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Studies on bioremediation of Zn and acid waters using *Botryococcus braunii*

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ABSTRACT

In the present article the effects of Zn(II) on *Botryococcus braunii* in terms of growth and the photosynthesis-respiration metabolism and the ability of this microalgae to remove zinc present in wastewaters is described. The photosynthetic and respiration rates are affected by increasing metal concentration in solution, and therefore *B. braunii* growth rate decreases to a half, nevertheless the maximum value of biomass reached ($770 \pm 40\ mg\ l^{-1}$) is the same and the biomass remains viable throughout the range of concentrations studied ($0–80\ mg\ l^{-1}$). *B. braunii* exposed the ability to reverse the acidic conditions of the medium, showing a pH increase from 5.2 till values above 8.0 favoring the precipitation of different zinc compounds. Zn(II) specific removal increases along with initial metal concentration. The net adsorption capacity was determined, and the Freundlich, Langmuir and Hill models were applied. The stoichiometric relationship between H^+ release and zinc uptake in slightly acidic conditions is 1:1, and the adsorption kinetics follows a pseudo-second order model. The amount of metal removed increase when metabolic processes are involved. Removal of Zn with successive additions was achieved along 200 days, reaching a value of zinc removal of $3.4\ g\ g^{-1}$. The remediation of heavy metals (zinc, nickel and copper) and nitrates present in a leachate obtained from a bioleaching process was successfully performed. The present work represents a new approach on the biotechnological potential of *B. braunii* to grow in acidic conditions and to remove zinc, while differentiates passive adsorption from metabolically active remediation.

1. Introduction

The development of new technologies for removing contaminants from natural environments has been, in recent years, subject of study by researchers around the world [1,2]. Much of the pollution of natural waters is due to the discharge of industrial effluents [3,4]. The main sources of heavy-metal pollution are mining, milling and surface finishing industries, releasing a variety of toxic metals such as Cd, Cu, Ni, Co, Zn and Pb into the environment [5]. Since many of these metals are used as raw materials for different industrial applications and have a significant cost, they represent a valuable resource, so their recovery from wastes and effluents, assumes even greater significance.

Different chemical, biological, biochemical, biosorptive and physico-chemical techniques have been used to remove heavy metals from wastewaters [6] and groundwater [7], but although the removal of toxic heavy metals from industrial wastewaters has been practiced for several decades, the cost-effectiveness of these common physico-chemical processes is limited. These disadvantages can become more pronounced and further aggravate the cost of the process in such cases as contaminated ground waters, mine tailings effluent and other industrial wastewaters due to voluminous effluents containing complex organic matter and low metal contamination [5].

The development of economical and efficient technologies for the remediation of effluents with high volumes and low concentrations of metals such as those found in many industries is fundamental. Several approaches based on pH monitoring and control in wastewater treatment plants were reported [8].

Resistance to metal ions has been observed in several microorganisms, such as microalgae; suspended and immobilized cultures of a given specie have shown that their cell response depends mainly on the metal (type, concentration and activity) [9]. *Botryococcus braunii* is a green colonial planktonic alga that lives in freshwater and has a worldwide distribution. This alga is a well-known specie characterized by the generation of hydrocarbons in the outer layers of the cell wall [10]. Its rather slow growth, is the primary reason why the practical use of *B. braunii* is still considered to be quite challenging [11]. The major target for any possible application is increasing *B. braunii* biomass productivity [12]. Furthermore, recently published papers described

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wastewaters as a promising culture media due to its greater nitrate and mineral contents [13–16]. Even though *B. braunii* has a great capacity to synthesize hydrocarbons, its biotechnological potential also resides in its ability to reverse the acidic conditions of an effluent and to remediate the presence of heavy metals [17]. In previous works, we have demonstrated the capability of this alga to remove copper from solution through metabolically coupled mechanisms [17,18], since microalgae use carbon in its inorganic form, the carbonaceous equilibrium and, hence, the pH will be influenced [19]. When biotechnological processes are applied for environmental technologies, biomass growth characterization in terms of kinetics is of crucial importance for system design and optimization. Respirometry is often used to accomplish this by measuring the production and consumption rate of O_2 and CO_2 which is then coupled to the kinetics of the organism. According to Nakamura et al. [11] primary metabolism including nutrient uptake and utilization relevant to growth of the alga has lacked substantial attention, and is therefore, still poorly understood.

Even though several authors studied the capacity of *B. braunii* for nutrient removal [14,20,21], few have demonstrated the potential of this microalgae to remediate the presence of heavy metals in waste waters, nor evaluated its toxicity on *B. braunii* [22–25] grew *Botryococcus* sp. in heavy metal contaminated textile industry wastewater for the potential Cr, Cu, As and Cd bio-removal. They found that *Botryococcus* sp. effectively reduce Cr, Cu, As and Cd concentrations up to 94%, 45%, 9% and 2%, respectively Ab Razak et al. [26], also uses *Botryococcus* sp. to remove heavy metals present in waste waters. Gani et al. [22], investigated the bio-removal of low concentrations (ppb) of heavy metals from wastewater by *Botryococcus* sp., and results demonstrated that zinc, iron, cadmium and manganese were successfully removed. Nevertheless, the authors did not study the remediation process in terms of the physicochemical nor biological aspects involved in the processes. Two main mechanisms are expected for the removal of heavy metals: the uptake by biomass which in turn is divided into adsorption and active uptake and precipitation processes due to changes induced by microbial metabolism, mainly an increase in pH [27]

The present work pretends to determine the toxicity of Zn(II) on *Botryococcus braunii* in terms of growth and the photosynthesis-respiration metabolism; to study the ability of this microalga to reverse the acidic conditions of the medium and remove zinc present in wastewater; to study the adsorption capacity of this metal by *B. braunii*; and to implement this technology for the removal of heavy metals present in a leachate obtained from a bioleaching process of sediments of the Reconquista River [28], one of the most contaminated streams in Buenos Aires Province, Argentina.

2. Materials and methods

2.1. Microalgae cultivation

B. braunii was obtained from the Patagonic National University, San Juan Bosco. It was identified as A-race based on its hydrocarbon profile. Stock cultures were maintained in solid and liquid Bold Basal Medium (BBM), regularly subculturing at 3/4-week intervals. Cultures were maintained at 25 ± 1 °C temperature, under 1000 lux light intensity and 16:8 h light-dark intervals.

Biomass growth was determined by optical density (O.D.) at 680 nm [29] and dry weight was measured after cells were filtered on a glass fiber filter (GF/C 45 mm diameter, Whatman), rinsed with distilled water and dried at 60 °C for 24 h (constant weight). To establish the correlation between O.D. and biomass dry weight (mg), 100 ml of BBM was added to each of 7 erlenmeyer flasks containing a sample of the original *B. braunii* inoculum (25%). Samples were incubated in batch mode at 25 ± 1 °C temperature, under 1000 lux light intensity and 16:8 h light-dark intervals during different time intervals (7, 9, 13, 16, 25, 30 and 40 days). Afterwards, O. D. (680 nm) and dry weight were measured in each sample.

The biomass productivity P ($mg\,l^{-1}\,day^{-1}$) was calculated from the variation in biomass concentration X ($mg\,l^{-1}$) within cultivation time (days) [30] using Eq. (1):

$$P = \frac{(X_1 - X_0)}{(t_1 - t_0)} \quad (1)$$

The specific growth rate (μ) was calculated according to the following equation:

$$\mu = \frac{\ln\, x_1 - ln\, x_0}{t_1 - t_0} \quad (2)$$

2.2. Media and culture conditions

The biomass productivity of *B. braunii* was evaluated in 4 culture mediums, differing mainly in their nitrogen content and/or the presence or lack of citrate: BBM, BG-11, Chu 13 and Chu modified medium (no citrate). Known amounts of alga biomass were incubated in batch mode in 250 ml erlenmeyer flasks containing 100 ml of BBM, BG11, Chu 13 or modified Chu 13 mediums, at 25 ± 1 °C, under 1000 lux light intensity and 16:8 h light-dark intervals. The cultures growths were determined in each flask along 30 days.

2.3. Metal solutions

All metal solutions used were of analytical reagent grade.

