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Optimization of strontium removal by *Tetraselmis chui* grown in bubble column photobioreactors

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ABSTRACT

Green microalgae of the class Chlorodendrophyceae have recently attracted the interest of researchers due to their ability to form micropearls (intracellular inclusions of amorphous calcium carbonate) highly enriched in Sr. The marine species *Tetraselmis chui* (included in the class Chlorodendrophyceae) shows high uptake of both stable and radioactive Sr isotopes and has been suggested as a potential candidate for the development of new bioremediation tools regarding radioactive ^{90}Sr pollution. In this study, we optimized Sr removal from seawater by growing *T. chui* in 1-L bubble column photobioreactors (PBRs) with and without CO_2 supply. Culturing *T. chui* in bubble column PBRs greatly improves cell production and Sr removal compared to previous studies. Furthermore, the addition of 10 mL L^{-1} h^{-1} CO_2 further accelerates *T. chui* growth and results in better Sr removal rates. This study presents promising results for the development of new bioremediation methods to treat ^{90}Sr pollution.

1. Introduction

Strontium is an alkaline earth metal whose stable isotopes (^{86}Sr, ^{87}Sr, and ^{88}Sr) are naturally occurring with low toxicity in the environment [1]. On the other hand, ^{90}Sr is an artificial radioactive isotope produced by fission in nuclear reactors. This radionuclide has a half-life of 28.9 years and emits ß rays [1,2]. It has been released into the environment due to military testing of nuclear weapons, effluent discharges from nuclear power plants, liquid effluents from fuel reprocessing plants, and nuclear plant accidents such as Chernobyl or Fukushima [3–5]. Strontium-90 is also produced for use in medicine and industry [6]. Due to its similarity to calcium (Ca), oral exposure to ^{90}Sr can cause its accumulation in vertebrate bones, leading to severe health problems in humans, including the destruction of the hematopoietic bone marrow, leukemia, and other types of cancer [6,7]. Harmful concentrations of ^{90}Sr have been detected in seas, rivers, and aquifers [4,8–10]. The United States Environmental Protection Agency [6] established a maximum ^{90}Sr concentration in drinking water of 0.3 Bq L^{-1}.

The methods traditionally applied to reduce radioactive Sr concentrations in water are analogous to those for Ca, and include techniques such as clarification or sedimentation, selective adsorption, ion exchange, nanofiltration, and reverse osmosis [11–15]. These techniques have high economic and energetic costs and may present serious efficiency limitations in cases of extensive contamination [11]. Therefore, there is a need to develop efficient and alternative sustainable remediation methods.

In recent years, there has been increasing interest in developing new ^{90}Sr bioremediation techniques involving the use of microalgae. For instance, the green algae *Closterium moniliferum* and *Scenedesmus spinosus* have been suggested as possible bioremediation agents to treat ^{90}Sr contamination due to their ability to sequester natural Sr [16–19]. Recently, several species within the class of green microalgae Chlorodendrophyceae have also been shown to exhibit high Sr uptake capacities [20–22]. In these organisms, Sr accumulates in intracellular mineral inclusions of amorphous calcium carbonate (ACC), called micropearls, which had been overlooked in the past. Microalgae within this class are widespread organisms that can live in diverse aquatic environments, including hypersaline water, seawater, brackish water, and

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freshwater [20,23]. Species within the genus *Tetraselmis* (included in the class Chlorodendrophyceae), especially *Tetraselmis suecica*, are easily cultivated in large-scale photobioreactors and have received an increased interest regarding their application for wastewater treatment, CO_2 mitigation, heavy metal bioremediation, as well as to produce biofuel and high-value products (carotenoids, antioxidants, etc.) [24–31].

Tetraselmis chui is a species phylogenetically close to *T. suecica*, both included in the same clade and showing similar biomineralization patterns [21,32,33]. Although *T. chui* is largely produced as an aquaculture feed [34,35], its application for bioremediation purposes has been little explored.

Strontium uptake by *T. chui* was recently investigated in small-scale laboratory cultures, using a growth medium prepared with synthetic seawater enriched with stable Sr [36]. This study showed that *T. chui* presents a high Sr uptake capacity that is directly related to the micropearl formation process and results in a significant decrease of Sr concentration in the growth medium within a short period of time. However, the initial Sr concentrations in the culture medium only decreased by half after two weeks of growth, suggesting that the culture technique could probably be optimized for higher Sr removal. A subsequent study demonstrated *T. chui* effectiveness in sequestering the radioactive isotope ^{90}Sr, highlighting its potential as a bioremediation agent to address ^{90}Sr water contamination [37]. However, the complete removal of ^{90}Sr from the culture medium was, again, not achieved because the growth conditions of *T. chui* were not optimal. Therefore, it is desirable to develop culture methods that optimize *T. chui* growth and Sr uptake with a view to using this organism for bioremediation purposes.

Bubble columns are versatile photobioreactors (PBRs) that provide controlled environmental conditions to achieve optimal microalgal growth on a wide range of production scales [38]. The design of this type of PBR allows a better light delivery into the cultures compared to traditional Erlenmeyer cultures. Moreover, aeration is provided from the bottom, which allows a good mixing of the cultures, reducing the formation of pH, nutrients, and temperature gradients, and preventing cell sedimentation [39,40]. This aeration system also allows CO_2 injections into liquid cultures, which can improve microalgal production since CO_2 is the carbon source of photoautotrophic organisms [39].

The present work aimed to optimize *T. chui* cultures to obtain a higher Sr removal from the culture medium using 1-L bubble column PBRs. Cultures were enriched with different concentrations of stable Sr and, in some cases, also with CO_2.

2. Material and methods

This study includes two sets of experiments: (A) testing *T. chui* growth and Sr uptake in bubble column PBRs with different Sr concentrations under brackish water salinity conditions and (B) testing the effect of CO_2 in *T. chui* growth and Sr uptake (also using bubble column PBRs) under seawater salinity conditions. The algal strain studied, *Tetraselmis chui* (8–6), was obtained from the Culture Collection of Algae of the University of Göttingen (SAG).

2.1. Bubble column photobioreactors: General specifications

The PBRs used for *T. chui* growth consisted of 1-L volume borosilicate glass cylinders (diameter of 6.5 cm and height of 47 cm). Sterile aeration was provided from the bottom part, allowing good mixing of the cultures. In some cases, CO_2 was also supplied (Fig. 1). The cylinders were placed at room temperature, 10 to 20 cm from LED cool white strips, under a photosynthetically active radiation (PAR) of 90 μmol photons m^{-2} s^{-1} (≈ 2000 lx intensity).

