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Effect of heavy metals on the antioxidant system of the acid-tolerant microalga *Coccomyxa onubensis*

María del Carmen Romero-Cruz [a], Antonio Leon-Vaz [a], Inmaculada Giráldez [b], José María Vega [c], Javier Vigara [a,*]

[a] Laboratory of Biochemistry, Faculty of Experimental Sciences, Marine International Campus of Excellence and REMSMA, University of Huelva, 21071 Huelva, Spain
[b] Department of Chemistry, Research Centre in Technology of Products and Chemical Processes, PRO2TECS, University of Huelva, 21071 Huelva, Spain
[c] Plant Biochemistry and Molecular Biology Department, Faculty of Chemistry, University of Seville, 41012 Seville, Spain

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ABSTRACT

Coccomyxa onubensis (*C. onubensis*) is an acid-tolerant microalga whose ecosystem (Tinto River, Huelva, Spain) contains high amounts of different heavy metals. However, the potential of this microalga to remove these pollutants from the environment has still not been studied. Thus, this work evaluates the tolerance and physiological response of this microalga in the presence of three heavy metals, copper, cadmium, and mercury, and the metalloids As (III) and As (V). The results demonstrated that *C. onubensis* is able to improve its growth parameters with the presence of copper in the culture medium until concentrations of 2 mM Cu^{2+} and also shows high tolerance to other heavy metals and metalloids tested, such as Cd^{2+}, As(III), As(V) or Hg^{2+}. Moreover, this microalga seems to be a high arsenic accumulator organism, showing its high potential for arsenic bioremediation processes. Finally, the characterization of three antioxidant enzymes and their evaluation under heavy metal stress was performed. The results showed that ascorbate peroxidase (APX, EC:1.11.1.11), catalase (CAT, EC:1.11.1.6), and glutathione reductase (GR, EC:1.6.4.2) were upregulated to different degrees when *C. onubensis* was cultivated under heavy metal stress.

1. Introduction

Soils and rivers are important sinks of contaminants due to human anthropogenic activities. Industries and mining are the main producers of these pollutants, including organic wastes, inorganic compounds, and heavy metals. Heavy metals are one of the most dangerous contaminants in those activities because of their toxicity, persistence, ubiquity and bioaccumulation capacity in different organisms [1]. The Iberian Pyrite Belt, located in southwestern Spain, is an excellent example of mining activity developed over a period of 3000 years. In this location, pyrite is one of the most abundant rocks, whose oxidation and reduction reactions with water and oxygen produce acid mine drainages [2]. These drainages include high concentrations of different pollutants, with approximately 1221 mg L^{-1} SO_4^{2-}, 67 mg L^{-1} Al^{3+}, 147 mg L^{-1} As, 107 mg L^{-1} Cd^{2+}, or 17 mg L^{-1} Cu^{2+}, and pH values of approximately 2.5–3 [3].

These conditions produce a unique ecosystem where only adapted microorganisms can survive. Some photosynthetic eukaryotic species have been detected in the Tinto River ecosystem, with green microalgae being the most abundant. Previous studies included the genera *Chlamydomonas*, *Dunaliella* and *Chlorella* as the most abundant eukaryotic microorganisms [4]. Likewise, other species of *Cyanidium*, *Ochromonas*, *Euglena* or diatoms, as well as prokaryotic microorganisms, have been detected in the river [5]. Another studied microalga isolated from the Tinto River ecosystem is *Coccomyxa onubensis* (*C. onubensis*). This microalga is an extremophile chlorophyte able to produce high amounts of carotenoids as a response to the natural stress produced by its habitat [6]. Additionally, it has been described that *C. onubensis* can accumulate linoleic and linolenic acid and lutein or increase its antioxidant activities when it is cultivated under different types of stress, such as salt stress, UV light or the presence of Fe^{3+} in its culture medium [7,8]. However, there are no studies with this microalga in the presence of other heavy metals that are present in the Tinto River ecosystem.

One of the main applications using microalgae isolated from contaminated environments is heavy metal bioremediation. Previous studies demonstrated that different microalgae of the *Chlorococcum*

* Corresponding author.
E-mail address: vigara@uhu.es (J. Vigara).

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genus isolated from polluted areas showed a high potential to remove lead or arsenic from the medium [9,10]. Other species, such as *Chlorella vulgaris* or *Spirulina platensis* were able to remove different contaminants, including heavy metals, from anaerobic digestion effluents [11,12]. Additionally, it has been shown [13] that *Desmodesmus* or *Chlorella* sp., isolated from a highly contaminated area of the Reconquista River (Argentina), can remove high amounts of zinc and copper, respectively. Thus, *C. onubensis* could have a high potential for heavy metal bioremediation processes.

Nevertheless, the presence of high concentrations of heavy metals in its habitat may also cause *C. onubensis* to face, putatively, a higher number of reactive oxygen species (ROS) than other microorganisms. This would activate the antioxidant system of this microalga, including different enzymes, or increase the levels of glutathione and proline, as was previously reported in *Scenedesmus vacuolatus* [14]. Among these enzymes, CAT catalyses the dismutation reaction of H_2O_2 without requiring any reductant, whereas APX utilizes ascorbate as an electron donor for the reduction of H_2O_2 into H_2O [15]. Finally, GR catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as an electron donor [16]. Thus, the objective of this study was to determine the tolerance of *C. onubensis* to different heavy metals, such as copper, cadmium, arsenite, arsenate, and mercury, as well as its bioaccumulation capacity. Furthermore, an enzymatic characterization of the antioxidant enzymes ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) and the effect produced by these heavy metals on these enzymes was also studied.