Stock zinc(II) solutions for the experiments were prepared by dissolving $ZnSO_4{\cdot}7H_2O$ in distilled water. Solution concentration ranged from 5.5 to 80 $mg\,l^{-1}$ at a pH of 5.25 ± 0.25 to avoid precipitation of different insoluble zinc species.

2.4. Zinc(II) toxicity on B. braunii

Cultures were exposed during 35 ± 2 days to different zinc concentrations (0, 5.5, 8.5, 20, 25, 35, 45, 65 and 80 $mg\,l^{-1}$). Aliquots were taken at different time intervals in order to evaluate the pH variation, the culture growth (O. D. 680 nm), biomass productivity (Eq. (1)), the biomass specific growth rate (Eq. (2)), and the final zinc concentration in solution.

The effects of metals on the growth of *B. braunii* were statistically evaluated using Kolmogorov-Smirnov and Barlett tests and were finally analyzed using one-factor ANOVA.

2.4.1. Respirometry assays

Known amounts of biomass were added to several Schott vessels containing 80 ml of BBM with different zinc concentrations (0, 5, 10, 20, 45, 65 and 80 $mg\,l^{-1}$) and incubated in batch mode with constant orbital agitation, at 25 ± 1 °C, initial pH of 5.2 ± 0.2, under 1000 lux light intensity and 16:8 h light-dark intervals. The gas concentrations were measured by a Micro-Oxymax gas analyzer (Columbus instruments©), with a high sensitivity in order to detect the small gas composition change by 80 ml of algal culture. The O_2 and CO_2 concentration of the gas phase were measured along 26 h and the changes in the concentrations were used to estimate gas consumption or production rates.

2.5. Zinc(II) removal by B. braunii. Living biomass

The zinc removal efficiency was evaluated as a function of contact time and initial metal concentration. Known amount of biomass were added to 10 erlenmeyer flasks with 100 ml of BBM and different zinc initial concentrations (0, 5.5, 8.5, 10, 20, 25, 35, 45 and 65 $mg\,l^{-1}$). Samples were incubated during 35 ± 1 days in batch mode with constant orbital agitation; at 25 ± 1 °C; initial pH of 5.25 ± 0.25; under 1000 lux light intensity and 16:8 h light-dark intervals and initial pH of 5.25 ± 0.20. Experiments were conducted in initial acidic conditions

because, most effluents contaminated with Zn are acids, and to evaluate the metabolic capacity of the biomass to raise the pH of the culture media and hence favors the metal precipitation. Aliquots were taken at different time intervals to evaluate the pH variation, the culture growth and the final zinc concentration in solution.

Two control experiments were conducted. 1) 250 ml erlenmeyer flask containing BBM (100 ml) with 20 mg l^{-1} of zinc (no biomass); 2) 250 ml erlenmeyer flask containing *B. braunii* biomass cultured in BBM (100 ml) with no metal.

2.6. Zinc(II) removal experiments with continuous Zn(II) addition

For this purpose, known amount of biomass were added to 4 erlenmeyer flasks with 150 ml of BBM and different zinc initial concentrations (0, 5.5, 8.5, and 20 mg l^{-1}). Samples were incubated during 197 ± 3 days in batch mode with constant orbital agitation; at 25 ± 1 °C; initial pH of 5.25 ± 0.25; under 1000 lux light intensity and 16:8 h light-dark intervals. Aliquots were taken at different time intervals to evaluate the pH variation, the culture growth, and the final zinc concentration in solution.

When the zinc concentration in solution decreased to constant values in the erlenmeyers, an aliquot of concentrated zinc solution (1000 mg l^{-1}) was added to each flask to reestablish the initial metal concentration (5.5, 8.5 or 20 mg l^{-1}) and to evaluate the capacity of *B. braunii* to continue removing the metal in solution through time. This procedure was repeated in each flask every time the concentration in solutions decreased to constant values.

Two control experiments were conducted. 1) 250 ml erlenmeyer flask containing 100 ml of BBM with 20 mg l^{-1} of zinc (no biomass); 2) 250 ml erlenmeyer flask containing *B. braunii* biomass cultured in 100 ml of BBM with no metal.

2.7. Zinc(II) removal by B. braunii. Sorption experiments with dead biomass

Batch sorption experiments were performed with 0.1 g of biomass suspended in 100 ml of Zn(II) solutions in a concentration range between 2.5 and 575 mg l^{-1}. Suspensions were kept in constant agitation at pH 5.25 ± 0.25 and 25 °C.

Algal biomass was separated from metal solutions by filtration with cellulose nitrate membrane filters (0.22 μm). Afterwards, the final metal concentrations in the filtered solutions were determined and metal uptake (q) was calculated using the following mass balance equation [31]

$$q = \left[\frac{(C_i - C_{eq})*V}{m}\right] \quad (3)$$

Where C_i is the initial metal concentration (mg l^{-1} or mM), C_{eq} the equilibrium metal concentration (mg l^{-1} or mM), V the solution volume (l) and m is the dry alga weight (g).

Control experiments were carried out in the absence of adsorbents to find out whether there was any adsorption on the container walls.

To determine the net sorption capacity of *B. braunii*, the biomass was collected and washed thoroughly with deionized water and protonated with an acidic solution of HNO_3 (0.1 M) in order to displace the light metal ions from the binding sites (i.e. carboxylic, sulfonic, and others). Sorption experiments were conducted using stirred batch experiments at 25 °C to analyze net sorption capacity of *B. braunii* (0.130 ± 0.05 g l^{-1}) protonated dead biomass. The initial Zn(II) concentration was 25 ± 2 mg l^{-1}, at initial pH of 4–6, that were adjusted using NaOH (0.020 ± 0.001 M). This pH range was chosen to avoid the precipitation of zinc complexes that would lead to an overestimation of the adsorption. After zinc(II) adsorption equilibrium was reached the pH remained constant, the metal concentration in solution was determined and initial metal concentration was restored by adding an aliquot of concentrated zinc(II) solution. At the end of each experiment the amount of NaOH added to each solution as well as the amount of metal removed from solution was calculated to determine if there was any correlation between the number of protons released from the biomass and the amount of zinc adsorbed. Experiments were carried out by duplicate for each pH value.

2.8. Surface characterization

Dehydrated raw (control) and adsorbed metal samples with initial zinc(II) concentration of 20 mg l^{-1} were fixed to 10 mm metal mounts using carbon tape and spit coated with gold under vacuum in an argon atmosphere. The surface morphology of the coated samples was visualized by a file emission gun scanning electron microscope Zeiss (FEG-SEM Zeiss LEO 982 GEMINI©) with combined energy dispersive X-ray analyzer (EDS) at a voltage of 5.0 kV. SEM permitted the identification of interesting structural features on the microalgae surface with EDS. INCA® software was used to determine the elemental composition of the surface before and after metal binding.

2.9. Live biomass cultured in contaminated effluent

Most of the metal removal studies are conducted using synthetic metal solutions [32,33] and when the removal potential using real effluent is tested, the efficiency turns out to be very low [5]. In order to evaluate the remediation potential of a real effluent by *B. braunii*, the alga was cultured for 7 days in a lecheate obtained after the bioleaching of a sediment from the Reconquista River [28]. Experiments were made in shake flasks with constant orbital agitation; at 25 ± 1 °C; under 1000 lux light intensity and 16:8 h light-dark intervals. The effluent was first chemically characterized and initial copper, zinc, iron, nickel, NO_3^-, pH and the presence of organic compounds (TOC) were determined.

Aliquots were taken at different time intervals to evaluate the pH variation, the culture growth (O.D. 680 nm), the final metals and NO_3^- and TOC concentrations in the effluent, as well as biomass productivity (Eq. (1)) in order to compare it with the productivity obtained when cultivated in BBM.

2.10. Metal quantification

Metal concentrations were determined by atomic absorption spectroscopy (AAS) using a (GBC©) spectrophotometer and a zinc, copper, nickel or iron cathode lamp.

2.11. Reproducibility and data analysis

Unless otherwise indicated, all data shown are the mean values from three replicate experiments. Where error bars are not visible, this means errors were smaller or equal to the symbols (standard deviations below 5%).

For statistically interpreting the results of *B. braunii* growth in different culture mediums and exposed to different zinc concentrations, one-factor ANOVA analysis was performed using the GraphPadPrism 7® software package. The assumption of normality was verified using the Kolmogorov-Smirnov test, and homocedacea assumptions were verified using the Barlett test.

