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Impact of heavy metals in the microalga Chlorella sorokiniana and assessment of its potential use in cadmium bioremediation

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Impact of heavy metals in the microalga *Chlorella sorokiniana* and assessment of its potential use in cadmium bioremediation

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Highlights:

- *C. sorokiniana* can tolerate high concentrations of Cu, Cd, As (III) and As (V)
- Ammonium consumption is affected only at high concentrations of heavy metals
- *C. sorokiniana* can accumulate up to 11,000 mg kg^{-1} of Cd
- Antioxidant enzymes are overexpressed in the presence of these metals
- N and S assimilation metabolism is also altered by the presence of heavy metals

Keywords: microalgae, heavy metal, nitrogen uptake, accumulation, enzymatic activity, gene expression

Abstract

The chlorophyte microalga *Chlorella sorokiniana* was tested for the bioremediation of heavy metals pollution. It was cultured with different concentrations of Cu^{+2}, Cd^{+2}, As (III) and As (V), showing a significant inhibition on its growth at concentrations of 500 µM Cu^{+2}, 250 µM Cd^{+2}, 750 µM AsO_3^{-3} and 5 mM AsO_4^{-3} or higher. Moreover, the consumption of ammonium was also studied, showing significant differences for concentrations higher than 1 mM of Cu^{+2} and As (III), and 5 mM of As (V). The determination of intracellular heavy metals concentration revealed that *Chlorella sorokiniana* is an outstanding Cd accumulator organism, able to accumulate 11,232 mg

kg^{-1} of Cd, and removing 65% of initial concentration of this heavy metal. Finally, antioxidant enzymes, such as catalase (CAT) and ascorbate peroxidase (APX), and enzymes involved in the production of glutamate and cysteine, such as glutamine syntethase (GS), glutamate dehydrogenase (GDH), O-acetylserine (thiol) lyase (OASTL) and NAD-isocitrate dehydrogenase (NAD-IDH) were studied both at gene expression and enzymatic activity levels. These enzymes exhibited different grades of upregulation, especially in response to Cd and As stress. However, GS expression was downregulated when *Chlorella sorokiniana* was cultured in the presence of these heavy metals.

1. INTRODUCTION

Heavy metals are hazardous pollutants especially dangerous for aquatic ecosystems. The mining and mineral treatment activities, which produce wastewater with Cu, Cd or As, among others, are the main cause of pollution by heavy metals of the aquatic ecosystems. These heavy metals have a high impact in all the organisms present in the polluted environments, especially in microalgae (Salama et al., 2019). It has been described that metal stress can induce the increase of reactive oxygen species (ROS) and the expression of enzymes involved in the elimination of these molecules, such as catalase (CAT), glutathione reductase (GR) or ascorbate peroxidase (APX) (Sabatini et al., 2009). Moreover, other cellular pathways involved in nutrients assimilation or metabolism are also affected by heavy metals (Devriese et al., 2001; León-Vaz et al., 2021). Additionally, alterations in the DNA, mutagenesis and other toxic effects have also been described in microalga and other aquatic organisms exposed to heavy metals (Salama et al., 2019). Thus, understanding these responses and the metabolic modifications produced under metal stress conditions is essential for the development of heavy metals phycoremediation procedures.

Different heavy metal remediation methods, such as chemical precipitation, ion-exchange, flocculation or membrane filtration, have been developed with different results (Salama et al., 2019). On the other hand, an increasing number of studies have shown the potential of bacteria and microalgae, in order to remove heavy metals from aquatic environments (Li et al., 2020). Microalgae have been reported to have several advantages for bioremediation of heavy metal compared to bacteria and fungi, because of their tolerance and high accumulation capacity. Although exposure to heavy metals may affect growth and metabolism of microalgae, many species are able to deal with high concentrations of these compounds and remove them from wastewater (Debelius et al., 2009). As a consequence, microalgae could be a cost-effective and ecologically safe alternative for remediation of heavy metals in aquatic environments and an excellent model, providing important information about the physiological impact of these contaminants in plant cells

In this context, the microalga *Chlorella sorokiniana* (*C. sorokiniana*) emerges as an excellent alternative to develop bioremediation strategies. This chlorophyte presents a wide range of advantages over other microalgae. Not only can *C. sorokiniana* tolerate a broad range of temperature (Yoshida et al., 2006), but also can grow under different adverse conditions, including wastewaters or heavy metal wastes (León-Vaz et al., 2019; Liang et al., 2017). In addition, the microalga shows a high growth rate compared to other chlorophytes explored for their use in phycoremediation, such as *Chlamydomonas reinhardtii, Chlorella minutissima.* or *Scenedesmus sp.* (Salama et al., 2019). As a consequence, *C. sorokiniana* could be an optimal candidate to study the response of plant cells to heavy metals stress and evaluate its accumulation capacity and its possible use in bioremediation.

The aim of the present study is to determine the response of the microalga *C. sorokiniana* to the presence of Cu^{+2}, Cd^{+2}, and the metalloid As (III and V), focussing on its bioaccumulation capacity, growth and ammonium consumption rates. These elements are common wastes generated in pyrite extraction (Taggart et al., 2006), an important activity in the pyrite belt located in the SW of the Iberian Peninsula . Moreover, the effect of these elements on the gene expression and the activity level of several enzymes involved in antioxidant and nitrogen and sulphur metabolism of the microalga has also been studied.