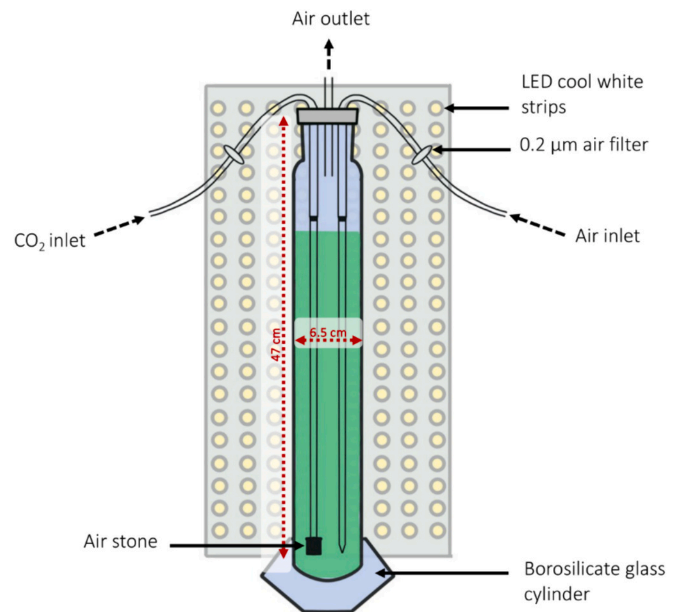

Fig. 1. Diagram of the bubble column photobioreactor specifications.

2.2. Experiments A: Setup and procedure

T. chui was grown in Algal Medium [23,41] that was prepared with sterile seawater from the Atlantic coast of South Portugal. Salinity was adjusted to 20 ‰ (by dilution in MilliQ water) before adding the concentrated (1000×) Algal nutrient solution. Cells were transferred from the original agar culture to 60 mL of Algal Medium in Erlenmeyer flasks. Cultures were placed for seven days in an incubator (Multitron Standard-Infors HT) at 20 °C with continuous light at a PAR of 75 μmol photons m^{-2} s^{-1} (≈ 1700 lx intensity) and 110 rpm shaking. They were then transferred to 300 mL of liquid culture medium in 1-L Erlenmeyer flasks that were placed back in the incubator. After one week, cultures were finally transferred into 1-L bubble column PBRs along with fresh culture medium to reach a total culture volume of 1 L. Each culture was grown for one week and was then sufficient to inoculate three new PBRs, at a cell density between 5×10^5 and 1.3×10^6 cell mL^{-1}, to start the experiments. Photobioreactors were exposed to continuous light and aerated with 0.2-μm-filtered air at a flow rate of 80 mL min^{-1}. *T. chui* cultures were enhanced with different concentrations of $SrCl_2 \cdot 6H_2O$: 0.04, 0.4, and 0.8 mM. The 0.04 mM concentration represents the natural Sr level in seawater after dilution in MilliQ water for culture medium preparation. The 0.4 and 0.8 mM Sr concentrations were selected based on previous studies investigating the effects of higher Sr concentrations on *Tetraselmis* behavior [36,42]. For each growth condition, cultures were grown in duplicate for one week (Fig. 2A). Twenty-five-milliliter samples of each *T. chui* culture were collected after 0, 1, 3, 5, and 7 days of growth. One mL of the sample was used to measure the optical density of the culture, 10 mL to measure its dry weight (DW), and another 10 mL for chemical analysis.

2.3. Experiments B: Setup and procedure

In this case, the Algal Medium was prepared with commercial sea salt from the Red Sea (Red Sea). To better replicate seawater conditions, the salinity was adjusted to 30 ‰ (30 g sea salt in 1 L MilliQ water). The scaling-up was performed as indicated for the set of experiments A. Photobioreactors were also exposed to continuous light and aerated with 0.2-μm-filtered air at a flow rate of 80 mL min^{-1}. For this set of experiments, *T. chui* cultures were enhanced with 0.5 mM $SrCl_2 \cdot 6H_2O$, and the effect of CO_2 enhancement on cell growth and the Sr uptake capacity of

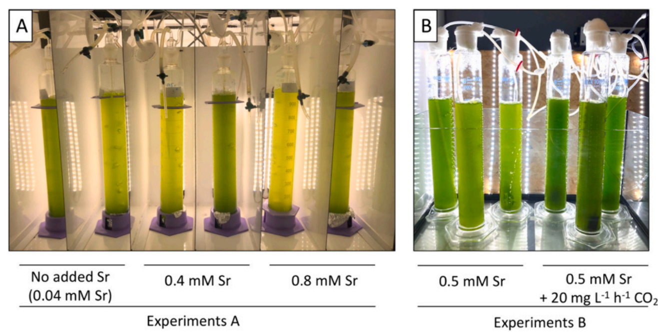

Fig. 2. *Tetraselmis chui* culture set-up in 1-L bubble column photobioreactors. (A) Culture set-up for experiments A. Cultures were performed in duplicate with initial Sr concentrations of 0.04, 0.4 and 0.8 mM. (B) Culture set-up for experiments B. Cultures were set in triplicate with an initial concentration of 0.5 mM Sr, with and without CO_2 enhancement (10 mL L^{-1} h^{-1} CO_2).

T. chui cells was tested by adding 10 mL L^{-1} CO_2 for one minute each hour (20 mg L^{-1} h^{-1}) in some of the cultures. Cultures were set in triplicate and grown for one week (Fig. 2B). Non-inoculated bubble column PBRs were also set in triplicate under the same culture conditions as controls. Twenty-five-milliliter samples were collected every day from each culture (including controls) for one week and processed as in experiments A. In addition, their pH was also measured. The remaining volume was used for Scanning Electron Microscopy (SEM) observation and Energy-Dispersive X-ray Spectroscopy (EDXS) analysis.

2.4. Cell growth and pH measurements

Cell growth was monitored by measuring both the optical density (OD) and the dry weight (DW) of the cultures. The OD was determined by measuring in triplicate the absorbance of 200-μL samples using a Biotek Synergy 4 (experiments A) or a Biotek Synergy HT (experiments B) microplate reader at a wavelength of 750 nm. Dry weight was determined by filtering 10-mL culture samples in previously weighed 0.7-μm glass microfiber filters (VWR). After filtration, following the method developed by Zhu and Lee [43], filters were washed with 10 mL NH_4HCO_3 (35 g L^{-1}) and dried for 48 h at 60 °C using a laboratory oven. Finally, filters were weighted using an analytical balance. Optical density and dry weight values were used to estimate the cell concentration of the cultures since a correlation between both parameters and cell density was previously established after cell counting in a hemocytometer.