2. Materials and methods

2.1. Algal strain and cultivation

The microalga used in this work was *C. onubensis* ACCV1 (SAG2510), isolated from acid drainages in the Tinto River [6]. *C. onubensis* was cultivated under continuous light (150 μmol m^{-2} s^{-1}) in modified K-9 medium [17], which contains (in g L^{-1}): 0.5 HK_2PO_4, 3.95 K_2SO_4, 0.10 KCl, 0.40 $MgSO_4{\cdot}6H_2O$, 0.01 $CaCl_2{\cdot}2H_2O$, 0.03 $FeCl_3$, 2.30 KNO_3 and 5 mL of trace solution containing (g L^{-1}): 10.0 EDTA, 2.28 H_3BO_3, 4.40 $ZnSO_4{\cdot}7H_2O$, 1.02 $MnCl_2{\cdot}4H_2O$, 1.00 $FeSO_4{\cdot}7H_2O$, 0.32 $CoCl_2{\cdot}6H_2O$, 0.32 $CuSO_4{\cdot}5H_2O$ and 0.22 $Mo_7O_{24}(NH_4)_6{\cdot}4H_2O$. After the addition of the medium, the pH was adjusted to 2.5. The microalgae were cultured at 25 °C and bubbled with 5 % CO_2-enriched air. Heavy metals were added as $CuCl_2$, $CdSO_4$, $NaAsO_2$, Na_2HAsO_4, or $HgCl_2$ at the indicated concentrations.

2.2. Analysis of chlorophylls

C. onubensis growth was determined by total chlorophyll (Chl) content as described by [18] with minor modifications. A 1 mL aliquot of culture was sampled and harvested by centrifugation at 4400 rpm for 10 min. After that, the supernatant was discarded, and the pellet was resuspended in 5 mL of methanol. The suspension was heated for 20 min at 80 °C for Chl extraction. The suspension was centrifuged for 5 min at 4400 rpm, and the total Chl concentration was calculated spectrophotometrically using the calculations provided by [19].

2.3. Bioconcentration factor calculation

The copper, cadmium, and arsenic contents in lyophilized cells or culture media of *C. onubensis* were determined by inductively coupled plasma–mass spectrometry (ICP–MS). Samples were measured in an Agilent 7700 spectrometer (Agilent Technologies, Santa Clara, CA, USA). The bioconcentration factor (BCF) was calculated as previously described [20] with the following equation:

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

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

2.4. Preparation of crude extract

At the end of the exponential phase, *C. onubensis* cells were harvested by centrifugation for 5 min at 4400 rpm, and traces of elements in the medium were removed by washing with Milli-Q water three times. Collected cells were mixed with glass beads (0.3 mm Ø, 3 mL g^{-1} fresh weight) in the presence of 1 mL g^{-1} wet weight of 50 mM Tris-HCl (pH 7) buffer and disrupted in a Bühler homogenizer (type Vi 4; Bühler, Tübingen, Germany) for 5 cycles of 1 min. The homogenate was filtered in nylon gauze, washed with 2 mL of buffer per gram of fresh weight, and centrifuged twice at 13,400 rpm for 30 min. The supernatant obtained was used as a crude extract source for the enzymatic studies. The total protein of the crude extract was determined using the Bio-Rad Bradford assay with BSA as a standard.

2.5. Enzyme assays

All assays were performed with the crude extract prepared as indicated above. Ascorbate peroxidase (APX) and catalase (CAT) assays were performed kinetically as previously described by [20]. The glutathione reductase (GR) assay was carried out as described by [16].

The determination of optimal temperature was performed in an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences, Amersham, UK) using a continuous flux of water provided from a thermostatic bath whose temperature was established between 0 and 80 °C.

For optimal pH determination, different buffer solutions were used: acetic acid/sodium acetate solution for pH values of 3.0–4.0, MES solution for pH values of 4.0–6.0, potassium phosphate solution for pH values between 6.0 and 8.0, and Tris-HCl solution for pH values of 8.0–13.0.

2.6. Statistical analysis

All measures were performed in triplicate and represented as the mean value ± SD. Significance of values was considered for $^{*}p < 0.05$ and $^{**}p < 0.01$. Statistical analyses were performed using IBM SPSS Statistics 27 software (Armonk, NY, USA) by comparing mean values using one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Tolerance of C. onubensis to heavy metals

C. onubensis is a microalga able to tolerate high concentrations of different heavy metals, such as copper or iron, due to the presence of these elements in its ecosystem. The influence of different heavy metals, including Cu^{2+}, Cd^{2+}, As(III), As(V), and Hg^{2+}, was studied in this microalga. The range of concentrations tested in the experiment was 50 μM-2 mM for Cu^{2+}, 50 μM–500 μM for Cd^{2+}, 50 μM–500 μM for As(III), 2.5 mM–20 mM for As(V), and 50 nM–500 nM for Hg^{2+}. The results showed that *C. onubensis* is a highly resistant copper species, showing better growth results with concentrations of 2 mM than in the control cultures (Fig. 1A). However, the optimal concentration of Cu^{2+} for this microalga was between 50 and 300 μM, with similar values of total Chl at these concentrations ($p < 0.05$). Other species are able to tolerate concentrations of approximately 300 μM copper, although their growth is altered at these concentrations. Previous studies by our research group showed that *Chlorella sorokiniana* can tolerate up to 1 mM with a 40 % control growth rate [20]. Additionally, *Chlorella protothecoides* can tolerate concentrations of approximately 1 mM copper in heterotrophic cultivation [21]. However, the authors are unaware of any other study in which microalgal species were able to grow with concentrations of Cu^{2+}

