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Fluctuation analysis to select for Samarium bio-uptaking microalgae clones the repurposing of a classical evolution experiment☆

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ABSTRACT

Rare Earth Elements (REE) increasing demand prompts the research of biotechnological approaches to exploit secondary resources. We made use of the adapted Fluctuation analyses experiment to obtain *Chlamydomonas reinhardtii* ChlA strains resistant to Samarium (Sm) as the reference REE. The starting hypothesis was that adaptation to metal-containing media leads to an enhanced metal uptake. ChlA was able to adapt to $1.33 \cdot 10^{-4}$ Sm M and pH~3 by pre-existing genetic variability, allowing the evolutionary rescue of 13 of the 99 populations studied. The rescuing resistant genotypes presented a mutation rate of $8.65 \cdot 10^{-7}$ resistant cells per division. The resulting resistant population contradicted the expected fitness cost associated with the adaptation to Sm, selection resulted in larger and faster-growing resistant cells. Among the three isolated strains studied for Sm uptake, only one presented uplifted performance compared to the control population (46.64 μg Sm g^{-1} of wet biomass and $3.26 \cdot 10^{-7}$ ng Sm per cell, mainly bioaccumulated within the cells). The selection of microalgae strains with improved tolerance to REEs by this methodology could be a promising solution for REES sequestration. However, increased tolerance can be independent or have negative effects on uptake performance and cellular features studied are not directly correlated with the metal uptake.

Summary sentence: Repurposing a classic laboratory evolution experiment to select for microalgae Samarium adapted strains for metals recovery and biotechnology approaches.

Data availability statement: All data generated or analyzed during this study are included in this published article (and its raw files).

1. Introduction

Rare Earth Elements (REEs) are a set of highly demanded metals due to the current global trend of consumption of these elements. As REEs importance gains momentum because of its multiple uses (e.g. industrial catalysts, fertilizers in agriculture, medical products, fluorescence bulbs, smartphones, clean energy, batteries, to mention a few (Migaszewski and Gałuszka, 2015) developing new efficient alternatives to extract and exploit those elements are required to meet the demand trends (Alonso et al., 2012). Research has therefore recently shifted towards technologies with a smaller environmental impact for the recovery of REEs. Biological methods are already widely used in activities including but

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Abbreviations: REE, Rare Earth Elements; EEE, Electric and Electronic Equipment; EDTA, Ethylenediaminetetraacetic acid; ICP-MS, Inductively coupled plasma mass spectrometry; DB, Dry Biomass; WB, Wet Biomass; BLM, Biotic ligand model.

☆ The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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not limited to bioleaching, bio-uptake, bio-reduction, bio-recovery, bioaccumulation, bio-precipitation, or biosorption (Ambaye et al., 2020; Čížková et al., 2019; Dunbar, 2017), to recover and extract REEs and other scarce elements. These technologies offer an alternative to physicochemical approaches (Ambaye et al., 2020; Fathollahzadeh et al., 2019).

A considerable amount of research has been conducted in the development of efficient methods for REEs recovery with different types of biomass or by microorganisms (Ambaye et al., 2020). Regarding microalgae, only a few studies have been published in REEs recovery. Birungi and Chirwa (2014) used six different microalgae species to study the La biosorption and desorption; Kucuker et al. (2017) studied the pH and temperature parameters to uptake Nd from real Nd-magnets with *Chlorella vulgaris* biomass. Kosak née Röhder et al. (2018) highlight the role of the *Chlamydomonas reinhardtii* wall in the sorption of Ce rather than an efficient transport route for its internalization. Tan et al. (2017) successfully modeled the competition between Sm^{3+} and different binding ligands (Mg^{2+} and Ca^{2+} and other three REE cations: La^{3+}, Ce^{3+}, and Eu^{3+}) with the biotic ligand model (BLM), suggesting also its extrapolation to other REE trivalent cations. Other studies assessing the BLM using *C. reinhardtii* had been made in the biouptake context of different REE like Eu (Yang et al., 2014), Nd (Yang and Wilkinson, 2018), or In (Yang et al., 2019). Čížková et al. (2019) exposed three different green microalgae species to red mud containing REE and they even observed a growing improvement suggesting them as a good method for biomining or bio-leaching lanthanides. However, a critical aspect of bio-recovery methods is the selectivity and efficiency of the process, which is directly related to the species and strains used. Biotechnology methods have demonstrated that adaptation to metal-containing media leads to an enhanced metal uptake by microalgae (Malik, 2004; Sandau et al., 1996; Suresh Kumar et al., 2015). This may have subsequent benefits, a driven selection could lead towards new strains able to thrive in artificial media with high metal concentrations and low pH, in the acidic conditions required to dissolve metals and in which normally REEs biosorption decreases (Boyanov et al., 2003; Karavaiko et al., 1996; Sastre de Vicente et al., 2004). Therefore, we propose the repurpose of a classic experimental evolution bioassay to select and identify more efficient strains for REEs recovery, the fluctuation analysis first published by Luria and Delbrück (1943).

