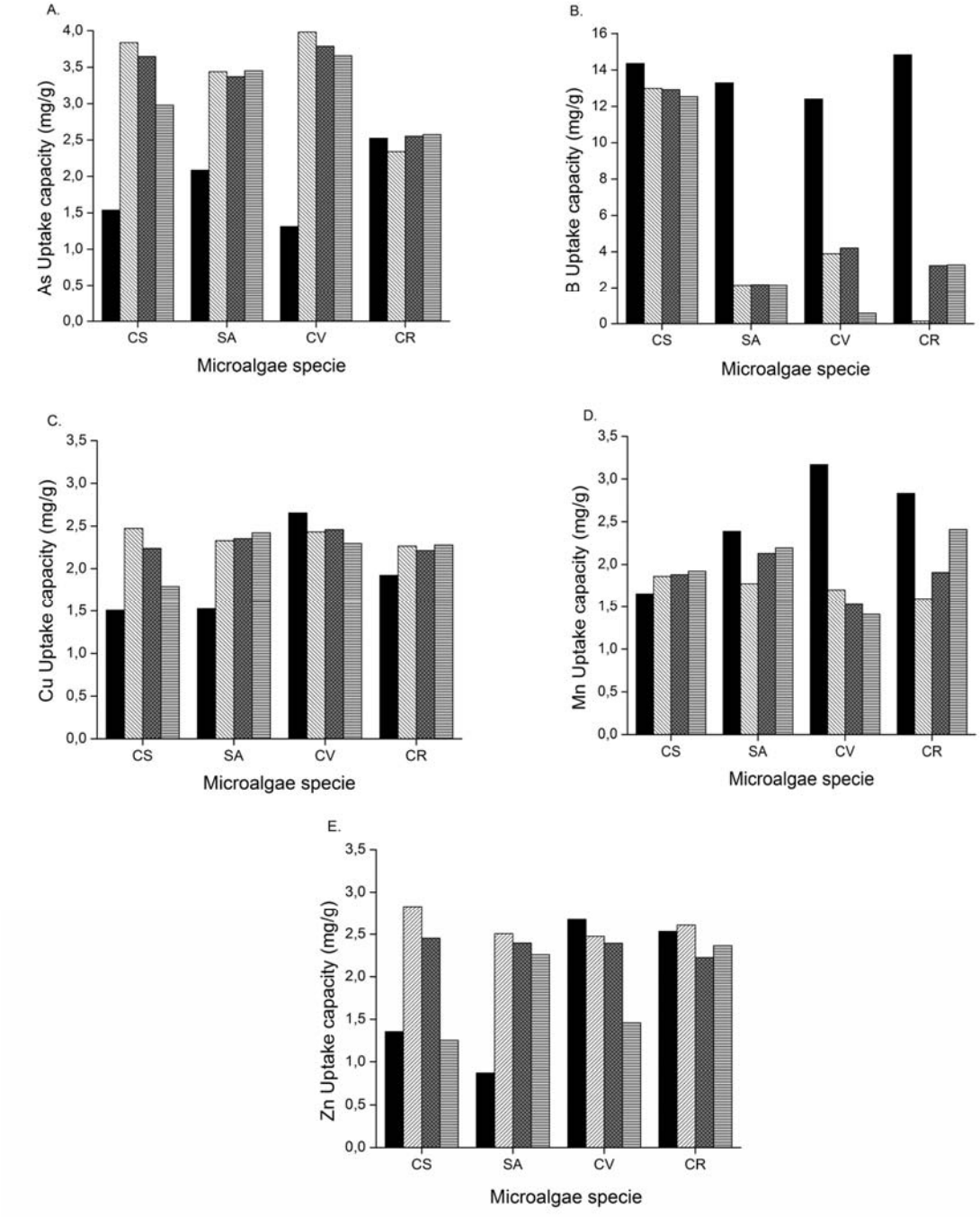
