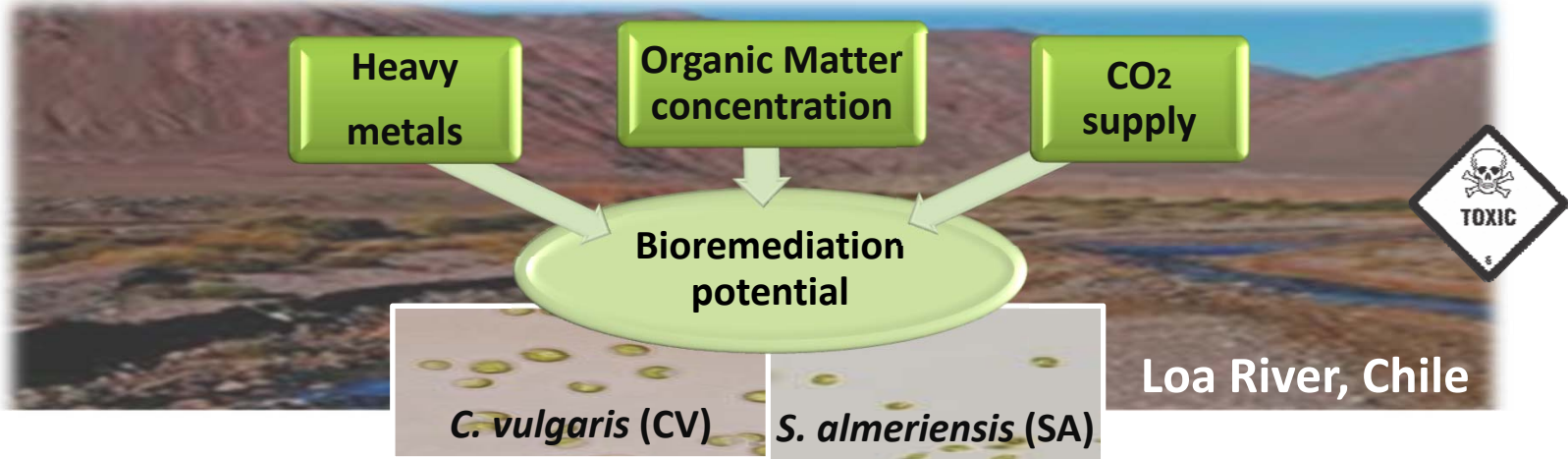
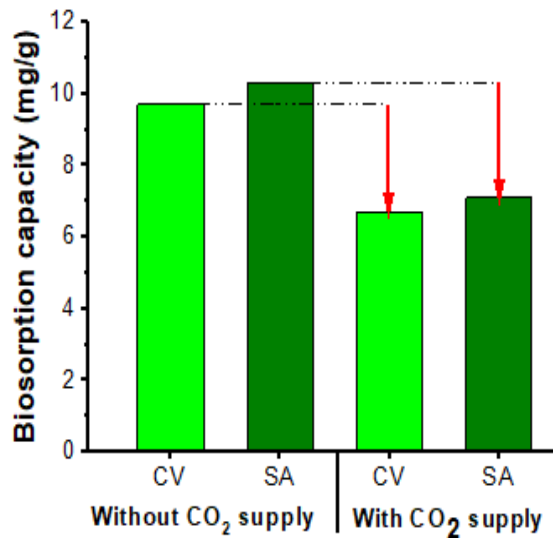


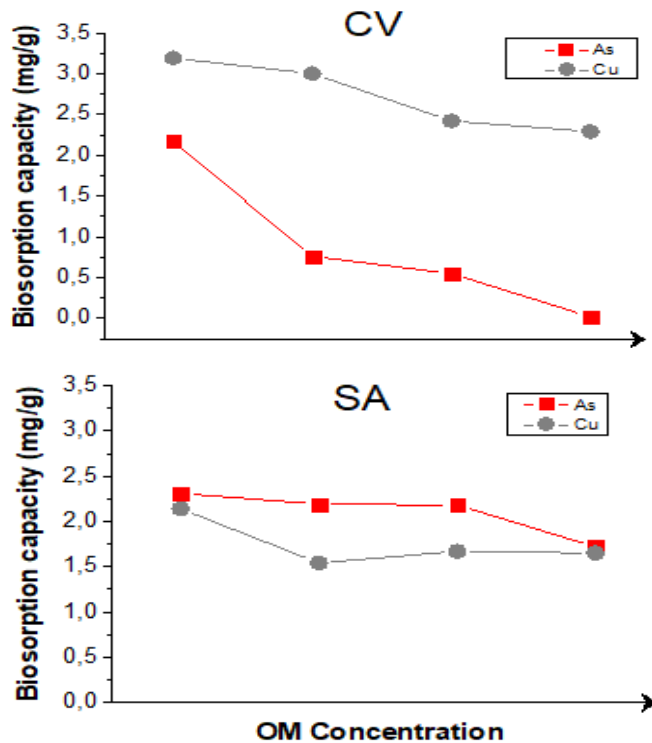
HM Bioremediation by microalgae



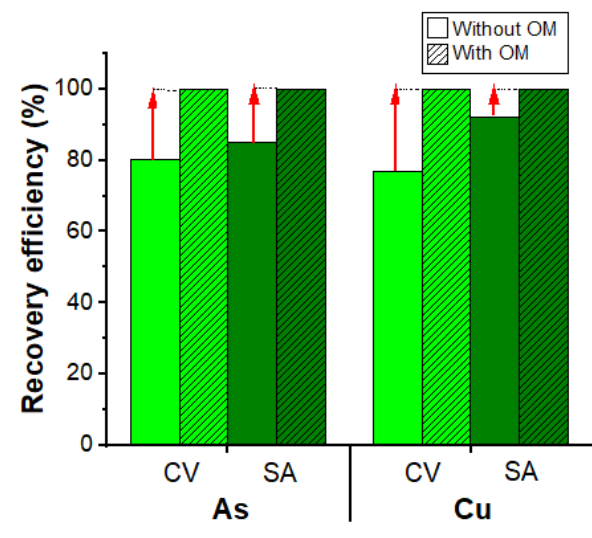
CO₂ supply



Organic Matter Influence



Recovery



HCl 0.1 M

**Influence of organic matter and CO₂ supply on bioremediation of heavy metals by
Chlorella vulgaris and *Scenedesmus almeriensis* in a multimetallic matrix**

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DECLARATIONS OF INTEREST: NONE

1 ABSTRACT

2 This research evaluated the influence of organic matter (OM) and CO₂ addition on the
3 bioremediation potential of two microalgae typically used for wastewater
4 treatment: *Chlorella vulgaris* (CV) and *Scenedesmus almeriensis* (SA). The heavy metal
5 (HM) removal efficiencies and biosorption capacities of both microalgae were determined
6 in multimetallic solutions (As, B, Cu, Mn, and Zn) mimicking the highest pollutant
7 conditions found in the Loa river (Northern Chile). The presence of OM decreased the total
8 biosorption capacity, specially in As (from 2.2 to 0.0 mg/g for CV and from 2.3 to 1.7 mg/g
9 for SA) and Cu (from 3.2 to 2.3 mg/g for CV and from 2.1 to 1.6 mg/g for SA), but its
10 influence declined over time. CO₂ addition decreased the total HM biosorption capacity for
11 both microalgae species and inhibited CV growth. Finally, metal recovery using different
12 eluents (HCl, NaOH, and CaCl₂) was evaluated at two different concentrations. HCl 0.1 M
13 provided the highest recovery efficiencies, which supported values over 85% of As, 92% of
14 Cu, and ≈ 100% of Mn and Zn from SA. The presence of OM during the loaded stage
15 resulted in a complete recovery of As, Cu, Mn, and Zn when using HCl 0.1 M as eluent.

16 Keywords: *bioremediation, CO₂, heavy metal, microalgae, organic matter, toxicity.*

17

18 HIGHLIGHTS

- 19 • “SA showed a better bioremediation potential than CV for HM-laden wastewater
20 treatment”
- 21 • “OM enhanced cell growth but decreased the HM removal 22.7% for CV, and 11.1%
22 for SA.”
- 23 • “CO₂ addition decreased the total biosorption capacity in a 27% for CV, and 35%
24 for SA.”

- 25 • *“As was the HM most remarkably affected by the OM presence and CO₂ addition.”*
- 26 • *“HCl 0.1M resulted more effective than NaOH, CaCl₂, and HCl 0.2M for HM*
- 27 *recovery.”*

28

29 **1. Introduction**

30 Heavy metal (HM) pollution in bodies of water is an environmental problem that affects
31 millions of people as well as important economic activities worldwide. Conventional
32 technologies such as chemical precipitation, adsorption, ionic exchange, electrochemical
33 treatment and reverse osmosis are expensive, not eco-friendly and not efficient for the
34 treatment of dilute HM laden effluents (<100 mg/L) (Montazer-Rahmati et al., 2011). In
35 addition, the increasingly stringent environmental regulations and public awareness of HM
36 pollution have triggered the quest for novel and cost-competitive remediation technologies.

37 In this context, biosorption has arisen as a green alternative to conventional
38 technologies for the treatment of effluents containing dilute metal concentrations (Suresh
39 Kumar et al., 2015). This technique is based on the high affinity and fast adsorption kinetics
40 exhibited by organisms such as bacteria, plant, microalgae species, or a mixture of them,
41 towards inorganic pollutants (Bilal et al., 2018; Li et al., 2018; Zhang et al., 2012). In this
42 context, microalgae have emerged as a promising biosorbent for water treatment due to the
43 presence of a high number of functional groups in their cell wall that contribute to the
44 removal of toxic elements from water in a simple, efficient, and economic manner
45 (Zeraatkar et al., 2016). Previous studies have demonstrated the ability of microalgae
46 species to adsorb HM ions from water, which confirms their high bioremediation potential
47 (Cameron et al., 2018; Torres et al., 2017). For instance, a metal biosorption of 22.3 mg Zn
48 (II)/g was obtained in a solution with 150 mg Zn/L, using the green microalgae

49 *Scenedesmus obliquus* (Monteiro et al., 2011). Moreover, *C. vulgaris* exhibited a
50 biosorption capacity of 45.4 mg As/g in a solution with 200 mg As/L in an experiment of 7
51 days of contact time (Jiang et al., 2011).