3. Results and discussion

3.1. Botryococcus braunii

growth rate and culture conditions

When correlation analysis between alga growth measured gravimetrically and spectrophotometrically (O.D. 680 nm) was performed results demonstrate the normal distribution of the data obtained

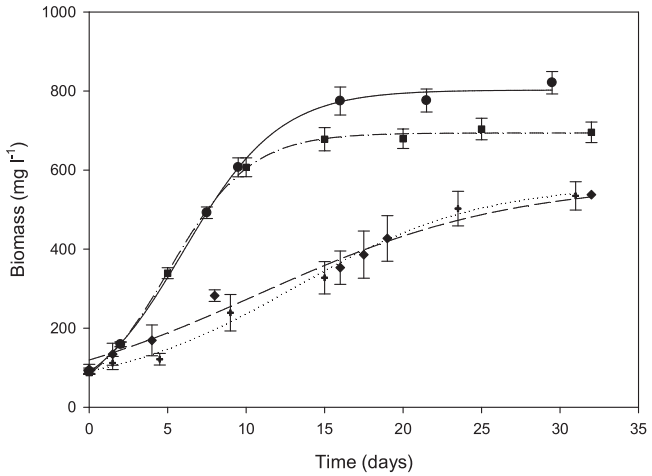

Fig. 1. *B. braunii* growth along 30 ± 2 days, in BBM (•); BG11 medium (■); Chu medium (+) and modified Chu medium (♦).

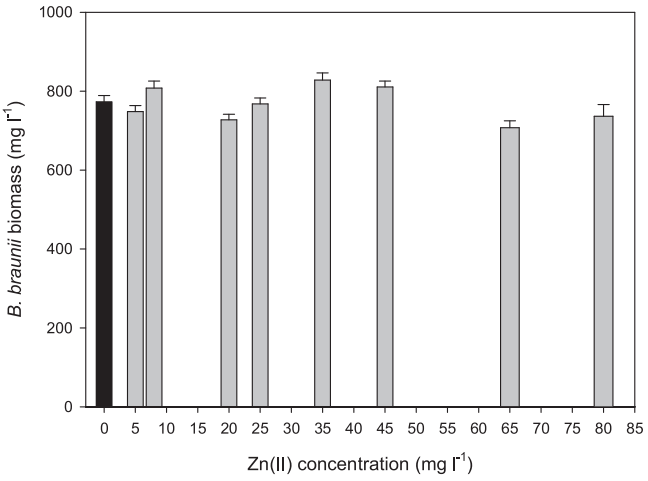

Fig. 2. Maximal growth of *B. braunii* when exposed during 35 ± 2 days to different Zn(II) concentrations: 0 or control (•) and 5.5, 8.5, 20, 25, 35, 45, 65 and 80 mg l^{-1} (●).

(P = 0.9928) and a linear correlation (R = 0.9778) between dry weight and O.D. The equation obtained was:

$$O.D. = 0.10379 \times C - 0.0791 \quad (4)$$

Where O.D. is the optical density measured at 680 nm for *B. braunii* growth and C is the alga dry weight (mg).

There are many autotrophic culture mediums for *B. braunii* culture maintenance and growth [34]. Each *B. braunii* strain grows to a different extent when it's cultivated in different kinds of media. Several suitable media for this alga have been reported in literature, such as modified BG-11 medium [35], modified Chu 13 medium [36], Bold basal medium (BBM) and BG-11 medium [34], among others. Hence when experimental conditions vary new experiments need to be conducted to find the adequate culture medium for rapid and sustained growth over time.

The extents of growth of *B. braunii* (A race), in different culture media and at room temperature (25 ± 1 °C), under 1000 lux light intensity and 16:8 h light-dark intervals are shown in Fig. 1. Even though the alga *B. braunii* can be cultivated in a varied range of culture conditions and various media, the microalgae cultured in BBM showed a greater and faster growth under the cultivation conditions of the present work, than in the other tested media. After 16 days, the culture growth reached a plateau and the maximum biomass obtained was 774 ± 37 mg l^{-1}, the values of P (Eq. (1)) and μ (Eq. (2)) obtained were 46 ± 4 mg l^{-1} $days^{-1}$ and 0.14 $days^{-1}$, respectively (Table A, Supplementary material). These results differ from others cited in literature, where other culture media were proposed as optimum for *B. braunii* autotrophic cultivation [34,37,38], or mixotrophic cultivation [39,40], where the biomass productivity (P) and/or the specific growth (μ) were smaller than those obtained for *B. braunii* cultivated in BBM (Table A, Supplementary material).

The four-different media compositions tested in the present study are shown in Table B (Supplementary material). BBM medium possess higher levels of K_2HPO_4, KH_2PO_4, $FeSO_4$, H_3BO_5, EDTA, $ZnSO_4$, $CuSO_4$ and $Co(NO_3)_2$. Even though the BG11 medium has 6 times higher $NaNO_3$, still the growth of *B. braunii* is higher in BBM than in BG11 culture medium. As it can be seen in Fig. 1 and Table B (Supplementary material), the modification in the Chu 13 medium, presence or absence of sodium citrate, did not affect the growth of the biomass through time, since the biomass productivity of the alga cultivated in both media did not present significant differences when statistically analyzed ($p < 0.05$).

3.2. Zinc (II) toxicity on B braunii. Respiration, photosynthesis and growth

Algae are susceptible to different contaminants, such as heavy metal, since they may produce metabolic changes that affect, respiration, photosynthesis and growth.

The effect of different concentrations of Zn(II) on the final biomass of *B. braunii* obtained after 35 days is shown in Fig. 2. The effects of the metal on both, the total growth and the specific growth rate of *B. braunii* were statistically evaluated using Kolmogorov-Smirnov and Barlett tests and were finally analyzed using one-factor ANOVA. Different Zn(II) concentrations (5.5, 8.5, 20, 25, 35, 45 65 and 80 mg l^{-1}) do not seem to affect the total growth of *B. braunii* after 35 days (Fig. 2), since there are no significant differences on the total alga growth exposed to the metal with respect to the control. Nevertheless, the specific growth rate (μ)decrease from 0.14 day^{-1} till values ≈ 0.06 day^{-1} when it is exposed to increasing concentrations of zinc, meaning that, even though zinc affects the growth kinetics of *B. braunii*, the maximum amount of biomass obtained is the same in all cases studied.

The capacity of *B. braunii* to grow under high Zn(II) concentrations represents an advantage over other microalgae species where Zn(II) is toxic at very low concentrations: *Scenedesmus* sp. [41], *Pseudokirchneriella subcapitata* [42], *Conticribra weissflogii* [43] among others.

These results demonstrate the capacity of *B. braunii* to grow under high Zn(II) concentrations, making it suitable for the remediation of effluents contaminated with this metal, such as those generated in the electroplating industry (among others).

The photosynthetic and respiration metabolism of *B. braunii* and how it is affected by the presence of Zn (II) in the medium was studied. Different Zn(II) concentrations (5.5–80 mg l^{-1}) affect *B. braunii* growth rate, even though do not seem to affect the amount of biomass obtained after 35 ± 2 days of culture, which corresponds to the maximum growth obtained for *B. braunii* (control) after 15 days when cultured in BBM (Fig. 1). These results are in agreement with those obtained when the photosynthetic and respiration metabolism is analyzed since the photosynthetic and respiration rates (CO_2 and O_2) are affected by increasing metal concentration in solution (Fig. 3A and B). Results show that in the light cycles Zn(II) affects the CO_2 fixation, and the O_2 generation (Fig. 3A).

In darkness, the respiration rate (CO_2 and O_2) decrease when Zn(II) concentration in solution increase (Fig. 3B). The net balance between the rates of consumption (photosynthesis) and production (respiration) of CO_2 in the presence of zinc, are in agreement with the growth curves obtained under the same conditions.