In concrete, we are going to study Samarium (Sm) that served us as a prior model in the research of the lanthanides within the REE. REE toxicity against microalgae has been demonstrated with no significant differences within REE elements (Tai et al., 2010). Samarium (Sm) is one of the most commonly used REEs (Chua, 1998). It is present in fertilizers, in microwave filters, it is used as permanent magnets or in control rods in the nuclear industry (Giese et al., 2019; Pang et al., 2002; Tyler, 2004), isotope ^{153}Sm is employed to treat pain in bone tumors in nuclear medicine (Giese, 2018), industrially it is used in dehydration and dehydrogenation of ethanol and the catalysis of polychlorinated biphenyls plastics (Emsley, 2011). Sm is considered a mildly toxic element when ingested and exposure to Sm can cause skin and eye irritation (Pagano et al., 2015). The maximum permissible concentration limit of Sm in drinking water is 8.2 ng mL^{-1} (Sneller et al., 2000) and human consumption through vegetable intakes are being studied (Zhuang et al., 2017). Environmentally, Sm is not considered a hazard to plants or animals. Sm has no significant biological role, but it has been noted to increase metabolism in certain organisms (Pang et al., 2002) and is regarded as an anthropogenic emerging REE contaminant (Kulaksiz and Bau, 2013). Its presence has been also determined in different organisms such as microalgae (Mann et al., 1988) or other aquatic flora like water hyacinth (Chua, 1998).

Here, we address the effect of Sm in *C. reinhardtii* growth and photosynthetic activity. Then, we obtained through selection different strains of *C. reinhardtii* resistant to high Sm concentrations. *C. reinhardtii* is a well-established eukaryotic model for phylogenetic, toxicological, and evolutionary studies (Salomé and Merchant, 2019). Knowledge already acquired indicates *C. reinhardtii* as a candidate with commercial purposes in metal-capture processes (Suresh Kumar et al., 2015). The additional benefits of photosynthetic organisms, low-cost harvesting, CO_2 sequestration, assimilation of other pollutants, combined with the selectivity in lanthanides deposition displayed by other Chlorophyta and Euglena species made it suitable for this study. Our objective was to obtain resistant strains adapted to Sm and acid conditions employing experimental evolution. The simultaneous verification of different traits and Sm accumulation could be used to establish an inventory of phenotypic features related to improve Sm biouptake. This work describes the potential of experimental evolution methodologies for biotechnological approaches.

2. Material and methods

2.1. Organisms and growth conditions

The experiments were carried out using the *C. reinhardtii* Dangeard (ChlA strain) wild type strain isolated from Doña National Park (Andalucía, Spain), obtained from the algae culture collection of the Universidad Complutense de Madrid, Spain. In the laboratory conditions, ChlA strain was transferred axenically every 20 days to maintain the mid-log exponential growth and cultured with 20 mL of bi-distilled water enriched with BG_{11} standard broth (Sigma-Aldrich®, Chemie, Taufkirchen, Germany) in 50 mL cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ). The cell cultures are grown under continuous light conditions at 80 μmol m^{-2} s^{-1} over the waveband 400–700 nm, with a controlled temperature of 22 °C ± 2 °C. All experiments were conducted in the same conditions unless otherwise indicated.

A commercial solution of samarium (Carl Roth GmbH + Co. Laboratories, Roti®Star, Karlsruhe, Germany) was used, where Sm_2O_3 was the starting material with a purity level of 99.99%. The 100 mL solution contained 1 g $^{-1}$ of samarium and 2% (v/v) HNO_3. Under these culture conditions, Sm^{3+} cation presents the highest bioavailability in dissolution among Sm ions (Tan et al., 2017). All Sm-containing media were prepared to add the volume required of the commercial solution to bi-distilled water enriched with BG_{11} culture broth. The pH of the solutions was measured with a pHmeter Sension+ph3 and adjusted with a solution NaOH (1 M) in case it was necessary.

2.2. Toxicity tests: dose-effect inhibition of cell growth and photosynthetic quantum yield

The toxic effects of Sm were evaluated employing two methods: cellular growth and photosynthetic yield inhibition ChlA strain was exposed to serial solutions of Sm ($2{\cdot}10^{-7}$; $6.65{\cdot}10^{-7}$; $2{\cdot}10^{-6}$; $6.65{\cdot}10^{-6}$; $2{\cdot}10^{-5}$; $6.65{\cdot}10^{-5}$ M) at pH~3 to ensure Sm availability in the dissolution. *C. reinhardtii* was not acclimated to acid pH prior to the experiments. This microalgae species was chosen due to its pH tolerance. Although a reduction of its growth can be observed at pH~3 (Crémazy et al., 2013; Lustigman et al., 1995), the growth inhibition does not occur until pH~2 (Messerli et al., 2005). Control groups did not include HNO_3 and starting pH was 7.1–8., address both the effect of the Sm and pH 3 in the growth rate and photosynthetic yield.

Sm cellular growth inhibition was estimated as follows: replicates of $1.2{\cdot}10^4$ cells per mL of ChlA strain at mid-log, exponential-growth phase was exposed to the different Sm concentrations, three replicate cultures were established for each dose of study, and three replicates unexposed were used as control populations. Cell densities were counted with a Beckman Coulter Z2 cell counter (Beckman Coulter® Inc. Particle Characterization Group, Florida) after 4 and 7 days of incubation respectively.

The effective quantum yield of the photosystem II ($Y(II)$) of the cultures was measured by a ToxY-PAM fluorometer (Dual-Channel Photosynthesis yield analyzer, Heinz Walz GmbH, Effeltrich, Germany). This saturation pulse method (Genty et al., 1989; Schreiber et al., 1986) permits us to identify if there is any substance in the liquid medium affecting the photosynthetic electron flow, considered as a crucial factor for algal health. Duplicates for each concentration were placed in falcon tubes of ten mL with an initial culture of 10^5 cells mL^{-1}. The measures were taken at 0 h, 2 h, 24 h, and 48 h of exposure. To calculate the percentage of inhibition, we took as reference an equal culture with the same culture conditions, but with no Sm addition, as follows:

$$Inh(\%) = [(Y(II)control - Y(II)sample)/Y(II)control]x100\% \tag{1}$$

where $Y(II)sample$ corresponds to the effective PSII quantum yield at each time of Sm exposure and $Y(II)control$ represents the effective PSII quantum yield of the control.