The pH of the cultures was measured in 2-mL samples using a Mettler Toledo Seven2GoTM Pro pH meter (Mettler Toledo) in experiments B.

2.5. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDXS)

T. chui cell samples for SEM observation and EDXS analysis were prepared by gently filtering 200 μL of the cultures with 1-μm pore size polycarbonate membranes (Whatman® Nuclepore™) using a manual vacuum pump. Membranes were subsequently dried at room temperature, mounted on Al stubs by using a double-sided carbon tape, and coated with a 10 nm Au layer by low vacuum pressure sputtering (Leica EM SCD 500).

Samples were imaged and analyzed with a JEOL JSM 70001F scanning electron microscope with a coupled EDXS detector (model EX-94300054L1Q, JEOL). Images were obtained using the detection of backscattered electrons. EDXS analysis was performed using a beam current of 7 nA, an accelerating voltage of 15 kV, and an acquisition time of 30 s. Micropearl length measurements were performed using the software ImageJ [44].

2.6. Strontium concentration measurements

The Sr concentration in the culture medium and *T. chui* biomass was measured using Microwave Plasma Atomic Emission Spectrometry (Agilent Technologies 4200 MP-AES, Santa Clara, CA, USA) (experiments A) and Inductively Coupled Plasma Mass Spectrometry (Agilent Technologies ICP-MS 7700×, Santa Clara, CA, USA) (experiments B). Several control samples were previously analyzed in both instruments to ensure that they provide comparable results.

Samples for MP-AES analyses were prepared based on the protocol presented in Segovia-Campos et al. [36]. Ten milliliters of the culture samples were centrifuged for 5 min at 4121 ×g in 15-mL sterile polypropylene centrifuge tubes (Falcon®). The supernatants were transferred to new sterile tubes, stabilized with the addition of 0.2 mL 65 % HNO_3, and stored at 4 °C for further analyses. Algae pellets were transferred to 2-mL Eppendorf tubes and rinsed by resuspending the pellets in 1 mL of a solution containing Milli-Q water, 15 mM Tris-HCl, and 0.1 M NaCl at pH 8.2. To remove the rinsing solution, the Eppendorf tubes were centrifuged for 5 min at 2350 ×g and the supernatant was discarded. The rinsing process was repeated twice. Wet pellets were digested with 320 μL 65 % HNO_3 and 80 μL 30 % H_2O_2 at 100 °C for 45 min. Digested biomass solutions were then diluted 100 times in 2 % HNO_3. The calibration of the instrument was performed over a range of Sr concentrations from 0 to 10 mg L^{-1} in a 5 % HNO_3 matrix. Strontium measurements were performed at wavelengths 407.771 and 460.733 nm.

Samples for ICP-MS analyses were prepared in a similar way. Supernatants were collected, stabilized, and stored as previously mentioned. Algae pellets were transferred to 2-mL Eppendorf tubes and rinsed by resuspending the pellets in 1 mL of a solution containing Milli-Q water, 0.1 M NaCl, and 2 mM HEPES at pH 8.2. Eppendorf tubes were centrifuged for 5 min at 2350 ×g and the supernatant was discarded. The rinsing process was repeated twice before freeze-drying (Alpha 2–4, Christ) the biomass for 24 h. Lyophilized pellets, as well as three samples of Certified Reference Material (CRM) No. 3 *Chlorella* (National Institute for Environmental Studies, Japan), were accurately weighed and digested with 320 μL 65 % HNO_3 and 80 μL 30 % H_2O_2 at 100 °C during 45 min. Before starting ICP-MS analysis, the digestion mixtures of *T. chui* cells and CRM samples were diluted 600 and 80 times, respectively, in 2 % HNO_3. Culture medium samples and the CRM *Sea Water* (High-purity standards) were diluted 100 times in 2 % HNO_3. Strontium calibration was performed over a range of concentrations from 0 to 1 mg L^{-1}. During the entire analysis process, an internal standard solution containing 50 μg L^{-1} Re and 50 μg L^{-1} Rh was injected into the instrument to detect any plasma fluctuation. Accuracies for Sr concentrations measured in CRM samples ranged between 90 and 110 % with a degree of precision (RSD) below 10 %.

2.7. Calculation of strontium uptake rates

Strontium uptake rate was calculated as [36,45]:

$$UR_j = \frac{\frac{[X]_i - [X]_j}{\overline{CD}_{ij}}}{t_j - t_i}$$

where t is time (in h), i and j two successive measurement times, [X] the concentration of the element X (in fmol L^{-1}) in the culture medium, $\overline{CD}$ the average cell density between times i and j (in cell L^{-1}).

2.8. Statistical treatment

Micropearl length and Sr/Ca mol% ratios were compared between *T. chui* cells grown with and without CO_2 enhancement by performing statistical tests. Micropearl length was compared using the unpaired *t*-test, while Sr/Ca mol% ratios were compared with the unpaired t-test

with Welch's correction (as variances were significantly different between the two compared groups). In both cases, we previously applied the Shapiro–Wilk statistical test to confirm the normal distribution of the dataset. These statistical tests were performed using the software GraphPad Prism 8 (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Cell growth

The growth of *T. chui* cultures in different Sr concentrations was followed (experiments A) as well as with and without CO_2 (experiments B) in bubble column PBRs. After seven days of growth, the cell density reached values higher than 2.4×10^6 cells mL^{-1} in all the cultures, regardless of the different culture conditions (Fig. 3A-C and 4A).

Cultures grown in natural seawater with salinity adjusted to 20 ‰ (experiments A), and enhanced with 0, 0.4, and 0.8 mM Sr, showed comparable growth patterns (Fig. 3A-C). However, specific growth rates during the exponential phase were lower in cultures with 0.4 and 0.8 mM Sr (Fig. 3D). Cultures grew exponentially during the first three days and continued growing at a slower rate from day 3 to day 7 (Fig. 3A-C). After one week of growth, the cell density of the cultures was $3.0 \times 10^6 \pm 0.4 \times 10^6$ cells mL^{-1}.