These results demonstrate that metal in solution possess a negative

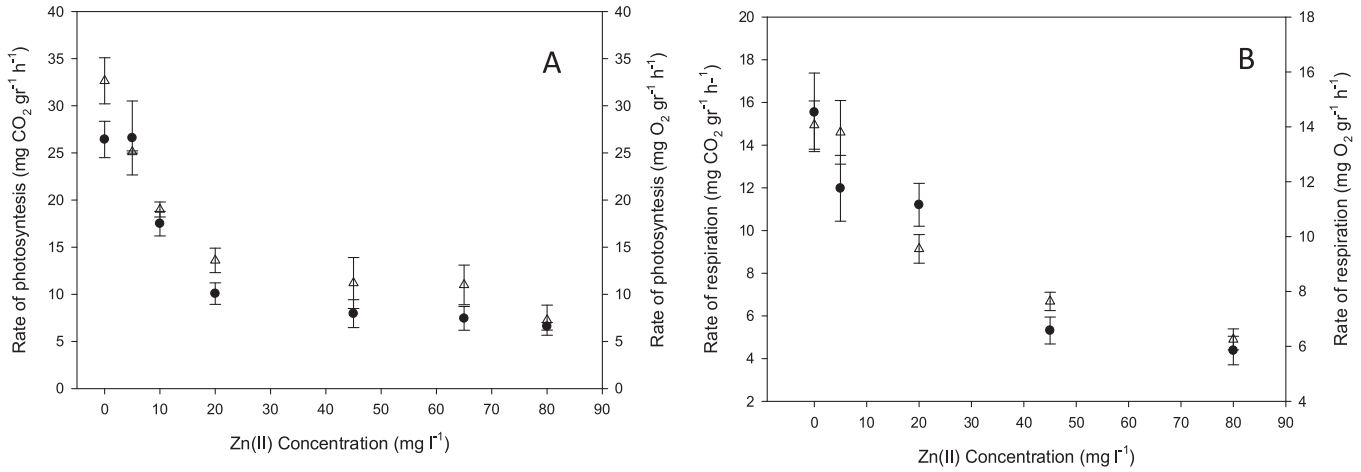

Fig. 3. *B. braunii* photosynthesis (A) and respiration (B) rate, in terms of CO_2 consumption/production (Δ) and O_2 production/consumption (•) (mg g^{-1} h^{-1}), when exposed to different Zn(II) concentrations (0, 5, 10, 20, 45, 65 and 80 mg l^{-1}).

effect on the metabolism of *B. braunii* even if there is no net effect on its growth along 30 days.

3.3. Zinc(II) removal efficiency by B. braunii. Live biomass

In order to evaluate the Zn(II) removal capacity of *B. braunii,* it is important to assess how metal removal varies with aqueous metal concentrations. Fig. 4 shows examples of the different profiles removal through time for low, medium and high Zn(II) concentrations, when cultured in BBM at room temperature (25 ± 1 °C), under 1000 lux light intensity and 16:8 h light-dark intervals.

Fig. 4 shows the metal removal by this alga after 35 ± 1 days, when cultured in BBM at room temperature (25 ± 1 °C), under 1000 lux light intensity and 16:8 h light-dark intervals. As it can be seen (Fig. 4) for all the initial concentrations tested the final Zn(II) concentration never reaches values lower than 5 mg l^{-1}. Control experiments without biomass showed no changes in Zn(II) concentration along time.

The decrease of zinc concentration in solution is expected to be mainly due to precipitation and adsorption mechanisms. As it will discuss later, the amount of zinc removed by adsorption at concentration of 5.5 mg l^{-1} may not be detectable (Fig. A, Supplementary material) and although it is not be strictly correct to analyze the results as in equilibrium systems (comparing with tabulated solubility constants),

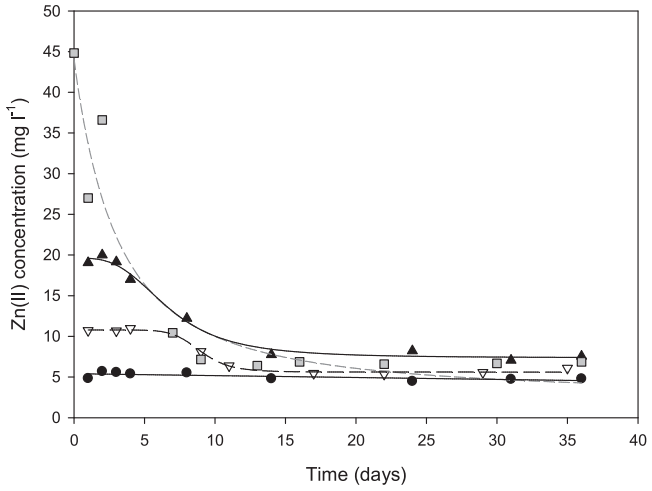

Fig. 4. Zn(II) removal profile for four different initial metal concentrations: 5.5 (•), 10 (▽), 20 (▲) and 45 (■) mg l^{-1}.

the results obtained suggest that in this experimental conditions, the minimum Zn(II) concentration necessary for solution metal saturation is close to 5.5 mg l^{-1}, since Zn(II) hydroxides, oxides and other complexes begin to precipitate.

This would explain why at initial metal concentration of 5.5 mg l^{-1} there is no metal decay in solution during the experiment (35 ± 1 days) (Figs. 4 and 5 A).

The variation of medium pH when *B. braunii* was grown in BBM with different Zn(II) concentrations (0, 5.5, 8.5, 10, 20, 25, 35, 45 and 65 mg l^{-1}) was studied. The variation of pH throughout different experiments does not seem to be affected by initial zinc concentration in solution (Fig. 5B), since results demonstrated that the pH of the medium increases to values above 8 ± 0.4, in all cases studied; it is well-known that the physiological activity of the phototrophic organisms, such as *B. braunii*, affect the medium pH, as Eqs. (5) and (6) [44] show, the consumption of dissolved CO_2 or HCO_3^- by microalgae results in a pH increase in the system as protons are consumed in the process.

$$106\ CO_2 + 122\ H_2O + 16\ NO_3^- + 18\ H^+ + HPO_4^{2-} \rightarrow\ C_{106}H_{263}O_{110}N_{16}P + 138\ O_2 \quad (5)$$

$$106\ HCO_3^- + 16\ H_2O + 16\ NO_3^- + 124\ H^+ + HPO_4^{2-} \rightarrow\ C_{106}H_{263}O_{110}N_{16}P + 138\ O_2 \quad (6)$$

In these experiments, the initial medium pH were slightly acidic (5.25 ± 0.25) to avoid the initial metal complexes precipitation, and to ensure that the decay of metal concentration in solution was due only to the presence of the microalga (metabolic and non-metabolic processes).

The maximum pH value of the media reached in the experiences where zinc concentration in solutions were higher than 5.5 mg l^{-1}, favors the precipitation of different zinc complexes, therefore, metal remediation is due to adsorption process as well as precipitation. We will discuss later in the present manuscript the extent of both mechanism on zinc removal by *B. braunii*.

Results demonstrate the capability of *B. braunii* to remove Zn(II) from solution, since the metal specific removal grows with initial metal concentration (Table 1), as well as the removal percentage, reaching values higher than 90% of the initial metal concentration (Fig. 5A and B). The Zn(II) removal efficiency obtained exceed those found in literature for other species: *Botryococcus* sp. [22]; *Phormidium luridum* [45]; *Scenedesmus obliguus* [46] and can be compared to those obtained for some other microalgae, such us: *Chlorella* sp. [47–49]; *Stichococcus bacillaris* [50] and *Spirulina maxima* [51].