52 On the other hand, many recent research efforts have focused on the integration of
53 wastewater treatment (WWT), CO₂ mitigation and bioenergy production processes in
54 microalgae-based photobioreactors (Gani et al., 2017; Rugnini et al., 2019). In this context,
55 domestic and agroindustrial wastewaters contain high concentrations of organic matter
56 (OM) and nutrients suitable for the growth of microalgae (B. Dong et al., 2014; Sahu et al.,
57 2013). The performance of a microalgae-based WWT system depends directly on the
58 capacity of microalgae to assimilate effectively inorganic carbon (CO₂), organic carbon,
59 and nutrients like N and P, with the purpose to produce clean water and low-cost biomass
60 that can be used for biofuels or for the generation of other bio-products (Abou-Shanab et
61 al., 2013; Salama et al., 2017). Nevertheless, typical industrial and livestock wastewaters
62 not only contain nutrients but also hazardous HM. The capacity of the microalgae used in
63 WWT photobioreactors to remove HM in these wastewaters has been widely reported. For
64 instance, a complete reduction of Cu and a 64.96% Zn removal were obtained in *C.*
65 *vulgaris* cultures in sewage after 10 days at neutral conditions (El-Sheekh et al., 2016).
66 Nevertheless, the biosorption capacity of microalgae in multimetal matrices has been
67 sparsely studied. In this context, the interference of OM and CO₂ supply on HM adsorption
68 has been reported in sediments and soils, but there is a limited understanding of these
69 phenomena in microalgae (Álvarez-Benedí et al., 2005; X. Dong et al., 2014). This work
70 will contribute to elucidate the potential of microalgae biomass for HM removal and the
71 effect of OM concentration and CO₂ supply on the biosorption process occurring in

72 photobioreactors devoted to WWT. This will provide valuable information about the
73 biosorption mechanisms, applicable also to other microbes.

74 Additionally, HM adsorption on microalgae biomass plays a key role during WWT but
75 can remarkably affect the further valorization processes and bioproduct applications
76 (Bădescu et al., 2018). The recovery of metals improves the economic viability of the
77 process, thereby valorizing the bioremediation process and subsidizing the bioremediation
78 process (Piccini et al., 2019). Therefore, recovery of HM might be needed before biomass
79 processing, which will entail the recovery of HM in a concentrated form (Gupta and
80 Rastogi, 2008). The recovery process can be carried out by proton exchange using eluents
81 such as acid solutions (i.e. HCl, HNO₃), chelating agents (EDTA) or exchange with other
82 ions (i.e. CaCl₂) (Vijayaraghavan and Balasubramanian, 2015). Previous studies with
83 *Scenedesmus quadricauda* using HNO₃ 0.5 M as the eluent revealed a desorption capacity
84 of 90% for Cd and Pb (Mirghaffari et al., 2014).

85 This research combines the study of the effect of the presence of OM and CO₂ supply
86 on the HM biosorption in a multimetallic solution using microalgae and the evaluation of
87 alternatives of eluents to recover the HM retained in the biomass. Two green microalgae
88 species currently used in studies of photobioreactors for WWT (i.e. *Chlorella vulgaris* and
89 *Scenedesmus almeriensis*) with proven metal adsorption capacity were used as model
90 microorganisms. The toxic elements As, B, Cu, Mn, and Zn were selected as model metals
91 for this study based on the maximum pollution levels recorded in the Loa River basin, the
92 main water source in the Antofagasta region (Chile): an arid region and mining zone with
93 serious problems of water shortage and toxic metal pollution (Romero et al., 2003;
94 Saavedra et al., 2018). Firstly, the effect of the OM concentration on the biosorption of HM

95 by microalgae was studied, comparing the results with and without CO₂ supply. Variables
96 such as microalgae growth, pH and TOC (total organic carbon) depletion were also
97 monitored for a comprehensive understanding of the OM effect in the bioremediation
98 process. Then, the recovery of toxic elements retained by harvested biomass using three
99 different eluents (HCl, NaOH, CaCl₂) at two concentrations (0.1 M and 0.2 M) was
100 evaluated, analyzing metal recovery along time and the effect of OM on the desorption with
101 HCl.

102

103 **2. Material and methods**

104 *2.1. Microalgae cultures and reagents*

105 The microalgae species used in this work were *Chlorella vulgaris* (obtained from the
106 collection of the University of Antofagasta, Chile) and *Scenedesmus almeriensis* (provided
107 by the University of Almeria, Spain). Microalgae biomass was cultivated under axenic
108 conditions using a Bristol medium (UTEX S.A) enriched with a trace metal solution from
109 F/2 of Guillard medium (UTEX S.A) supplying pure CO₂, as reported by (Saavedra et al.,
110 2018). LEDs lamps at 1000 μE/m²/s were used in a 12:12 h:h photoperiod. Cell culture
111 viability and purity were periodically checked by optical microscopy analysis.

112 Multimetallic solutions of toxic elements were prepared by dissolving a stock solution
113 with 600 mg As /L (Na₂HAsO₄·7H₂O, Sigma Aldrich), 3000 mg B /L (H₃BO₃, Sigma
114 Aldrich), 150 mg/L of Cu, Mn, and Zn (CuCl₂·2H₂O, MnCl₂·4H₂O, and ZnCl₂, Sigma
115 Aldrich, Germany) and ultra-pure water under acidic conditions (pH < 3). The stock
116 solution was periodically analyzed and stored at 4°C for quality assurance purposes.

117 Synthetic sewage waters (SSW) were prepared based on typical wastewater
118 compositions by adding (per L of deionized water) 30 mg urea, 28 mg K_2HPO_4 , 7 mg
119 NaCl, 4 mg $CaCl_2 \cdot 2H_2O$, and 2 mg $MgSO_4 \cdot 7H_2O$ and varying amounts of peptone and
120 meat extract: COM_3 = 160 mg of peptone and 110 mg of meat extract; COM_2 = 80 mg of
121 peptone and 55 mg of meat extract; COM_1 = 40 mg of peptone and 27.5 mg of meat extract,
122 and COM_0 = neither peptone nor meat extract (control test) (Alcántara et al., 2015).

123 NaOH (0.1 M) and HCl (0.1 M) were used for pH adjustment. All the chemicals
124 employed in this study were analytical grade (PANREAC, Barcelona). Plastics and glass
125 containers were immersed in diluted HNO_3 (10% v/v) for 24 h and rinsed three times with
126 Milli-Q water ($R > 18M\Omega \cdot cm$) before use.

127

128 *2.2. Multimetallic biosorption experiments*

129 Biosorption experiments were performed batchwise for both microalgae species at
130 different OM concentrations. The microalgae culture was centrifuged at 4500 rpm for 7
131 min, washed with Milli-Q water to remove the growth medium and centrifuged again prior
132 to the determination of the biomass concentration by gravimetric analysis of the total solid
133 (TS). Synthetic sewage waters of different OM concentrations were added to 4 mL of
134 multimetallic stock solution in order to obtain 200 mL of solution with initial
135 concentrations of toxic elements 12 mg As/L, 60 mg B /L, and 3 mg/L of Cu, Mn, and Zn.
136 Toxic elements and their concentrations were selected according with the composition and
137 maxima concentrations of HM found in the Loa river (Saavedra et al., 2018). The
138 calculated weight of washed and centrifuged biomass were added to these multimetallic
139 solutions to obtain 1 g/L microalgae suspension. The pH value was adjusted to 7.0 in order

140 to simulate the environmental conditions that exist in WWT photobioreactors (Posadas et
141 al., 2015). The tests were conducted in 500 mL glass flasks at 25°C and 1000 $\mu\text{E}/\text{m}^2/\text{s}$
142 under a 12:12 h:h photoperiod and magnetic agitation at 250 rpm. Control experiments
143 (without biomass) were conducted at operating pH conditions to check multimetallic
144 solubility. The total contact time was 72 h based on the typical hydraulic residence time
145 used for WWT photobioreactors (Acién et al., 2012). Samples of 8 mL were taken at time
146 0, 3, 24, and 72 hours to determine the pH and HM concentrations. After pH determination,
147 samples were filtered through 0.22 μm membrane filters. Aliquots of 4 ml were acidified
148 with 30 μL of HNO_3 (0.1 M) and stored at 4°C prior to metal quantification, while the
149 remaining sample was immediately used for TOC determination. An additional test series
150 was conducted under identical conditions but a constant flow of pure CO_2 was supplied (v
151 $> 99.9\%$, Abello Linde, Spain). The total suspended solid (TSS) concentration (after
152 filtration) in the liquid medium was measured at time 0 and 72 h in order to determine the
153 microalgae growth. TOC removal was determined according to Eq. (1):

$$154 \text{ TOC removal (\%)} = (\text{TOC}_0 - \text{TOC}_t) / \text{TOC}_0 \times 100 \quad \text{Eq. (1)}$$

155 where TOC_0 and TOC_t are the TOC concentrations at the initial and sampling times (t),
156 respectively, in (mg/ L). On the other hand, the removal efficiency of each element (i) at
157 time t ($\text{RE}_{i,t}$) was determined by Eq. (2):

$$158 \text{ RE}_{i,t} = (C_{i,0} - C_{i,t}) / C_{i,0} \times 100 \quad \text{Eq. (2)}$$

159 where $C_{i,0}$ and $C_{i,t}$ are the concentrations at the initial and sampling times (t), respectively,
160 for each toxic element (i) (mg/ L). On the other hand, the biosorption capacity of
161 microalgae for each toxic element ($q_{i,72}$) was calculated by Eq. (3):

$$162 q_{i,72} = V \times (C_{i,0} - C_{i,72}) / W_{72} \quad \text{Eq. (3)}$$

163 where $q_{i,72}$ is the biosorption capacity for each toxic element i : As, B, Cu, Mn, and Zn at 72
164 h (mg toxic element/g of biomass), V is the volume of suspension (L), $C_{i,0}$ and $C_{i,72}$ are the
165 initial and final toxic elements concentrations (mg/L), respectively, and W_{72} is the mass of
166 dry microalgae at 72 h (g).