2. MATERIALS AND METHODS

2.1. Algal strain and culture conditions

C. sorokiniana 211-32 was kindly provided by the Institute of Plant Biochemistry and Photosynthesis (IBVF; Seville, Spain). The microalga cells were grown at 27 ºC in TAP (Tris-acetate-phospate) media with acetate and ammonium concentrations previously optimized (León-Vaz et al., 2019), under continuous agitation (150 rpm) and white light irradiation (150 $\mu E\ m^{-2}\ s^{-1}$). When indicated, different concentrations of $CuCl_2$, $CdCl_2$, $NaAsO_2$ or Na_2HAsO_4 were added to the culture medium and pH was adjusted out to 6.5-7 before autoclaving.

2.2. Analytical determinations

Dry weight (DW) was determined as previously described in León-Vaz et al. (2019), using 5 mL of culture, and drying the filters overnight at 90 °C.

For ammonium determination in culture medium, total ionic strength was calculated with an ammonium ion selective electrode, as described in León-Vaz et al. (2019).

2.3. Bioconcentration factor (BCF) calculation

Cooper, cadmium and arsenic content in lyophilized cells or culture media of *C. sorokiniana* was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Freeze-dried cells (0.05 g) were digested as described in León-Vaz et al. (2021). Samples were measured in an Agilent 7700 spectrometer (Agilent Technologies, Santa Clara, CA, USA). The BCF was calculated as described in Ellison et al., (2014), with the equation:

$$\boldsymbol{BCF} = \frac{C_b}{C_w}$$

where C_b is the concentration of the heavy metal in dry algal biomass (mg kg^{-1}) and C_w is the concentration in culture medium (mg L^{-1}).

2.4. Preparation of crude extract

At 42h of culture, *C. sorokiniana* cells were harvested by centrifugation and traces of heavy metals in culture media were eliminated by washing with Milli-Q water. Cells were then resuspended in 50 mM Tris-HCl pH 8.0 buffer at 0.2 g fresh weight mL^{-1}, in a bashing beads tube. The cells were disrupted by agitation with glass beads in a Digital Disruptor Genie® (Scientific Industries, Bohemia, NY, USA) for 3 cycles of 30 s, and the homogenate was centrifuged twice at 14000 x g for 20 min. The supernatant obtained was used as the crude extract source. Bio-Rad Bradford assay was used to determined protein in crude extract according to the manufacture protocol, using BSA as standard.

2.5. Enzyme assays

All the assays were performed with the crude extract prepared as above indicated. APX and CAT assays were performed as previously described Daud et al. (2014) and Martins and English, (2014), respectively. GDH was measured kinetically as described Gronostajski et al. (1978) for *C. sorokiniana*. GS and OASTL activities were determined colorimetrically, measuring the γ-glutamyl hydroxamate and L-cysteine formed respectively, as described in Devriese et al. (2001). Finally, NAD-IDH activity was measured as described by Domínguez et al. (2003).

2.6. qRT-PCR analysis

C. sorokiniana total RNA was extracted according to Arriola et al. (2018) using Direct-zol RNA Kit (Zimo Research, Irvine, CA, USA). Gene expression experiments were carried out in a Mx3000P Multiplex Quantitative PCR qPCR Equipment (Stratagene) using 1 μL of the cDNA as template and the SYBR® Premix Ex Taq, Bulk Mix (Takara Bio, Kusatsu, Japan), as previously described (Rengel et al., 2018). Each qPCR measurement was carried out in triplicate using specific primers for the *APX*, *CAT*, *GS*, *GDH*, *OASTL* and *NAD-IDH* encoding genes (Table 1). The 18s gene (KF673387), which expression was constitutive under the different conditions used, as has been previously stated (Wan et al., 2014), was used as hosekeeping gene to normalize mRNA abundance. The fold change in gene expression was calculated using the relative quantification $2^{-\Delta\Delta CT}$ approach (Pfaffl, 2001).

Table 1. Oligonucleotides used as primers for quantitative RT-PCR experiments

Primer Name	Gene Bank Accession number no.	Name	Sequence
APX	*PRW20193.1*	qAPXF	GTTCCACGACGCCGGCTCCTACA
		qAPXR	CTGCCGCCCCACTGCCACCTTGAT
CAT	*PRW57738.1*	qCATF	CCCACCTGCGGCGTCAAGTTCCT
		qCATR	CGGTGGCGCTGCGTGTCCTGGTA
GS	*PRW34037.1*	qGSF	CACCGGCCCCCTGGAGACC
		qGSR	GCAGGGGGATGCGGATGGAG
GDH	*PRW44353.1*	qGDHF	AGATGGGCGGCCGCGTGGTAGC
		qGDHR	GCCGCCGCCGTTGGTGAAGATG
NAD-IDH	*PRW57886.1*	qNADIDHF	GTGGTGCCCGGCGTGGTGGAGTC
		qNADIDHR	CCCGTTGGCGCCGATGTTGC
OASTL	*PRW20938.1*	qOASTLF	GCAACCCGGGGCCCCACAAGAT
		qOASTLR	GGAACAGCGCGGACGACAGGTAGC
18sCS	*KF673387.1*	q18sCSF	TCCGCCGGCACCTTATGAGAAATC
		q18sCSR	CGCGTGCGGCCCAGAACA

APX, ascorbate peroxidase 6; *CAT*, catalase isoenzyme 1*; GS*, glutamine synthetase*; GDH*, glutamate deshydrogenase 1 isoform X1*; NAD-IDH*, isocitrate dehydrogenase [NAD] catalytic subunit mitochondrial*; OASTL*, cysteine synthase chloroplastic; *18sCS, Chorella sorokiniana* 18s ribosomal gene.