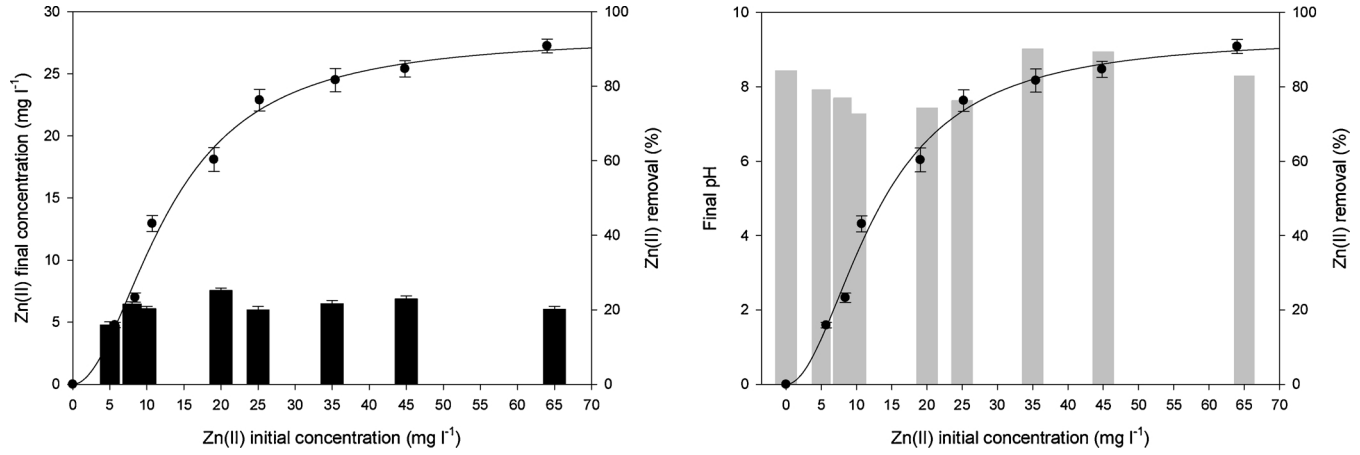

Fig. 5. A: Total metal removal (%) (•), and final Zn(II) concentration (mg l^{-1}) (black columns) B: Total metal removal (%) (•) and final pH (grey columns). The data in both figures were obtained when *B. braunii* was cultured during 35 ± 1 days in BBM with different Zn(II) initial concentrations (5.5, 8.5, 10, 20, 25, 35, 45 and 65 mg l^{-1}).

Table 1
Total Zn(II) specific removal (mg g^{-1}) by *B. braunii* exposed to different Zn(II) initial concentrations (0, 5.5, 8.5, 10, 20, 25, 35, 45 and 65 mg l^{-1}).

Initial Zn(II) concentration (mg l^{-1})	Zn(II) Specific Removal (mg g^{-1})
0	0
5.7 ± 0.2	1.2 ± 0.1
8.4 ± 0.2	2.4 ± 0.1
10.7 ± 0.3	5.6 ± 0.2
19.06 ± 0.50	16.6 ± 1
25.25 ± 0.25	25.2 ± 2
35.5 ± 0.4	33.4 ± 2
44.8 ± 0.1	45.3 ± 3
64 ± 0.5	95.6 ± 2

3.4. Zinc(II) removal experiments with sequential Zn(II) addition

The capability of *B. braunii* to remove Zn(II) when it is incubated for 197 ± 3 days with different initial Zn(II) concentrations, 5.5, 8.5 and 20 mg l^{-1} and with constant metal addition through time are shown in Fig. 6(A)–(C), respectively.

As it can be seen in Fig. 6A, Zn(II) when initial metal concentration in the culture medium was 5.5 mg l^{-1}, there was no metal removal, and therefore, there was no need to add more metal in subsequent cycles. As mentioned in Section 3.3, the minimum Zn(II) concentration necessary for solution metal saturation is close to 5.5 mg l^{-1}, since Zn(II) hydroxides, oxides and other complexes begin to precipitate. Instead when initial Zn(II) concentration was 8.5 mg l^{-1} (Fig. 6B), The Zn concentration drops following pH rising and the necessary calculated Zn(II) amount was added to the systems to reestablish the original zinc concentration. A total of 11.8 mg l^{-1} of metal were added to the culture medium throughout 3 metal removal-addition cycles. The metal removal along the experience corresponds to 28.8% (165 mg zinc gr biomass^{-1}) of the total zinc. added to the culture medium (Fig. 6D).

In the experience were initial metal concentration was 20 mg l^{-1} (Fig. 6C), five removal-addition cycles of metal were done. As in the previous experiment, in each cycle, the necessary calculated Zn amount was added to the systems to reestablish the original zinc concentration (20 mg l^{-1}). However, the initial metal concentration could not be reestablished in any of the addition cycles due to the rise in the pH value of the solution (> 8), since this chemical condition causes zinc precipitation when is added to the medium. For this reason, at the last metal addition cycle a total of 34 mg l^{-1} of zinc was added, achieving a total zinc removal of 31.3 mg l^{-1}. In this experience a total of 54.62 mg l^{-1} were added in the first four metal removal-addition cycles, removing till this point 60% of the total metal added in the experience. Along this experience, a total of 89.62 mg $l^{-1}$of zinc was added to the solution and the total metal removal was 79.52 mg l^{-1} (Fig. 6D), which corresponds to a total metal removal of 88.7% (3.4 gr zinc gr biomass^{-1}).

Results obtained from control experiments, demonstrate that metal concentration and the pH of the culture medium (5.2 ± 0.2) remain constant through time when there is no algae present in solution; and when *B. braunii* is cultivated in the culture medium with no zinc, the pH of the solution rises to values above 8.

These results support those obtained in Section 3.3 for open systems; in the experiments with initial zinc(II) concentration of 8.5 and 20 mg l^{-1} (Fig. 6B and C, respectively) the minimum solute concentration necessary for solution metal saturation is reached, since metal complexes begin to precipitate (because of pH rise in the culture medium), and metal concentration measured in solution begin to decay. This would explain why at initial metal concentration of 5.5 mg l^{-1} (Fig. 6A) there is no metal decay in solution during the experiments (200 days). From these results a theoretical mean solubility constant was calculated for a hypothetical hydroxide in the present system, KSP = 2,47 × 10^{-6}, this calculated value is similar to the KSP of the $ZN(OH)_2$.

3.5. Biosorption experiments. Dead biomass

3.5.1. Biosorption kinetics and kinetic models

Fig. 7 shows the Zn(II) metal uptakes by *B. braunii* versus time at pH 5.5 and room temperature. The initial metal concentrations were 22.5 ± 0.5 mg l^{-1}. The metal uptake (q) was calculated using Eq. (3) and increases with the time of contact, it is relatively fast and in a few minutes the equilibrium plateau is reached. The maximum experimental zinc uptake obtained was 8.5 ± 0.2 (mg g^{-1}).

The time profile of metal uptake is a single, smooth, and continuous curve leading to saturation. Two kinetic models, pseudo first order and pseudo second order were applied to interpret the experimental results.

3.5.1.1. Pseudo-first-order model. This kinetic model is based on a pseudo-first order rate expression of Lagergren

$$\ln(q_e - q_t) = \ln q_e - k_1 t \quad (7)$$

Where k_1 is the pseudo first order sorption rate constant, q_e is the amount of metal ion adsorbed at equilibrium by the biomass and q_t is the amount of metal ion adsorbed at any time t. Both, q_e and q_t were expressed in mg g^{-1} units. The overall rate constant k_1, in hours^{-1}, was calculated (Table 2).

