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Sirakov, Maria, Palmieri, Maria Palmieri, Iovinella, Manuela et al. (5 more authors) (Accepted: 2021) Cyanidiophyceae (Rhodophyta) tolerance to precious metals: metabolic response to Cl4K2Pd and AuCl4K. Plants. ISSN 2223-7747 (In Press)

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Communication

Cyanidiophyceae (Rhodophyta) Tolerance to Precious Metals: Metabolic Response to Cl_4K_2Pd and $AuCl_4K$

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Abstract: Cyanidiophyceae are polyextremophilic red algae adapted to live in geothermal and volcanic sites with a high concentration of heavy and rare metals and can mobilize and adsorb metals selectively. In this work, we assessed the capacity of 3 strains of *Galdieria* (*G. maxima*, *G. sulphuraria*, *G. phlegrea*) and one strain of *Cyanidium caldarium* to tolerate different concentrations of rare metal as Cl_4K_2Pd and $AuCl_4K$ by monitoring algal growth in cultures exposed to the metals and investigating algae potential oxidative stress induced by them.

Keywords: metal tolerance; *Galdieria sulphuraria*; precious metal;s metabolic response

Citation: Sirakov, M.; Palmieri, M.; Iovinella, M.; Davis, S.J.; Petriccione, M.; Di Cicco, M.R.; De Stefano, M.; Ciniglia, C. Cyanidiophyceae (Rhodophyta) Tolerance to Precious Metals: Metabolic Response to Cl_4K_2Pd and $AuCl_4K$. *Plants* **2021**, *10*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

In the last decade, there was a remarkable and growing demand for recovering elements and energetic resources from waste streams [1]. In particular, high levels of concern were directed towards Rare Earth Elements (REEs) due to their extensive use in superconductors, catalysts, and the electronic industry. Conversely, the discharge in the environment and the suitability of recycling REE from e-waste are relevant topics because of their hazard for the environment and health, besides their economic value. These issues become evident to both governments and electronic industries, which are increasingly prone to develop new methods to remove REEs from the environment and possibly recycle them back into a "closed-loop economy" production cycle [2–6] while simultaneously achieving energy optimization goals [7,8]. Recently, biological methods were developed to ensure the recovery of small quantities of these metals from wastewater systems [5], using mainly bacteria [9–12] or plants known for their ability to immobilize heavy metals in the cell wall and compartmentalization in vacuoles [13]. Interestingly, polyextremophilic algae have intrinsic properties that make them capable of selective removal and concentration of metals, thanks to their adaptation to live in geothermal and volcanic sites [14–17]. Geothermal fluids leach out of the hot volcanic rocks and are enriched by enormous amounts of minerals and metals, including lithium, sulphur, boric acid, and precious metals such as gold, platinum, palladium, and silver [18].

Cyanidiophyceae, unicellular red algae, survive in extreme conditions, very low pH (0.0–3.0) and high temperatures (37–55 °C), and colonize acid and hydrothermal sites but also rocks and muddy soil around hot ponds [19]. They are divided into 3 genera: *Cyanidioschyzon*, *Cyanidium* and *Galdieria*, which differ in size, cellular shape, and growth conditions. *Cyanidioschyzon merolae*, the only species belonging to the *Cyanidioschyzon*

genus, differs from the other two taxa in the lack of a cell wall and division by binary fission [20]. *Cyanidioschyzon*, *Cyanidium* and *Galdieria* can grow both on ammonia and nitrate. *Cyanidioschyzon* and *Cyanidium* species are obligatory autotrophs, while *Galdieria* ones can grow auto-, mixo-, and heterotrophically and tolerate high concentrations of salts [21], thus making *Galdieria* more suitable for biotechnological applications [14]. The ability of *Galdieria sulphuraria* of recovering REEs was already assessed [5,22] and confirmed by an approved patent [23]. In this report, we focused in deep for the first time on the ability of different *Galdieria* species (*Galdieria maxima*, *Galdieria sulphuraria*, *Galdieria phlegrea*) and *Cyanidium caldarium* to tolerate different concentrations of REEs (such as palladium-Cl_4K_2Pd and gold-$AuCl_4K$). We also investigated the metabolic response and possible oxidative stress induced by these metals by monitoring superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) activities.