2.7 Statistical analysis

All measures were carried out by triplicate and represented as mean value ± SD. Significance of values was considered for $*p < 0.05$ and $**p < 0.01$. Statistical analyses were performed using

STATISTICA 8.1 software (Dell, Round Rock, Tx, USA), by comparing mean values using one-way analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1. Effect of heavy metals exposure on *C. sorokiniana* growth

The influence of different concentrations of Cu, Cd, As (III) and As (V) on the growth of *C. sorokiniana* was studied, being these concentrations between 0.1-5 mM, 50-500 μM, 0.25-5 mM, and 2.5-50 mM for Cu, Cd, As (III) and As (V), respectively. As shown in Fig.1A, *C. sorokiniana* growth was affected at a concentration of 500 μM of Cu, with a significant decrease of total biomass reached at 72 h, being 20 % lower than control culture ($p < 0.05$). However, the microalga could grow even in the presence of 1 mM Cu, reaching 70% of the biomass obtained in the control culture. These results show the high tolerance of *C. sorokiniana* to Cu, higher than other microalgae such as *Chlamydomonas reinhardtii* and *Scenedesmus acuminatus* (Table 2). High tolerance to this heavy metal has been also described for organisms which live in Cu contaminated soils (Table 2).

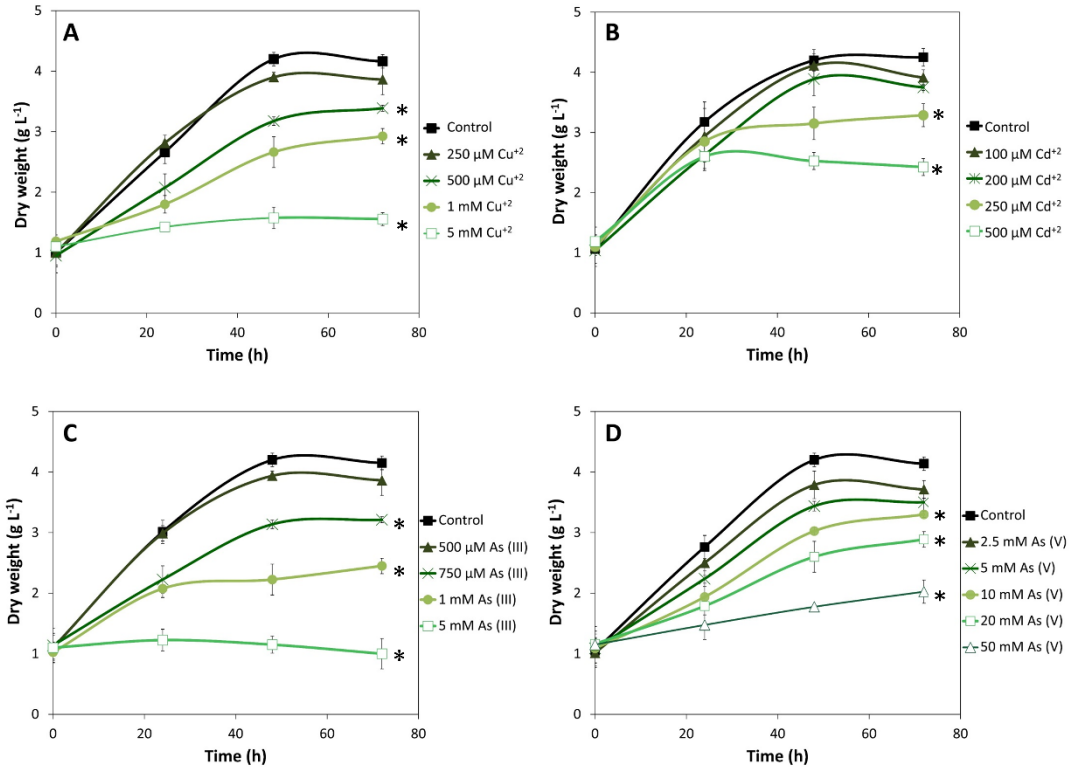

Fig.1: Growth curve of *C. sorokiniana* cultured with different concentrations of Cu^{+2} (A), Cd^{+2} (B), As III (C), and As V (D). Cells were cultured in optimized TAP media, as described in Materials and Methods. * Significant differences in biomass between control and heavy metal treatment at $p < 0.05$.

On the other hand, the tolerance of *C. sorokiniana* to Cd was lower than to Cu (Fig.1B). The microalga has, in the presence of Cd concentrations until 200 µM Cd, growth rates similar to the control. At higher concentrations, despite growing with a normal rate during the first 24 h, the cultures experimented a considerable growth inhibition thereafter. Moreover, significant differences in total biomass obtained at 72 h were observed for cultures with 250 or 500 µM of Cd (75 and 55 % of the control biomass, respectively). This could be due to the higher toxicity of Cd, which is able to inhibit the synthesis of chlorophylls and the photosynthesis, or induce the production of ROS, as has been described in our previous studies with *Chlamydomonas reinhardtii* (Domínguez et al., 2003). However, *C. sorokiniana* is more tolerant to Cd than other microalga species, such as *Chlamydomonas reinhardtii*, *Skeletonema marinoi* or *Thalassiosira baltica* (Table 2).