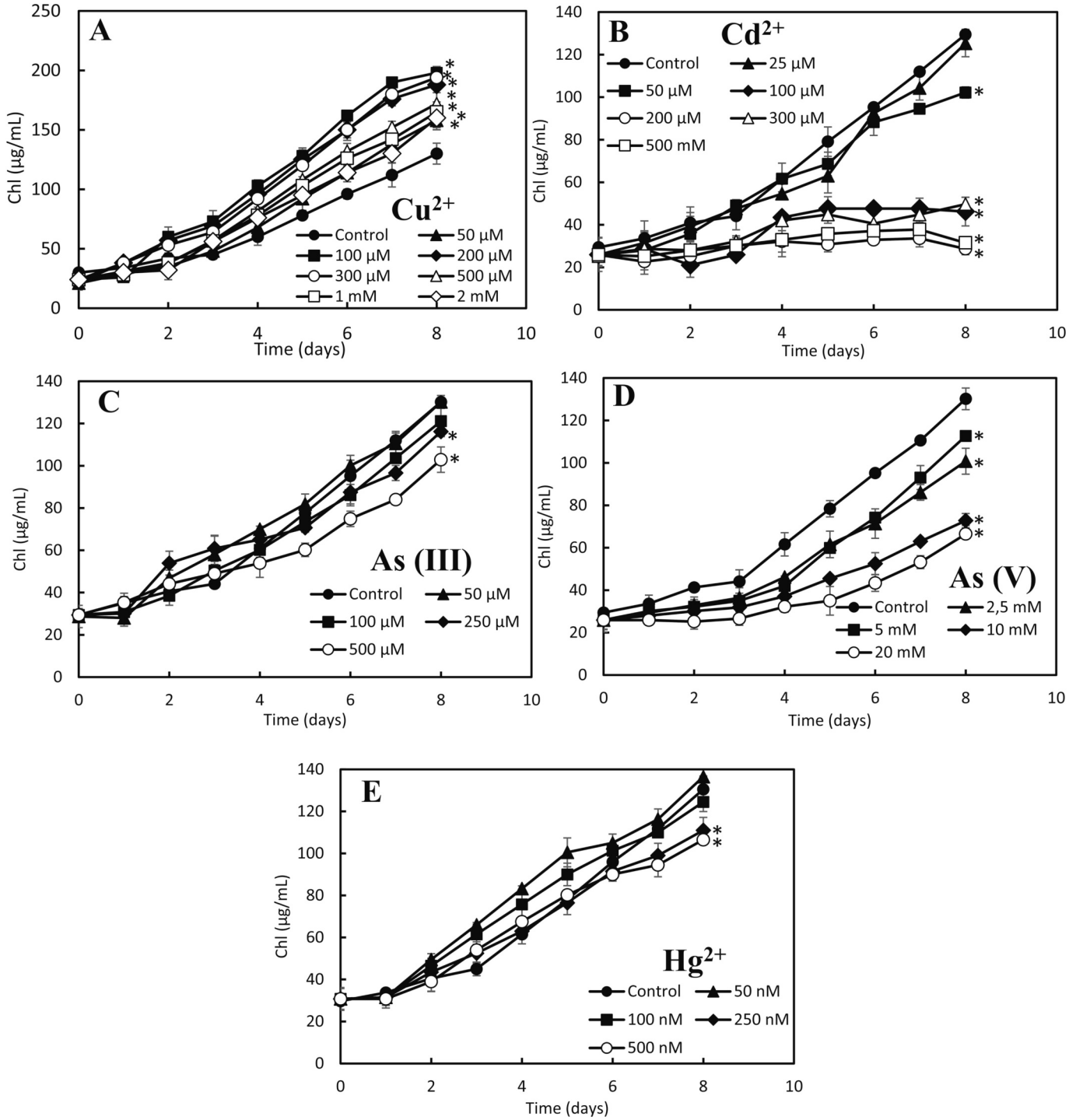

Fig. 1. Growth curve of *C. onubensis* cultured with different concentrations of Cu^{2+} (A), Cd^{2+} (B), As(III) (C), As(V) (D), and Hg^{2+} (E). Cells were cultured in modified K9, as described in the Materials and Methods. * Significant differences in biomass between control and heavy metal treatment at $p < 0.05$.

of 2 mM, as Fig. 1A shows for *C. onubensis*.

Despite its high tolerance to copper, *C. onubensis* does not exhibit the same behaviour as the other heavy metals tested. For cadmium, Fig. 1B shows that this microalga could tolerate concentrations up to 50 µM without a significant decrease in biomass. Concentrations up to 300 µM of Cd^{2+} were toxic for this microalga, although its tolerance for this cation is higher than that reported in previous studies with other microalgae, such as *Dunaliella salina*, *Chlamydomonas reinhardtii* or *Skeletonema marinoi*, and lower tolerance than that reported for *Chlorella sorokiniana* [20,22–24].

For the metalloid As, *C. onubensis* was able to tolerate concentrations of arsenite up to 500 µM without significant differences (Fig. 1C). However, arsenate inhibited its growth at concentrations up to 5 mM (Fig. 1D). The concentrations of arsenate used in this study were higher than those of arsenite due to its lower toxicity. Additionally, arsenic concentrations along the habitat of *C. onubensis* (Tinto River) increase in the upper part of the river, with an arsenate/arsenite ratio of approximately 25 [25]. This also explains the high tolerance that *C. onubensis* presents to this metalloid, which is higher than that of other species, such as *Chlamydomonas reinhardtii*, *Chlorococcum* sp. or *Skeletonema*

costatum [10,26,27].