167

168 2.3. HM Recovery studies

169 Suspensions of 200 ml containing 1g/L of microalgae, 12 mg/L of As, 60 mg/L of B,
170 and 3 mg/L of Cu, Mn, and Zn were stirred at 250 rpm and 25 ± 1 °C under dark conditions
171 for 3 h in order to load the microalgae with metals. At the end of the contact time, the
172 aqueous phase was removed by centrifugation (4500 rpm, 7 min), and 4 ml of supernatant
173 was filtered through 0.22 μ m membrane filters, acidified with 30 μ L of HNO₃ (0.1 M) and
174 stored at 4° C for the determination of the HM concentrations. Subsequently, 50 mL of the
175 selected eluents (HCl 0.1 M and 0.2 M, NaOH 0.1 M and 0.2 M, CaCl₂ 0.1 M and 0.2 M)
176 were added to the centrifuged microalgae. The suspensions were stirred at 250 rpm and 25°
177 C \pm 0.1 for 60 minutes. Samples of 4 mL were taken at time 10, 20 and 60 min, filtered
178 through 0.22 μ m membrane filters, acidified with 30 μ L of HNO₃ (0.1 M) and stored at 4°C
179 prior to the analysis of element concentrations. All the experiments were performed in
180 duplicate. The recovery efficiency ($Y_{R,i,t}$) was defined as follows (Eq. (4)):

$$181 Y_{R,i,t} = (C_{R,i,t} \times V_e) / (W_0 \times q_{i,3}) \times 100 \quad \text{Eq. (4)}$$

182 where $C_{R,i,t}$ (mg/L) is the concentration of each toxic element (i) in the solution after
183 recovery at sampling time (t), V_e is the volume of eluent (L), W_0 is the initial mass of dry
184 microalgae (g), and $q_{i,3}$ is the initial specific element content of the microalgae (mg/g).

185 In order to analyze the effect of the presence of OM in the metal loading step on metal
186 recovery, the experiment supporting the highest metal recovery efficiency was repeated
187 under identical operation conditions using COM₃ SSW instead of ultrapure water to prepare
188 the multimetallic solution used for the load of toxic elements in the microalgae.

189

190 *2.4. Analytical procedures*

191 The aqueous concentrations of As, B, Cu, Mn, and Zn were determined by inductively
192 coupled plasma optical emission spectrometry (ICP-OES) (HP 7500 cc, Agilent, USA)
193 according to the internal procedures of the Instrumental Techniques Laboratory (LTI –
194 UVa). For quality assurance, two reference water materials were included in the ICP-OES
195 analysis as quality control (QC) samples: ICP multielement Calibration Standard Solution,
196 100 mg/l Scharlau (26 elements in HNO₃ 5%), and a certified Reference Material
197 (Environment Canada TMDA-64.2 LOT 0313, HNO₃: 0.2%) as trace element fortified
198 calibration standard. QC samples were measured every 10 samples, considering a range
199 within 10% of the true value for valid acceptance. Concentrations of dissolved TOC were
200 measured using a Shimadzu TOC-VCSH analyzer (Japan) according to (Marín et al., 2018).
201 The pH was measured using a pH-meter Basic 20+ (Crison, Spain). Determination of TS
202 and TSS concentrations were performed according to standard methods (E.W. Rice, R.B.
203 Baird, A.D. Eaton, 2017).

204

205 *2.5. Statistical analysis*

206 The influence of TOC concentration, pH, CO₂ supply and ICP-OES analysis was
207 statistically evaluated by correlation and interference analysis (ANOVA). Additionally,

208 results of HM recovery studies were analyzed by one-way ANOVA and t-test ($P < 0.05$).

209 The data were analyzed using the software Minitab 18.

210

211 **3. Results and discussion**

212 *3.1. Effect of OM on the biosorption of toxic elements from the multimetallic solution*

213 *without CO₂ supply*

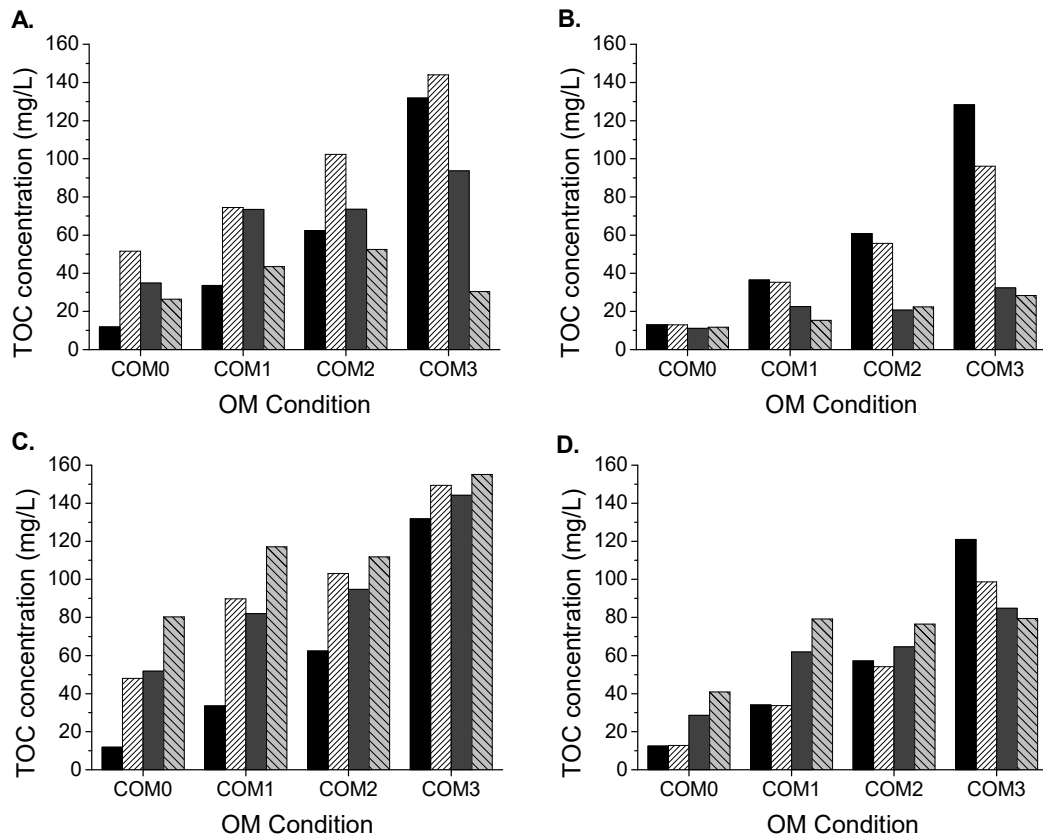
214 The pH value in *C. vulgaris* cultures remained approximately constant, slightly
215 increasing in solutions with high OM concentrations (values of 7.1 and 7.3 at 72 h in COM₂
216 and COM₃, respectively). These results were consistent with the biomass growth recorded
217 during the experiments: no growth in the control solution COM₀ and increases in TSS of
218 2.0% in COM₁, 16.3% in COM₂ test and 27.6% in COM₃ tests at 72 h. Interestingly, TOC
219 concentrations increased remarkably at 3 h in the biosorption experiments with *C. vulgaris*
220 regardless of the OM concentrations (Fig. 1A). However, the TOC concentration decreased
221 in all the experiments after 24 h of incubation, although positive TOC removals at 72 h
222 were only achieved in COM₂ and COM₃ test (15.9% and 76.9%, respectively). It is therefore
223 clear that the presence of HM in the medium significantly influenced SSW treatment.

224 On the other hand, the pH in *S. almeriensis* biosorption experiments experienced a
225 remarkable initial increase up to pH 8 at 3 h regardless of the OM concentrations. This fast
226 change in medium pH can be related to the high physiological activity associated to the
227 phototrophic metabolism under this experimental condition (Cabello et al., 2015). From 24
228 h onwards, pH values increased slightly at high OM concentrations (i.e. pH of 8.3 in COM₂
229 at 72 h) and pH decreased in solutions at low OM concentrations (pH of 7.1 in COM₀ at 72
230 h). An increase of *S. almeriensis* biomass concentration was recorded in all biosorption

231 experiments, evidencing that the experimental methodology applied did not affect cell
232 viability, and cell growth was correlated with the OM concentration in the solution. Thus,
233 *S. almeriensis* growth at 72 h ranged from 30.6% in COM0 to 40.8% in COM3. It is known
234 that microalgae support different metabolic processes in order to avoid the high toxicity
235 generated by the presence of HM in the medium. Indeed, extracellular sequestration,
236 intracellular sequestration, active export and enzymatic detoxification rank among the most
237 common processes (Yin et al., 2018). The expression of proteins such as Glutathione S-
238 transferases (GSTs) play an important role in the cell detoxification process, contributing to
239 the protection against oxidative stress generated by the presence of HM (Yin et al., 2016;
240 Zhang et al., 2013). Previous studies showed different growth responses of microalgal
241 species to HM stress. Thus, *Scenedesmus acuminatus* presented a higher antioxidant
242 capacity, lower membrane damage, and higher tolerance than *Chlorella sorokiniana* to
243 stress induced by Cu ions, demonstrating a better tolerance against Cu (Hamed et al., 2017).
244 Therefore, the difference in the pH and cell growth observed between the microalgae
245 studied can be explained by the different metabolic mechanisms used to alleviate HM
246 toxicity.