Table 2. Comparison of tolerance to heavy metals in different organisms

Heavy metal	Organism	Tolerance	Reference
Cu	*Chlamydomonas reinhardtii*	200 µM	(Nowicka et al., 2016)
	Scenedesmus acuminatus	50 µM	(Hamed et al., 2017)
	Elsholtzia haichowensis	50-6300 mg kg^{-1} soil	(Li et al., 2007)
	Chlorella sorokiniana	1 mM	This study
Cd	*Chlamydomonas reinhardtii*	50-300 µM	(Domínguez et al., 2003)
	Skeletonema marinoi	3.3-13 µM	(Andersson et al., 2020)
	Thalassiosira baltica	1.3-5.2 µM	(Andersson et al., 2020)
	Chlorella sorokiniana	50-500 µM	This study
As III	*Nannochloropsis sp.*	1 mM	(Upadhyay et al., 2016)
	Chlorella sp.	300 µM	(Levy et al., 2005)
	Monoraphidium arcuatum	200 µM	(Levy et al., 2005)
	Chlorella sorokiniana	1 mM	This study
As V	*Chlorella vulgaris*	2.7 mM	(Jiang et al., 2011)
	Dunaliella salina	20 mM	(Wang et al., 2017)
	Chlorella sp.	60 µM	(Levy et al., 2005)
	Monoraphidium arcuatum	30 µM	(Levy et al., 2005)
	Chlorella sorokiniana	50 mM	This study

As Fig.1C shows, *C. sorokiniana* tolerated concentrations until 750 μM As (III), without a significant decrease of growth rate or final biomass reached, being capable to live even at the concentrations of 1 mM (60 % of control dry weight, at 72 h). The tolerance observed for As (V), was higher than to arsenite, and the decrease of the growth was proportional to concentration of this metalloid, with significant differences in final biomass obtained at 5 mM (Fig.1D). Furthermore, *C. sorokiniana* is able to tolerate a concentration of 50 mM As (V) in the culture medium, showing only a partial yet substantial inhibition of its growth rate. It has been described that microalgae have different mechanisms to cope with As toxicity, including oxidation from As (III) to As (V), methylation of As species or formation of arsenosugars (Wang et al., 2015). Similar adaptation mechanisms can be the cause of the enormous tolerance of *C. sorokiniana* to arsenate, which is much higher than the observed in *Chlorella vulgaris* (Table 2) and similar to marine microalgae *Dunaliella salina* or *Nannochloropsis sp.* (Table 2).

Fig.2 shows the relation between heavy metal concentrations and growth rates, calculated as previously described (León-Vaz et al., 2019). In Cd cultures, two responses were observed; one until 200 μM, where growth rate was maintained at 80% of that of the control; and another one upon this concentration, where there was a quick inhibition of relative growth rate. In fact, at 500 μM Cd^{+2}, the microalga growth rate was 40 % of the value observed in the control culture, much higher tolerance than that reported for other microalgae species (Table 2). Cu and As (III) caused a lower effect on the growth rate of *C. sorokiniana*, with values of 70% for Cu and 90% for As (III), of the observed in the control culture at this concentration. In addition, at concentrations lower than 200 μM, the microalga kept over 90 % of the growth rate with all the heavy metals tested. These results indicated that *C. sorokiniana* is shown as a robust microalga able to tolerate high concentrations of heavy metals and metalloids, compared to other microalgae such as *Chlamydomonas reinhardtii*, *Scenedesmus acuminatus*, *Chlorella sp.* or *Dunaliella salina* (Table 2).

Furthermore, in the concentration ranges showed in Fig.2A, As (V) did not provoke a significant effect on growth rate. This demonstrated that *C. sorokiniana* can tolerate higher concentrations of As (V) than As (III), being the growth rate with 20 mM As (V) over 40 % of the values observed in the control culture (Fig.2B). It has been described that AsO_4^{-3} is analogue to PO_4^3, so it is incorporated into cells via phosphate transporters. As a consequence, there could be an uptake competition between these ions (Wang et al., 2017). TAP medium has a high concentration of phosphate compared to other culture media (2.16 g L^{-1} PO_4^{-3}), which means a low ratio As/P and, consequently, this competition could explain the high arsenate tolerance and the behaviour of the growth rate.