2.3. Fluctuation analysis experiment

We performed a modified fluctuation analysis as previously described by López-Rodas et al. (2001). Fluctuation analysis experiment adapted to liquid cultures and possible outcomes are shown in Fig. 1 (see Supplemental Experimental Procedures for detailed information).

The fluctuation test also permits to estimate the frequency of a spontaneous mutation to appear in a population. This value is known as the mutation rate (μ) and is calculated with the proportion of tubes were no alive cells were seen, by using the following equation:

$$P_0 = e - \mu(N_t - N_0) \tag{2}$$

N_0 and N_t (10^2 and 10^5) are the initial and the final population size contained in the tubes before the toxic (Sm) exposure took place. Luria and Delbrück P_0 estimator is the proportion of tubes with no resistant cells. Aliquots were monitored throughout the experiment for alive cells with fluorescence microscopy.

2.4. Sm-resistant strains characterization

Resistant strains obtained from the set 2 of the Fluctuation analyses experiment were characterized by Malthusian fitness under not selective conditions (Crow and Kimura, 1970). The coefficient of selection s was calculated to the equation of Ayala and Kiger (1980). Morphological and stereological features were measured in between 50 and 75 cells from each strain. For each strain, we obtained four morphometric traits: the area, perimeter, major axis (D), and minor axis (d). These values were used to calculate as an estimator of asymmetry the coefficient of the form of the diameters (C.F.d.) and as an estimator of roundness the coefficient of form for circular profiles (C.F.c.) (equations and extended methods can be found in Suppl. materials).

2.5. Sm biouptake determination

Three of the strains obtained, SmR9, SmR17, and SmR59, were selected to study their Sm biouptake capacity (see results for rationale). We exposed the three selected strains and ChlA (control) never exposed before to Sm, to a solution of BG_{11} with Sm concentration of $3{\cdot}10^{-7}$ M. A concentration of $\approx 10^{-7}$ M Sm was chosen for being similar to the one found by other authors in waters near mining activities all around the world (Gammons et al., 2003; Li et al., 1998; Mann et al., 1988). The pH was maintained at 4.5 (at 20 °C) to assure the viability of the control strain and the bioavailability of Sm.

The experiment was carried out for 27 days, when cellular pellets and supernatants were harvested. rom the two pellets obtained per strain; one was directly stored to be analysed and the other pellet was washed and resuspended three times with 5 mL of a BG_{11} solution enriched with 0.05% ethylenediaminetetraacetic acid (EDTA) for 15 min. All chemical analysis was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) by the CAI of geological techniques of the Complutense University of Madrid (detailed description of the experimental procedures and chemical analyses can be found in supplemental materials).

2.6. Statistical analyses

Statistical analysis and graphs were performed with the software

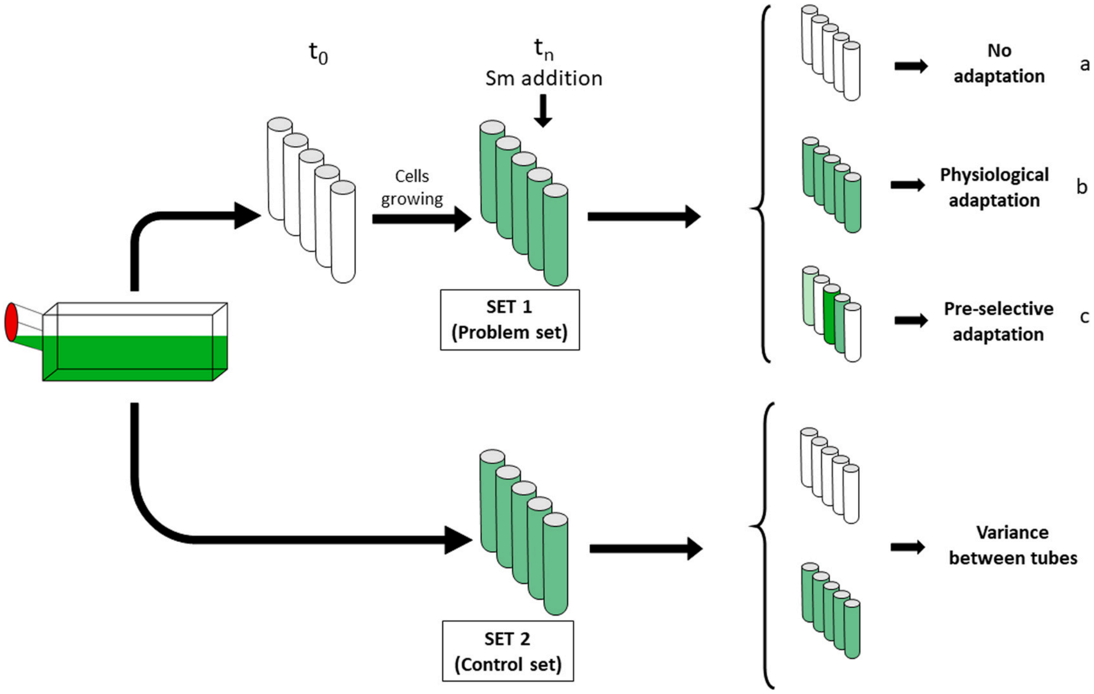


Fig. 1. Schematic representation of the Fluctuation analysis experiment. From the same parental culture, set 1 and set 2 were established at different times: set 1 at t_0 and set 2 at t_n when the Sm was added. Three different results in set 1 are possible although only three are observable: a. No adaptation: no alive cells will be found in any tube. b. Physiological adaptation, all the replicate populations present living cells, or c. Pre-selective adaptation: replicate populations present tubes without living cells and tubes with different cellular densities. Set 2 acts as a control, and all the tubes should present grown cells in a similar density.