2. Results

Polyextremophilic microalgae, such as Cyanidiophyceae, have a high intrinsic capacity to uptake metals, involving active and passive mechanisms [5]. Heavy, rare, or precious metals can influence algae physiology in various ways, likely inhibiting different physiological processes. To evaluate the suitability of Cyanidiophyceae other than *G. sulphuraria* for biotechnological application to recover REEs effectively, we tested the tolerance to Cl_4K_2Pd and $AuCl_4K$ by monitoring the growth and metabolic response of 4 different taxa, exposed to each of these metals at a concentration in the range 1–10 g/L. As more deeply discussed in Section 4, the growth was evaluated after 4 days (96 h) since the single metal exposure. The results were expressed in the form of Maximum Growth Rate (MGR).

As shown in Figure 1, the presence of $AuCl_4K$ significantly reduced cellular duplication in G. maxima at all the concentrations tested; Cl_4K_2Pd did not negatively affect cell growth, and no statistical difference was recorded between MGR in control and tests (Figure 1A). Regarding *G. phlegrea*, both metals induced a trend of reduction in growth rate at both concentrations (Figure 1B). Viceversa, in G. *sulphuraria*, $AuCl_4K$ reduced cell growth at the maximum concentration, while the MGR appeared not affected by Cl_4K_2Pd, as shown by the MGR values at 10 g/L comparable to control. A decrease in growth rate was recorded at lower concentration (1 g/L); presumably, the highest amount of palladium was beneficial for the growth of this strain, or even the improvement of the cell duplication should be interpreted as a defence of the algal strain. Finally, *C. caldarium* showed a high tolerance to Cl_4K_2Pd, whereas $AuCl_4K$ significantly inhibited cell duplication as metal concentration increased (Figure 1D). The highest concentration of palladium (10 g/L) improved the growth, and in *G. maxima* and *G. sulphuraria*, the MGR values outperformed the controls.Subsequently, it was decided to evaluate the ROS scavenging activities of SOD, CAT and APX in all algae tested in the presence of Cl_4K_2Pd and $AuCl_4K$ at a concentration of 1 g/L, after an incubation period of 24 h. The reason behind this choice was that the antioxidant activity could be considered a measure of the cell effectiveness in responding to the impact of metals, increasing their tolerance as a protective mechanism necessary to remove ROS before they can damage sensitive parts of the cellular machinery. In particular, the SOD, which catalyses the dismutation of O_2^- (singlet oxygen) to O_2 and H_2O_2, was defined as the first cellular defence against ROS production. Meanwhile, CAT catalyses the production of H_2O from the degradation of H_2O_2 and ROOH, respectively. Finally, APX reduces H_2O_2 to H_2O using the ascorbate as an electron donor. The strain/metal-specific metabolic responses were quite diverse, as shown in Figure 2. Indeed, APX activity significantly increased only in *G. maxima* in response to Cl_4K_2Pd, while in the presence of $AuCl_4K$, all the enzymatic activities appeared reduced (Figure 2A). Concerning the other strains, in G. *phlegrea*, all the enzymes tested activity decreased in the presence of Cl_4K_2Pd and increased in the presence of $AuCl_4K$ (Figure 2B); in *G. sulphuraria*, both metals induced an

enzymatic activity decrease (Figure 2C). Finally, we observed a significant increase of all the enzymes in *C. caldarium* in the presence of Cl_4K_2Pd, but not in $AuCl_4K$ (Figure 2D).