C. onubensis is also able to tolerate Hg^{2+} concentrations up to 100 nM without significant differences in growth (Fig. 1E). Moreover, it can tolerate concentrations up to 500 nm of this toxic element with a decrease of 25–30 % in total chlorophylls. High levels of mercury are quite toxic to plants because this cation can substitute for the central magnesium atom in chlorophylls, which should significantly alter the content of chlorophyll in microalgae [28]. The concentration of this metalloid that *C. onubensis* tolerates is higher than that reported for *Microcystis aeruginosa* but lower than that reported for *Chlorella vulgaris*, also showing a prominent diminution of chlorophyll content in these species after Hg^{2+} exposure [28,29].

3.2. Removal of heavy metals

The ecosystem of *C. onubensis* could make this microalga a potential organism for heavy metal bioremediation due to the permanently high concentrations of these compounds to which it is exposed. Thus, the accumulation capacity of this microalga after 8 days of growth in the presence of Cu, Cd, Zn and/or As was determined by ICP–MS.

C. onubensis showed the highest cumulative capacity for arsenate, obtaining a BCF value of 1437.27 (Table 1), thus highlighting the possible application of microalgae in arsenic phytoremediation processes. The BCF values obtained for Cu^{2+} and Cd^{2+} were very similar, at 8.39 and 9.00, respectively. Zn^{2+} yielded a lower BCF value, although it should be noted that part of the accumulated Zn^{2+} came from the culture medium itself, as the control data show. In addition, when *C. onubensis* was exposed to the combined presence of Cu^{2+}, Cd^{2+} and Zn^{2+}, the observed accumulation was similar to that of the separate cultures, without affecting cell viability.

C. onubensis showed a higher BCF of arsenic than other microalgae [20,30], although the BCF values for Cu^{2+}, Cd^{2+} and Zn^{2+} were lower [20]. The low accumulation of these metals may be due to the low pH value in the *C. onubensis* culture medium.

Apparently, pH affects the conformation of membrane proteins and can affect transporters, so that as the pH of the medium decreases, the flow of metal into the cell also decreases [31]. It has been shown that *Scenedesmus* sp. showed a great inhibitory effect on Cd^{2+} and Pb^{2+} removal at low pH values [32]. The same results were also obtained using *Chlorella vulgaris* for As(III) removal [31]. However, *C. onubensis* is an extremophile organism whose potential in phytoremediation would be indicated in effluents with high metal contents, especially arsenic, which also has a very acidic pH, an environment in which other organisms could not live.

3.3. Characterization of antioxidant enzymes of C. onubensis

The presence of heavy metals in the culture medium of *C. onubensis* generates oxidative stress in this microorganism and, consequently, produces an increase in reactive oxygen species (ROS) compounds. High amounts of ROS species in cells cause damage to lipids, nucleic acids, proteins, or carbohydrates, endangering cell viability [33]. One of the first responses to ROS increase in microalgae under heavy metal stress is the production of antioxidant molecules, such as reduced glutathione, phytochelatins or antioxidant enzymes, including ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) [20]. To carry out the studies of metallic stresses in the antioxidant system of *C. onubensis*, a prior characterization of the activity assay of CAT, APX and GR was performed.

Table 1
Intracellular dry weight accumulation, bioconcentration factor (BCF) and heavy metal concentration (μM) levels in the culture media in *C. onubensis* after 8 days of culture.

	Accumulation (mg kg^{-1})	BCF
Control		
+ Cu^{2+}	<LD	–
+ Cd^{2+}	<LD	–
+ As(V)	<LD	–
200 μM Cu^{2+}	106.7 ± 5.4	8,39
200 μM Cd^{2+}	202.4 ± 6.1	9,00
200 μM Zn^{2+}	54.4 ± 5,4	4,15
10 mM As(V)	1076.8 ± 19.5	1437,27

3.3.1. Ascorbate peroxidase

APX is an enzyme able to reduce ascorbate and H_2O_2 with high affinity in the ascorbate glutathione cycle. The characterization of this enzyme in *C. onubensis* was performed with a series of *in vitro* experiments to obtain the optimal values of pH, temperature and K_m of APX substrates (Table 2). For this microalga, K_m values for ascorbate and H_2O_2 were 37.0 ± 1.1 μM and 1.2 ± 0.1 mM, respectively, demonstrating a high affinity of this enzyme for ascorbate. These values of ascorbate K_m were lower than those reported for other microalgae, such as *Chlamydomonas reinhardtii* or *Chlorella vulgaris* [34,35] (Table 2). Thus, APX seems to play a fundamental role in the reduction of ascorbate in *C. onubensis* due to its natural growth conditions.

The influence of temperature and pH on APX enzymatic activity was also determined in *C. onubensis* (Fig. 2 A and B). The maximum activity was found at 50 °C and pH values of 7.0 in 50 mM potassium phosphate buffer, as shown in Table 2 and Fig. 2 A and B, respectively. It should be noted that APX activity was stable under a wide range of temperature and pH values, retaining >45 % of its enzymatic activity in the ranges of 1.5–65 °C and pH values between 5 and 9. This low dependence on temperature is noteworthy because most of the described APXs in microalgae, such as *Chlamydomonas reinahrdtii* or *Chlorella vulgaris*, showed a lower percentage of activity in this range of temperatures and lower values of optimal temperature than *C. onubensis* [34,35] (Table 2).