GraphPad Prism 8.4.2 for Windows (GraphPad Prims, n.d.) and R-statistical software (R Core Team, 2019). To study differences between the 13 resistant strains and the controls in fitness we performed a parametric analysis, one-way ANOVA, and Tukey HSD post-hoc. Morphometric data were compared with a Kruskal-Wallis test with a post hoc Wilcox test. For the biouptake experiment, we look for correlations between each parameter measured for the four strains used. We performed a non-parametric Spearman test, to study if there are tendencies of any predictor value to assure a better capture. A p-value less than 0.05 was used as a cutoff for determining significance.

3. Results

3.1. Toxic effect of Sm on C. reinhardtii

Sm at pH 3 was mildly toxic at low doses to *C. reinhardtii,* but at high doses, we can observe growth and photosynthesis inhibition. At the lowest Sm doses, $2 \cdot 10^{-7}$ M and $6.65 \cdot 10^{-7}$ M, growth was mildly inhibited on day 4, but the population totally recovered on day 7 and even presented 10% of increased growth compared to the control population. We observed a 50% growth inhibition at $2 \cdot 10^{-6}$ Sm M maintained after 7 days of exposure. After 7 days of exposure, we observed growth inhibition of ~80–90% at concentrations of $6.65 \cdot 10^{-6}$ M, $2.1 \cdot 10^{-5}$ M, and $6.65 \cdot 10^{-5}$ M compared with the control replicates. Moreover, there was a decrease in the cell density at $6.65 \cdot 10^{-5}$ M from day 4 to day 7, suggesting that not just growth is impaired but that the cells might be dying.

The extent of *Y(II)* inhibition compared to a control population measured by Eq. (1) indicated a dose-dependent relationship of Sm toxicity effect in PS II. No inhibition of the *Y(II)* was observed at the doses $\leq 2 \cdot 10^{-6}$. At the concentration of Sm $6.65 \cdot 10^{-5}$ M, there was a mean inhibition of 30.45% ± 7.67 at T_0, reaching a mean inhibition of 99.76% ± 0.01 during the first 2 h (see Supplemental Table 1 for a detailed description of the toxicity analyses results).

3.2. Fluctuation test

We doubled the highest Sm amount chosen for the toxicity assays to assure a population dye-off, selecting a final concentration of $1.33 \cdot 10^{-4}$ M for the fluctuation analysis experiment. The resume of the fluctuation analyses results obtained is shown in Table 1 grouped according to cells' densities. At the beginning of the fluctuation experiment, massive die-offs were observed in both sets. But, over the 120 days of incubation, 13 out of 99 tubes of set 1 and all the tubes of set 2 presented living cells due to the proliferation of Sm resistant variants. We calculated the covariance of each set, resulting in significantly higher covariance in set 1 (One Tail Z-test= 10.14, $p < 0.0001$). The strong variation in the cell densities among set 1 tubes, in comparison to the variance presented in the set 2 control trial, suggests that the resistant variants arose employing pre-selective adaptation. We estimated *a posteriori* the mutation rate for the strain and Sm concentration studied following Eq. (2) as $8.65 \cdot 10^{-7}$ resistant cells per division.

Table 1

Fluctuation analysis experiment results. We grouped the replicated population of each set per number of cells mL^{-1}. We found a greater variance in the cell density counted between the tubes of set 1 than in tubes of set 2. Significant differences between Set1 and Set2 were observed and preselected mutations were found. Mutation rate (μ) was calculated with the proportion of tubes where no growing cells were identified, and it is expressed as mutations per cell per generation.

C. reinhardtii	**Set1**	**Set2**
N° of replicate cultures	99	50
0	86	0
$15 * 10^3 - 1 * 10^5$	2	49
$1 * 10^5 - 5 * 10^6$	6	1
$> 5 * 10^6$	5	0
Mean	$4.87 \cdot 10^5$	$2.32 \cdot 10^4$
Variance	$1.56 \cdot 10^6$	$2.32 \cdot 10^4$
CV (Variance/mean)	3.20	1.39
One Tail Z-Test	10.14****	
Fluctuation	YES	NO
Mutation Rate (μ)	$8.65 \cdot 10^{-7}$	

**** Signif. codes: 0.0001.

One Sm resistant strain from each active population of the Fluctuation analyses set 2 was re-isolated by serial dilutions and named as SmRX being X the number of the tube in which they were cultured: SmR1, SmR5, SmR9, SmR13, SmR17, SmR27, SmR51, SmR52, SmR55, SmR57, SmR59, SmR63, and SmR77.