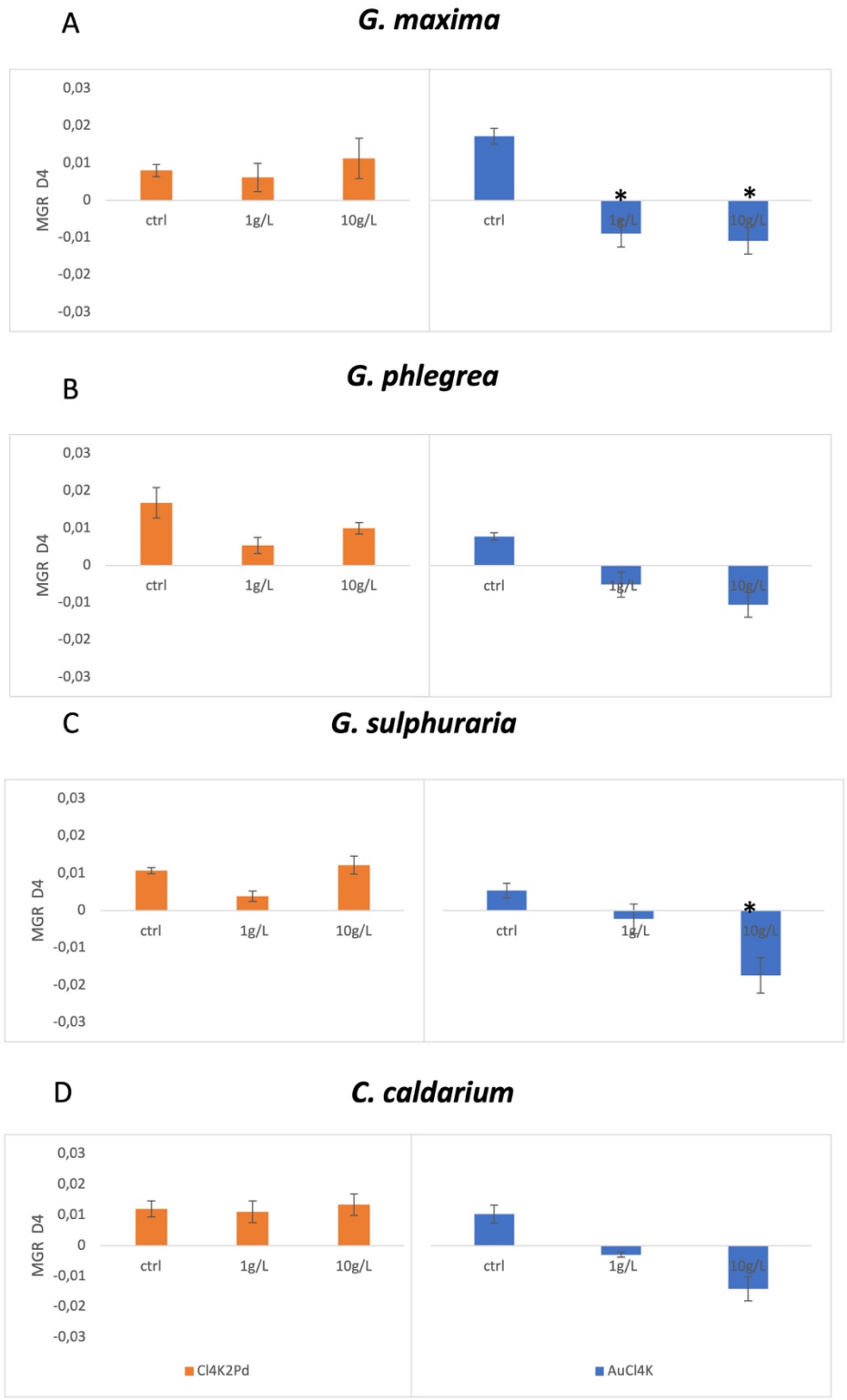

Figure 1. Evaluation of metal tolerance through MGR monitoring after 4 days (96 h). Maximum growth rate in presence of different concentrations of palladium (Cl_4K_2Pd-orange-left panel) and gold ($AuCl_4K$-blue-right panel), for the species *G. maxima* (**A**), *G. phlegrea* (**B**), *G. sulphuraria* (**C**) and *C. caldarium* (**D**). Error bars represent standard deviation of three replicates; (*) = *p*-value ≤ 0.000000001 calculated by *T*-test.