247 Contrary to the results recorded for *C. vulgaris*, *S. almeriensis* biosorption tests
248 revealed TOC removal from the beginning of the experiment, with a greater removal at
249 higher initial OM concentrations (Fig. 1B). At 3 h, TOC removal was low in all *S.*
250 *almeriensis* biosorption tests, ranging from 0.3% for COM0 to 25.1% for COM3. From 24 h
251 onwards, TOC removal increased remarkably up to 10.4%, 57.9%, 63.2% and 77.9% at 72
252 h in COM0, COM1, COM2, and COM3 tests, respectively. TOC removal in the medium can be
253 directly associated to the ability of microalgae to use organic carbon as energy source for

254 mixotrophic growth (Shen et al., 2015). Therefore, the results obtained in *S. almeriensis*
 255 tests can be associated to the higher cell growth of this microalga, which supported a
 256 superior tolerance over the multimetallic solution compared to *C. vulgaris* during the
 257 treatment of wastewater contaminated with metals.



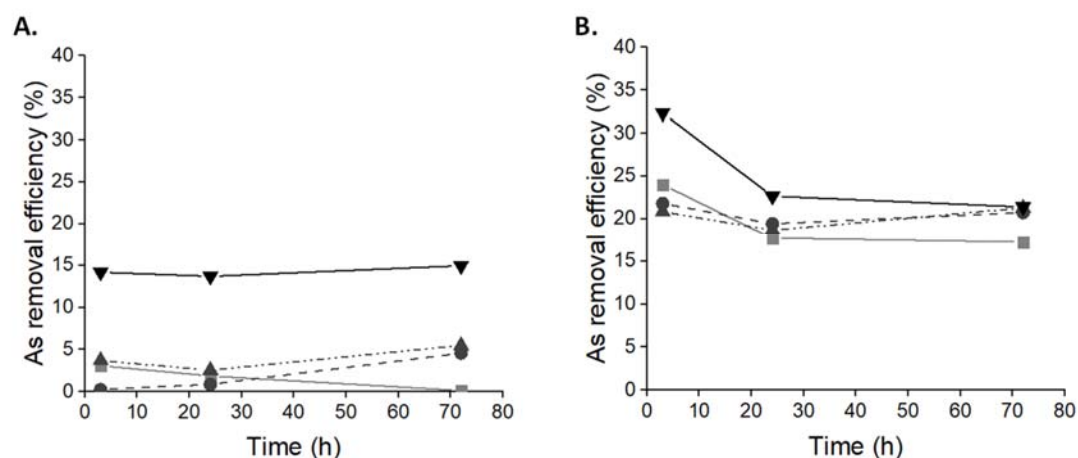
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259 **Fig. 1.** Time course of the TOC concentration (mg/L) in biosorption studies of *Chlorella*
 260 *vulgaris* (CV) and *Scenedesmus almeriensis* (SA) conducted in a multimetallic solution
 261 with different initial OM concentrations at 1 g TSS/L and pH 7.0. A) CV – without CO₂
 262 supply; B) SA – without CO₂ supply; C) CV – with CO₂ supply; D) SA – with CO₂ supply.
 263 (■) T₀ (Initial time), (▨) 3 h; (■) 24 h; (▨) 72 h.

264

265 The influence of OM on the As biosorption in *C. vulgaris* tests was remarkable from the
266 first sampling time at 3 h (with $RE_{As,3}$ 14.2% in C_{OM0} and 3.7% in C_{OM1}). The highest As
267 removal efficiencies were obtained in control tests, where no growth of biomass was
268 observed (Fig. 2A). Interestingly, the As removal efficiencies remained constant from 24 h
269 onwards. The effect of OM was higher in terms of As biosorption capacity of *C. vulgaris*
270 ($q_{As,72}$ of 2.2 mg/g, 0.8 mg/g, 0.5 mg/g, and 0.02 mg/g in C_{OM0} , C_{OM1} , C_{OM2} , and C_{OM3} ,
271 respectively) than in terms of As removal. Therefore, the presence of OM decreased As
272 removal but promoted biomass growth, thus increasing biomass concentration.

273 Overall, the As removal efficiencies for *S. almeriensis* were higher than for *C. vulgaris*
274 (Fig. 2B). The highest As removal efficiency of *S. almeriensis* was obtained in the control
275 solution C_{OM0} at 3 h (32.4%), which decreased with the contact time to ~ 20% by 72 h,
276 similar to the observations in the other SSW tested. Differences in the final calculated $q_{As,72}$
277 in *S. almeriensis* tests were inversely correlated with cell growth, descending from 2.3 mg/g
278 in C_{OM0} to 1.7 mg/g in C_{OM3} . The lower influence of OM on the As biosorption capacity of
279 *S. almeriensis* compared to *C. vulgaris* could be related to its higher TOC removal capacity,
280 which supported similar final OM concentrations in all the experiments.



281

282 **Fig. 2.** Time course of the removal efficiency (%) of As in biosorption studies without CO₂
 283 supply at different initial OM concentrations by *Chlorella vulgaris* (A), and *Scenedesmus*
 284 *almeriensis* (B) cultures at 1g TSS/L and pH 7.0. (—▼—): COM₀ (control); (- -▲ -): COM₁ (40
 285 mg peptone /L and 27.5 mg meat extract /L); (- ♦ -): COM₂ (80 mg peptone /L and 55 mg
 286 meat extract /L), and (-■-): COM₃ (160 mg peptone /L and 110 mg meat extract /L).

287 The As biosorption capacities obtained in the control tests in this study were lower than
 288 those previously reported by Saavedra et al, (2018) in multimetallic solutions at the same
 289 metal concentrations, but without the addition of urea and mineral salts. It has been
 290 hypothesized that some of the added compounds such as urea or phosphates, besides
 291 enhancing the growth and resistance of *S. almeriensis*, could compete with As for the
 292 adsorption sites, decreasing the As uptake by microalgae cells (Yamani et al., 2016).

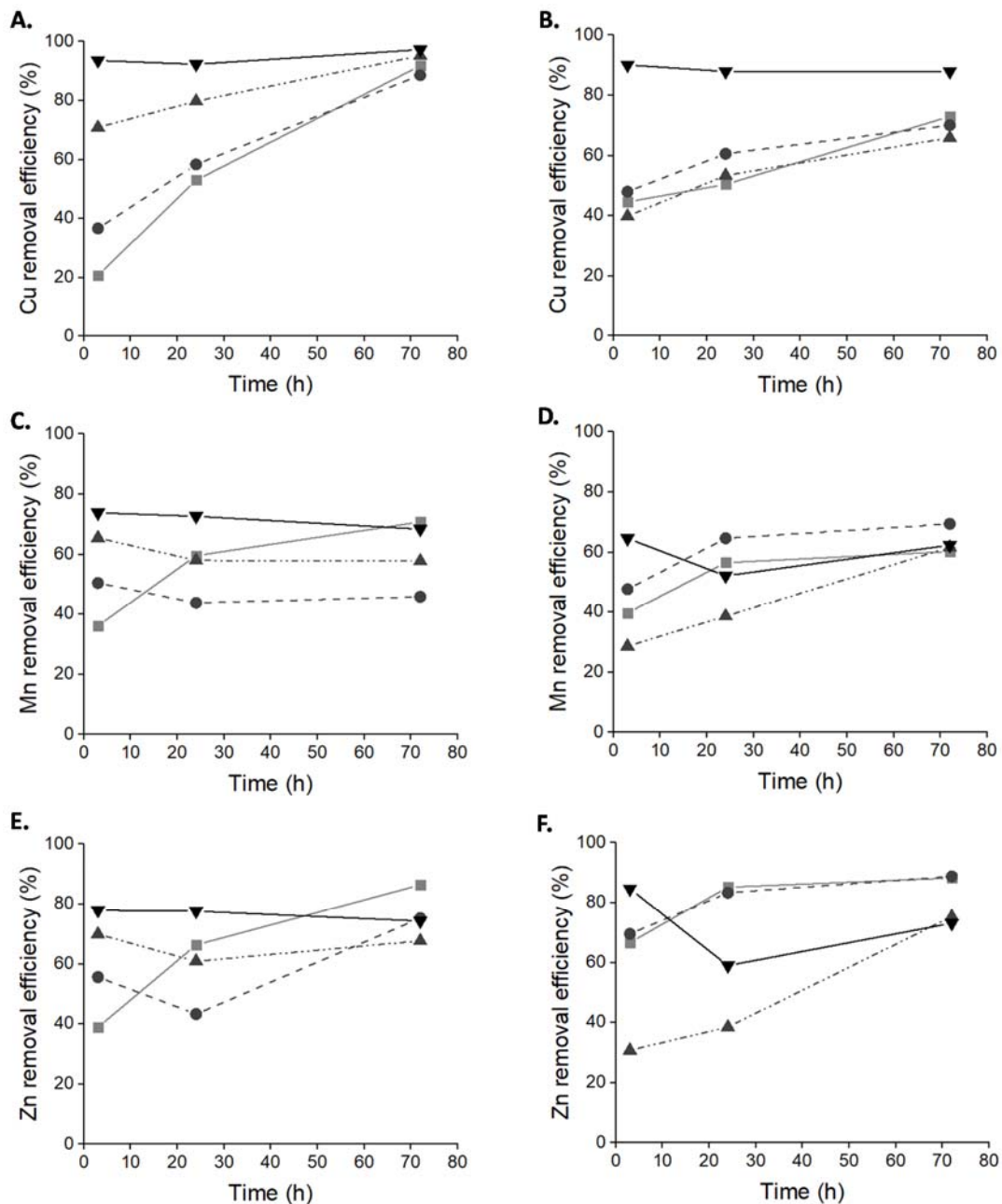
293 B removal efficiencies were low and relatively stable over time, with a maximum of
 294 8.6% in RE_{B,24} for *S. almeriensis* at COM₁ (Supplementary material). The B biosorption
 295 capacity of *C. vulgaris* increased with the OM concentration of the SSW (1.4 mg/g, 2.1
 296 mg/g, 2.3 mg/g and 2.6 mg/g in COM₀, COM₁, COM₂, and COM₃, respectively). These results
 297 agree with previous studies that reported an uptake capacity of 2.7 mg B/g for *Chlorella* sp.