3.3. Sm resistant strains characterization

The results for the individual traits per strain are summarized in Fig. 2. Regarding the fitness values obtained under not selective conditions, we observed that all of the resistant strains isolated grow faster than the control strain (Fig. 2B-2D). We found significant *p* values between the control versus all the strains except SmR1, SmR17, and SmR59 (one-way ANOVA (F(13,28) = 4.003, $p < 0.05$; Tukey HSD) (Suppl. Table 2). Strain SmR9 presented the highest fitness with less error among the isolated strains, which on the other hand, presented the lowest cell density among the set 1 tubes at the end of the fluctuation analysis. We obtained negative *s* values in a medium without Sm, suggesting that these variants are not selected against at all.

As said in material and methods, four measures of the cells per strain were obtained from the photographs: area, perimeter, major axis (*D)* and minor axis (*d*); and two form coefficients were calculated; *C.F.d.* for measuring the asymmetry and *C.F.c.* to measure the roundness (Fig. 2A). For the C.F.d., seven strains, SmR5, SmR9, SmR55, SmR57, SmR59, SmR63, and SmR77, were significantly different from the control strain (K-W chi-squared=60.05, df=13, $p < 0.5$). For C.F.c. coefficient all the strains were significantly different compared with the control strains, the control presenting the highest C.F.c. (K-W chi-squared=116.87, df=13, $p < 0.5$) (see Suppl. Table 2 for traits mean and SD values).

Among the 13 strains, SmR9 (Fig. 2A), SmR17, and SmR59 were selected for the biouptake studies. Both SmR9 and SmR17 (Fig. 2B and 2C) presented traits performance above 50% for all the traits, SmR9 presenting the highest minor axis (*d*) and among the highest fitness and SmR17 presents the higher area. SmR59 strain was selected as a representative strain of the intermediate phenotype, presenting mid values for most of the traits, except for C.F.d. that was high indicating asymmetry.

3.4. Sm uptake

The Sm nominal and measured concentrations in abiotic conditions at the beginning of the experiment presented no significant differences (Sm measured values were of 45.5 ± 12 ng Sm mL^{-1} (~$3.03 \cdot 10^{-7}$ M). The four strains, the control, and the three evolved strains were able to uptake Sm, and at the end of the experiment most of the Sm, between 70% and 85%, was removed from the dissolution (Table 2). Concerning Sm's effect on cellular growth, the control population presented less cellular density compared to the resistant strains. Among the four strains, SmR17 presented the highest uptake performance among the strains studied, with a mean of 46.64 μg Sm g^{-1} of wet biomass (WB) and $3.26 \cdot 10^{-7}$ ng Sm per cell. Surprisingly, the control strain presented the second-best performance (Table 2). We also found that most of the Sm were not released by the EDTA extraction, reaching up to 86% of the total Sm remaining in the cellular pellet after EDTA treatment of SmR17, indicating that most of the Sm might be sequestered inside the cells.

Regarding Fig. 3 we can observe the *r* factor obtained from the Spearman test. We didn't find a strong relation between Sm uptake and fitness as we expected. An *r* factor of − 0.19 is found comparing these two variables, which means that a slower growth slightly benefits the

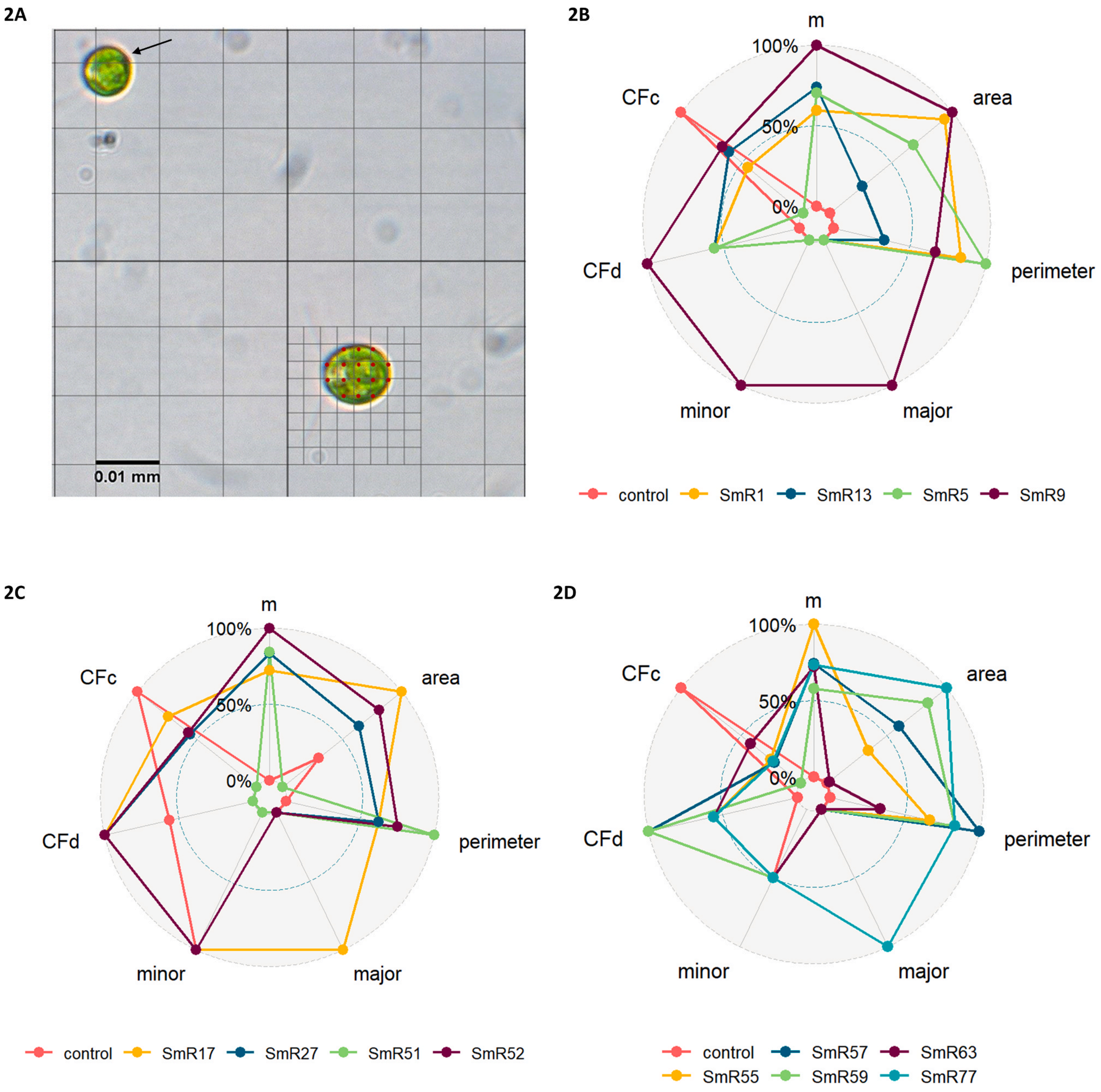
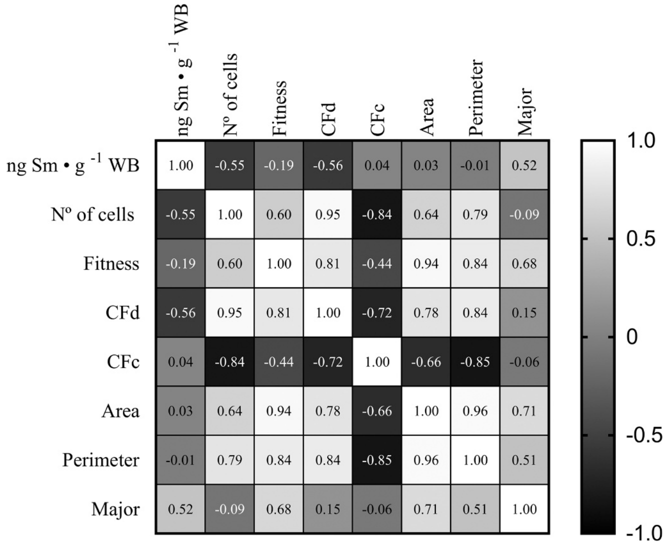