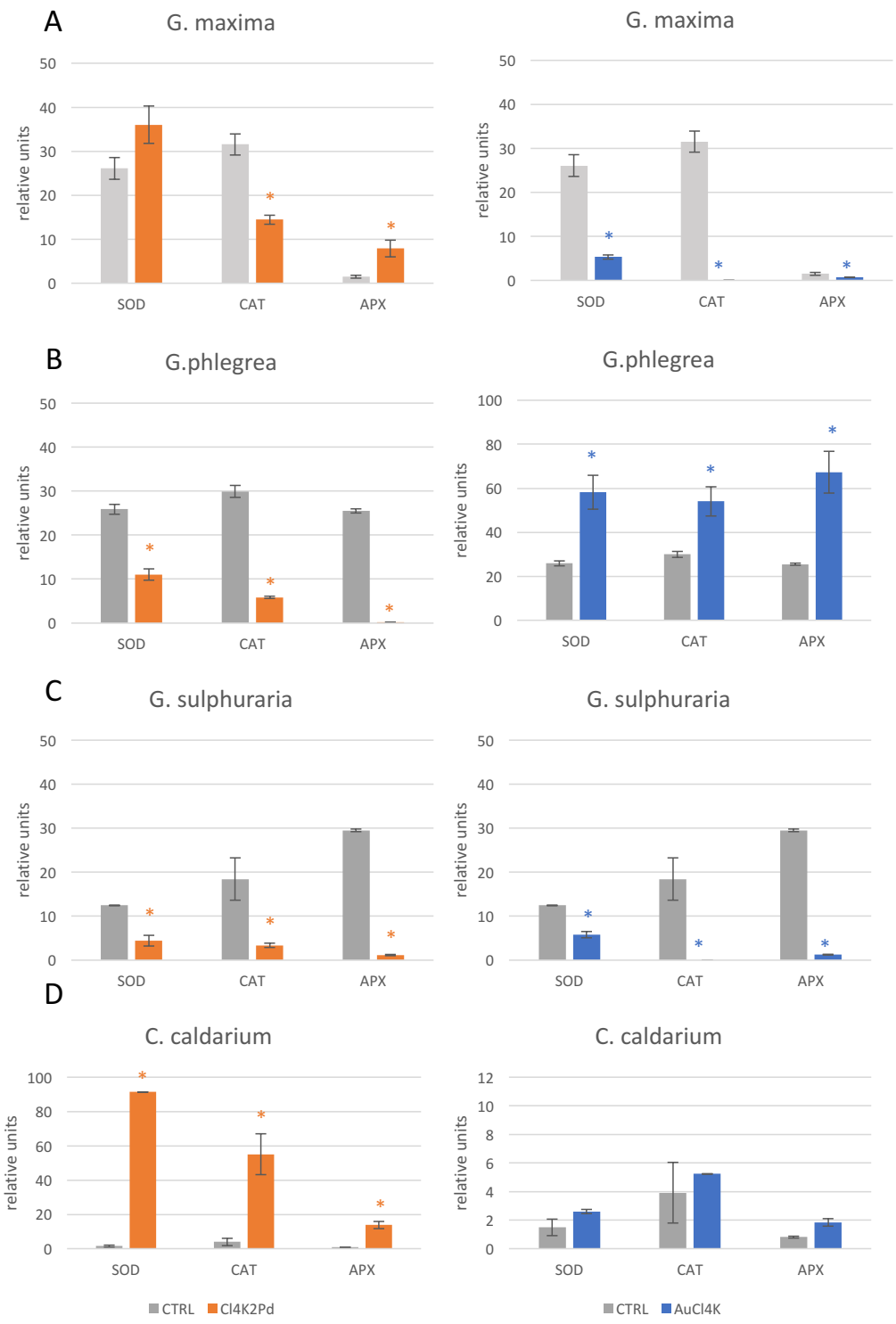

Figure 2. Evaluation of enzymatic activities after metals exposure. Relative units represent: enzymatic activity as units/g of dry weight (SOD); enzymatic activity as nmol H_2O_2/g of fresh weight (CAT); enzymatic activity as µmol ascorbate/g of fresh weight (APX). Enzymatic activities were monitored in *G. maxima* (**A**), *G. phlegrea* (**B**), *G. sulphuraria* (**C**) and *C. caldarium* (**D**), treated with 1 g/L of palladium (Cl_4K_2Pd—orange bars) and gold ($AuCl_4K$—blue bars) after 96 h. Mean (± SD) was calculated from three replicates. (*) = *p*-value ≤ 0.05 calculated by *T*-test.

