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Bio-removal of Yttrium (III), Cerium (III), Europium (III) and Terbium (III) from single and quaternary aqueous solutions using the extremophile *Galdieria sulphuraria* (Galdieriaceae, Rhodophyta)

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Abstract: The lanthanides are among the Rare Earth Elements (REEs), which are indispensable constituents of modern technologies and are often challenging to acquire from natural resources. The demand for REEs is so high that there is a clear need develop efficient and environmentally friendly recycling methods. In the present study living cells of the extremophile *Galdieria sulphuraria* were used to remove four REEs, Yttrium, Cerium, Europium and Terbium, from single and quaternary metal aqueous solutions. Two different strains, SAG 107.79 and ACUF 427, were exposed to solutions buffered at pH 2.5, 3.5, 4.5 and 5.5. Our data demonstrated that the removal performances were strain and pH dependent for all metal ions. At lower pHs, ACUF 427 outperformed SAG 107.79 considerably. Increasing the pH of the solutions, there was a significant surge in the aqueous removal performance of both strains. The same trend was highlighted using quaternary metal solutions, even if quantities of metal removed were significantly lower. The present study provided the first insight into the comparative removal capacity of *Galdieria sulphuraria* strains. The choice of the appropriate operational conditions, such as the pH of the metal solutions, is an essential step in developing efficient, rapid, and straightforward biological methods for recycling REEs.

Keywords: Recycling, Rare Earth Elements; extremophile, *G. sulphuraria*; bio removal, pH

1. Introduction

The successful application of metals in a great variety of fields, being machinery, energy, transportation, building and construction, relies on their characteristic features such as high robustness, thermal and electrical conductivity, and great performances at high temperatures [1]. Metals can be repeatedly recycled, decreasing the necessity to extract them from mines [1]. According to the report of the Working Group on the Global Metal Flows to UNEP's International Resource Panel, the recycling rates of "base metals" (iron, copper, zinc, etc.) are above 50%. In contrast, a large number of metals used in small amounts in new technologies, such as red phosphorus, permanent magnets, solar cells and computer chip, are rarely (< 1%) recycled [1,2]. At the same time, however, the high popularity of these machineries is causing the demand and price increment of their components, especially of the irreplaceable Rare Earth Elements, REEs [3].

REEs, which comprise 17 metallic elements (15 lanthanides, plus Scandium and Yttrium) with the same chemical properties, were classified as "critical raw materials" by the European Commission because of their high supply risk [4]. In many cases, obsolete electrical and electronic equipment can be re-used for advanced technological applications. In these cases the equipment can be resold or donated to schools or charities without further modifications, alternatively, in particular computers, they can be regenerated - or disassembled into different components, cleaned, repaired and reassembled - and put back on the market, in order to prolong their "life cycle" and reduce the amount of WEEE. When this is not possible, the recycling and recovery of WEEE components become an essential process to reduce the costs of disposal and production of the equipment and to minimize the environmental and health risks connected to them.

Physicochemical methods are often used for the recycling and recovery of REEs [5,6], even if some weaknesses exist with these techniques: 1) they are ineffective when the metal concentration is deficient; 2) a large amount of toxic waste will be produced, requiring further treatments. This increases operating costs and environmental responsibility; 3) some physicochemical methods are costly, such as ion exchange and membrane technologies; 4) incomplete metal adsorption [7]. New methods are thus warranted.

Biosorption and bioaccumulation are biological approaches emerging as promising methods in replacing the physicochemical ones, being environmentally and cheaper methods [8]. Biosorption is the absorption of metal particles on the cell surface, which

could be not viable (microbial and algal biomass, proteins, and other biomaterials), producing small agglomerates. Even if the selectivity is not high, the process has significant effectiveness. Still, metal ion uptake is affected by the pH, since it is related to the metal ion complexation chemistry in solution, and behavior of many different functional groups present in the surface of algal cells [9,10]. Bioaccumulation is the intracellular accumulation of metals, and it occurs when cells actively transport them inside the protoplast through an energy (ATP)-driven process [7]. Operating conditions (e.g., temperature, pH and nutrients) need to be strictly controlled to maintain the cell viability, but the selectivity of the process is high [7].

Modern technological components and the contaminated effluents used for their production are often made of multiple Rare Earth Elements [11]. Most scientific studies regarded the bio-removal efficiency in a single-metal system, and little has been examined in a multiple-metal system [12]. The lack of data produces an uncertain estimation of the actual capacity of organisms to recover metals. Mixed-metals effects can not be predictable from the effects of the single metals since they depend on extrinsic factors, such as the used organism, temperature, pH, metal ion and biomass concentration [12,13]. In general, a mixture of metals can induce three kinds of behaviour: 1) synergism, when the effect of the mixture is greater than the sum of the individual metal; 2) antagonism when the effect of the mixture is less than the sum of the individual effects; 3) lack of interaction when the effect of the mixture is equivalent to the sum of the individual effects [12,14].