Fig. 2. Sm resistant strains fitness and morphological scores. (2A) Micrograph (40x) of Sm resistant *C. reinhardtii* Sm resistant (SmR9 strain) representing the point-counting procedure based on planimetry techniques for morphometric analyses (the arrow points to the photoreceptor-organelle eyespot). In (^{2}B–^{2}D) we show the polar plots of the different resistant Sm strains against the sensible control. Polar plots go radically from 0 (lower scored trait value), in the center, to 100% (higher scored trait value), among the strains included in each of the plots. We refer to the threshold value of 50% to indicate that the strains present high and low performance per trait.

Table 2
Sm biouptake experiment results. Sm quantification is expressed as the analytical value and the analysis error. Values of ng Sm per g^{-1} WB and total cells are expressed as mean and SD.

Strain	Total ng Sm Pellet	Total ng Sm Pellet (EDTA)	ng Sm ml^{-1} Supernatant	µg Sm g^{-1} WB	ng Sm·per cell	N° of total cells
Control	11 ± 1	8.4 ± 08	6.9 ± 2.26	28.89 ± 2.63	2.68·10^{-7}	41.12 ± 0.57 (·10^6)
SmR9	7.7 ± 0.8	5 ± 0.5	11 ± 1	14.82 ± 1.54	1.54·10^{-7}	50.1 ± 0.42 (·10^6)
SmR17	15 ± 1	13 ± 1	13.5 ± 0.5	42.64 ± 2.84	3.26·10^{-7}	45.96 ± 0.23 (·10^6)
SmR59	8.8 ± 0.5	7.5 ± 0.8	12.5 ± 2.1	15.61 ± 1.59	1.63·10^{-7}	54.09 ± 0.41(·10^6)

uptake. A similar, but positive tendency is found between *s* and Sm uptake, we observe a direct relation of 0.19. It is interesting the inverse correlation obtained between the number of cells at the end of the biouptake experiment and Sm uptake, − 0.55. Not a higher number of cells will suppose a higher inside taking of Sm which suggests that biological internalization is restricted or that cultures should not reach the stationary stage. With the morphometric values of the major and minor axis, ng of Sm uptake is related in a positive way with the first one



	ng Sm • g^{-1} WB	Nº of cells	Fitness	CFd	CFc	Area	Perimeter	Major
ng Sm • g^{-1} WB	1.00	-0.55	-0.19	-0.56	0.04	0.03	-0.01	0.52
Nº of cells	-0.55	1.00	0.60	0.95	-0.84	0.64	0.79	-0.09
Fitness	-0.19	0.60	1.00	0.81	-0.44	0.94	0.84	0.68
CFd	-0.56	0.95	0.81	1.00	-0.72	0.78	0.84	0.15
CFc	0.04	-0.84	-0.44	-0.72	1.00	-0.66	-0.85	-0.06
Area	0.03	0.64	0.94	0.78	-0.66	1.00	0.96	0.71
Perimeter	-0.01	0.79	0.84	0.84	-0.85	0.96	1.00	0.51
Major	0.52	-0.09	0.68	0.15	-0.06	0.71	0.51	1.00

Fig. 3. Physiological, morphological and, Sm biouptake parameters *r* correlation (Spearman test) for the Sm resistant strains SmR9, Sm17, SmR59, and, the wildtype strain.