3. Discussion

A significant increase of the enzyme compared to the control suggests a high scavenging activity of the singlet oxygen in peroxide of hydrogen, which can be expressed as an evident tolerance of these algae to the metal under examination. An increase in the activity of both antioxidant enzymes is necessary to reduce the concentrations of both singlet oxygen and hydrogen peroxide, minimizing the risks. In general, the modulation of antioxidant enzymes is an essential adaptive response to counteract adverse conditions; in fact, maintaining a high antioxidant capacity in the cells can be correlated with increased tolerance against different types of environmental stress [24].

Our results indicate that rare and precious metals can be tolerated by all the strains tested, even if there is a clear higher tolerance to Cl_4K_2Pd vs. $AuCl_4K$ considering growth rates. Comparing the growth rate in the presence of the different concentrations of the metals, it seems clear that the growth and the metabolism of *G. phlegrea* appear to be more affected by the presence of both metals, showing a decrease of both growth and metabolic responses. The contribution to the oxidative equilibrium of the examined extremophile microalgae and the induction of antioxidant enzymes could result from the adaptation of the cell to the development of intracellular ROS. However, there is no clear correlation between any enzymatic activity and the better performing growth of the other 3 strains tested.

Although metals generally induce inhibition in microalgal growth, several reports also suggest their positive roles. It is known that metals at small concentrations are useful for microalgal metabolism since they participate in the synthesis of proteins involved in photosynthesis, nitrogen assimilation, phosphorous acquisition, CO_2 fixation and DNA transcription [25]. Algae can develop efficient defence mechanisms to counteract the toxicity and improve their survival, even at high metal concentrations [26]. One of the defence strategies is the accumulation of the metals, which consists of the metal adsorption on the cell surface (biosorption), followed by their entry into the cell protoplast (bioaccumulation). When metals are accumulated inside the cell, the algae activate molecular mechanisms as other defence strategies to reduce their toxicity [26]. *G. sulphuraria* can survive in harsh environments rich in heavy and rare metals by detoxifying and transforming them into less toxic derivatives [27]. The defence strategies developed by algae to prevent the toxic effect of some metals represent a good opportunity for biotechnological purposes. The study from Ju et al. (2016) showed the ability of *G. sulphuraria* to recover both Cl_4K_2Pd and $AuCl_4K$ inefficiently [5]. However, the authors did not test this strain's tolerance to growth in the presence of these metals. In contrast, we consider that tolerance and growth capacity is an essential parameter to take in account for the biotechnological application, such as REEs recovering.

4. Materials and Methods

4.1. Strains Cultivation

The algal strains used in this study belong to the algal collection of the University of Campania "L. Vanvitelli" derived from the University of Naples (www.acuf.net), namely ACUF 3.4.5 (*G. maxima*), ACUF 7.6.21 (*G. phlegrea*), ACUF 9.2.11 (*G. sulphuraria*) and ACUF 626 (*C. caldarium*). All the strains were inoculated in Allen medium containing $(NH_4)_2SO_4$ as nitrogen source, at pH 1.5 by adding H_2SO_4 [28] and cultivated at 37 °C, kept mixed on an orbital shaker under a photon irradiance of 150 μmol photons m^{-2} s^{-1} with 16/8-light/dark cycle provided by cool-light fluorescent lamps (Philips TLD30w/55). Cell densities of the algal cultures were assessed, recording the Optical Density (OD) at 750 nm with a spectrophotometer (Bausch & Lomb Spectronic 20).