On the other hand, cultures grown in culture medium prepared with commercial sea salt with adjusted salinity to 30 ‰ (experiments B) amended with 0.5 mM Sr, with and without CO_2, showed different growth trends (Fig. 4A, B): while cultures without CO_2 supply showed a cell density of $2.4 \times 10^6 \pm 0.4 \times 10^6$ cell mL^{-1} after one week of growth, the cell density in cultures supplemented with CO_2 was significantly higher, reaching $3.7 \times 10^6 \pm 0.8 \times 10^6$ cell mL^{-1}.

3.2. Strontium removal

Strontium removal efficiency of *T. chui* cells under the tested culture conditions was evaluated. After seven days of growth, *T. chui* cultures with an initial Sr concentration of 0.04 (= non-added Sr), 0.4, and 0.8 mM Sr, showed a Sr removal of 100, 94, and 72 %, respectively (Fig. 3A-C). *T. chui* cultures enhanced with 0.5 mM Sr without CO_2 supply showed a Sr removal of 81 % after seven days, while 97 % of the initial Sr concentration was already internalized by *T. chui* after four days of

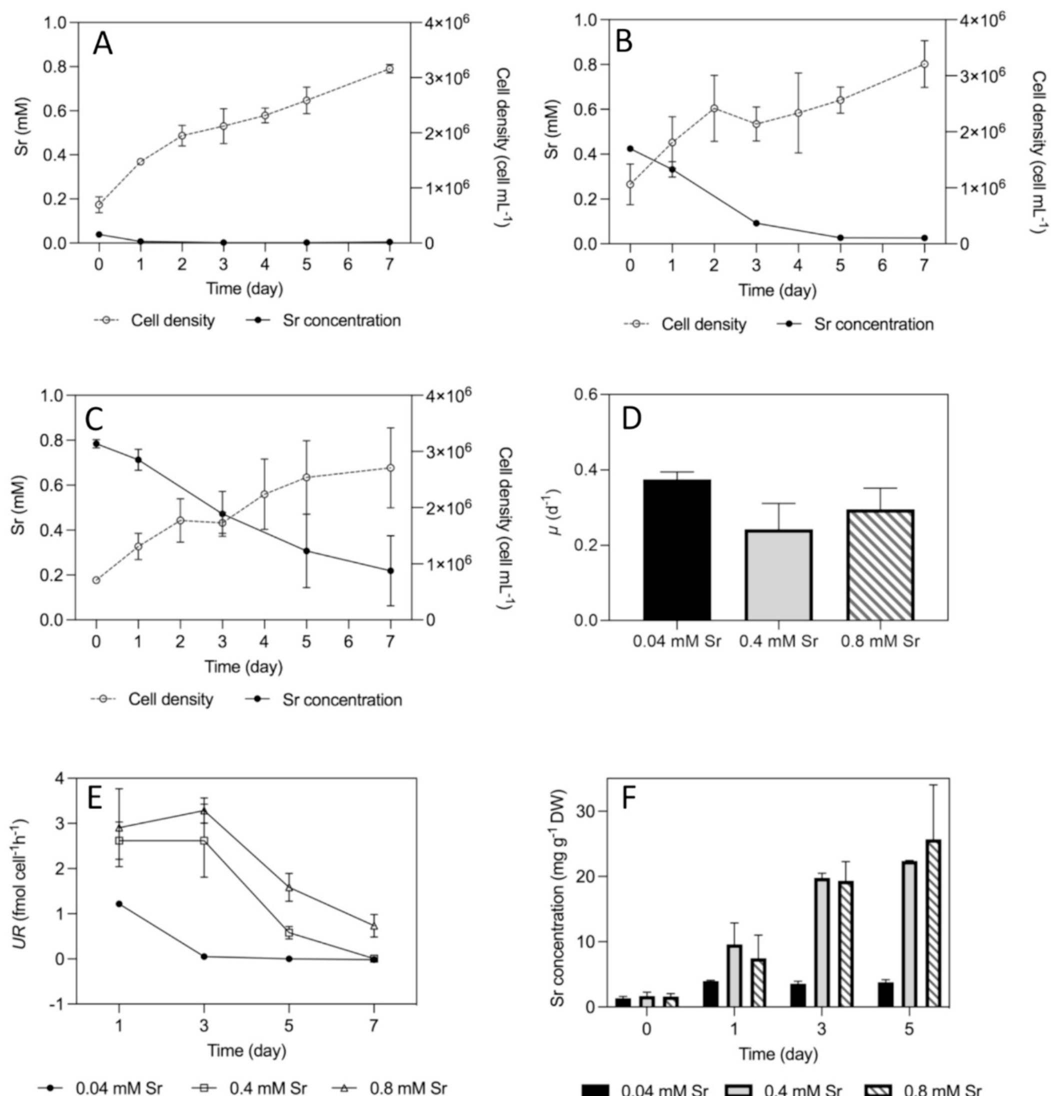

Fig. 3. Results of experiments A. (A-C) Time evolution of cell densities and dissolved Sr concentrations of *T. chui* cultures grown with an initial Sr concentration of (A) 0.04, (B) 0.4, and (C) 0.8 mM. (D) Specific growth rate during the exponential growth phase (from day 0 to day 3) and (E) daily Sr uptake rates in cultures with initial Sr concentrations of 0.04 (closed circles), 0.4 (open squares), and 0.8 (open triangles) mM. (F) Time evolution of the Sr concentration in *T. chui* dry biomass from cultures with an initial Sr concentration of 0.04 (black bars), 0.4 (grey bars), and 0.8 (stripped bars) mM. Error bars represent standard deviations between two replicates. In some cases, error bars are not visible as they are shorter than symbols.