(0.52) and in a negative way with the second one (−0.52). This seems to be consistent with the fact that no strong relationship has been found with the roundness coefficient, C.F.c. (0.04). Neither the Area (0.03) nor the Perimeter (−0,01), show an interesting relation with the ng of Sm uptaken. Lastly, the greatest value of *r* related to the Sm uptake is obtained with the C.F.d. in inverse relation, – 0.56, suggesting that this allometric coefficient between the axis could be interesting for future predictions (see Suppl. Table 3 for all the traits correlation r values).

4. Discussion

Sm and other REEs, at low concentrations, are known to be plants growing promoters (Tyler, 2004) and they are widely used to improve fertilization in China (Pang et al., 2002; Zhang and Shan, 2001). A few studies have addressed the positive effects of REEs on microalgae growth (Evseeva et al., 2010; Goecke et al., 2015; Gong et al., 2011). We observed a growth increase in ChlA strain exposed to doses equal or below 6.65·10^{-7} M in the toxicological studies. Parallel patterns were observed in the photosynthetic inhibition study, at the lower doses studied the photosynthetic yield was higher in the exposed populations compared to the control. The mechanisms to these positive effects have been related to nutrient limitation alleviation (for example Ca $^{2+}$, Mg $^{2+}$ or Mn $^{2+}$ deficiencies), but it is not clear whether other mechanisms are implicated (such as scavenging of oxygen- free radicals (Valcheva--Traykova et al., 2014), or as a stress compensation (Volland et al., 2014)). To date there is no commercial use of Sm and other lanthanides for microalgae biomass production, growth promotion could be a favorable feature for developing an optimal biocapture system of these elements, especially if REE concentrations do not reach toxic levels in the water object of bioremediation.

These beneficial effects are concentration-dependent, growth and photosynthetic yield inhibition occurred at higher concentrations. We observed a significantly reduced growth rate in ChlA strain, resulting in a ~50% reduction of growth, at 2·10^{-6} Sm M after 72 h. Similar toxic effects had been observed in other microalgae exposed to Sm and single lanthanides (Tai et al., 2010). The photosynthetic yield was completely inhibited after 48 h in the 6.65·10^{-5} Sm M exposition, but yield decrease could be due to the extracellular pH. REEs are classified as low toxicity. However, they are gaining attention due to their residual footprints in natural environments (Adeel et al., 2019). There are already studies pointing to anomalies measured in Europe in natural resources, due to anthropogenic contributions, like with Gd in the Garonne River (Lerat-Hardy et al., 2019) or Gd, La, and Sm in the Rhine River (Kulaksiz and Bau, 2013). Information about REEs toxicity in microalgae stills limited and incomplete (reviewed by Goecke et al. (2015)), but there is substantial evidence that REEs influence the normal physiological functions of the cells affecting membrane permeability and enzymes (Brown et al., 1990; Palasz and Czekaj, 2000). Microalgae are primary producers, due to their trophic and environmental importance, further investigation of REEs toxicity needs to be addressed to establish guidelines and guide environmental policies.

One of our main objectives was achieved by obtaining different Sm resistant strains to high Sm concentrations. Only 13 out of 99 replicate tubes were able to adapt to 1.33·10^{-4} M Sm concentration and pH 3. We obtained a μ of 8.65·10^{-7} mutants per cell division resulting among the lowest mutation rates obtained utilizing fluctuation analyses in microalgae chlorophytes (Suppl. Table 4). In previous studies using the same strain of *C. reinhardtii*, exposed to beryllium sulfate and Uranium mining tailings showed the closer values, 9.61·10^{-6} (Baselga-Cervera et al., 2016) and 3.8·10^{-7} (García-Balboa et al., 2013), respectively. This rate is in the order of magnitude observed in studies addressing other metals and other stressful environments, suggesting that the adaptation to Sm acid waters would be possible by the evolutionary rescue of the population. Here, we have studied the resulting strains from set 1 separately to address if different Sm adaptation pathways were taking place and, if we could identify among the adapted strain phenotypic traits related to biotechnological purposes, concretely Sm biouptake. Surprisingly, most of the resistant strains isolated from set 1 presented increased fitness compared to the control population under no selective conditions (Table 2). These results contradict the expected fitness cost associated with the adaptation to Sm. This result could be influenced by traces of Sm presented in the resistant strains that increase cell growth. All the resistant isolates were bigger in area and volume than the ancestral strain, except SmR51. The coefficients of the form indicated that the resistant cells presented a less circular form. Sm pressure selected for resistant cells with higher fitness rates, larger size, and more asymmetry. Adaptation to metal contamination entails multiple tolerance mechanisms to alleviate the toxic effects. Defence mechanisms in microalgae change physiological, structural and enzymatic activities at both extracellular and intracellular level (Priyadarshini et al., 2019). The molecular basis of metal resistance response are controlled by multiple genes in eukaryotes (Gutiérrez et al., 2003; Siripornadulsil et al., 2002) and there is little genomic information available on genes related to metal-resistance in microbial eukaryotes (Balzano et al., 2020). Phenotyping microalgae resistant strains, identifying traits that could be related to metals-uptake, might open avenues to identify phenotypic related resistant features that facilitate mining and finding novel metallothioneins or enzymes for a variety of biotechnological applications.