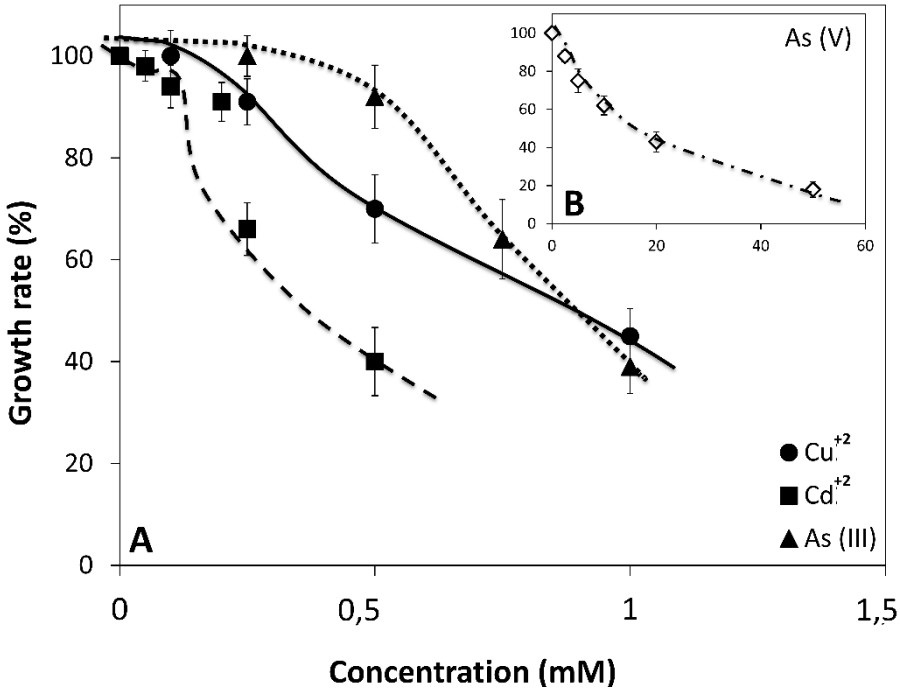

Fig.2: Growth rate (%) compared with control culture of *C. sorokiniana* cultured with Cu^{+2}, Cd^{+2}, arsenite (A) and arsenate (B). Growth rates were calculated based on Fig.1 growth curves. The value of 100% in control cultures is 0.06 h^{-1}.

3.2. Nitrogen uptake of *C. sorokiniana* cultured with heavy metals

Several previous studies report the effect of heavy metals on the photosynthetic assimilation of nitrogen, especially in higher plants (Anu et al., 2018; Devriese et al., 2001). Thus, we have studied how these elements affect the uptake of ammonium by *C. sorokiniana*. Table 3 shows the ammonium consumption rate (mM h^{-1}) of *C. sorokiniana*, in the presence of different heavy metals, measured during the first 24 h of culture. Although the tendency was a lower consumption rate for higher concentrations of heavy metals, significant inhibitions ($p < 0.05$) only appeared at concentrations over 5 mM Cu, 1 mM As (III) and 5 mM of As (V). No significant differences were found in N uptake in *C. sorokiniana* cultures under Cd stress.

Table 3. Ammonium consumption rate of *C. sorokiniana* during the first 24 h of culture with different heavy metals. * Significant differences between control culture and treatments at $p < 0.05$.

Cu^{2+} (mM)	v (mM h^{-1})	As (III) (mM)	v (mM h^{-1})
Control	0.887 ± 0.075	**Control**	0.930 ± 0.065
0.25	0.874 ± 0.031	**0.50**	0.887 ± 0.012
0.50	0.705 ± 0.028	**0.75**	0.772 ± 0.058
1	0.601 ± 0.003	**1**	0.738 ± 0.029*
5	0.437 ± 0.054*	**5**	0.434 ± 0.009*

Cd^{2+} (μM)	ν (mM h^{-1})	As (V) (mM)	ν (mM h^{-1})
Control	0.751 ± 0.058	**Control**	1.013 ± 0.072
100	0.741 ± 0.133	**2.5**	0.938 ± 0.062
200	0.691 ± 0.083	**5**	0.776 ± 0.058*
250	0.702 ± 0.027	**10**	0.751 ± 0.030*
500	0.608 ± 0.040	**20**	0.581 ± 0.028*
		50	0.537 ± 0.035*

Comparing N uptake (Table 3) with growth curves (Fig.1), it is possible to assert that concentrations over 5 mM of Cu and As (III) were able to inhibit both, the growth and the ammonium uptake of *C. sorokiniana*, suggesting that the inhibition of growth could be influenced by the limitations of nitrogen assimilation. However, the remarkable growth inhibition observed for concentrations lower than 5 mM of Cu and As (III) and 500 μM of Cd should be due to the effect of metals on other metabolic pathways, since at these concentrations ammonium consumption rate is not significantly affected in *C. sorokiniana* (Torres et al., 2008). For As (V) 5 mM, there was a significant inhibition in N uptake (Table 3). This concentration of As (V) also had a slight inhibitory effect on the final biomass reached (Fig. 1D).

3.3. Accumulation capacity of heavy metals in *C. sorokiniana* cells

Bioremediation of heavy metals by microalgae is one of their main potential applications (Raikova et al., 2019). Thus, the accumulation capacity of *C. sorokiniana* with different concentrations of heavy metals has been tested at the end of its exponential phase (72 h). The intracellular heavy metal concentrations, the bioconcentration factors (BCF) and the final heavy metal concentrations in culture media were calculated as indicated in Materials and Methods, and the results presented in Table 4. *C. sorokiniana* can accumulate considerable amounts of Cu, (Table 4), however, this BCF value is lower than the reported for other microalgae, such as *Phaelodactylum tricornutum* which has a BCF factor of 200-1500 (Atay et al., 2013). Additionally, at high concentrations of Cu (500 μM), *C. sorokiniana* can remove over 35% of Cu present in the culture medium (Table 4). Similar Cu removal capacity was observed in other microalgae, such as *Nannochloropsis gaditana* or *Isochrysis galbana* (Debelius et al., 2009). However, lower values have been described for other microalgae species, such as *Rhodomonas salina* (23%), while higher Cu removal values have been reported for *Tetraselmis chuii* (64%) or *Chaetoceros sp.*(50%) (Debelius et al., 2009).

Table 4. Intracellular dry weight accumulation, bioconcentration factor (BCF) and heavy metal concentration (µM) levels in the culture media in *C. sorokiniana* after 72 h of culture.