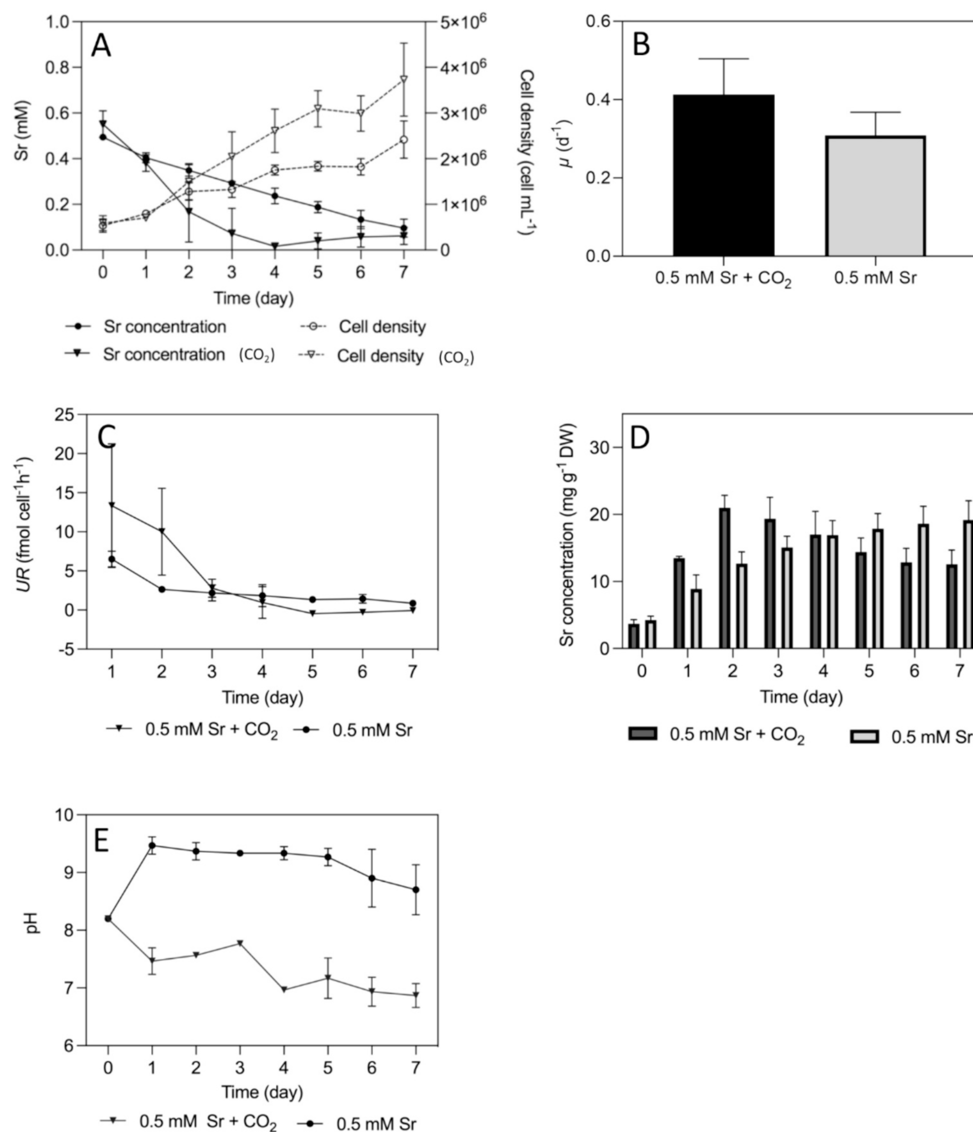

Fig. 4. Results of experiments B (cultures with and without CO_2 supply). (A) Time evolution of cell densities (continuous lines) and dissolved Sr concentrations (discontinuous lines) of *T. chui* cultures grown with 0.5 mM Sr, with and without CO_2 supply (triangles and circles, respectively). (B) Specific growth rates during the exponential growth phase (from day 0 to day 3). (C) Daily Sr uptake rates. (D) Time evolution of the Sr concentration in *T. chui* dry biomass. (E) pH evolution of *T. chui* cultures. Error bars represent standard deviations between triplicates. In some cases, error bars are not visible as they are shorter than symbols.

growth in cultures supplemented with CO_2 (Fig. 4A).

Maximum Sr uptake rates were observed after one day of growth in cultures without added Sr (1.2 ± 0.0 fmol cell^{-1} h^{-1}) as well as in cultures supplemented with 0.4 mM Sr (2.6 ± 0.4 fmol cell^{-1} h^{-1}) (Fig. 3E). Cultures enhanced with 0.8 mM Sr, showed their maximum Sr uptake rate after three days of growth (3.3 ± 0.3 fmol cell^{-1} h^{-1}).

Cultures grown with 0.5 mM Sr, with and without CO_2, showed maximum Sr uptake rates after one day of growth (Fig. 4C). However, the Sr uptake rate in cultures enhanced with CO_2 was higher than that of cultures without CO_2 injections during the first three days of growth. Analysis of the dissolved Sr in control cultures amended with 0.5 mM Sr with and without CO_2 input showed a slight increase over time of the Sr concentration in the culture medium probably related to evaporation (Fig. S1A, Supplementary Material).

Chemical analysis of *T. chui* cells showed that Sr constituted up to 2 % of the total dry biomass after three to five days of growth in cultures enhanced with 0.4 and 0.8 mM Sr (Fig. 3F). Dry biomass of cultures enhanced with 0.5 mM Sr without CO_2 supply showed a maximum Sr concentration of 1.9 % after seven days of growth, while the dry biomass of cultures supplemented with 0.5 mM Sr and CO_2 showed a maximum Sr concentration of 2.1 % after only two days of growth (Fig. 4D). However, in the latter case, from day 2 to day 7, the Sr concentration of the dry biomass gradually decreased until it reached a value of 1.3 %.

3.3. Changes in pH

The effect of CO_2 supplementation on culture pH was analyzed every 24 h in cultures supplemented with 0.5 mM Sr (Fig. 4E). Cultures supplemented with CO_2 showed acidification over time: the pH ranged from 8.2 ($t = 0$ days) to 6.9 ($t = 7$ days), and a strong decrease was observed between day 3 and 4 (from pH 7.8 to 7). The pH of cultures without CO_2 injections increased from 8.2 to 9.5 during the first day of growth and then slightly decreased over time, reaching a pH of 8.7 after one week of growth. The pH of control cultures without CO_2 slightly decreased during the first day, going from 8.2 to 7.9, and then remained stable (Fig. S1B, Supplementary Material). Control cultures with CO_2 supply showed a stronger acidification during the first two days, from pH 8.2 to 6.9. Then, pH increased to 7.4 and remained stable over time.

3.4. Effect of CO_2 supplementation on micropearl size

SEM observation of *T. chui* cells revealed a difference in micropearl size between cultures grown with and without CO_2 (Fig. 5A). After three days of growth, *T. chui* cells cultured with CO_2 supply, presented micropearls 18 % larger than those observed in cells cultured without CO_2 addition ($p < 0.0001$, $n > 108$). Analysis of the chemical composition using EDXS, also showed a difference between the Sr/Ca mol% ratio in micropearls between the two conditions, being higher in cultures with CO_2 supply ($p = 0.02$, $n = 25$).