Sm-uptake performance experiment indicated that SmR17 strain sequestered the largest amount of Sm up to 46.64 μg Sm g^{-1} of (WB) and 3.26·10^{-7} ng Sm per cell. Even more, our data shows that the control strain, never exposed before to samarium, sequestered larger amounts per cell than the other two resistant strains, SmR59 and SmR9. Contrary to expectation, SmR9, the resistant strain with the greatest Malthusian fitness, was also the one that less Sm retained despite presenting the highest density at the end of the biouptake experiment. Correlation analyses showed that the size of the major axis and the selection coefficient has a positive correlation with the total ng of samarium bioabsorbed. A negative r factor of 0.6 in the correlation between the ng of samarium up-took and the final number of cells in the culture could be explained by the phase of the culture, at the end of the experiment cultures were in a late stationary phase. Although specific experiments with Samarium (or other REE) are needed, based on a previous work where statistically significant differences of the biomass uptake for uranium was only observed during the mid-log exponential growth of the culture (Baselga-Cervera et al., 2018). After the exponential growth phase, microalgae cells start binding to other cells, reducing the cell wall surface available for metal binding (Gupta and Rastogi, 2008), or metal will be released into the dissolution related to cell death. These results indicate that the bottlenecks and selective pressures imposed in set 1 were selected for larger and faster-growing resistant cells, but not increased Sm uptake.

The Sm sequestration potential of the resistant strains was addressed at 3·10–7 Sm M and pH 4.5, pH at which Sm^{3+} is proposed to be the highest cation in dissolution among other Sm ions. Previous studies of Sm sequestration by phytoplankton described that the BLM can be assumed to be Sm^{3+} at pH below 6, but other species like hydroxo complexes gain importance at higher pH (Rowell et al., 2018). The complexation of Sm with natural organic matter resulted in enhanced Sm biouptake in the case of *C. reinhardtii,* contrary to classic BLM predictions (Zhao and Wilkinson, 2015), and a significant decrease in the Sm internalization flux (Rowell et al., 2018). Therefore, taking into account that pH and organic matter are among the principal factors affecting the REE speciation, Sm uptake studies at different conditions need to be conducted to predict their bioavailability in aquatic environments. As the analytical approach demonstrated, Sm is mainly bioaccumulated within the cells. EDTA washed weakly surface-adsorbed metal bound to functional groups in the cell surface. The higher percentage of Sm (up to 85%) was not released using the EDTA extraction, indicating the Sm internalization. Previous bioavailability Sm studies showed that free metal was a better predictor of Sm internalization. Several studies have shown that lanthanides accumulate in chloroplasts (Shen et al., 2002, 2003; Guo et al., 2000; Kang et al., 2000), and selective deposition of individual REEs in the cytoplasm or chloroplast have been described in green algae (Goecke et al., 2015). REEs properties similarity shared with calcium, ionic radii, electrostatic interaction, or oxygen ligands affinity, might enable Sm to be adsorbed and replaces calcium functions (Corrêa et al., 2017; dos Remedios, 1981; Dressler et al., 2014). There is evidence reported by Tan et al. (2017) of lanthanides competition with Mg^{+2} and Ca^{2+}, although for Mg^{2+} only with high concentrations were observed. It also has coherence with the fact that trivalent lanthanides could bind to the calmodulin protein (specific to Ca^{2+} binding), creating less rigid binding sites with different configurations than with Ca^{2+} binding (Edington et al., 2018). Although more research is needed, because calcium is essential for good cell development, these theories could also explain REE toxicity at high doses. Moreover, Zhao and Wilkinson (2015) observed enhanced biouptake of several REEs in the presence of ligands, hypothesising that ternary complexes will be formed and contributed to the biouptake.

Increased tolerance to REEs can be induced by exposing the population to metal concentrations or metal-rich dissolutions (Ipatova et al., 2015; Marvá et al., 2014). Another possibility is the bioprospection of microorganisms in contaminated environments, inhabitant strains present increased tolerance to selective pressure they are exposed to (Garbayo et al., 2012; Miazek et al., 2015; Yoshida et al., 2006). Microalgae populations with improved tolerance to REEs can be a promising solution for bioremediation of polluted water and sequestration of REEs. However, increased tolerance can be independent or have negative effects on uptake performance. For further improvement in these technologies and their implementation as an industrial process, in situ tests, as well as bioavailability relations in aquatic environments between Sm and microalgae species are needed.

5. Conclusions

Fluctuation analysis classical experiment can be a good method for obtaining different resistant strains to high concentrations of Sm in acid pH solutions, but uplift tolerance did not necessarily increase the Sm uptake ability. Only one of the three resistant clones isolated increased Sm biouptake compared to the wildtype strain. These strategies can be used alone or in combination, such as gradual increasing selection, to increase Sm and pH tolerance. Physiological and morphometry features could be a promising tool to identify phenotypic related resistant features of interest for biotechnological applications, however, it is necessary to carry out more studies.

CRediT authorship contribution statement

Paloma Martínez-Alesón García: Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing**, Camino García-Balboa**: Definition, Methodology, Resources, Investigation, Writing - Review & Editing**, Julia Romero-López:** Writing - Review & Editing, **Victoria López-Rodas:** Definition, Supervision, Funding acquisition, **Eduardo Costas:** Definition, Supervision, Funding acquisition, **Beatriz Baselga-Cervera:** Definition, Conceptualization, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112134.

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