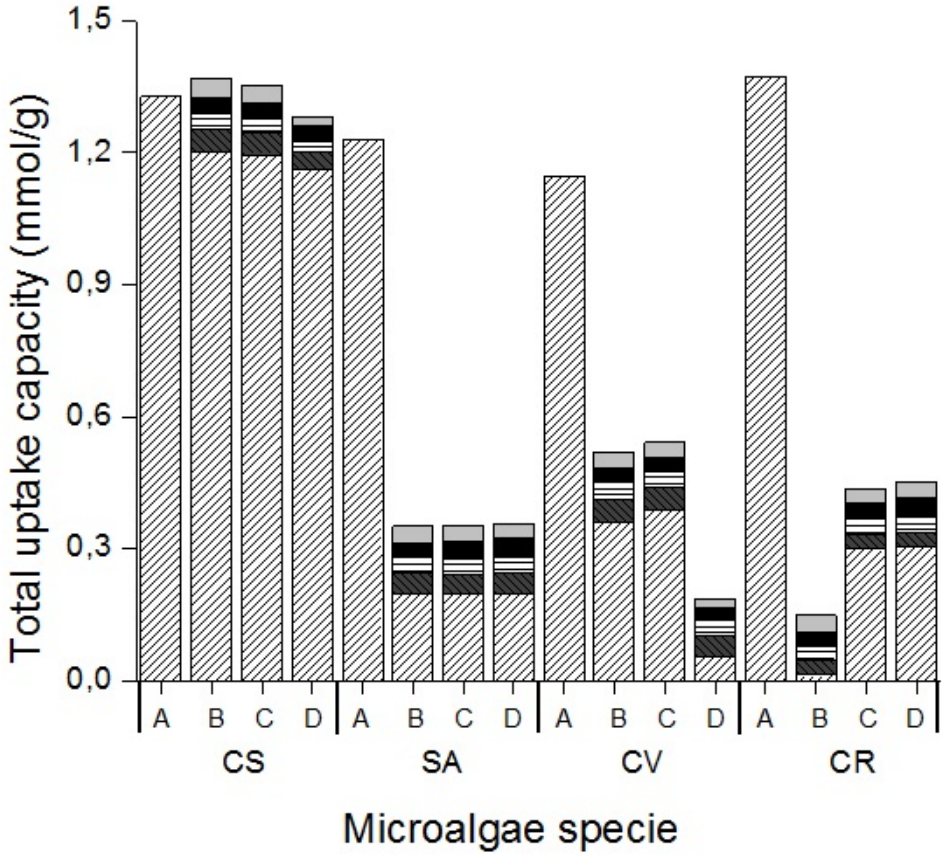