4. Discussion

4.1. Optimization of the culture growth: A key strategy for strontium removal

A recent study where *T. chui* was cultured in Erlenmeyer flasks with synthetic seawater showed that 50 % of the initial Sr concentration of the cultures was internalized by *T. chui* cells after six to nine days of growth [36] (Table 1). The present study shows that Sr removal by *T. chui* cells can be drastically improved by growing these organisms in bubble column PBRs. Although Sr concentrations in *T. chui* dry biomass obtained from cultures enriched with 0.5 mM Sr (without CO_2 supply) are similar in both studies (1.7 ± 0.2 % vs 1.9 ± 0.2 %), the cell density in bubble column PBRs is twice that obtained in the study previously cited (Table 1), allowing the nearly complete removal of Sr from the cultivation medium. Therefore, our results unequivocally confirm that Sr removal increases with higher cell densities as reported in previous research [37].

Bubble column PBRs are culture systems that typically provide high algal production and productivity. However, the composition of the culture medium also plays an important role in culture growth. Other *Tetraselmis* species such as *T. suecica* have been proved to show optimal growth in cultures where the culture medium is prepared with natural seawater instead of synthetic seawater [46]. Thus, the use of Algal Medium prepared either with natural seawater or with natural Red Sea salt has probably improved cultures growth compared to the work cited above [36]. From a bioremediation perspective, the fact that *T. chui* cultures show higher cell densities in a culture medium mainly composed of natural seawater or natural sea salt is a promising result as it indicates their potential use for the treatment of contaminated seawater.

4.2. CO_2 supply and pH

Microalgae use CO_2 as a direct source of carbon for photosynthesis but, due to its slow water solubility, CO_2 availability in microalgal cultures is often a major limiting factor to obtain the desired biomass productivity. In addition, at pH 8.2 (initial pH of our cultures), <1 % of dissolved inorganic carbon (DIC) is as CO_2(aq) whereas 90 % is present as HCO_3^- (due to the carbonate equilibria). Therefore, to avoid limiting cell growth and productivity in microalgae cultures, it is necessary to supply CO_2 to cultures at a partial pressure higher than 0.2 kPa (3.3 mg L^{-1}) [38,47]. Consistent with this, our results show higher cell density in *T. chui* cultures supplemented with 20 mg L^{-1} h^{-1} (10 mL L^{-1} h^{-1}) of CO_2 (Fig. 4A), leading to higher Sr removal efficiencies during the first days of growth compared to cultures without CO_2 supply (97 % vs 51 % in 4-days-old cultures with a Sr initial concentration of 0.5 mM).

pH is another essential parameter to consider in microalgae culture systems, as low and high pH can have inhibitory effects on microalgae growth [27,39,48]. Optimal growth of different *Tetraselmis* strains has been shown to occur at pH 6.5–8.5 [28,48,49]. In our study, the pH of

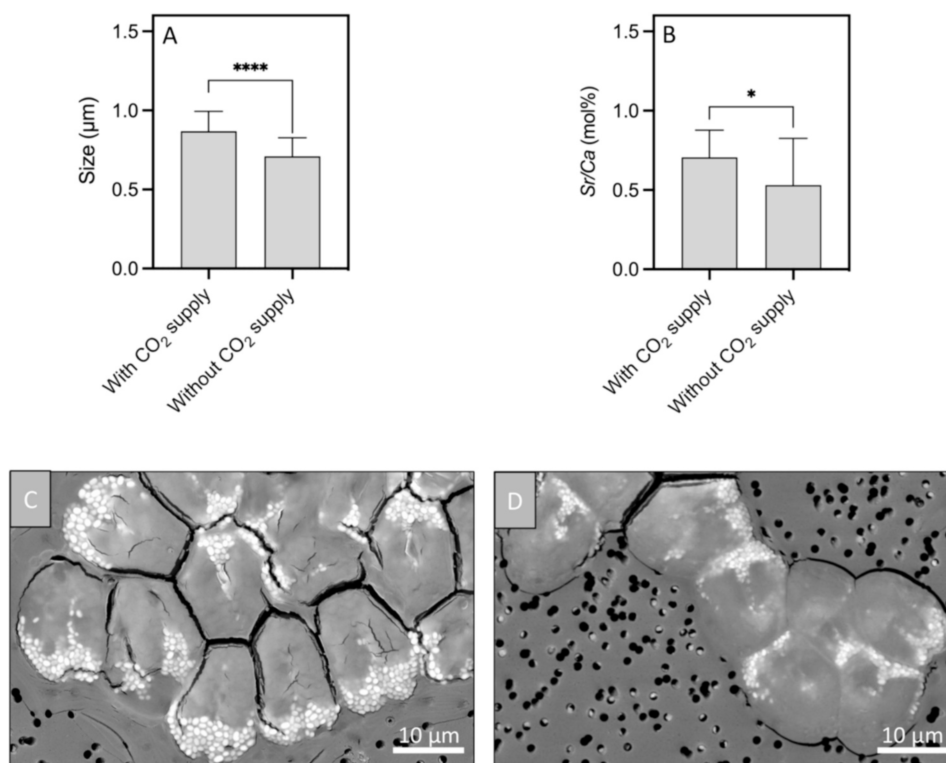

Fig. 5. (A) Comparison of micropearl size between *T. chui* cells grown for three days with CO_2 supply (10 mL L^{-1} h^{-1} CO_2) and without ($n > 108$). The statistical unpaired *t*-test shows significant size difference between the tested conditions (****$p < 0.0001$). (B) Comparison of micropearl Sr/Ca mol% ratio between *T. chui* cells grown for three days with CO_2 supply (10 mL L^{-1} h^{-1} CO_2) and without ($n = 25$). The statistical unpaired t-test with Welch's correction shows a significant difference between the tested conditions (*$p < 0.02$). (C—D) SEM observation of *T. chui* cells grown for three days (C) with CO_2 supply (10 mL L^{-1} h^{-1} CO_2) and (D) without. Micropearls are observed as white inclusions (≤ 1 µm) in the anterior part of the cells. Black dots in the background correspond to the pores of the membrane used for sample preparation.

Table 1
Results obtained in this study and a previous study [36] with *T. chui* cultures grown under different culture conditions.