Based on these premises, in the present study, two different strains of the extremophile *Galdieria sulphuraria* were tested for aqueous bio-removal of Yttrium (Y^{3+}), Cerium (Ce^{3+}), Europium (Eu^{3+}) and Terbium (Tb^{3+}), important REEs constituents of the phosphorus lamps, in a single and quaternary system. *G. sulphuraria* species are unicellular microalgae strains thriving in geothermal sites, where the ecological conditions are very extreme, such as low pH (0.5-3.0), high temperature (50°C-55°C) and vast amounts of heavy, precious and rare earth metals [15–18]. The coexistence of *G. sulphuraria* and huge amounts of metals in their natural environments makes this microalga one of the best candidates for the biological recovery of metals [19]. Indeed, in the last decade, the interest in using *G. sulphuraria* in the bio-uptake of metals was rapidly growing, thanks to the promising results and the increasingly comprehensive knowledge of their genomes [20–23]. Data produced in this study primarily aimed to highlight the comparative evaluation of two *G. sulphuraria* strains in terms of total metal removed in a single metal system. Quaternary-metal solutions were then used to analyse the influence of the mixed metals on the removal capacity for each metal.

2. Results

2.1. Removal of Y^{3+}, Ce^{3+}, Eu^{3+} and Tb^{3+} in single metal system: the influence of the initial pH

In the present paper, the bio removal of rare metals by two different *G. sulphuraria* strains was assessed at different initial pHs. The pH values were monitored for 24h both in control and in treated samples; a slight decrease (less than 0.5 ± 0.02) was recorded only in treated samples and remained constant until the end of the experiments (data not shown).

Both *G. sulphuraria*, strain SAG 107.79 and ACUF 427, were able to extract solute metals from the surrounded medium even if the extent of the ability was strain-dependent and metal-dependent. Tests performed in acidic conditions (pH 2.5) highlighted a significant difference in the removal performance amongst the two strains for all the treatments. While strain SAG 107.79 was unable to remove appreciable amounts of each metal (< 2.5 μmol/g, Figure 1a), strain ACUF 427 removed 22.43 ± 2.05 μmol/g of Y^{3+}, 20.98 ± 0.72 μmol/g of Ce^{3+}, 23.49 ± 0.55 μmol/g of Eu^{3+} and 22.26 ± 2.42

μmol/g of Tb^{3+} (Figure 1b). Increasing the medium pH, a gradual rise of the total metal removed was observed in SAG 107.79. At pH 3.5, 4.13 ± 0.13 μmol/g of Y^{3+}, 5.39 ± 0.40 μmol/g of Ce^{3+}, 7.18 ± 0.19 μmol/g of Eu^{3+} and 6.60 ± 0.10 μmol/g of Tb^{3+} were removed from the test solutions (Figure 1a). The improved removal performances induced by pH, just described for SAG 107.79, were not highlighted in ACUF 427, which indeed accumulated comparable metal quantity to those at pH 2.5 (Y^{3+} = 20.13 ± 1.48 μmol/g, Ce^{3+} = 25.08 ± 0.83 μmol/g, Eu^{3+} = 24.66 ± 1.78 μmol/g and Tb^{3+} = 24.14 ± 1.08 μmol/g; Figure 1b). Further pH increase induced an appreciable boost toward significant quantities of metals removed for both the strains, showing also significant differences among the metals (p-value < 0.05). At pH 4.5, SAG 107.79 was able to recover 14.26 ± 2.23 μmol/g of Y^{3+}, 17.82 ± 4.21 μmol/g of Ce^{3+}, 32.45 ± 7.23 μmol/g of Eu^{3+} and 35.21 ± 6.56 μmol/g of Tb^{3+} (Figure 1a), while ACUF 427 removed 28.36 ± 3.68 μmol/g of Y^{3+}, 29.82 ± 1.90 μmol/g of Ce^{3+}, 36.78 ± 5.95 μmol/g of Eu^{3+} and 40.58 ± 1.47 μmol/g of Tb^{3+} (Figure 1b). The increment of the metals removed from the solutions became even more evident when the tests were performed at the initial pH of 5.5. Indeed, the removed quantities for SAG 107.79 were 31.31 ± 3.28 μmol/g of Y^{3+}, 32.91 ± 1.87 μmol/g of Ce^{3+}, 43.02 ± 0.32 μmol/g of Eu^{3+} and 36.12 ± 2.26 μmol/g of Tb^{3+} (Figure 1a) and for ACUF 427 were 25.25 ± 5.87 μmol/g of Y^{3+}, 42.60 ± 4.28 μmol/g of Ce^{3+}, 42.91 ± 6.80 μmol/g of Eu^{3+} and 34.24 ± 3.13 μmol/g of Tb^{3+} (Figure 1b).