A.
As Uptake capacity (mg/g)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CS
SA
CV
CR
Microalgae specie
B.
B Uptake capacity (mg/g)
16
14
12
10
8
6
4
2
0
CS
SA
CV
CR
Microalgae specie
C.
Cu Uptake capacity (mg/g)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CS
SA
CV
CR
Microalgae specie
D.
Mn Uptake capacity (mg/g)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CS
SA
CV
CR
Microalgae specie
E.
Zn Uptake capacity (mg/g)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CS
SA
CV
CR
Microalgae specie

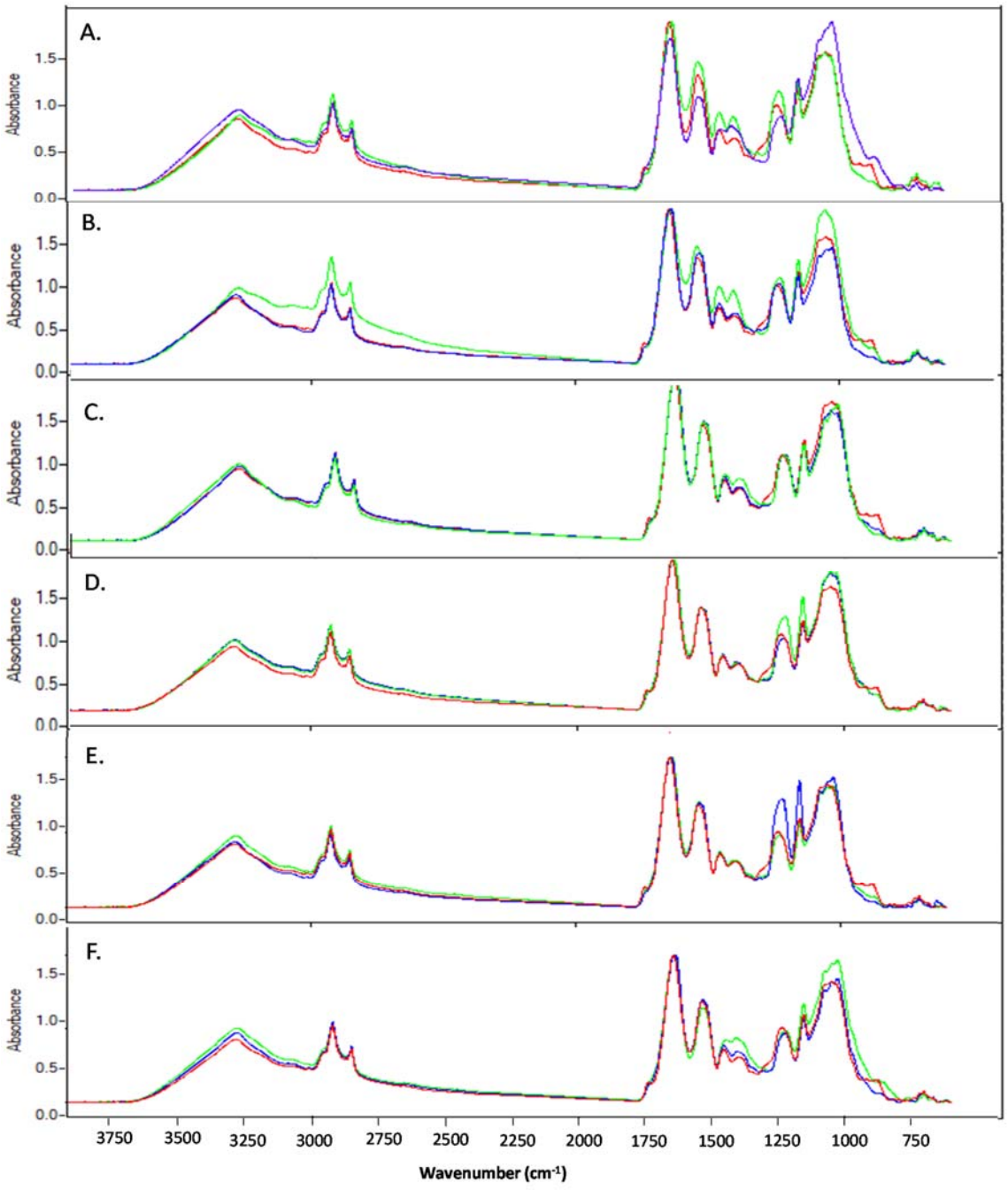
Figure 5.


Total uptake capacity (mmol/g)
1,5
1,2
0,9
0,6
0,3
0,0
A B C D A B C D A B C D A B C D
CS
SA
CV
CR
Microalgae specie

Supplementary material

Figure 1. FTIR spectrum comparison among raw *C. reinhardtii* biomass (Red) and *C. reinhardtii* biomass incubated with different solutions 10 min (Green) and 3 h (Blue). A) Multimetallic solution [As-B-Cu-Mn-Zn], B) Cu monometallic solution, C) Mn monometallic solution, D) Zn monometallic solution, E) Quaternary solution [As-Cu-Mn-Zn], F) Quaternary solution [B-Cu-Mn-Zn].

Figure 1.


A.
B.
C.
D.
E.
F.
Absorbance
1.5
1.0
0.5
0.0
3750
3500
3250
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
Wavenumber (cm-1)