Study	Culture type	Growth medium	Added Sr (mM)	CO_2 (mL L^{-1} h^{-1})	Maximum Sr removal (%)	Cell density (cell mL^{-1})	Sr concentration in dry biomass (%)
[36]	Erlenmeyer flask	ASP-H modified	0.1	0	50 (day 9)	$(1.0 \pm 0.2)x10^6$ (day 9)	1.1 ± 0.05 (day 9)
[36]	Erlenmeyer flask	ASP-H modified	0.5	0	50 (day 6)	$(1.2 \pm 0.1)x10^6$ (day 6)	1.7 ± 0.2 (day 6)
This study	Bubble column PBR	Algal Medium (sea salt)	0.5	0	81 (day 7)	$(2.4 \pm 0.4)x10^6$ (day 7)	1.9 ± 0.2 (day 7)
This study	Bubble column PBR	Algal Medium (sea salt)	0.5	10	97 (day 4)	$(2.6 \pm 0.4)x10^6$ (day 4)	1.8 ± 1.1 (day 4)
This study	Bubble column PBR	Algal Medium (seawater)	0.4	0	94 (day 7)	$(3.2 \pm 0.4)x10^6$ (day 7)	2.2 ± 0.0 (day 5)
This study	Bubble column PBR	Algal Medium (seawater)	0.8	0	72 (day 7)	$(2.7 \pm 0.7)x10^6$ (day 7)	2.6 ± 0.8 (day 5)

non-CO_2-supplied cultures increased to 9.5. At this pH, the growth of species within the genus *Tetraselmis* is strongly affected [48]. This alkalinization of the medium is due to the photosynthetic activity of microalgae, as a result of: (i) CO_2 uptake from the bicarbonate-carbonate buffer system and/or (ii) HCO_3^- uptake followed by its conversion into CO_2 and OH^- by the carbonic anhydrase, with concurrent OH^- efflux to the extracellular medium [49–51] (Fig. 6A).

In this study, we also observed a clear CO_2-induced acidification of the cultures when CO_2 is supplied (Fig. 4E). This results, in practice, in a pH controlling effect (i.e., avoiding culture alkalinization) that can also explain the higher cell densities observed in *T. chui* cultures with CO_2 supply, as the pH of these cultures was closer to the optimal values for *Tetraselmis* cell growth (Fig. 4A). However, too acidic pH values (i.e., below 7.5) have also been shown to affect calcium carbonate biomineralization in several calcifying microorganisms, such as the coccolithophore *Emiliania huxleyi* [52]. The alteration in the biomineralization seems to be due to the closure of voltage-regulated H^+ channels, directly related to calcification and pH homeostasis [53–55]. The micropearl formation in *T. chui* cells also appears to be affected by a culture pH below 7.5 (Fig. 6C): An increase in Sr concentration in the culture medium (Fig. 4A) and a decrease in Sr concentration in *T. chui* cells were observed after four days of culture with CO_2 (Fig. 4D) coinciding with a sharp drop in culture pH (from pH 7.8 to pH 7.0) (Fig. 4E).

Hence, the addition of CO_2 into the cultures not only represents a major source of inorganic carbon available for photosynthesis but also contributes to maintaining an optimal pH for cell growth. However, its

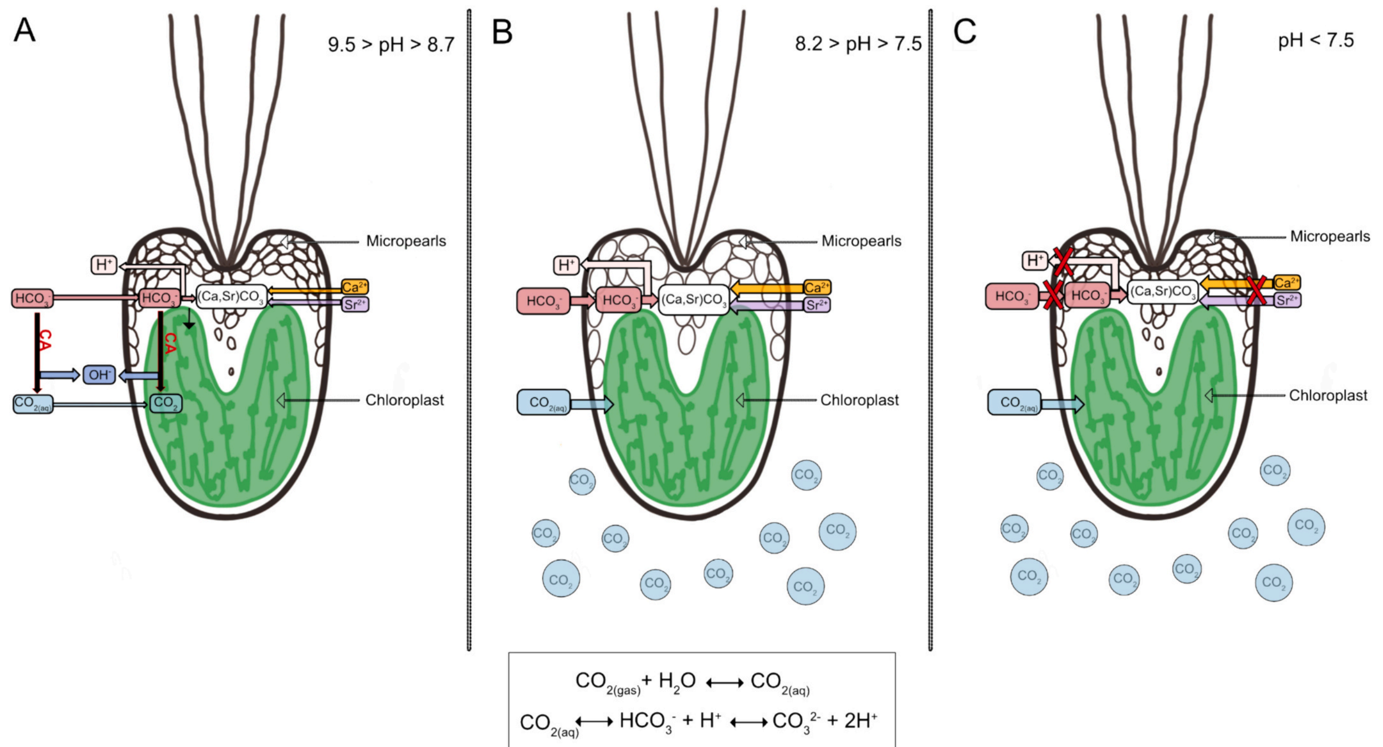

Fig. 6. Simplified outline of CO_2, HCO_3^-, Ca^{2+}, Sr^{2+}, and H^+ pathways possibly associated with micropearl formation in *T. chui* under different growth conditions. (A) Without CO_2 supply, the pH of the culture medium increases due (i) to the CO_2 consumption related to photosynthesis, shifting the carbonate equilibrium of the culture medium to the left, and (ii) to the conversion of HCO_3^- into CO_2 by the carbonic anhydrase (CA), producing OH^-. (B) The addition of 10 mL L^{-1} h^{-1} CO_2 for a short period of time shifts the carbonate equilibrium to the right, increasing HCO_3^- and H^+ concentration in the culture medium and decreasing the pH. Added CO_2 can be directly used for photosynthesis, while the increase of HCO_3^- in the medium represents a higher input of CO_3^{2-} for micropearl formation. This could enhance their formation, explaining the larger size of micropearls and the higher Sr uptake by *T. chui*. (C) An uncontrolled addition of CO_2 in the *T. chui* cultures (in our study, the addition of 10 mL L^{-1} h^{-1} CO_2 for more than three days) can over-acidify the culture medium. This acidification could inactivate ion transporters located in the cell membrane, inhibiting micropearl formation.