By comparing each metal quantity obtained with the initial pH of 3.5, 4.5 and 5.5 with those obtained at pH 2.5, it was possible to highlight the influence of the pH solution on each metal species related to its removal efficiency. Figure 2a and 2b demonstrated significant differences between some of the metals as the pH increased to more subneutral values. Using SAG 107.79, Y^{3+} removal increments were a 2.1 fold increase (FC) at pH 3.5, 7.5 FC at pH 4.5 and 16.3 at pH 5.5 (Figure 2a). Similar increments were also obtained with Ce^{3+} (2.7 FC at pH 3.5, 8.9 FC at pH 4.5 and 16.3 at pH 5.5). Higher pHs remarkably affected the Eu^{3+} removal from the solutions, as the data demonstrated an increase of the metal removed of 3.4 FC at pH 3.5, 15.3 FC at pH 4.5 until it reached the increment of 20.3 FC at the highest pH tested (Figure 2a). As happened for the Eu^{3+}, Tb^{3+} removal was hugely affected from the pH 4.5, while at pH 5.5 there was no further increase (3.1 FC at pH 3.5, 16.3 FC at pH 4.5 and 16.9 at pH 5.5; Figure 2a).

Unlike SAG 107.79, pH affected the removal efficiency to a less extent when using the strain ACUF 427. Y^{3+} results obtained at pH 3.5 were highly comparable with those at pH 2.5, demonstrating a reduction of the uptake, even if not significant (p-value > 0.05). Higher pHs induced a slight increase, but again not significant (FC < 1.27 for both pH 3.5, 4.5 and 5.5; Figure 2b). Little increments were observed instead for the other metal species: Ce^{3+} removal showed a FC = 1.20 at pH 3.5, 1.42 at pH 4.5 and 2.03 at pH 5.5 (Figure 2b).

Figure 1. Metal removed from single metal aqueous solutions by *G. sulphuraria*, strain SAG 107.79 (a) and ACUF 427 (b). Data are divided based on the pH of the solutions. Different letters in the same experiment indicate significant difference, $p < 0.05$; Symbol (*) indicates $p < 0.05$ significant difference compared to the pH 2.5.

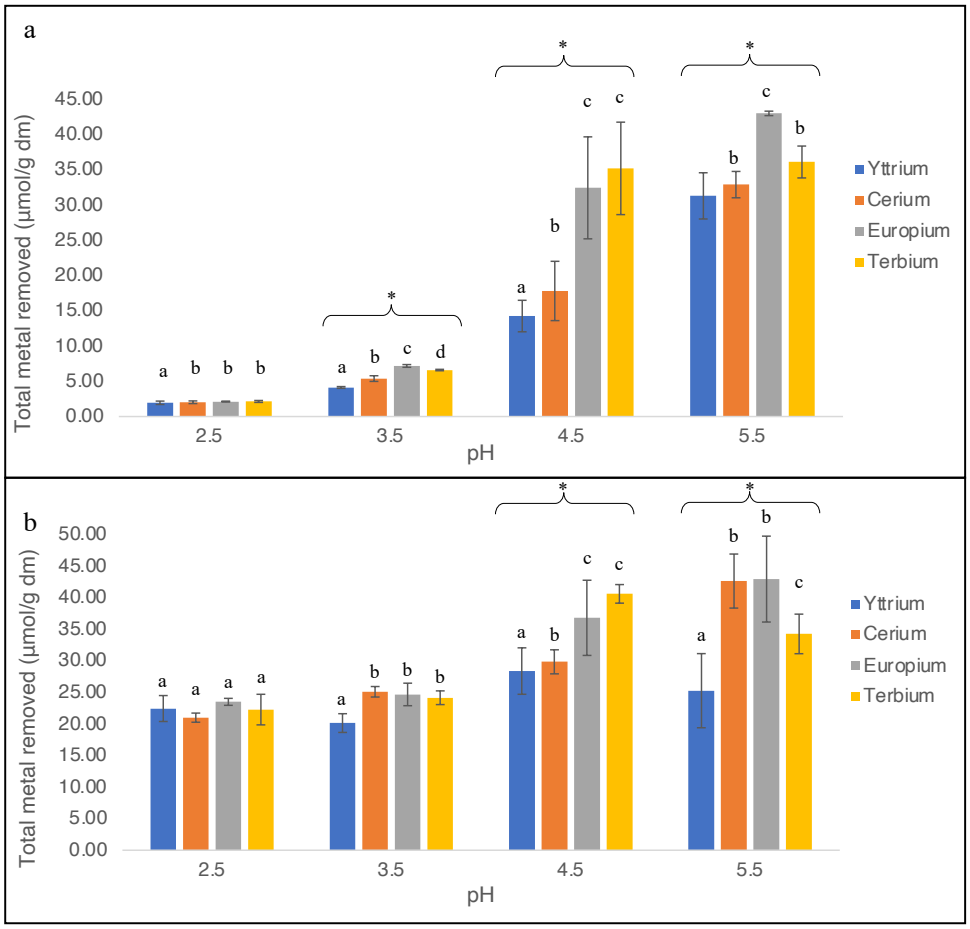
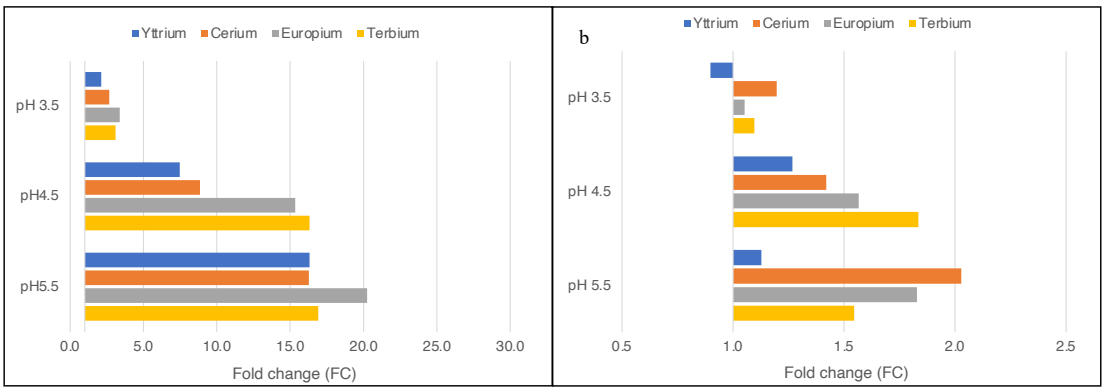