298 at pH 7.0 (Taştan et al., 2012). Nevertheless, no clear influence of the initial OM
299 concentration was observed in B biosorption capacities of *S. almeriensis*, with $q_{B,72}$ values
300 ranging from 3.2 mg/g in COM_0 to 3.7 mg/g in COM_2 . The biosorption capacities for B in the
301 control tests were higher than those previously found in the multimetallic solution without
302 the addition of urea and minerals (Saavedra et al., 2018), but these differences were lower
303 for the overall bioremediation process.

304 *C. vulgaris* showed an inverse correlation between RE for Cu, Mn, and Zn and the
305 initial OM concentration at the first sampling time (Fig. 3A, C, and D). For instance, the
306 $RE_{Cu,3}$ accounted for 93.6%, 70.9%, 36.7% and 20.7% in COM_0 , COM_1 , COM_2 , and COM_3 ,
307 respectively. Nevertheless, this inverse correlation between OM and RE was not
308 maintained over time. In *C. vulgaris*, the RE_{Cu} increased with the contact time, especially in
309 the COM_2 and COM_3 tests ($RE_{Cu,72} > 88.6\%$), although $q_{Cu,72}$ decreased from 3.2 mg/g in COM_0
310 to 2.3 mg/g in COM_3 . Mn and Zn removal efficiencies in *C. vulgaris* also increased with
311 time in COM_3 solutions, achieving values of 70.8% and 86.4% at 72 h, respectively. The
312 increase observed in Cu, Mn, and Zn removal efficiencies by *C. vulgaris* in high OM
313 concentration tests was likely due to the gradual decrease in TOC concentration (Hussain et
314 al., 2017), but also to cellular growth, which enhanced biosorption by increasing the
315 adsorption sites. Finally, *C. vulgaris* growth likely mediated differences in the Mn and Zn
316 biosorption capacities, with values of $q_{Mn,72}$ of 2.4 mg/g in COM_0 and 1.5 mg/g in COM_2 , and
317 $q_{Zn,72}$ values ranging from 2.7 mg/g in COM_0 to 2.3 mg/g in COM_2 .

318 Remarkable differences in the RE of Cu, Mn, and Zn in *S. almeriensis* biosorption tests
319 were recorded as a function of the initial OM conditions during the first 3 h (Fig. 3B, D,
320 and F). The highest RE_{Cu} was measured in the control tests, with values almost independent

321 of contact time ($RE_{Cu,3}$ of 90.1% and $RE_{Cu,72}$ of 88%). Similar RE_{Cu} values in C_{OM1} , C_{OM2} ,
322 and C_{OM3} were reported in *S. almeriensis* cultures, which increased over time from 39.8%
323 at 3 h in C_{OM1} to 73% at 72 h in C_{OM3} . Similarly, Cu biosorption capacities of this
324 microalga reached a maximum $q_{Cu,72}$ of 2.1 mg/g in the control test, which was higher than
325 the $q_{Cu,72}$ of 1.5 mg/g, 1.7 mg/g and 1.6 mg/g determined in C_{OM1} , C_{OM2} , C_{OM3} , respectively.
326 These lower values on RE_{Cu} demonstrated that the OM effect produced a decrease on Cu
327 removal, occurring independently of the the initial OM concentration in the SSW with added
328 peptone and meat extract.



329

330 **Fig. 3.** Time course of the removal efficiency (%) of copper (Cu), manganese (Mn), and
 331 zinc (Zn); in biosorption studies without CO₂ supply at different initial OM concentrations
 332 by *Chlorella vulgaris* (CV) and by *Scenedesmus almeriensis* (SA) cultures at 1g TSS/L and
 333 pH 7.0. A) CV-Cu; B) SA-Cu; C) CV-Mn; D) SA-Mn; E) CV- Zn; F) SA-Zn. (—▼—): COM₀
 334 (control); (-▲-): COM₁ (40 mg peptone /L and 27.5 mg meat extract /L); (-●-): COM₂ (80

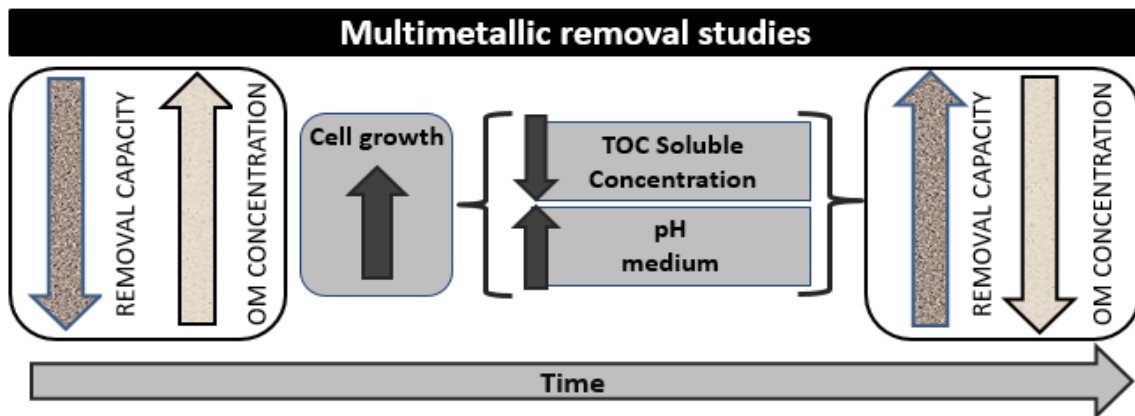
335 mg peptone /L and 55 mg meat extract /L), and (—■—): COM3 (160 mg peptone /L and 110
336 mg meat extract /L).

337

338 Although RE_{Mn} and RE_{Zn} increased in the tests conducted with time in SSW with added
339 peptone and meat extract, the removal efficiency of Mn and Zn by *S. almeriensis* decreased
340 with the contact time in the control tests. The lowest Mn and Zn removal efficiencies were
341 obtained at 3 h in COM1 (28.6% and 30.7%, respectively). The Mn removal efficiencies and
342 biosorption capacities at 72 h reached similar values, independently of the initial OM
343 concentration (\approx 63% and 1.6 mg/g). Larger differences were obtained in the Zn
344 biosorption test using *S. almeriensis*: RE_{Zn,72} of \sim 74% and q_{Zn,72} of \sim 2.0 mg/g in COM0 and
345 COM1, and RE_{Zn,72} of \sim 88% and q_{Zn,72} of 2.4 mg/g in COM2 and COM3.

346 The biosorption capacities based on the total mass of elements adsorbed at 72 h revealed
347 similar values for both microalgae studied. Interestingly, the COM0 test achieved the
348 maximum biosorption capacity in both microalgae species. At 72 h, the total biosorption
349 capacity of *C. vulgaris* ranged from 11.9 mg/g in COM0 to 9.2 mg/g in COM3, whereas that of
350 *S. almeriensis* ranged from 11.2 mg/g in COM0 to 10.4 mg/g in COM3. The results showed a
351 clear negative influence of the OM concentration on microalgae biosorption capacity for
352 the multimetallic solution under investigation. Nevertheless, correlation of various factors
353 associated with the biosorption process produced a combined effect, which modified the
354 extent of this influence over time (Fig. 4). At higher OM concentration in the SSW, the
355 toxic effect of HM on microalgae decreased. This triggered microalgae growth, which
356 slight increased the pH of the medium. When cellular growth is favored, TOC was
357 subsequently consumed. Therefore, the TOC concentration decreased along the test and the

358 negative effect associated with OM concentration in the biosorption process also decreased.
 359 The final differences in the total biosorption capacity observed between COM₀ and COM₃
 360 at 72 h can be directly related to the severe effect that OM induced in As biosorption, which
 361 was the only toxic element that experienced the interference of OM in the biosorption
 362 process over time.



363
 364 **Fig. 4.** Schematic correlation of organic matter (OM), cell growth, TOC concentration, and
 365 pH medium in the removal capacity of microalgae biomass for a multimetallic system.

366
 367 Finally, no precipitation was observed in the multimetallic control samples, probably
 368 due to the increase in the solubility of the cationic metals produced by the high borate
 369 concentration present in the multimetallic solution (Beckett, 2016; Graff et al., 2017).
 370 Future studies should be addressed to analyze possible metabolic responses of microalgae
 371 to HM, as the activation of desorption mechanisms to alleviate HM toxicity.