addition should be controlled and the pH setpoint maintained to avoid over-acidification of the cultures.

Finally, although cultures without CO_2 supply showed a lower cell production than cultures supplemented with CO_2, the final cell density, as well as the Sr removal efficiency, were, nevertheless, high under these culture conditions, proving a high pH tolerance of *T. chui*.

4.3. CO_2 supply and micropearl formation

T. chui cultures with 0.5 mM Sr showed higher Sr uptake rates during the first two to three days of growth when supplemented with CO_2 compared to cultures without CO_2 addition (Fig. 4C). At the same time, the Sr content in the biomass was also higher when CO_2 was added to the cultures (Fig. 4D). SEM observation of *T. chui* cells cultured for three days with 0.5 mM Sr showed that micropearls were 18 % larger in cells grown with CO_2 supply compared to those observed in cultures without CO_2 (Fig. 5). Taken together, these observations show that CO_2 supply to the culture media favors micropearl formation at pH 7.5 or higher. This is easily explained by the increased concentration of HCO_3^- in the system, favoring its uptake by *T. chui* cells for the intracellular formation of $(Ca,Sr)CO_3$ (Fig. 6B). Therefore, at pH 7.5 or higher, CO_2 supply to *T. chui* cultures enhances cell growth and promotes Sr uptake resulting in higher Sr removal efficiencies compared to cultures without increased CO_2.

4.4. Salinity and algal growth

Species within the genus *Tetraselmis,* including *T. chui,* have demonstrated tolerance to a broad range of salt concentrations [23,48,56,57]. The results of this study are consistent with previous research, as the growth rates of *T. chui* were high and comparable under the two salinities tested, 20 ‰ and 30 ‰ (Fig. 3D and 4B), corresponding to brackish and saline water environments, respectively.

4.5. Light conditions and algal growth

The light conditions have been improved compared to previous investigations in which the ^{90}Sr uptake capacity of *T. chui* was studied to assess the potential of this species as a bioremediation agent [37]. While the previous study was carried out with a light intensity of 1500 lx, in the present work we used a PAR of 90 μmol photons m^{-2} s^{-1}, which corresponds to 2000 lx with our type of light (cool white LED). It is possible that this increase in light intensity also had a positive effect on the growth of our cultures, due to a higher photosynthetic efficiency [56,58], and, consequently, on the Sr removal. In future research, the study of the effect of light on the formation of micropearls could provide further insights on this process.

4.6. Tetraselmis: A potential bioremediation agent

T. chui has proven to be a robust species, able to reach high cell densities in environments with different pH and salinities, which is an essential requirement for an organism to be used as a bioremediation agent. A recent study showed the capacity of *T. chui* to internalized radioactive ^{90}Sr and estimated that a cell concentration higher than 1.5 $\times 10^6$ cell mL^{-1} would be enough to remove almost all added ^{90}Sr from the culture medium [37]. Results presented here are promising since bubble column PBRs allowed to obtain *T. chui* cell concentrations that are twice the concentration needed for the almost complete removal of ^{90}Sr. In addition, this culture system can be reproduced on a larger scale and cultures can be transferred into other PBR types such as tubular PBRs [28,39,47,59]. Moreover, since the suitability of *T. suecica* and *Tetraselmis* sp. for CO_2 mitigation has been demonstrated [27,28,60], we can expect that this will also be the case for *T. chui* due to its high similarity to these species. A cultivation system coupled to a CO_2 source would not only contribute to CO_2 mitigation but would also favor Sr removal from water if the pH of the culture is controlled.

Our results also suggest the use of other species within the class Chlorodendrophyceae for bioremediation purposes regarding ^{90}Sr pollution as it has been shown that Sr uptake is related to micropearl formation [36]. Indeed, high concentrations of Sr have already been reported in *T. suecica* biomass [42]. Since micropearl-forming species live in very diverse environments, including hypersaline water, seawater, brackish water, and freshwater, the field of application of this bioremediation technology could be extremely broad [20,22].

5. Conclusions

The present study shows high Sr removal efficiencies in *T. chui* cultures grown in vertical bubble column PBRs with a culture medium mainly prepared either with natural seawater or natural sea salts. Strontium removal from the growth medium increases with the cell density of the culture, demonstrating the importance of optimizing culture growth for bioremediation applications.

CO_2 supply in *T. chui* cultures prevents excessive increase in culture pH, increases cell growth, and may favor the Sr uptake by the cells. However, a pH below 7.5 could alter the micropearl formation process, decreasing the Sr uptake capacity of the cells. It is, therefore, necessary to control the pH of the cultures when supplying CO_2 to avoid excessive acidification of the cultures. This is easily achieved on an industrial scale by using a pH-controlled CO_2 supplementation. In addition, a culture system coupled to a controlled CO_2 source would not only favor the removal of Sr from water but also contribute to CO_2 mitigation.

This work demonstrates that *T. chui* is a strong candidate for the development of bioremediation techniques to treat ^{90}Sr contamination. Since the ability to sequester Sr is related to the micropearl formation capacity of the species, it is likely that other species within the class Chlorodendrophyceae, including freshwater species, could be used for the same purpose. If proven, bioremediation techniques involving the use of Chlorodendrophyceae could be applied in a wide variety of environments.

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CRediT authorship contribution statement

Inés Segovia-Campos: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Montserrat Filella:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Muhammad Saad Bin Zahid:** Writing – review & editing, Investigation. **Luísa Barreira:** Writing – review & editing, Supervision, Resources, Methodology. **Karl Perron:** Writing – review & editing, Supervision, Resources. **Daniel Ariztegui:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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