Figure 2. Fold change of the total metal removed from the single metal aqueous solutions by *G. sulphuraria*, strain SAG 107.79 (a) and ACUF 427 (b). The fold change was calculated by comparing the µmol/g dm of every metal obtained at pH 3.5, 4.5 and 5.5 with the µmol/g dm measured at pH 2.5.



2.2. Removal of Y^{3+}, Ce^{3+}, Eu^{3+} and Tb^{3+} in quaternary metal system: the combined effect of the pH and the simultaneous presence of the metals

The removal capacity of both strains was also evaluated using quaternary solutions in which the metals were present in equimolar quantities. Total metals removed from the solutions were lower than the single metal systems, but still, significant differences were highlighted among the metal species and the pHs. At pH 2.5, strain SAG 107.79 confirmed the inability to recover metals in appreciable quantities (less than 1.03 µmol/g, Figure 3a), while strain ACUF 427 reached more significant values (Y^{3+} = 5.08 µmol/g, Ce^{3+} = 5.16 µmol/g, Eu^{3+} = 10.01 µmol/g, Tb^{3+}= 11.47 µmol/g; Figure 3b). As happened for the single solutions, increments of the pH increased metal bio removal, when SAG 107.79 was used for the assays. Indeed at pH 3.5, 1.14 ± 0.06 µmol/g of Y^{3+}, 1.65 ± 0.15 µmol/g of Ce^{3+}, 3.79 ± 0.14 µmol/g of Eu^{3+}, 3.35 ± 0.14 µmol/g of Tb^{3+} were measured in the biomass (Figure 3a). The same increments were not registered for ACUF 427 at the same pH (Y^{3+} = 3.56 ± 0.66 µmol/g, Ce^{3+} = 3.92 ± 0.88 µmol/g, Eu^{3+} = 8.47 ± 2.07 µmol/g, Tb^{3+} = 9.02 ± 2.20 µmol/g; Figure 3b).

Further increase of the pH at 4.5 deeply influenced the removal rates for both strains as already happened in the single metal systems, even if to a lesser extent. Metals quantities removed by SAG 107.79 at pH 4.5 were 4.69 ± 0.60 µmol/g of Y^{3+}, 6.28 ± 0.84 µmol/g of Ce^{3+}, 18.63 ± 2.39 µmol/g of Eu^{3+} and 18.48 ± 2.26 µmol/g of Tb^{3+} (Figure 3a). Comparable quantities were also registered in ACUF 427 (Y^{3+} = 4.92 ± 1.15 µmol/g, Ce^{3+} = 7.64 ± 1.40 µmol/g, Eu^{3+} = 15.17 ± 3.61 µmol/g, Tb^{3+} = 15.20 ± 3.50 µmol/g; Figure 3b). Finally, at pH 5.5, a slight increase of the metals accumulated was recorded, even if not significantly compared to the previous pH. The total metals removed were 5.26 ± 0.91 µmol/g of Y^{3+}, 9.32 ± 1.41 µmol/g of Ce^{3+}, 20.98 ± 2.17 µmol/g of Eu^{3+} and 20.85 ± 2.72 µmol/g of Tb^{3+} for SAG 107.79 (Figure 3a) and 4.58 ± 0.12 µmol/g of Y^{3+}, 6.59 ± 0.38 µmol/g of Ce^{3+}, 13.50 ± 0.68 µmol/g of Eu^{3+} and 13.74 ± 0.64 µmol/g of Tb^{3+} for ACUF 427 (Figure 3b).