4.2. Experimental Procedure

Microalgal cultures at exponential phase were inoculated into fresh Allen medium enriched with Cl_4K_2Pd and $AuCl_4K$ at concentrations ranging from 1 to 10 g/L. Growth

rates were calculated within 96h using spectrophotometric measurements of the optical density (OD 550 nm, Bausch & Lomb Spectronic 20), which were then used in the following equation for the Maximum Growth Rate (MGR):

$$MGR\ (1/d) = (Ln(Nt) - Ln(N0))/((t - t0))$$

where:

Nt is the optical density at the final time
N0 is the optical density at the initial time
T is the final time (days)
T0 is the initial time (days)

All analyses were performed in triplicates.

4.3. Enzyme Extraction and Assays

Algal cultures grown in the presence of the minimal dose of palladium and gold (1 g/L) were harvested by centrifugation at 14,000 rpm for 10 min after 96 h of exposure. The algal pellets were washed using KH_2PO_4 (0.1 M pH7.8) followed by centrifugation at 12,000 rpm for 4 min at 4 °C, twice. Proteins were extracted, homogenizing the sample with liquid nitrogen using a mortar and a pestle. The obtained powder was resuspended in 3 mL of Lysis Buffer (KH_2PO_4 0.5 M pH7.8, DTT 2 mM, EDTA 1 mM, PMSF 1 mM, PEG 1.25 mM) and centrifuged at 14,000rpm for 20 min at 4 °C. The supernatant was used for measurement after Bradford quantification.

SOD (EC 1.15.1.1) activity was assayed by the photochemical inhibition nitroblue tetrazolium (NBT) method [6]. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA and 30 µL of enzyme extract and 2 mM riboflavin. The reaction was started by switching on the light (two 15 W fluorescent lamps) for 15 min, and the absorbance was measured at 560 nm. Two samples without the enzymatic extract and illumination were used as controls. One SOD unit was defined as the amount of enzyme corresponding to 50% inhibition of the NBT reduction. The enzyme activity was expressed as units per 1 mg of protein (U mg^{-1} protein).

CAT (EC 1.11.1.6) activity was assayed according to Aebi (1984) [29], with minor modifications. The H_2O_2 decrease was determined after the reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction was monitored, measuring the decrease in the absorbance at 240 nm for 100 s. The CAT activity was calculated according to the molar extinction coefficient of H_2O_2 (39.4 mM^{-1} cm^{-1}) and expressed as nmol H_2O_2 min^{-1} mg^{-1} protein.

APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada (1981) [30]. The ascorbate oxidation was determined using the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA-Na2, 0.5 mM ascorbic acid and 100 µL of crude enzyme extract. The reaction started by adding 0.1 mM H_2O_2, monitoring the decreasing absorbance at 290 nm for 100 s. The APX activity was calculated according to the molar extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}) and is expressed as nmol di H_2O_2 min^{-1} mg^{-1} protein.

Each condition for each experimental approach was tested 3 times independently.

5. Conclusions

Our observations strongly suggest that other strains than *G. sulphuraria* can be used to recover REEs due to their high tolerance to precious and heavy metals. Nevertheless, further studies will be necessary to clarify the biological mechanisms underlying the tolerance capacity of *Cyanidiophyceae* and their strategies to respond to metal toxicity for future biotechnological applications.

Author Contributions: Conceptualization, M.S. and M.I.; Formal analysis, M.P. (Maria Palmieri), M.P. (Milena Petriccione) and M.R.D.C.; Methodology, M.P. (Maria Palmieri), M.P. (Milena Petriccione) and M.R.D.C.; Supervision, C.C.; Visualization, S.J.D., M.D.S. and C.C.; Writing—original draft, M.S. and M.I.; Writing—review & editing, M.S. and M.I. All authors have read and agreed to the published version of the manuscript.

Funding:

Institutional Review Board Statement:

Informed Consent Statement:

Data Availability Statement:

Conflicts of Interest: The authors declare no conflict of interest.

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