372
 373 *3.2. Effect of OM on the biosorption of toxic elements from the multimetallic solution with*
 374 *CO₂ supply*

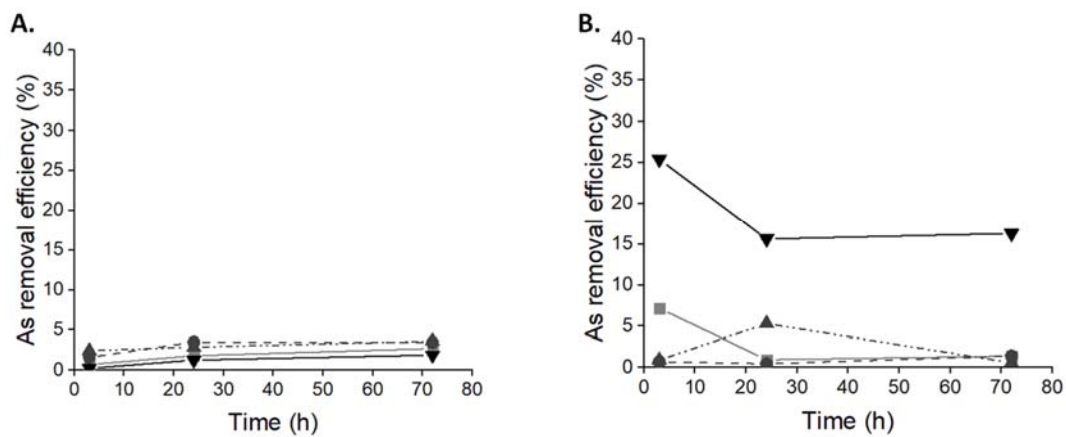
375 The addition of CO₂ in the biosorption experiments resulted in a decrease in the pH
376 from initial neutrality to \approx 5.2 at 24 h, regardless of the microalgae species. This medium
377 acidification can be directly attributed to CO₂ dissolution in the cultivation broth (Hu et al.,
378 2012). Slight differences in a range of pH of 5.1 – 5.5 were found at 72 h, where the highest
379 pH values were recorded in the test carried out at highest initial OM concentrations.

380 A negative biomass growth was recorded in the *C. vulgaris* biosorption tests conducted
381 with CO₂ addition. The TSS decreased by 10.2% in COM₀, 15.3% in COM₁, 8% in COM₂ and
382 4.1% in COM₃. These results suggest a cell decay in cultures mediated by the high
383 concentrations of CO₂ in the medium and by the low pH (<5.5) (Hussain et al., 2017).
384 Interestingly, *S. almeriensis* was able to grow under all conditions tested in spite of the low
385 pH value. Indeed, the TSS concentration increased by 19.4% in COM₀ and by 53.1% in
386 COM₃ at 72 h. These results confirmed the higher tolerance and growth capacity of *S.*
387 *almeriensis* (compared to *C. vulgaris*) previously found without CO₂ addition.

388 TOC concentrations increased with the time course in *C. vulgaris* experiments. At 72 h,
389 TOC concentrations reached values 676.4%, 348.4%, 179.1%, and 117.7% higher than
390 those observed at time 0 in COM₀, COM₁, COM₂, and COM₃, respectively (Fig. 1C). These
391 findings were likely due *C. vulgaris* decay in excess of CO₂ and the TOC generated can be
392 associated with the activation of protection mechanisms, such as metabolic export
393 processes to control the oxidative stress (Mohamed, 2008). Lower increases in TOC
394 concentrations (327.7%, 232.1% and 133.8% at 72 h in COM₀, COM₁ and COM₂, respectively)
395 were recorded for *S. almeriensis* (Fig. 1D). A positive TOC removal efficiency of 34.4%
396 was found in COM₃ at 72 h. The moderate increase in TOC concentration, together with the
397 *S. almeriensis* growth observed, could be associated to a partial cellular death but also to

398 the excretion of metabolites, in a lower extent than that observed in *C. vulgaris* (Wang et
399 al., 2010). Interestingly, similar final TOC concentrations of ~ 78 mg/L were detected at 72
400 h in COM1, COM2, and COM3 experiments.

401 Arsenic biosorption was severely affected by CO₂ addition, especially in *C. vulgaris*
402 experiments (Fig. 5A), Low As removal efficiencies (< 4 %) were obtained for *C. vulgaris*
403 regardless of the OM in SSW, resulting in a maximum As biosorption capacity of 0.6 mg/g
404 in COM1 at 72 h. CO₂ addition also decreased As removal efficiencies in *S. almeriensis* in
405 SSW with added peptone and meat extract, especially at high contact times ($q_{As,72}$ of 0.06
406 mg/g in COM1, and 0.1 mg/g in COM2 and COM3) (Fig. 5B). Only the control experiments
407 supported a significant As biosorption, which decreased from 25.4% at 3 h to 15.6% at 24
408 h, resulting in $q_{As,72}$ of 1.9 mg/g).



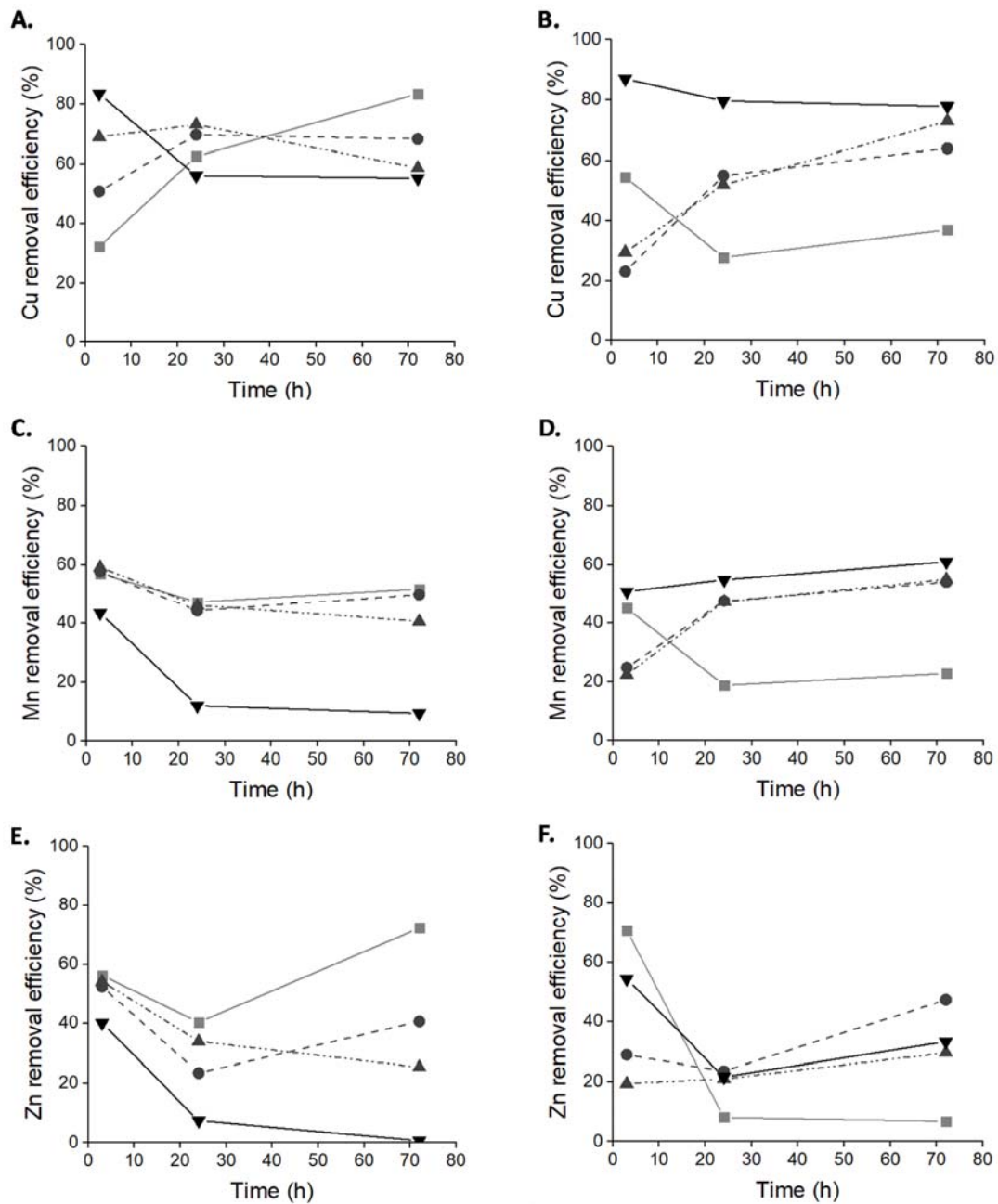
409
410 **Fig. 5.** Time course of the removal efficiency (%) of As in biosorption studies with CO₂
411 supply at different initial OM concentrations by *Chlorella vulgaris* (A), and *Scenedesmus*
412 *almeriensis* (B) cultures at 1g TSS/L and pH 7.0. (—▼—): COM0 (control); (- -▲- -): COM1 (40
413 mg peptone /L and 27.5 mg meat extract /L); (- ◆ -): COM2 (80 mg peptone /L and 55 mg
414 meat extract /L), and (-■-): COM3 (160 mg peptone /L and 110 mg meat extract /L).

415

416 Similarly, CO₂ addition resulted in even lower B removal efficiencies than those
417 reported in Subsection 3.1 for *C. vulgaris* with RE_{B,72} of ≈ 3 % and a maximum q_{B,72} of 1.8
418 mg/g in C_{OM3} (Supplementary material). The removal of B was less impacted by CO₂
419 addition in *S. almeriensis* experiments, with a slight variation of RE_B from 4.0% in C_{OM2} at
420 3 h to 6.4% in C_{OM0} at 72 h. The final B biosorption capacity of *S. almeriensis* decreased
421 with the OM concentration of SSW from 3.3 mg/g in C_{OM0} to 2.4 mg/g in C_{OM3}
422 (considering cell growth). The lower effect of CO₂ addition on B biosorption studies could
423 be associated with the high solubility of the B complexes in moderately-acidic solutions
424 (Guan et al., 2016). Hence, differences in B removal can be only associated with H⁺
425 competition for the active sites on the surface adsorption process.