Using the strain SAG 107.79, the comparison of the metals quantities at pH 3.5, 4.5 and 5.5 with those at pH 2.5 highlighted a significant increase of the Yttrium removal at pH 4.5 (FC = 6.3), and it remained almost unchanged at pH 5.5 (FC = 6.6). Unlike Yttrium, the ulterior increase of the pH to 5.5 more deeply affected the removal of Ce^{3+}, Eu^{3+} and Tb^{3+}, even if the rates changed among the metal species (Figure 4a). Indeed, Cerium quantities increased 2.3 FC at pH 3.5, 8.6 FC at pH 4.5 and 12.8 FC at pH 5.5. Significant higher quantities were registered for Europium, whose removal rates increased 4.7 times at pH 3.5, 23.3 times at pH 4.5 and up to 26.1 times at pH 5.5 (Figure 4a). As happened for the other metals, the pH solution of 4.5 most influenced the removal of Tb^{3+}, increasing the metal quantity 18.1 FC, while the removed Terbium at pH 5.5 was comparable to that at previous pH (20.3 FC; Figure 4a).

A different performance was highlighted when ACUF 427 was used for the assays, demonstrating a weak influence of the pH solution on the metal removal rates. Among the metal species, Yttrium was removed from the microalgae in the same way regardless of the pH (pH 3.5 = 0.7; pH 4.5 = 1.0; pH 5.5 = 0.9; p-value > 0.05; Figure 4b). On the contrary, Ce^{3+}, Eu^{3+}, Tb^{3+} removal rates were slightly affected by the increase of the pH to 4.5 (Ce3+ = 1.5 FC, Eu^{3+} = 1.52 FC and Tb^{3+} = 1.33 FC). Small increments of the metals quantities were also registered at pH 5.5, reaching a FC of 1.29, 1.36 and 1.21 respectively for Ce3+,, Eu^{3+} and Tb^{3+} (Figure 4b).

To analyse the effect of the simultaneous presence of the four metals in equimolar quantities on the bio removal capacity of both strains of *G. sulphuraria*, the total amounts of removed metals were calculated and compared with those obtained with the single-metals solutions. The total metals removed by SAG 107.79 were 148.08 µmol/g at pH 4.5 and 56.41 µmol/g at pH 5.5 (Table S1). On the contrary, the total metal quantities calculated in ACUF 427 at different pHs did not always exceed those of every single metal. In particular, at pH 2.5, the total amount (31.72 µmol/g) was slightly higher than the single metal quantities; at pH 3.5, the total amount (24.96 µmol/) was comparable to

single metal (Ce^{3+}, Eu^{3+} and Tb^{3+}; Table S2). At pH 4.5, the total amount of metals removed from the quaternary solution was 42.93 μmol/g, which was statistically higher when compared to the Y^{3+} and Ce^{3+} quantities at the same pH from the single-metal system (Table S2). Finally, at pH 5.5, the total metal removed was 38.41 μmol/g, which did not reach the Eu^{3+} and Ce^{3+} quantities removed from the single-metal system (Table S2).

Figure 3. Metal removed from quaternary metal aqueous solutions by *G. sulphuraria*, strain SAG 107.79 (a) and ACUF 427 (b). Data are divided based on the pH of the solutions. Different letters in the same experiment indicate significant difference, $p < 0.05$; Symbol (*) indicates $p < 0.05$ significant difference compared to the pH 2.5.