Table 2
Physicochemical properties of the ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) enzymes in different microalgae and plants.

Organism	Enzyme	Km (mM)	Temperature (°C)	pH	Reference
C. onubensis	APX	0.037 asc 1.2 H_2O_2	50	7.0	This study
Chlamydomonas sp. *W80*	APX	0.342 asc 0.052 H_2O_2	42	6.8	[35]
Chlorella vulgaris	APX	0.111 asc 0.020 H_2O_2	36	6.1	[34]
C. onubensis	CAT	17.1 H_2O_2	60	12.5	This study
Vigna mungo	CAT	16.2 H_2O_2	40	7.0	[36]
C. onubensis	GR	0.059 GSSG 0.022 NADPH	60	7.5	This study
Chlamydomonas reinhardtii	GR1	0.053 GSSG 0.007 NADPH	49	8.2	[37]
Chlamydomonas reinhardtii	GR2	0.058 GSSG 0.028 NADPH	49	8.5	[37]
Phaeodactylum tricornutum	GR	0.060 GSSG 0.014 NADPH	n.d.	8.0	[38]

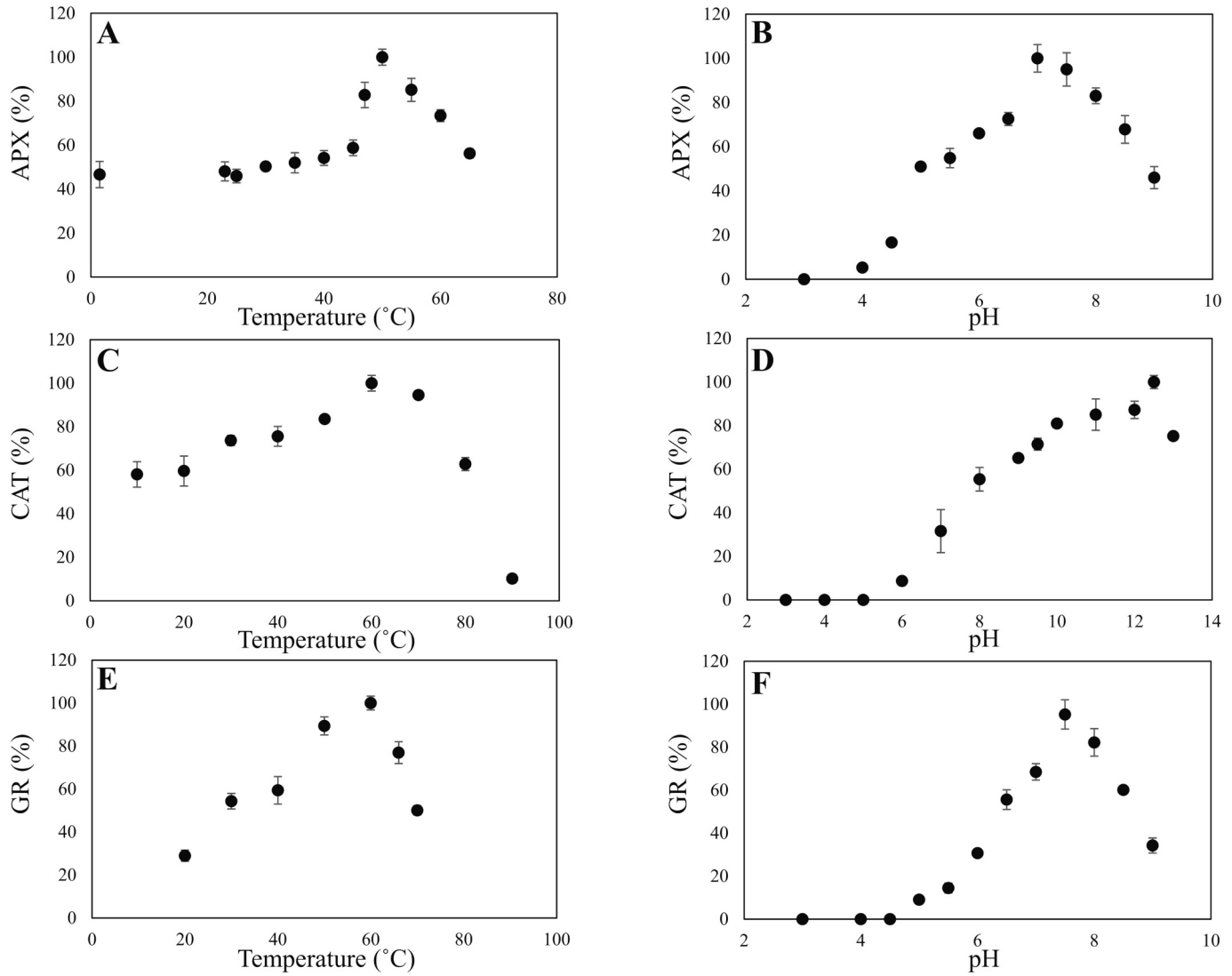

Fig. 2. Effect of temperature and pH on APX, CAT and GR enzymatic activity in *C. onubensis*. Relative APX, CAT and GR activities at different temperatures (A, C and E, respectively) and different pH values (B, D and F). One hundred percent of the total activity corresponding to relative activity was defined as the percentage of maximum activity; for each case, it was 601.85 ± 15.2 U mg^{-1} for APX, 9984.59 ± 98.3 U mg^{-1} for CAT and 17.85 ± 1.2 U mg^{-1} for GR.