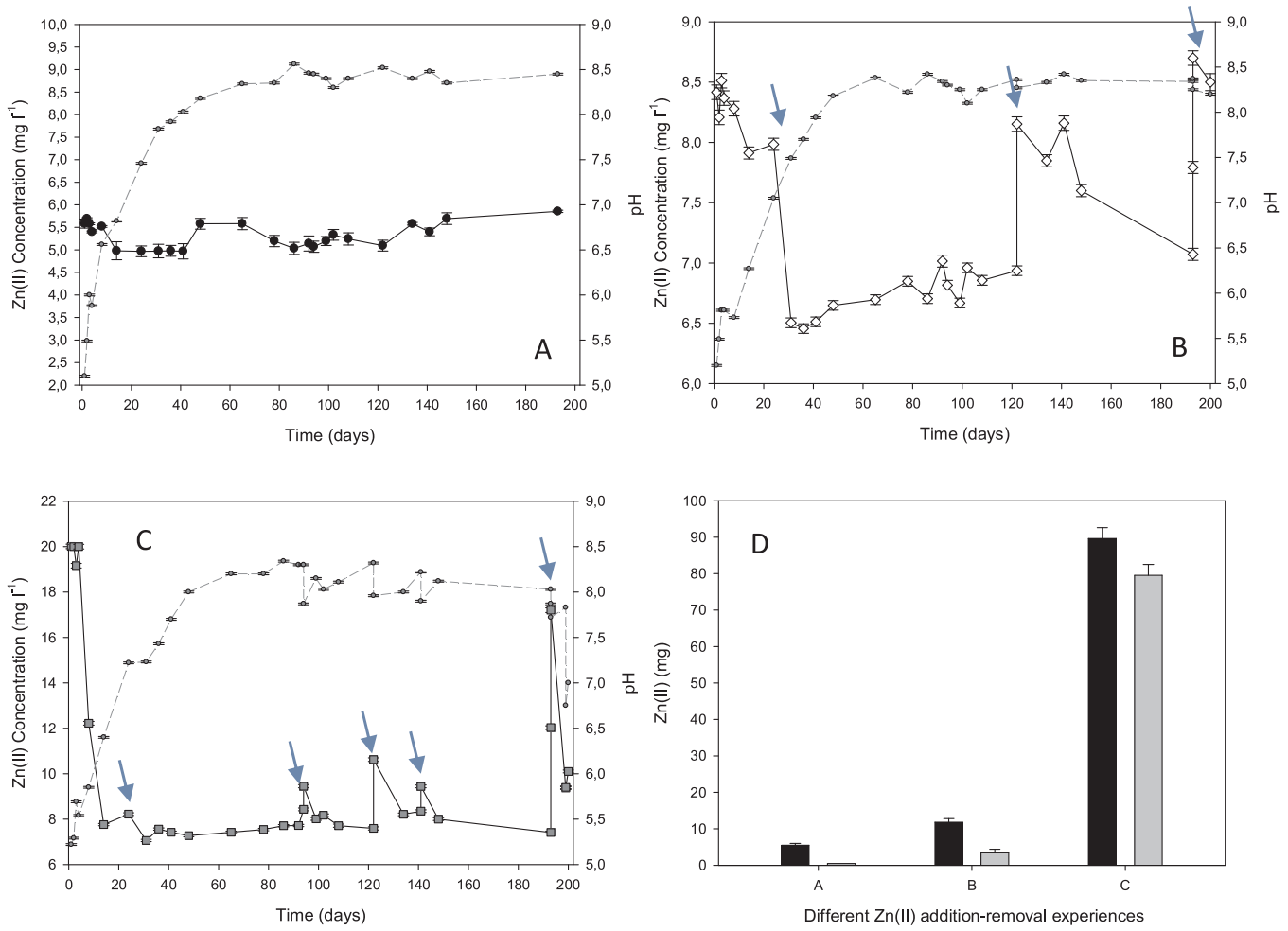

Fig. 6. Metal addition-removal profiles for different Zn(II) initial concentrations: (A) 5.5 mg l^{-1}; (B) 8.5 mg l^{-1} and (C) 20 mg l^{-1}. Arrows indicate metal addition. D: Total Zn(II) (mg) added (black) and removed (grey) in different experiments (A, B and C).

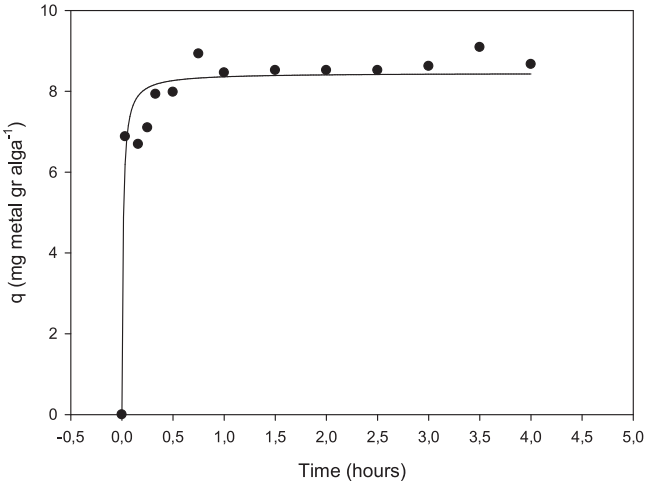

Fig. 7. Biosorption kinetics of zinc(II) uptake at pH = 5.5 and room temperature biosorbed onto *B. braunii*. Solid lines were calculated using binding ligand kinetics model (Eq. (10)).

3.5.1.2. Pseudo-second-order model. Several systems respond to a second order kinetics model for sorption reactions, and are represented by the following equation [52]:

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad (8)$$

Table 2
Pseudo-first and pseudo-second-order kinetic parameters for zinc(II) adsorption at room temperature and pH 5.5. q_{eq}: maximum coverage concentration, k_1: pseudo-first-order constant, k_2: pseudo-second-order constant.

Pseudo-First- Order	q_{e1} (mg g^{-1})	2.1 ± 0.2
	k_1 (h^{-1})	2.9 ± 0.2
	R^2	0.9705
Pseudo Second- Order	q_{e2} (mg g^{-1})	8.80 ± 0.01
	k_2 (g h^{-1} mg^{-1})	0.35 ± 0.02
	R^2	0.9986

where k_2 is the rate of pseudo second order adsorption and q_e and q_t are the amount of metal ion adsorbed at equilibrium and the amount adsorbed at any time, respectively. The sorption rate can be calculated as the initial sorption rate when t approaches 0. Integrating and rearranging, the pseudo-second-order equation can be written as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (9)$$

The solid lines in Fig. 8 were plotted using the binding ligand model (Eq. (10))

$$q = \frac{B_{max} * t}{(k + t)} \quad (10)$$

Eq. (10) is equivalent to Eq. (9) when $B_{max} = q_e$ and k2-1 = k*B_{max}. The fitting parameters for both pseudo-first and pseudo-second order equations are listed in Table 2. The correlation factor of the pseudo

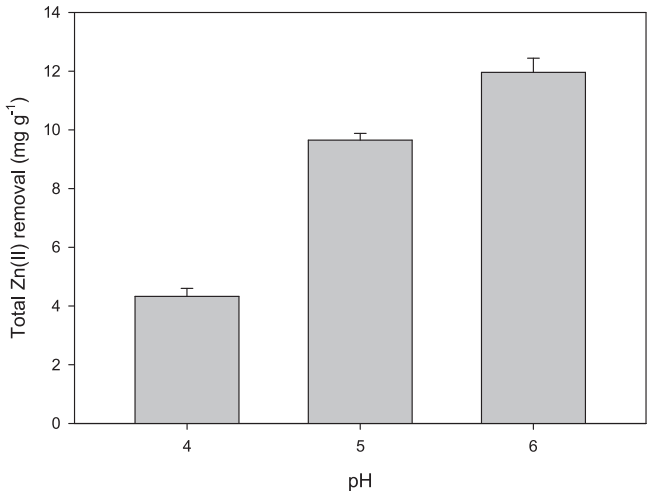

Fig. 8. Total Zn(II) removal efficiency by dead *B. braunii* biomass at different pH values (4–6).

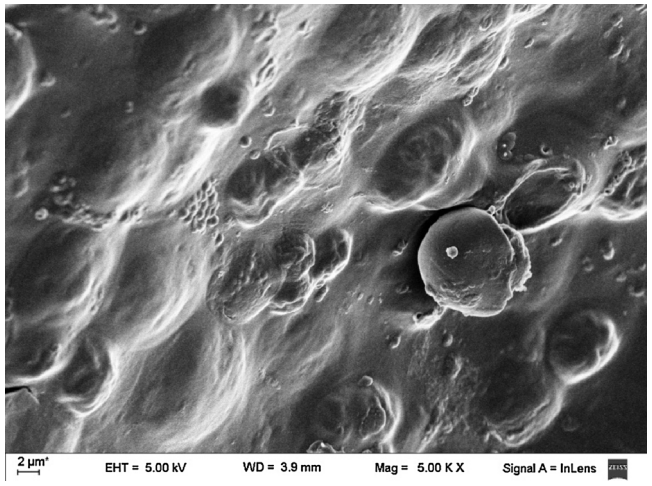

Fig. 9. *B. braunii* Scanning Electron Microscopy (SEM) at 5000X and 5.0 kV.

second order kinetics model obtained was 0.998. The analysis of the results shows that the correlation factors obtained were not as good for the pseudo-first order model, as they were for the pseudo-second order kinetic model, which were very good. Moreover, the values of q_e calculated from the pseudo-first order kinetics are not like the experimental values, while those calculated from second-order model are in good agreement with the experimental values in Fig. 7. Thus, experimental data are better fitted by the second-order equation than by the pseudo-first order equation. The pseudo second order model has already been successful when applied to the description of heavy metal biosorption by different bioadsorbents. The obtained pseudo second order kinetic parameters are in accordance with those found in the literature for the adsorption of Zn(II) by other species [53–55].

Table 3
Initial chemical composition of a contaminated effluent.