	Accumulation (mg/kg)	BCF	Concentration in culture media (µM)
Control			
Cu	51 ± 1	-	-
Cd	<LD	-	-
As	<LD	-	-
250 µM Cu^{+2}	1409 ± 9	89	199±26
500 µM Cu^{+2}	2232 ± 98	70	326±38
250 µM Cd^{+2}	2940 ± 60	105	148±28
500 µM Cd^{+2}	11232 ± 348	200	180±32
500 µM As III	60 ± 2	1.6	475±18
750 µM As III	59 ± 2	1.04	731±15
10 mM As V	50 ± 1	0.07	9.9±0.5 mM
20 mM As V	145 ± 1	0.10	19.87±1 mM

C. sorokiniana was also able to accumulate high amounts of Cd (11232 mg kg^{-1}), when the concentration of this metal in the culture medium was 500 µM (Table 4). These results can explain the data showed in Fig.2, where a strong inhibition of growth rate after 200 µM was observed; and also in Fig.1B, where after 24 h the tendency of growth curves changed. This behaviour could be due to the high amounts of intracellular Cd, which can affect other metabolic pathways, such as photosynthesis or respiration (León-Vaz et al., 2021), and may inhibit growth capacity of *C. sorokiniana*. Although cadmium BCF was lower to other micro and macroalgae, such as *Pavlova viridis* (500) (Chen et al., 1998) or different species of *Oedogonium* genus (between 290 and 750) (Ellison et al., 2014), total intracellular concentration of Cd for *C. sorokiniana* was much higher (11232 mg kg^{-1}) than these ones. Moreover, the initial concentrations of Cd tested for *Pavlova virdis* and *Oedogonium* species were much lower (45 and 22 µM, respectively) than the concentrations tested for *C. sorokiniana* in this study (50-500 µM), which could alter the BCF value. Additionally, in 500 µM Cd cultures, only above 35% of initial concentration of Cd was detected after algal treatment (Table 4). Thus, *C. sorokiniana* can remove 65% of Cd in three days, which is more than the values recently reported by Chandrashekharaiah et al., (2021), for the green microalgae *C. pyrenoidosa* (45.45%), and for *Scenedesmus acutus* (57.14%), after eight days of culture with lower initial concentrations of Cd (136 µM). Thus, *C. sorokiniana* might be a promising candidate for bioremediation of Cd in aquatic environments, especially under high concentrations of this heavy metal. This microalga combines two excellent advantages: its

tolerance and its accumulation capacity, which support their capacity for bioremediation of ecosystems with high concentrations of Cd, where other microorganisms cannot survive (Santiago-Martínez et al., 2015).

Finally, *C. sorokiniana*, which has not shown the ability to accumulate As, can grow at high concentrations of this metalloid (Table 4). The high tolerance of *C. sorokiniana* to As, which is much higher than that reported for other microalgae, such as *Chlorella sp* or *Monoraphidium arcuatum* (Table 2), could be explained by the low intracellular concentrations of As found in *C. sorokiniana*. In addition, the high tolerance to As shown by the microalga could let its growth in polluted environments with different heavy metals, at which other microorganisms could not grow.

3.4. Effect of heavy metals in gene expression and enzymatic activity in *C. sorokiniana*

The response to heavy metals stress is highly dependent on the organism and the element, and it can involve different cellular pathways which are severely affected. Photosynthetic organisms have developed different responses and resistant strategies to mitigate heavy metal stress. Regulation of the ROS species homeostasis, alterations in glutathione-ascorbate cycle or activation of catalase enzymes, involved in H_2O_2 elimination, are some of the most important responses. In addition, reduced glutathione (GSH) or phytochelatins (PCs) synthesis are also other usual responses (Gill and Tuteja, 2010).

For that reason, we have studied the gene expression in *C. sorokiniana* and the enzymatic activity levels of two enzymes related to antioxidant response, such as ascorbate peroxidase (APX) and catalase (CAT). Furthermore, other four enzymes from N and S metabolism, that are involved in the biosynthesis of Glu and Cys, required for the synthesis of GSH and PCs, such as glutamine synthetase (GS), O-acetylserine (thiol) lyase (OASTL), glutamate dehydrogenase (GDH) and NAD isocitrate dehydrogenase (NAD-IDH) were also studied. The determinations were carried out after 42 h of culture in the presence of different heavy metals. The concentrations tested for each heavy metal or metalloid were: 500 μM Cu^{+2}, 250 μM Cd^{+2}, 750 μM As (III) or 10 mM As (V), concentrations at which cell growth was significantly affected (Fig.1).

3.4.1. Effect on antioxidant enzymes

The effect of several heavy metals and metalloids on the enzymes APX and CAT has been investigated both at enzymatic activity (Fig. 3) and gene expression levels (Fig. 4).

APX gene expression increased in all the cases studied (Fig.4). This is in agreement with our previous proteomic studies in the microalga under Cd stress (León-Vaz et al., 2021), which demonstrated that APX was upregulated 1.8-fold under Cd stress. Nevertheless, a decrease in the APX activity level in Cu and Cd cultures was observed (Fig.3). These alterations may be due to an inhibition of APX enzyme activity, probably because of the high amounts of intracellular Cu and Cd that *C. sorokiniana* accumulate (Table 4). In order to confirm this hypothesis, different concentrations of Cu or Cd were added to the APX activity assay carried out with control crude extract. This experiment confirmed that APX activity was totally inhibited with 25 μmol of Cu and 100 μmol of Cd in the assay (data not showed). Similar response has been previously described in Cu and Cd hyperaccumulator plants, where APX activity is inhibited due the high intracellular amounts of these heavy metals (Daud et al., 2014). In the presence of As (III and V), the APX activity levels were 1.5 times higher than in the control (Fig. 3). This indicates that this enzyme is involved in the elimination of possible ROS species produced by metal stress. These results have been previously reported in plants and microalgae treated with As (Praveen et al., 2019; Upadhyay et al., 2016), showing that APX plays a leading role in elimination of ROS species.