426 Furthermore, CO₂ addition remarkably reduced the Cu, Mn, and Zn removal efficiencies
427 of *C. vulgaris*, with the major impact being in the control tests (Fig. 6A, C, and E). Thus,
428 Cu, Mn, and Zn removal by *C. vulgaris* in C_{OM0} under CO₂ supply at 3 h accounted for
429 83.5%, 43.4%, and 40.1%, and decreased to 55.2%, 9.3%, and 0.6% at 72 h, respectively.
430 The effect of CO₂ addition was less pronounced in SSW with higher OM concentrations,
431 where RE_{Cu}, RE_{Mn}, and RE_{Zn} remained higher than in the control test, but lower than those
432 recorded in the absence of CO₂ supply. These lower RE of Cu, Mn and Zn could be
433 associated with cell decay mediated by pH modification caused by CO₂ addition. Cu
434 removal at 3 h was the only exception to the general trend, with efficiencies decreasing
435 with the OM concentration of the SSW and values similar or even higher to those obtained
436 without CO₂ addition. The trends in the biosorption capacities were identical to those in the
437 removal efficiencies (increasing with OM) as a result of the decrease in *C. vulgaris*

438 concentration in this set of experiments. The final biosorption capacities ranged from 2.0
 439 mg/g in C_{OM0} to 2.8 mg/g in C_{OM3} for Cu; from 0.3 mg/g in C_{OM0} to 1.8 mg/g in C_{OM3} for
 440 Mn; and from 0.03 mg/g in C_{OM0} to 2.9 mg/g in C_{OM3} for Zn.



441
 442 **Fig. 6.** Time course of the removal efficiency (%) of copper (Cu), manganese (Mn), and
 443 zinc (Zn); in biosorption studies with CO₂ supply at different initial OM concentrations by

444 *C. vulgaris* (CV) and by *S. almeriensis* (SA) cultures at 1g TSS/L and pH 7.0. A) CV-Cu;
445 B) SA-Cu; C) CV-Mn; D) SA-Mn; E) CV- Zn; F) SA-Zn. (—▼—): C_{OM0} (control); (- -▲- -):
446 C_{OM1} (40 mg peptone /L and 27.5 mg meat extract /L); (- ◆ -): C_{OM2} (80 mg peptone /L and
447 55 mg meat extract /L), and (—■—): C_{OM3} (160 mg peptone /L and 110 mg meat extract /L).

448

449 On the other hand, the reduction in Cu, Mn, and Zn removal efficiencies by *S.*
450 *almeriensis* under CO₂ addition increased with the OM concentration of the SSW (Fig. 6B,
451 D, and F). The results at 3 h of contact time represented the only exception, with higher Cu,
452 Mn, and Zn removal efficiencies in C_{OM3} with supply of CO₂. In these tests, the removal
453 efficiencies decreased from 54.6%, 45.0%, and 70.6% at 3 h to 27.8%, 18.8%, and 8.0% at
454 24 h, respectively, with Zn being the toxic element most affected by CO₂ addition
455 regardless of the OM concentration. The Cu, Mn, and Zn biosorption capacities in *S.*
456 *almeriensis* exhibited an inverse correlation with the initial OM concentration, with values
457 decreasing from 2.1 mg/g in C_{OM0} to 0.8 mg/g in C_{OM3} for Cu, from 1.7 mg/g in C_{OM0} to 0.5
458 mg/g in C_{OM3} for Mn, and from 1.0 mg/g in C_{OM0} to 0.2 mg/g in C_{OM3} for Zn. These values
459 were lower than those obtained without CO₂ supply, with the highest decrease being
460 recorded in the C_{OM3} tests.

461 Contrary to the expected effect of stimulation of microalgae growth by CO₂ supply, the
462 results evidenced the negative influence of CO₂ addition on the removal of the target
463 elements by *C. vulgaris* and *S. almeriensis*. The decrease in cell growth was associated to
464 medium acidification, as well as to a metabolic response to the oxidative stress. The
465 increase in OM concentration in the SSW resulted in the opposite effect on the total
466 biosorption capacities in both microalgal species. Thus, whereas the total biosorption

467 capacity of *C. vulgaris* increased linearly with the initial OM concentrations, from 4.4 mg/g
468 in C_{OM0} to 9.7 mg/g in C_{OM3}, the total biosorption capacity of *S. almeriensis* decreased from
469 9.7 mg/g in C_{OM0} to 3.9 mg/g in C_{OM3}.

470 No positive effects on biomass growth or nutrient removal by CO₂ addition were
471 founded in studies of *Chlorella* sp. cultivated in wastewater containing high OM
472 concentrations in a closed photobioreactor system (Min et al., 2011). Indeed, an optimal
473 CO₂ concentration of 6.5% was identified in studies with *Chlorella vulgaris* P12 (Anjos et
474 al., 2013). In contrast, higher growth rates were founded at higher CO₂ concentration
475 (~20%) in cultures of *Scenedesmus* (Tang et al., 2011). Therefore, these results confirmed
476 that high CO₂ concentrations can induce different metabolic pathways between species and
477 determine the OM influence on the overall HM biosorption processes. The differences
478 found between the two pure microalgae species on this study warn about the risk of
479 extrapolating biosorption results of pure species to consortia of different microalgae and
480 bacteria, as those growing in WWT photobioreactors. These results indicate that microalgae
481 could remove efficiently HM from water with low OM content as the river water, but its
482 effectiveness could decrease in WWT photobioreactors with high OM content and, usually,
483 working with CO₂ addition. Further in-situ experiments of HM biosorption in real WWT
484 photobioreactors are required to study the biosorption of toxic elements in these plants.
485 Nevertheless, microalgae could be potentially used as a low-cost complement for the
486 purification step presented in the final stage of the decontamination process.

487

488 3.3. Recovery of toxic elements from microalgae

489 High recovery efficiency yields of As, Cu, Mn, and Zn from *C. vulgaris* ($Y_R > 70\%$)

490 were obtained with no significant differences ($P > 0.05$) using HCl at 0.1 M and 0.2 M as
491 eluent, regardless of the sampling times (Fig. 7A). The maximum recovery was achieved
492 for As with 0.1 M HCl at 60 min ($Y_{R,As,60}$ of 89 %). Cu, Mn, and Zn recovery increased
493 slightly with the HCl concentration, with maximum Y_R of 81%, 80%, and 78% for Cu, Mn,
494 and Zn, respectively, using 0.2 M HCl at 60 min. A slight increase on Cu recovery
495 efficiency with increasing the HCl concentration from 0.1M (~ 80%) to 0.2M (88%) at 60
496 min, in studies of *Halimeda gracilis*, was also previously reported (Jayakumar et al., 2015).

497 By contrast, B recovery from *C. vulgaris* with HCl was low (Y_R of 15-20% regardless of
498 time). HCl at 0.1 M also mediated the highest recoveries of As, Cu, Mn, and Zn from *S.*
499 *almeriensis*, with an almost complete metal recovery of Mn and Zn (> 99%) at 10 min (Fig.
500 7B). The recovery of B using HCl from *S. almeriensis* was low regardless of the
501 concentration used. In this context, (Vannela and Verma, 2006) reported the highest
502 recovery efficiencies (> 95%) of Co^{+2} , Cu^{+2} , and Zn^{+2} from *Spirulina platensis* using
503 inorganic acids (0.1 M HCl) in an eluents screening study comparing the performance of
504 inorganic acids, inorganic salts, chelating agents and organic acids. Unexpectedly, the
505 increase in the HCl concentration decreased the recovery efficiencies from *S. almeriensis*
506 with significant differences ($P = 0.001$) for all toxic elements by ~ 50 %, except B, likely
507 due to damage to the cell wall structure caused by the high acid concentration (Abdolali et
508 al., 2015; Kołodyńska et al., 2017).

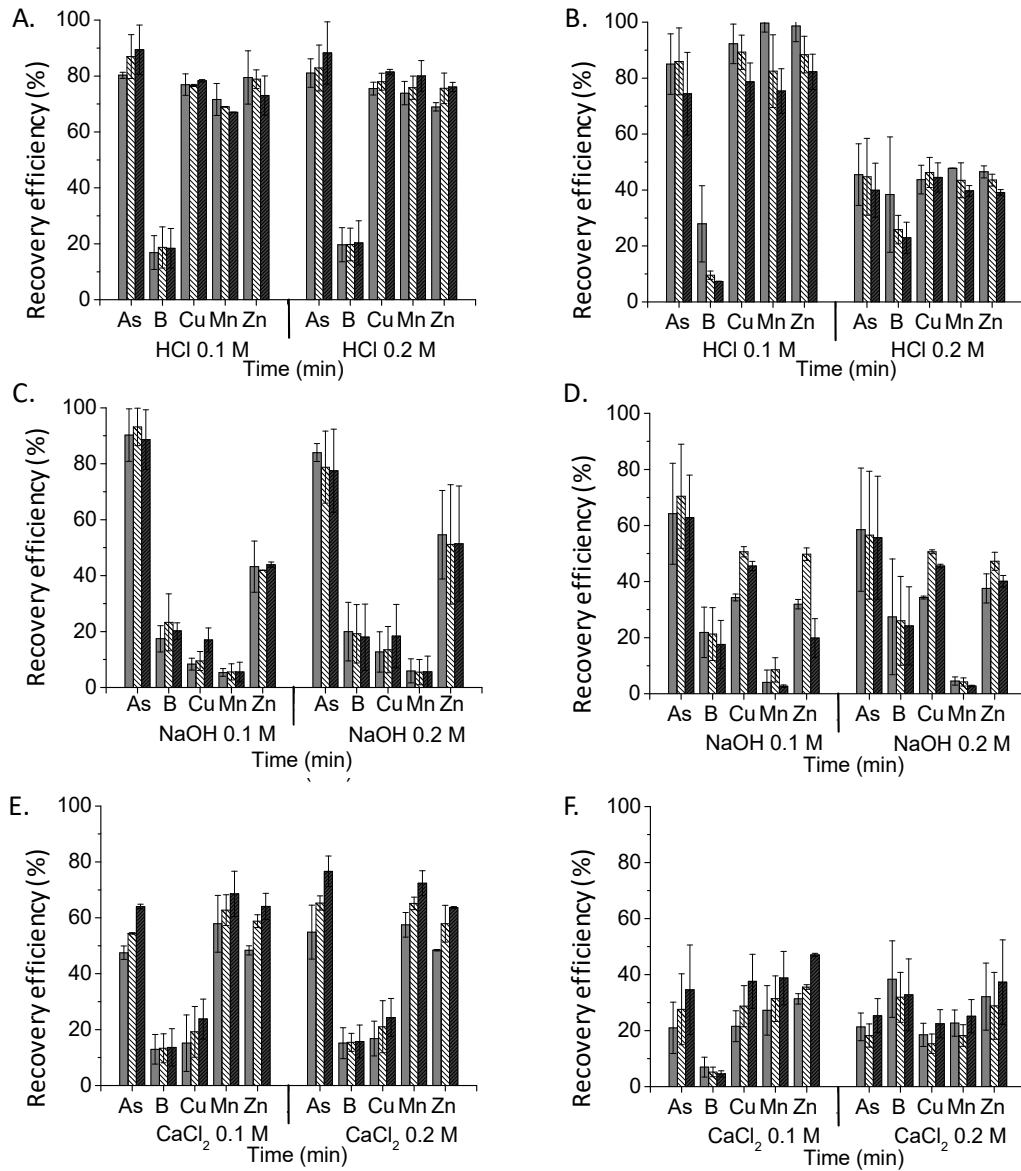
509 NaOH at 0.1 M provided the highest As recovery efficiency from *C. vulgaris* (93% at 20
510 min), a moderate recovery of Zn ($Y_{R,Zn}$ of 40%) and low recovery efficiencies for the rest of
511 the elements tested (Fig. 7C). The increase in the NaOH concentration to 0.2 M improved
512 Zn recovery ($Y_{R,Zn,10}$ of 55%) from *C. vulgaris*, although it slightly decreased the As