Further experiments on APX activity were performed at 50 °C and pH 7.0.

Additionally, the effect of different cations, chelating agents or DTT on APX enzymatic activity was also studied. The presence of monovalent cations, such as Na^+ and K^+, or Mg^{2+} provoked a low inhibition of APX activity in *C. onubensis* (Fig. 3). However, the divalent cation Mn^{2+} produced a significant decrease in APX at a concentration of 5 mM, with a decrease of 81 % of the control activity. Similar results were reported in the case of DTT, KCN and NaN_3, with a decrease between 70 and 100 % of APX at concentrations of 1 mM of these compounds (Fig. 3). This significant decrease with KCN and NaN_3 is related to the heme group of APX, while the inhibition in APX activity with DTT could be produced due to the amino acid Cys-32 of this enzyme, as was described for APX in other organisms [35].

3.3.2. Catalase

CAT is an enzyme able to reduce H_2O_2 without any extra cofactor. The characterization of this enzyme in *C. onubensis* (optimal temperature, pH, and Km value) is also indicated in Table 2. The CAT K_m value in *C. onubensis* for H_2O_2 was 17.1 ± 1.9 mM, while its optimal temperature was 60 °C and its optimal pH value was 12.0 in 50 mM Tris-HCl. Although there are no data about the optimal conditions of this enzyme in microalgae, the K_m value of *C. onubensis* was similar to that reported by [36] in *Vigna mungo* (Table 2). On the other hand, the optimal temperature was higher than that reported for *Vigna mungo* (Table 2). It is also remarkable that the CAT enzymatic activity of *C. onubensis* maintained >60 % of the total activity in the range of 40–80 °C (Fig. 2C), showing that it is a thermotolerant enzyme with high potential to be applied in industrial processes. CAT enzymatic activity also showed a high dependence on pH, having an optimal pH value of 12.5 and losing >50 % of total activity at pH values below 8.0 (Fig. 2D). Thus, further CAT experiments were carried out at a temperature of 60 °C using Tris-HCl buffer at a pH of 12.0.

As Fig. 3 shows, monovalent cations and Mg^{2+} only slightly affected CAT activity, with a decrease of 15–20 % of total activity at concentrations of 5 mM. However, at this concentration, Mn^{2+} totally inhibited CAT activity in *C. onubensis,* while at 1 mM, the inhibition was not significant (Fig. 3). KCN and NaN_3 caused total inhibition of this enzyme at a concentration of 1 mM, as was previously reported for *Vigna mungo* [36], probably because CAT enzymes are heme proteins. DTT was not able to inhibit CAT activity by >25 %, even at concentrations of 5 mM, demonstrating that sulfhydryl groups of CAT in *C. onubensis* are protected to avoid inhibition.

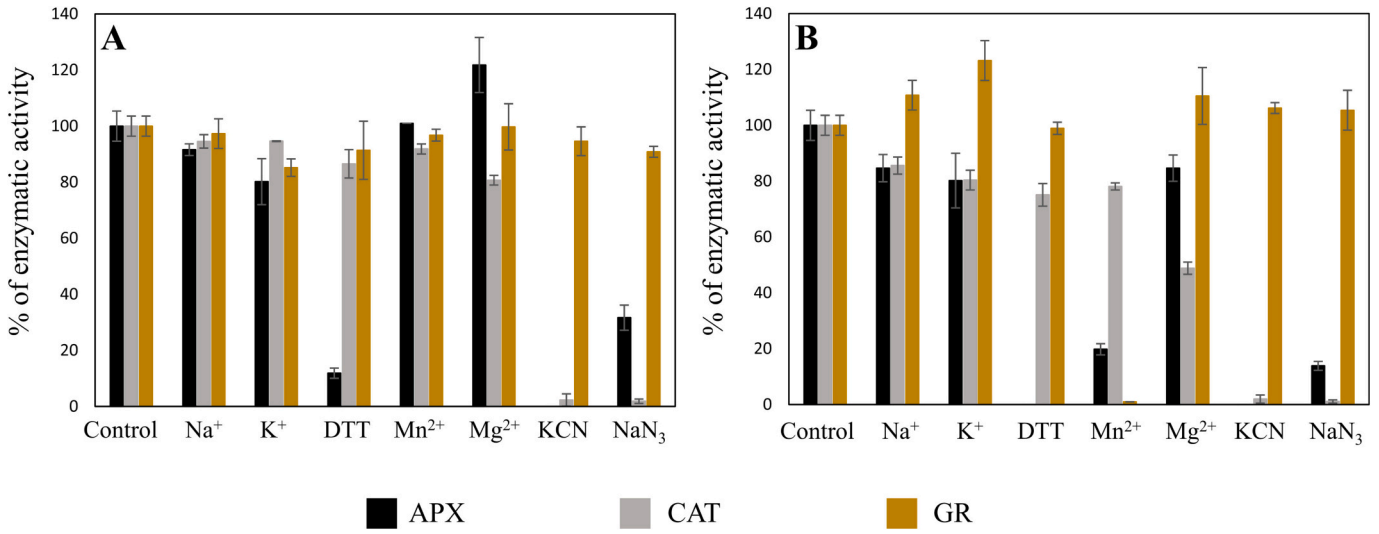

Fig. 3. Effect of different inhibitor agents on APX, CAT and GR in *C. onubensis* enzymatic activity with concentrations of 1 (A) and 5 (B) mM of each agent. Relative activity was defined as the percentage of maximal activity concerning the control with no additives. The control activity was 559.71 ± 13.4 U mg^{-1} for APX, 8633.94 ± 103.6 U mg^{-1} for CAT and 19.80 ± 1.6 U mg^{-1} for GR.