Parameter	Value
pH	4.7 ± 0.1
TOC	4.0 ± 0.2 mg l^{-1}
Nitrates	182 ± 2 mg l^{-1}
Zinc	6.04 ± 0.01 mg l^{-1}
Nickel	0.40 ± 0.01 mg l^{-1}
Copper	0.44 ± 0.01 mg l^{-1}
Iron	0.45 ± 0.01 mg l^{-1}

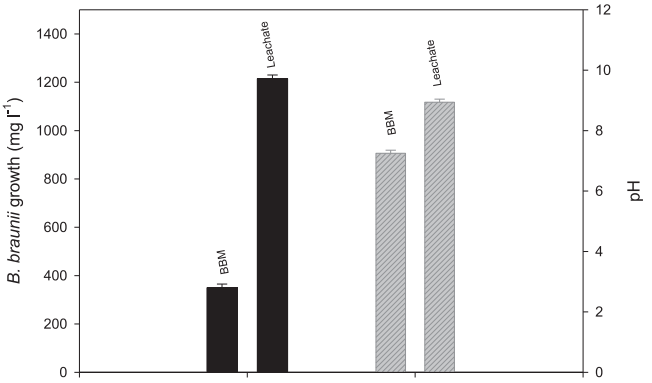

Fig. 11. *B. braunii* final growth (•) and solution pH (•) when cultivated for 7 days in BBM and in a contaminated leachate.

3.5.2. *Botryococcus braunii* adsorption capacity

In order to elucidate the extent of non-metabolic processes in the zinc removal capacity of *B. braunii*, the metal adsorption capacity of the microalgae was studied.

Many chemical groups present in the cell walls of the alga contribute to the biosorption of free ions in solution. The occupation of the surface will depend on the total number of sites, the concentration of the adsorbate in solution, the pH and the temperature.

The zinc adsorption isotherm by *B. braunii* (Fig. A, Supplementary material), cannot be described by typical isotherm adsorption models (Langmuir, Freundlich, D-R, among others) (Figs. B and C Supplementary material). The Hill model (Eq. (11)) [56] was applied to equilibrium data, this model assumes that the adsorption capacity is a cooperative phenomenon.

$$q_{eq} = q_0 + \frac{q_{SH} C_{eq}^{n_H}}{K_D + C_{eq}^{n_H}} \quad (11)$$

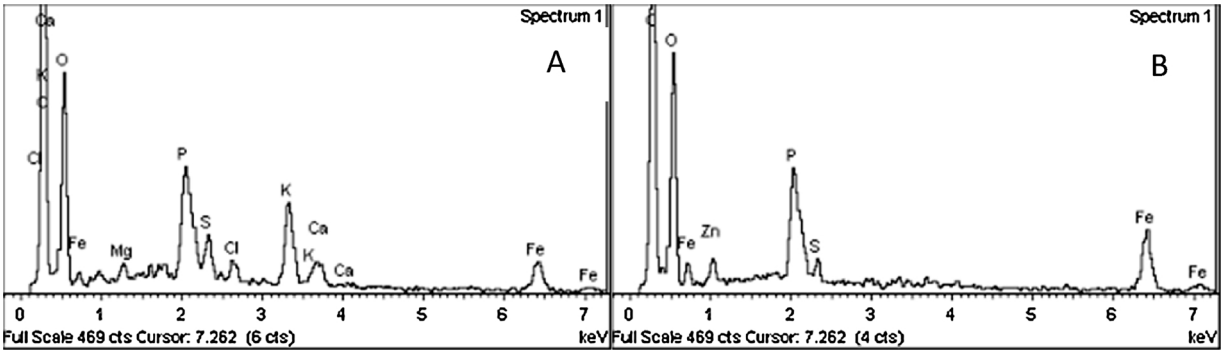

Fig. 10. *B. braunii* EDS images: (A) before treatment and (B) after Zn(II) removal experiment (initial concentration 20 mg l^{-1}).

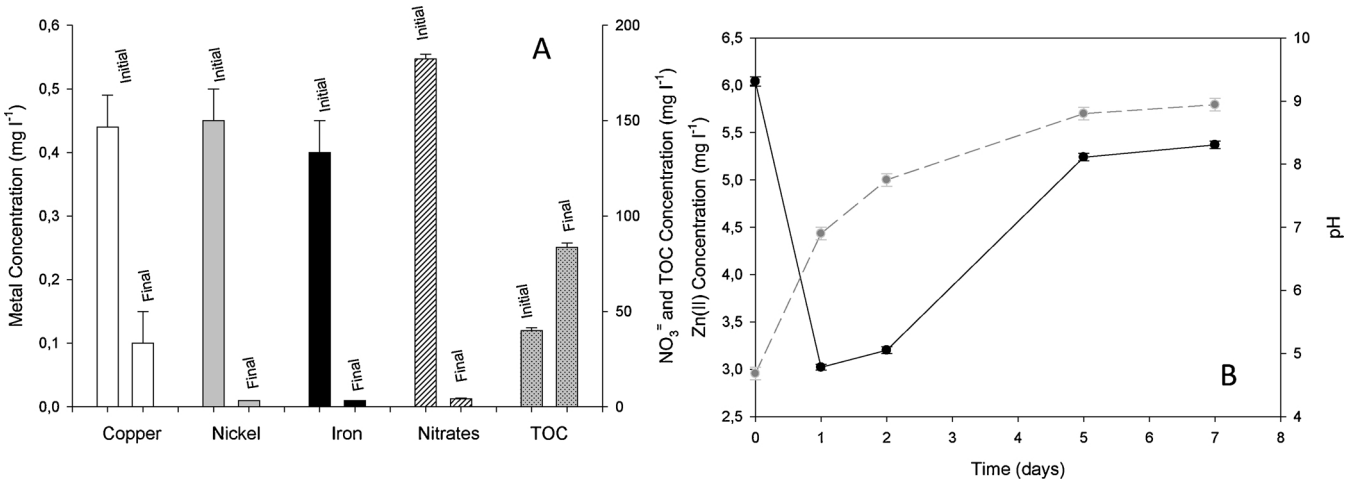

Fig. 12. (A): Copper (□), nickel (▒), iron (■), nitrate (▭) and TOC (▒) concentrations in the leachate before and after being treated with *B. braunii*; (B): Leachate Zn (II) concentration (——) and pH (- - -) profile along 7 days, when used for *B. braunii* cultivation.

Where q_{eq} is the specific adsorption at equilibrium (mg g^{-1}), C_{eq} is the metal concentration at the equilibrium on the aqueous media (mg l^{-1}), q_{SH} refers to the maximum metal uptake (mg g^{-1}), K_D is the Hill constant (mg l^{nH}), n_H is the Hill's cooperative coefficient of the binding interaction and q_0 is the adsorption at very low initial metal concentrations.

The Hill isotherm fitted well ($R^2 = 0.988$) the experimental data. This model would assume the existence of possible cooperative interactions via chemical bonding between the Zn complexes and the microalgae surface. The model illustrates when a ligand would be capable of binding with a macromolecule [57]; also influencing the binding ability of other ligands onto the same macromolecule [56]. Hence, cooperative interactions could occur on the surface of the microalgae creating strong chemical bonding between the functional groups present on the surface of *B. braunii* and more than one zinc complex present in solution. This effect is quantified by the Hill cooperativity coefficient (n_H), in the present study a positive cooperativity would seems to exist ($n_H > 1$) [58] since $n_H = 7.8$. *B. braunii* maximum sorption capacity (q_{SH}) obtained through the Hill equation was 71 ± 5 mg g^{-1} and $q_0 = 7 \pm 2$ mg g^{-1}. This value is very close to the minimum solute concentration necessary for Zn saturation at the studied pH (5.5 mg l^{-1}). Results (Fig. A, Supplementary material) demonstrate that the net adsorption at initial zinc concentrations smaller than 5 mg l^{-1} may not be detectable due to experimental conditions. These results explain those obtained in Sections 3.3 and 3.4 where there is no zinc removal at concentrations lower than 5.5 mg l^{-1}.

In the present work zinc adsorption by treated *B. braunii* biomass at pH values of 4, 5 and 6 were studied. Treated biomass implied the protonation of the biomass with a strong acid (HNO_3, 0.1 M). Most of the binding sites have acid-base properties: when the solution pH exceeds the pK_a values of the functional groups, there is a higher amount of binding sites that are deprotonated; under these conditions they are neutral when protonated and are negatively charged when deprotonated, and the cation adsorption is favored [59].