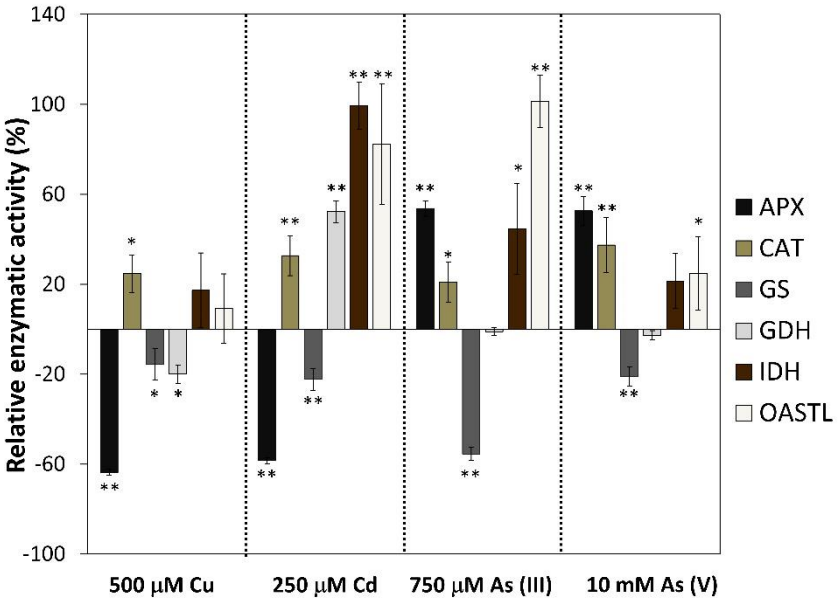

Fig.3: Relative enzymatic activity of antioxidants and N and S enzymes from *C. sorokiniana* under metallic stress measured after 42 h of culture. Ascorbate peroxidase (APX), catalase (CAT), glutamine sinthetase (GS), glutamate dehydrogenase (GDH), NAD isocitrate dehydrogenase (IDH) and O-acetylserine (thiol) lyase (OASTL). * Significant differences between control and heavy metal treatment at $p < 0.05$ and ** at $p < 0.01$.

On the other hand, CAT activity level in *C. sorokiniana* was increased between 1.2 (Cu) and 1.4 (As V) times (Fig.3) and its gene expression between 2 (Cu) and 14 (As III) times (Fig.4),

compared to the control culture. The highest levels of CAT gene expression were observed in the presence of Cd and both As species, showing high significant differences ($p < 0.01$). The higher *CAT* gene expression in arsenite cultures than arsenate (1.4-fold) is in agreement with the high toxicity of the metalloid at this oxidation state, as observed in Fig.1 (Wang et al., 2015). Moreover, CAT overexpression in Cd could compensate the enzymatic inhibition that APX activity suffers (Fig.3), which situates CAT as the main ROS eliminator enzyme. Similar results were reported in the proteomic study performed with this microalga (León-Vaz et al., 2021), with a significant upregulation of CAT under Cd stress. Finally, the slight overexpression of CAT in Cu culture is in agreement with previous studies in microalgae, both at the level of gene expression and enzymatic activity (Hamed et al., 2017; Nowicka et al., 2016).

3.4.2. N and S metabolism enzymes

The GS-GOGAT cycle, together with the enzymes GDH and IDH, are involved in the biosynthesis of glutamate, required for the production of PCs, which are responsible of the accumulation of heavy metals into vacuoles (Gill and Tuteja, 2010). Thus, heavy metals effects in *C. sorokiniana* have been studied in order to understand the role of these enzymes under this abiotic stress. Metal stress provokes a decrease in GS levels, both at enzymatic activity (between 40 and 80 % of the control culture), and gene expression (between 30 and 90 %), As (III) cultures being the most affected (Fig. 3 and 4). Nevertheless, no significant differences in gene expression were observed with Cu, Cd and As (V). These results are similar to other previously reported in microalgae (Devriese et al., 2001; Domínguez et al., 2003), which suggest that metal stress provokes a partial block of the GS-GOGAT cycle. Furthermore, the GS gene expression in the presence of heavy metals depends on the concentration and the element (Praveen et al., 2019), which could explain the results of Fig.4 for this enzyme.