3.3.3. Glutathione reductase

GR is an enzyme that takes part in the ascorbate-glutathione cycle as a reduced glutathione donor. The characterization of this enzyme showed a high affinity for its two substrates, with K_m values of 59.7 ± 3.5 and 22.2 ± 2.3 μM for reduced glutathione (GSSG) and NADPH, respectively (Table 2). Similar K_m values were reported for *Phaeodactylum tricornutum* and *Chlamydomonas reinhardtii* (Table 2) [37,38], showing a consistent affinity of this enzyme for its substrates throughout the microalgae phylogeny.

GR enzymatic activity also showed tolerance to high temperature and low dependence on this parameter, with its optimal temperature at 60 °C and maintaining >60 % of total activity in the range of temperatures of 20–66 °C (Fig. 2E). The optimal temperature for *C. onubensis* was higher than that reported for *Chlamydomonas reinhardtii* or other GR in plants (Table 2) [37]. The optimal pH for GR in *C. onubensis* was 7.5 in 50 mM Tris-HCl. However, the dependence of GR on pH was high, obtaining enzymatic values higher than 65 % of optimal activities only in the range 7–8 (Fig. 2F). These results led to the use of a temperature of 60 °C and Tris-HCl buffer (pH 7.5) for the following GR activity experiments. Finally, the presence of different cations, chelating agents or DTT did not significantly affect GR enzymatic activity (Fig. 3). The only significant inhibition was performed for Mn^{2+} at a concentration of 5 mM (100 % inhibition). These data showed the resistance of the GR active group in *C. onubensis*, which was not affected by the presence of other substances.

3.4. Effect of heavy metals on the antioxidant enzymes of C. onubensis

The presence of heavy metals in the culture medium of *C. onubensis* could alter the enzymatic effect of antioxidant enzymes in microalgae. Thus, the enzymatic activity of APX, CAT and GR was also studied in the microalga *C. onubensis* cultivated under different heavy metal stresses after 8 days of cultivation.

3.4.1. Ascorbate peroxidase

The effect of heavy metals on APX activity levels was tested at the same concentrations of pollutants as for growth studies (Fig. 4). APX enzymatic activity increased significantly in the presence of Cu^{2+} in the culture medium of *C. onubensis* up to concentrations of 200 μM. However, there was a decrease in APX activity at concentrations up to 300 μM Cu^{2+} (Fig. 4A). These data are not in agreement with the growth data of *C. onubensis*, which is able to improve its growth until concentrations of 2 mM (Fig. 1A). Thus, APX is not the main enzyme of *C. onubensis* metabolism to deal with oxidative stress produced by copper. Previous studies demonstrated that APX activity could be inhibited by the presence of Cu^{2+} in *Chlorella sorokiniana* cells, showing that this enzyme could be very sensitive to the presence of this cation [20]. Nevertheless, APX enzymatic activity levels increased significantly under Cd^{2+} stress in *C. onubensis*, showing values between 40 and 60 % higher than those of the control culture (Fig. 4B). This APX induction produced by cadmium is in agreement with that reported for *Chlorella sorokiniana* or *Chlamydomonas reinhardtii* [20,23].

In the presence of arsenic, *C. onubensis* APX enzymatic activity levels were increased at concentrations up to 200 μM for arsenite, maintaining similar values to the control cultures at higher concentrations (Fig. 4C), and significantly increased 20–40 % at concentrations up to 10 mM for arsenate (Fig. 4D). This increase could be explained based on the report of [39], which demonstrated that APX plays an important role in ROS elimination when the microalga *Nannochloropsis* sp. was in the presence of As. Finally, APX activity suffered a slight activation at a concentration of approximately 200 nM of Hg^{2+}, with a significant decrease in its activity at higher concentrations, as was also reported for its biomass production (Fig. 1E).

3.4.2. Catalase

In the case of CAT activity, the presence of heavy metals produced a general overexpression in the presence of the cations. For Cu^{2+}, there was a significant increase in the enzymatic activity level until concentrations of 500 μM, with CAT activity 4 times higher at 300 μM Cu^{2+} than in the control culture (Fig. 5A). However, at concentrations of 1–2 mM, there was a slight decrease in CAT activity. The increase in CAT activity in the presence of copper, higher than that reported for APX, demonstrates that CAT plays a leading role in ROS elimination with this heavy metal, similar to what was reported for *Chlorella sorokiniana* [20] and contrary to the data reported for *Chlamydomonas reinhardtii* [15]. The presence of Cd^{2+} also significantly increased CAT activity at all the concentrations tested between 1.2 and 2.1 times (Fig. 5B). This significant increase is in agreement with previous studies of our research group with other microalgae, such as *Chlamydomonas reinhardtii* or *Chlorella sorokiniana* [20,40]. In the case of As, there was an increase of approximately 2 times more CAT activity than that in the control culture at concentrations of 100–300 μM arsenite (Fig. 5C). However, arsenate only provoked a significant increase in CAT activity at concentrations up to 10 mM (Fig. 5D), probably due to its low toxicity compared to the