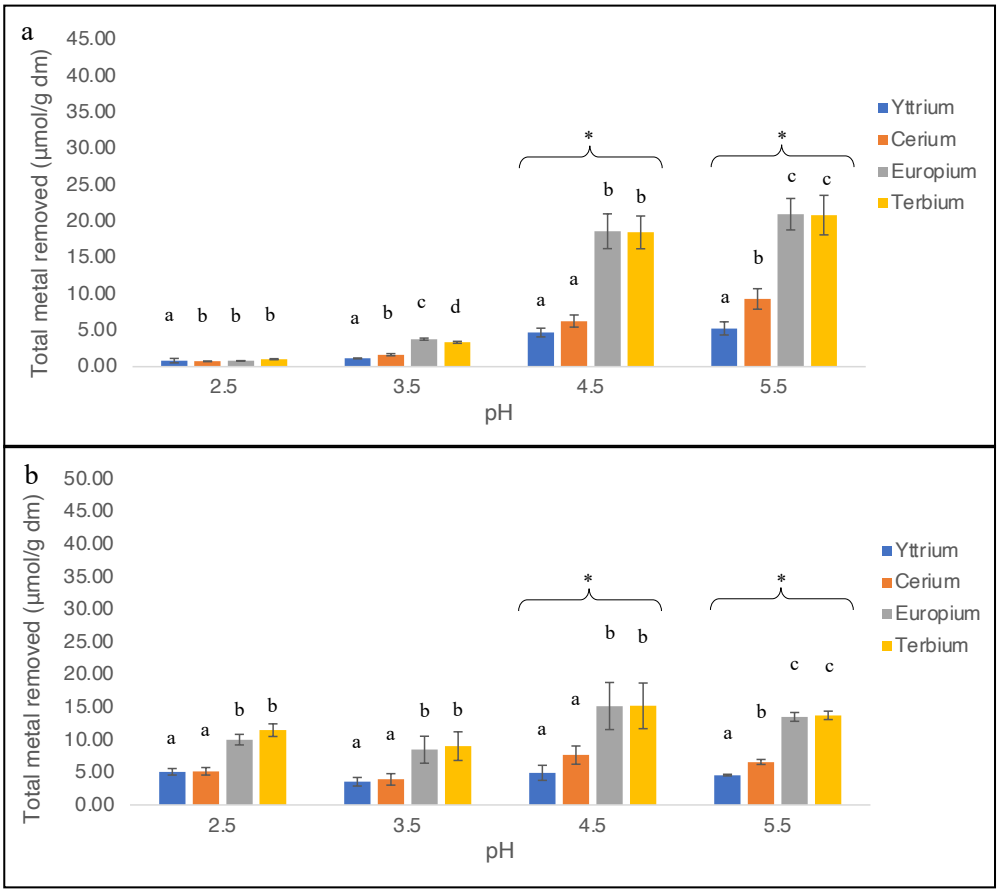
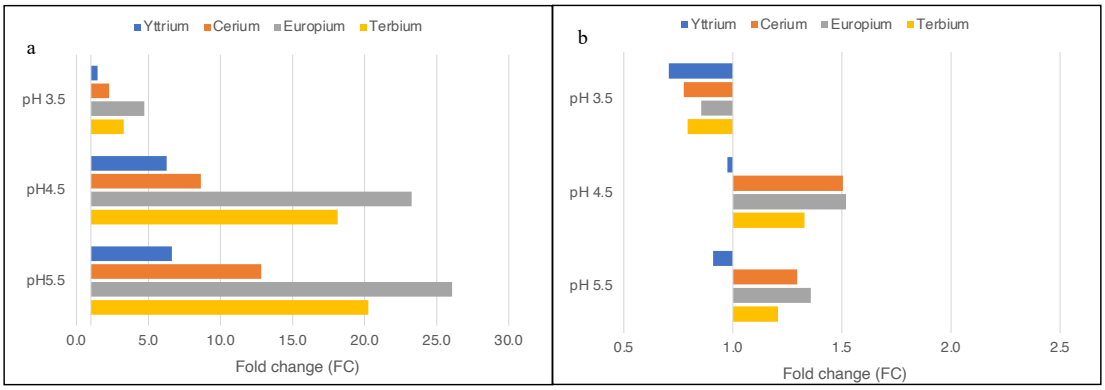


Figure 4. Fold change of the total metal removed from the quaternary metal aqueous solutions by *G. sulphuraria*, strain SAG 107.79 (a) and ACUF 427 (b). The fold change was calculated by comparing the μmol/g dm of every metal obtained at pH 3.5, 4.5 and 5.5 with the μmol/g dm measured at pH 2.5.



3. Discussion

The utility of microalgae for the bio-recycling of REEs from End-Of-Life products has been predominantly evaluated in a single-metal context. However, in light of a scale-up application of these systems, the main practical interest is the assessment of the microalgal bio-recovery capacity in a multi-metal system, which is more likely the scenario of industrial effluents [12]. In the present study, we investigated the ability of *G. sulphuraria*, strain SAG 107.79 and ACUF 427, to accumulate four Rare Earth Metals, Y^{3+}, Ce^{3+}, Eu^{3+} and Tb^{3+} from aqueous solutions at a wide range of pH (from 2.5 to 5.5) in single and quaternary metal systems. We found that strain ACUF 427 was superior a highly acidic conditions and that both strains performed well under more weakly acidic contexts.

When single metal solutions were used, significant differences were highlighted between the two strains (p-value < 0.05). At pH 2.5, ACUF 427 outperformed SAG 107.79, being its removed metal quantities 10 fold higher than those of the other strain (Figure 1a-b). Higher pHs induced a remarkable increase in metals removal, especially for the strain SAG 107.79. The fold change, calculated by comparing the quantity of the metals per grams of dried biomass at the chosen pH and the pH 2.5, highlighted significant differences among the metal species removed from the solutions. On the contrary, increments of the metals removed by ACUF 427, based on the pH solutions, were much lower than those by SAG 107.79, thus hypothesising that the capacity of metal removal of this strain is less affected by the pH (Figure 2a-b). Few data were produced in the research on *G. sulphuraria* to study the accumulation of different kinds of metals in single metal solutions. *G. sulphuraria*, strain ACUF 074, was used for the recovery of a great variety of REEs, varying various parameters, such as the concentration of the metal ions, the pH of the experimental solutions, and the preincubation of the microalgal stock solutions [20]. In particular, the authors described a reduction of the REEs uptake with increasing pH of the solutions, which contrasts with the results of the present study. Afterwards, they efficiently recovered gold and palladium (over 80%) from aqua regia-based metal wastewater, using 1.4 mg/mL of biomass (dry matter) and an exposition time of 30 minutes. They also identified a suitable modality for the desorption of metal ions from the biomass, using a solution of 1 M thiourea and 0.1 M HCl [21]. *G. sulphuraria* biomass grown under mixotrophic conditions was able to recover the radionuclide Cesium as well, reaching a recovery percentage of 52 ± 15%, after 10 days of exposition to the metal [22].