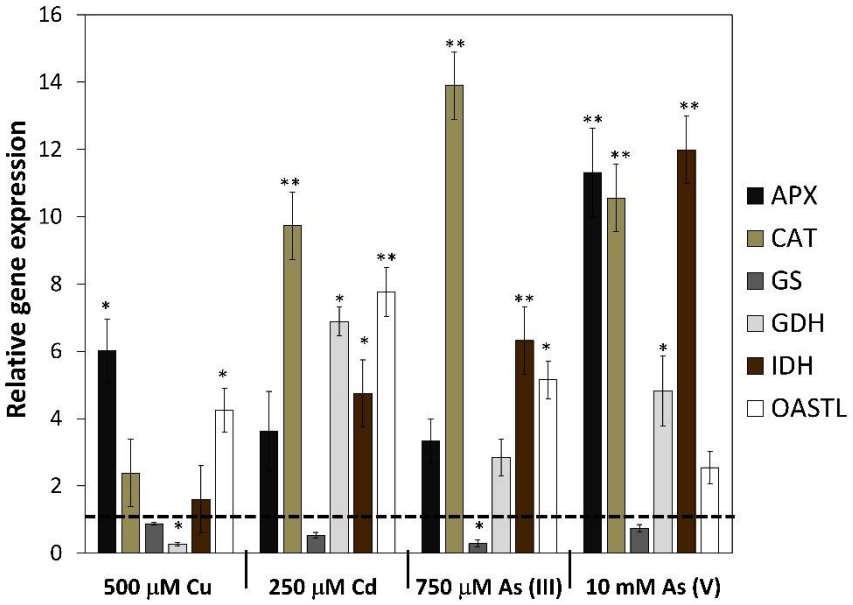

Fig.4: Relative gene expression of antioxidants and N and S enzymes from *C. sorokiniana* under metallic stress measured after 42 h of culture. Ascorbate peroxidase (*APX*), catalase (*CAT*), glutamine sinthetase (*GS*), glutamate dehydrogenase (*GDH*), NAD isocitrate dehydrogenase (*IDH*) and O-acetylserine (thiol) lyase (*OASTL*) gene expression was measured after 42 h of culture. * Significant differences between control and heavy metal treatment at $p < 0.05$ and ** at $p < 0.01$.

It has been previously described that, under abiotic stress situations, the amination activity of GDH to produce L-glutamate increases, indicating its important role in the adaptive mechanisms of the microalga in response to metallic stress conditions (Devriese et al., 2001). Moreover, it has been widely described that this enzyme plays a leading role in N assimilation when microorganisms are cultured in a rich ammonium media (Vega, 2019). All these data corroborate the lead role of GDH under abiotic stress, and are in agreement with the increases showed in Fig.3 and Fig.4. This is remarkable in Cd cultures, which show 7 times the *GDH* gene expression and 1.6 times the GDH enzymatic activity than the control. These results agree with previous proteomic studies of our research group that postulated an upregulation of glutamate biosynthesis when *C. sorokiniana* is cultivated with 250 μM Cd (León-Vaz et al., 2021). For As (III) and (V) there was also a slightly upregulation at GDH gene expression level (Fig. 4), whereas did not affect at enzymatic level (Fig. 3). Similar behaviour has been reported in *Brassica juncea* cultivated with As (Praveen et al., 2019). However, it is important to indicate that in the presence of Cu, *C. sorokiniana* GDH suffered an inhibition, both on gene expression and enzymatic activity level.

Another enzyme involved in the synthesis of L-glutamate is NAD-IDH, which supplies the α-ketoglutarate skeleton carbon needed (Vega, 2019). Fig.3 shows a significant increase of NAD-

IDH activity in Cd and As (III) cultures, compared to the control. In addition, there is a significant increase of gene expression in Cd, As (III) and As (V) cultures, being 5, 6 and 12 times higher than in the control, respectively (Fig.4). Previous studies have demonstrated a similar behaviour of IDH activity in the model microalga *Chlamydomonas reinhardtii* under Cd-stress (Domínguez et al., 2003), probably due to the demand of α-ketoglutarate needed for the glutamate and PCs synthesis. Although there are no many studies with IDH in these stress conditions, previous works showed the importance this enzyme in alleviating the oxidative stress produced by As (Rodríguez-Ruiz et al., 2019).

On the other hand, OASTL is an enzyme involved in S metabolism which catalyses the biosynthesis of cysteine (Vega, 2019), another amino acid needed for the synthesis of PCs. Fig.3 shows an increase in OASTL activity, between 1.2 and 2 times more than control, being significant in Cd, As (III) and As (V). In addition, Fig.4 shows similar results for gene expression in these cultures, being between 4 and 8 times the gene expression of the control cultures. The OASTL activation and the GDH and IDH upregulation observed in Cd cultures, demonstrates that the supply of Glu and Cys needed for PCs synthesis and vacuolar transport mediated by PCs are guaranteed. This is in agreement with the Cd accumulation data previously mentioned (Table 4), and the proteomics results reported by León-Vaz et al. 2021, where an increase in size and number of *C. sorokiniana* vacuoles have been reported when the microalga was cultured under Cd stress. In the same way, the increase of Cys content under As stress has been previously reported in plants and microalgae in order to alleviate oxidative damages (Rodríguez-Ruiz et al., 2019; Upadhyay et al., 2016). Finally, there was a minor increase of OASTL gene expression and enzymatic activity when *C. sorokiniana* was cultivated with Cu, following the same tendency that the other enzymes studied.

5. CONCLUSIONS

This study demonstrates that *C. sorokiniana* is a robust microalga able to tolerate high concentrations of Cu, Cd and As, without significant growth rate inhibition. We have observed in the microalga an important upregulation of the antioxidant enzymes and the enzymes involved in synthesis of the amino acids glutamate and cysteine, which are precursors of the phytochelatins, as response to metal stress. As a consequence of these metabolic alterations, *C. sorokiniana* is able to cope with such high heavy metal concentrations. The high Cd accumulation capacity of this microalga, its capacity of removal it, and its high tolerance to this element, makes of *C. sorokiniana* an excellent organism for the potential bioremediation of this heavy metal.

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