513 recovery. Alkaline solutions supported lower recovery efficiencies from *S. almeriensis* than
514 from *C. vulgaris*, with maximum Y_R of 70% for As at 20 min using 0.1 M NaOH, and 51%
515 for Cu at 20 min using 0.2 M NaOH (Fig. 7D). No significant differences ($P > 0.05$) on
516 metal recovery were observed between NaOH concentrations in *S. almeriensis*. The
517 recovery of Mn was low when using NaOH solutions as eluents, with Y_R values lower than
518 8% for both microalgae species.

519 Finally, the recovery efficiencies using CaCl_2 increased over time in all the experiments,
520 for all the elements except for B (Fig. 7E, F). The increase of CaCl_2 concentration to 0.2 M
521 achieved the maximum recoveries for As and Mn from *C. vulgaris*, reaching Y_R values of
522 of 77% and 72% at 60 min, respectively. On the other hand, a low recovery of all the
523 elements from *S. almeriensis* was obtained. Only, CaCl_2 0.2 M supported a relevant B
524 recovery efficiency of 38% at 10 min, the highest achieved for this element.

525 From the above results, biomass elution with 0.1M HCl supported the best metal
526 recovery performance of the target metals in both microalgae species. Therefore, these
527 conditions were applied during the evaluation of the effect of the OM on the recovery of
528 toxic metals. In this context, a complete recovery of As, Cu, Mn, and Zn using 0.1 M HCl
529 as eluent was achieved in both *C. vulgaris* and *S. almeriensis* loaded with the multimetallic
530 solution in COM_3 SSW. The above described decrease in the removal capacity of the
531 biomass induced by the presence of OM in the medium likely facilitated the recovery
532 process. It was noteworthy that kinetics of metal recovery were very fast (data not shown),
533 supporting a total recovery (~100%) of As, Cu, Mn, and Zn within 10 min. The presence of
534 OM in the multimetallic solution used to load the microalgae slightly increased the B

535 recovery efficiency from *C. vulgaris* (26% at 10 min), but decreased B recovery from *S.*
 536 *almeriensis* (18% at 10 min).



537

538 **Fig. 7.** Time course of the recovery efficiency of toxic elements (%) in *C. vulgaris* (CV),
 539 and *S. almeriensis* (SA) cultures at 1g TSS/L and pH 7.0, using different eluents (HCl,
 540 NaOH, and CaCl₂) at two concentrations (0.1 M, 0.2 M). A) CV – HCl, B) SA – HCl, C)
 541 CV – NaOH, D) SA – NaOH, E) CV – CaCl₂, F) SA – CaCl₂. (■): 10 min, (▨): 20 min, (■):
 542 60 min.

543

544 **4. Conclusions**

545 *S. almeriensis* resulted in better performance in HM tolerance, arsenic removal and
546 TOC removal in multimetallic solutions of HM than *C. vulgaris*. A negative effect in RE
547 was associated with OM presence, but this effect decreased along time. The highest arsenic
548 removals (14.9% for CV; 32.4% for SA) were obtained in absence of OM and without CO₂
549 addition. Boron removal was low (<8.5%), with no clear influence of the presence of OM
550 and CO₂. Overall, the presence of OM decreased Cu, Mn, and Zn removals, but facilitated
551 metal recovery under acidic elution. CO₂ addition decreased the total biosorption capacity
552 from average values of 10.1 and 11.0 mg/g to 7.1 and 6.6 mg/g for CV and SA,
553 respectively. HM biosorption by microalgae can be useful for remediation of water with
554 low OM content, but further in-situ studies of HM biosorption in WWT photobioreactors
555 are recommended. HCl 0.1 M resulted the best eluent for metal recovery, despite
556 supporting low boron recovery.

557

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565

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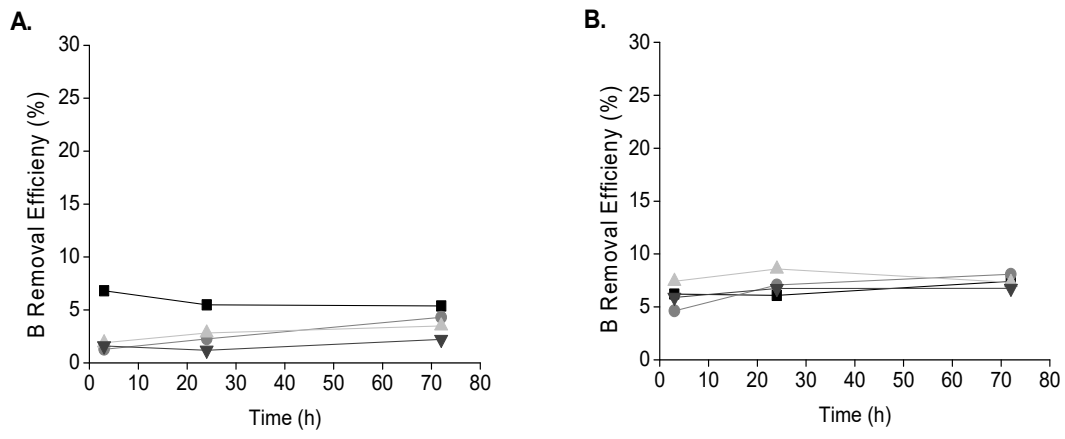
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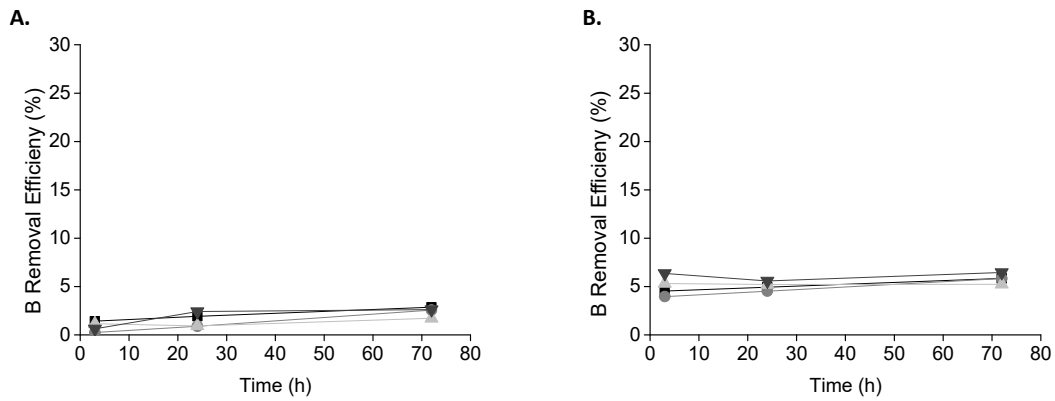


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772 **Figure S1.** Time course of the removal efficiency (%) of B in biosorption studies without
 773 CO₂ supply at different initial OM concentrations by *Chlorella vulgaris* (A), and
 774 *Scenedesmus almeriensis* (B) cultures at 1g TSS/L and pH 7.0. (▼) COM0 (control); (▲)
 775 COM1 (40 mg peptone /L and 27.5 mg meat extract /L); (■) COM2 (80 mg peptone /L and
 776 55 mg meat extract /L) and (■) COM3 (160 mg peptone /L and 110 mg meat extract /L).

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780 **Figure S2.** Time course of the removal efficiency (%) of B in biosorption studies with CO₂
 781 supply at different initial OM concentrations in cultures of *Chlorella vulgaris* (A) and
 782 *Scenedesmus almeriensis* (B) at 1g TSS/L and pH 7.0. (▼) C_{OM0} (control); (▲) C_{OM1} (40
 783 mg peptone /L and 27.5 mg meat extract /L); (■) C_{OM2} (80 mg peptone /L and 55 mg meat
 784 extract /L) and (■) C_{OM3} (160 mg peptone /L and 110 mg meat extract /L).

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