From the quaternary solutions of the metals, a significant reduction of the bio removal was highlighted for both strains (p-value < 0.05), but still, SAG 107.79 outperformed ACUF 427 when considering the calculated fold change (Figure 4a). Unlike the results of SAG 107.79, in ACUF 427, the simultaneous presence of four metals at pH 3.5 resulted in a reduction of all the metals even if not significant. ACUF 427 reached values significantly lower at higher pHs than those calculated for SAG 107.79 (Figure 4b). Data from the present study confirmed the results obtained by Čížková et al., 2021, which used *G. phlegrea* to evaluate the simultaneous removal of REEs using luminophores. The concentration of the included metals was not in equimolar quantities, but it reflected precisely the measured metal of the luminophores [24]. Considering the results of every single metal, the authors measured up to 1.98 mg/g, except for Yttrium (11.14 mg/g), already present in high concentration in the initial solution, 178.65 mg/g [24].

When the experimental design includes mainly a biosorption approach, a high variety of physicochemical and biological parameters, such as metal ionic characteristics (e.g., atomic weight, ion radius, valence, etc.), nature of the biosorbents (e.g., cell age), and biosorption conditions (e.g., pH, temperature, contact time, etc.) must be

considered. These represent the main reason for a different removal rate by biomass [25]. Among the metal ion characteristics, the most important are the atomic weight, electronegativity, ionic radius and covalent index [25,26]. Previous data demonstrated the positive correlation between the biosorption rates and the atomic weight, being larger ions capable of binding sites with two distant functional groups [26,27]. An increased biosorption was also observed when more electronegative metal ions were used [28]. Different biosorption rates for a high variety of metals were explained by the influence of the covalent index (X^2mr) with freeze-dried cells of *Rhizopus arrhizus* [29], and *Saccharomyces cerevisiae* [25].

In the present study, a correlation approach was used to understand the influence of the above-mentioned physiochemical properties on the bio removal degree of *G. sulphuraria*. A positive correlation with the covalent index and the electronegativity was registered at pH 2.5 when strain ACUF 427 was used ($R^2 = 0.93$, p-value < 0.05), while no significant correlations were found with the other metal properties and at higher pHs (data not shown). Different results were obtained with the strain SAG 107.79, where only positive correlations were obtained between the metal removal and the ionic radius at higher pHs ($R^2 > 0.91$, p-value < 0.05; data not shown). The incongruent results obtained from the correlation analysis suggested a more complicated system than the easier biosorption approach. The uptake level also depends on the composition and the specific properties of the cell wall of the microalgae. Important microalgal cell wall components are peptidoglycan, polysaccharides and proteins [30]. Most of these molecules carry charged groups, such as carboxyl, phosphate, hydroxyl, or amine, which could be protonated or deprotonated, depending on the media pH. Oxygen, sulphur and phosphorus atoms present in these groups perfectly react with rare earth trivalent ions, being the last classified as Pearson hard acid [31]. This electrostatic attraction plays an essential role in the recovery process and could explain the differential uptake of the two strains of *G. sulphuraria*.

Recovery of rare earth can be also achieved by bioprecipitation, thanks to the release of organic phosphates, which cause the precipitation of the metal in form of phosphate (Dev et al., 2020). Moreover, ions could be either attached to the cell surface or transported and accumulated inside the cell, in various contexts [31]. Bioaccumulation is generally enhanced by specific cysteine-rich peptides, such as glutathione, metallothioneins, lipopolysaccharides and phytochelatins [9]. The different removal rates amongst the two strains, especially at pH 2.5, could be also ascribed to different transportation systems activated by the microalgae. In general, the transport across the membrane is affected by the physiochemical parameters as well, *e.g.* molecular size, and polarity [32–34]. Inside the cell, metal ions could be accumulated in vacuoles or bound by specific molecules for storage or detoxification [31,33,35]. At this regard, genomic analyses identified in *G. sulphuraria* enzymes such as arsenite methyl transferases and mercury reductase employed in the biotransformation into less toxic and metal derivates [36]; these findings give a reasonable explanation of the high metal resistance by this extremophilic alga.

4. Materials and Methods

4.1 Metals stock solutions

In this study the removal capacity of Ce^{3+}, Eu^{3+}, Y^{3+} and Tb^{3+} as single, and quaternary systems (Table 1) was studied using living algal biomass of *G. sulphuraria*, at constant and equimolar concentration of 178 μmol/L. Y^{3+}, Ce^{3+}, Eu^{3+} and Tb^{3+} were acquired from Alfa Aesar (USA) in the form of chloride salt monohydrate (MetalCl3.H2O, 99.9%). Stock solutions were prepared, dissolving 2 grams of each metal salt in 1 litre of Milli-Q water and acidified at pH 2.5, 3.5, 4.5 and 5.5 using sulphuric

acid (98%). All the solutions were then sterilised with a 0.45 μm filter. To prevent interferences with the chemical analyses, all materials were previously rinsed with nitric acid and deionized water prior to use. In addition, the initial concentration (C_i) of the pH adjusted REE solutions were verified by ICP-MS before experiments to be sure that there is no precipitation involved for the tested REE concentration (178 μmol/L). pH of the metal solution was measured before and after sterilization (pHmeter Mettler-Toledo GmbH Process, Switzerland).