The observed release of protons balanced the uptake of heavy metal from solution leading to a change in the pH of the solution to lower values. In order to maintain the pH of the solution at a constant value (4, 5 or 6) and to calculate the number of protons released from the surface of the microalga, pH was kept constant by NaOH (0.020 ± 0.001 M) addition. The amount of base added is proportional to the proton released. This was interpreted as ion-exchange between zinc and protons at the binding sites. Ion-exchange is not the only binding mechanism involved, but yet the main-one in Zn biosorption by *B. braunii*. Other binding mechanism(s) may range from physical (i.e. electrostatic or London–van der Waals forces) to chemical binding (i.e. ionic and covalent) [60]. Results demonstrated that a 1:1 stoichiometric relationship exist between H^+ release and zinc uptake by protonated biomass. The speciation diagram (MINEQL®) for Zn complexes in BBM (Fig. D Supplementary material) shows that the main species at the working concentration (26 ± 2 mg l^{-1}) and pH range (4–6) are $ZnSO_4$ (aq), $Zn(SO_4)^{2-}$, $ZnHEDTA^{1-}$, $ZnEDTA^{2-}$ and Zn^{2+}. The Eqs. (12)–(15) describe the formation reactions of the most important complexes in the working pH range.

$$Zn^{2+} + H_4EDTA \rightarrow ZnHEDTA^{1-} + 3H^+ \quad pK_f = 21.4 \tag{12}$$

$$Zn^{2+} + H_4EDTA \rightarrow ZnEDTA^{2-} + 4H^+ \quad pK_f = 16.5 \tag{13}$$

$$Zn^{2+} + SO_4^{4-} \rightarrow ZnSO_4^{2-} \quad pK_f = 3.28 \tag{14}$$

$$Zn^{2+} + SO_4^{2-} \rightarrow ZnSO_{4(aq)} \quad pK_f = 2.34 \tag{15}$$

The isoelectric point (IEP) of most algal cell walls lies between pH 3 and 4 [59]. Metals such as Zn(II), are biosorbed at pH values above the IEP, near the apparent dissociation constant of carboxylic acids (pKa around 5), where the overall surface charge becomes negative, resulting in weaker electrostatic interactions mostly through ion-exchange mechanisms between the surface groups and metal cations but also in covalent bonds, if the pH dependence is slight. Thus, a pH range of 4–7 is widely accepted as being optimal for metal uptake for almost all types of biomass [61]. Then, zinc, which is positively charged at the working pH range, is adsorbed at pH values over the IEP of the microalga, this process led to an acidification of the media as was experimentally observed.

In the present work, the zinc adsorption capacity increased along with the pH, as shown in Fig. 8. These results demonstrate that pH affects the adsorption capacity of *B. braunii* when the pH is slightly acidic.

The amount of Zn(II) removed by adsorption (non-metabolic process) at initial concentration of Zn(II) of 25.2 ± 0.2 mg l^{-1}, was 4.3 ± 0.3, 9.6 ± 0.2 and 12 ± 0.5 mg g^{-1}, at constant pH values of 4, 5 and 6, respectively. Initial solution pH played a significant role on metal biosorption, with maximum uptake at pH values of six (Fig. 8). In acidic conditions, the functional groups of the cell walls are protonated, which means that most of the binding sites are occupied by protons therefore decreasing the microalga metal biosorption capacity.

In contrast, when the microalga is metabolically active, the pH of the medium increases to values above 8.0 ± 0.4, in all cases studied (Fig. 5B); as depicted early, and then the zinc removal by *B. braunii* increase till values of 25 ± 2 mg g^{-1}. These results demonstrate that when metabolic processes are involved, the amount of zinc(II) removed from solution increase significantly, with respect to net adsorption (non-metabolic process).

3.6. Surface characterization

Scanning electron microscopy (SEM) images were used for the surface analysis of the alga as shown in Fig. 9.

Fig. 9 reveals the superficial structure of the cell walls of *B. braunii* imbibed in the polysaccharides matrix, where zinc cations could be adsorbed. To establish the extent of adsorption in the zinc remediation experiments the energy-dispersive X-ray spectroscopic (EDS) images for the alga before and after experimental procedures for zinc removal when initial metal concentration of 20 mg l^{-1}, are presented in Fig. 10A and B, respectively. *B. braunii* does not present any metal adsorbed on the surface before being cultured in BBM with zinc (Fig. 10A). EDS after being exposed to the metal, reveal the presence of zinc on the surface of the microalga (Fig. 10B). Results also revealed that the amount of metal present on the surface of the alga was less than 30% of the total metal added originally to the medium. These results are consistent with those presented in Section 3.5.2, where the amount of zinc removed by *B. braunii* was ≈ 35%.

3.7. Contaminated leachate remediation

The capacity of *B. braunii* to growth and to remediate a contaminated effluent was evaluated in the present work. The chemical composition of a real effluent obtained from bioleaching process applied to a contaminated sediment [28] is described in Table 3.

Fig. 11 shows the final alga growth as well as the final pH of the effluent after 7 days of being cultivated in the contaminated leached. As it can be seen, the growth of *B. braunii* cultivated in the leachate was 3 times higher than the control after the same period of time (7 days), with a biomass productivity (P) of 179.7 mg l^{-1} day^{-1} in the leachate vs 50.0 ± 4.0 mg l^{-1} day^{-1} in the control experiment. The pH increase in the system with leachate is also higher than in the control system with BBM (9 vs 8.3).

Along the 7 days experiment it was possible to remove most of the copper and all iron and nickel initially present in the leachate (Fig. 12A). Nitrates were consumed by the alga as a nitrogen source, since the final concentration in solution was 4.23 mg l^{-1}. Besides the pH value of the leachate, the only parameter which grew was the TOC concentration in solution, which may be due to the metabolites that *B. braunii* produces and excrete to the media.

The zinc concentration was reduced in the first two days by half in ranges of pH 7–8; after 7 days (pH = 9), the solution metal concentration rises to values above 5 mg l^{-1} (Fig. 12B), reaching the minimum solute concentration necessary for solution metal saturation as demonstrated in Sections 3.3 and 3.4. This variation of Zn(II) concentration in solution with pH is because the amphoteric behavior of Zn (II). Zn(II) complexes (Section 3.5.2) begin to precipitate at pH values above 6 and when it reaches values near 9, the zinc precipitated is resuspended ones again as $Zn(OH)_4^{-2}$, as it can be seen in Fig. 12B. Results demonstrate not only the capability of *B. braunii* to grow in contaminated effluents, but to remediate the presence of Zn(II) and low concentrations of nickel, copper, iron and nitrates from the effluent due to metabolic and non-metabolic remediation pathways (precipitation-adsorption).

4. Conclusions

The present work demonstrates the ability of *B. braunii* to grow in acidic environments contaminated with high concentrations of zinc, even when the metal affects the rates of photosynthesis and respiration. Which represents a new approach on the biotechnological potential of this microalgae, that have not been previously addressed.

Botryococcus braunii can remove Zn(II) from aqueous solutions by non-metabolic (adsorption) and mainly by metabolic (precipitation) processes, due to the capacity of this algae to reverse the acidic conditions of the medium, which favors the precipitation of different zinc insoluble species.

B. braunii can successfully remove zinc over long periods of time (months) with the subsequent addition of metal, which implies an advantage over other remediation mechanisms.

EDS confirmed the presence of Zn(II) ions on the biomass surface when exposed to high metal concentrations.

It was demonstrated that *B. braunii* can grow in a contaminated leachate obtained from a bioleaching process of anaerobic sediments of a contaminated river (Reconquista River, Argentina) while successfully remediate the presence of zinc, copper, nickel, iron and nitrates in it, due to the biomass capacity to revert the acidic conditions of the leachate and to metabolize de nitrates in less than 48 h.

Results demonstrate that pH must not exceed 8.5 along the remediation process to avoid metals (such as zinc) complexes to resuspend.

These results suggest the biotechnological potential of *B. braunii* to be used in the remediation process of acidic contaminated effluents containing heavy metals such as Zn, Ni and Cu; and a new approach on the study of this microalgae.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jece.2018.05.041.

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