4.2 Microalgal cultures preincubation

Two *G. sulphuraria* strains, genetically distant from each other, were used for this study. Strain ACUF 427 was obtained from the algal collection of the University of Naples "Federico II" (www.acuf.net) and was formerly collected from the acidic soil of the thermal station in Gunnuhver, Southwest Iceland. Strain SAG 107.79, originally sampled from a very hot acidic water in Sonoma, California, was obtained from Culture Collection of Algae at Göttingen University (https://www.uni-goettingen.de/en/culture+collection+of+algae+%28sag%29/184982.html). Both algal cultures were further isolated by streaking the colonies three times across Allen agar plates, starting from a diluted solution of the cultures. The ultimate colonies were eluted in Allen medium at pH 2.5 and grown at a temperature of 37°C and constant light intensity (50 μmol photons/m^2 s). The cultures were refreshed weekly with a new medium until the microalgae reached the logarithmic growth phase.

4.3 Experimental design

The metal uptake experiments were performed in triplicate, in 24-wells plates (FalconTM) 2 mL solutions, stirred on a tilting shaker (MR-1 Biosan, BioScientifica, Italy) and kept in a climatic chamber (ThermoFisher Scientific) at 37°C (Figure S1). A defined volume of microalgal culture was centrifuged at 13.000 rpm for 2 min at 4ºC; the supernatant discarded and the algal pellet was washed twice with autoclaved deionized water and then added to the experimental metal solution (water + metal), in order to achieve an initial optical cell density of 5 OD (ƛ=550nm; Secomam spectrophotometer Prim light). Positive controls (metal solutions without microalgal biomass) and negative controls (algal biomass without metals) were considered as well. pH of each metal solution was measured at the beginning and at the end of exposure (24h) (pHmeter Mettler-Toledo GmbH Process, Switzerland).

After 24 h treatment, samples were collected and centrifuged at 10000 rpm to separate the biomass fractions from the supernatant ones. Supernatant samples were filtered with a 0.2 μm filter, while the biomass samples were washed twice with H_2O at the corresponding pH and digested using aqua regia (HNO_3:HCl = 3:1 v/v). Digestion was conducted in a microwave oven (Milestone OneTouch, Italy) at 175 °C for 10 minutes, following US Standard recommendations (US-EPA 3051A). Metals concentrations were finally measured in the supernatant and the digested samples through inductively coupled plasma mass spectrometry, using an ICP-MS (Aurora M90 Bruker Daltonics). The evaluation of the metal uptake was done, measuring the total metal removed and the removal efficiency. The first measure was done using the following formula:

$$\text{Total metal removed (mg/g dw)} = C_{biomass} \times V / M$$

where $C_{biomass}$ is the metal concentration measured in the biomass fraction, V is the volume of the test solutions, and M is microalgal biomass (g, dry matter).

4.4 Statistical analysis

All the experiments were performed in triplicates and the data were expressed as mean ± standard deviation. Metal recovery values were analysed through one-way analysis of variance (ANOVA). A multiple comparison Tukey test was then used to evaluate the significance of the differences among the treatments.

5. Conclusion

Knowing the effect of the pH is one step toward fully understanding the mechanisms involved in the bio removal of rare earth metals, and the findings of this study represent an added value for developing an efficient system. Based on the data produced and willing to speculate the best conditions, in terms of pH solution and chosen strain of *G. sulphuraria* for each metal and using equimolar quaternary solutions, all four metal species were best removed from SAG 107.79 at pH 5.5. In the necessity to use more acidic solutions to remove these metals, the best choice lay on ACUF 427. Further data on the cellular localization of the metal ions could represent an important step in understanding the contribution of biosorption and bioaccumulation to the bio removal of REEs using *G. sulphuraria* biomass.

Supplementary Material: Figure S1. Example of the triplicates of treated and untreated samples (green wells) in 24-wells plates. The plates were kept on a rotary shaker at 37°C for 24 hours. Transparent wells contained 2 mL metal solutions set up as positive control. Table S1. Total metal removed from single and quaternary metal aqueous solutions by *G. sulphuraria*, strain SAG 107.79. Data are expressed as μmol/g dry matter. The total metal removed quantities were calculated by adding the amount of every metal component (Y^{3+} + Ce^{3+} + Eu^{3+} + Tb^{3+}). Table S2. Total metal removed from single and quaternary metal aqueous solutions by *G. sulphuraria*, strain ACUF 427. Data are expressed as μmol/g dry matter. The total metal removed quantities were calculated by adding the amount of every metal component (Y^{3+} + Ce^{3+} + Eu^{3+} + Tb^{3+}).

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