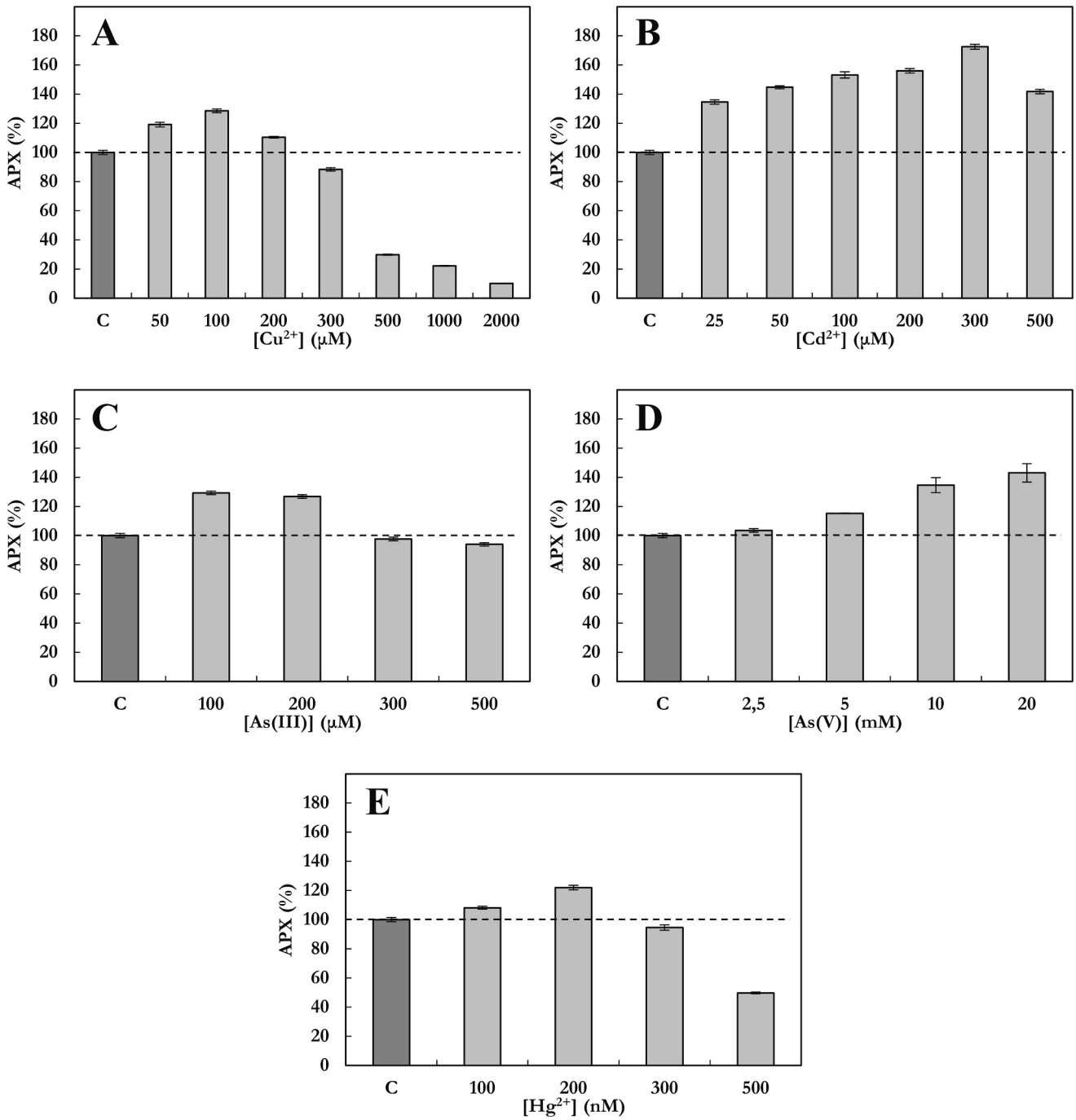

Fig. 4. Effect of different heavy metals, such as copper (A), cadmium (B) and mercury (E), as well as metalloids arsenite (C) and arsenate (D), on APX in *C. onubensis* enzymatic activity after 8 days of growth. The 100 % APX activity corresponded to 525.14 ± 14.63 U mg^{-1}.

oxidation state III of this element. Additionally, CAT enzymatic activity was unaffected in the presence of Hg^{2+} in the culture medium (Fig. 5E). This could be possible due to the assimilation pathways of this element in microalgae [41].

3.4.3. Glutathione reductase

GR activity was also affected by the presence of heavy metals in the culture medium of *C. onubensis*. In the presence of copper, GR activity was overexpressed until concentrations of 500 µM were reached. However, GR enzymatic activity was not significantly affected at concentrations of 1–2 mM, in contrast to APX or CAT activities (Fig. 6A). These results point to GR as one of the main factors responsible for the growth results reported in this work (Fig. 1A), where *C. onubensis* is able to increase its chlorophyll content at concentrations between 1 and 2 mM of Cu^{2+}. The GR activity of *C. onubensis* was also significantly increased in the presence of Cd^{2+}, As(III), and Hg^{2+} until concentrations of 200 µM for Cd and As and 300 nM for Hg^{2+}, showing a significant decrease at concentrations up to these (Fig. 6B, C and E). This significant decrease in GR activity at high concentrations of heavy metals, with an increase in GR activity at low concentrations, was also reported for the microalga *Chaetoceros calcitrans* [42], which means that GR is not able to reduce ROS species with high levels of stress. Nevertheless, no significant alterations were reported for GR activity in the presence of As(V) in the culture medium (Fig. 6D).

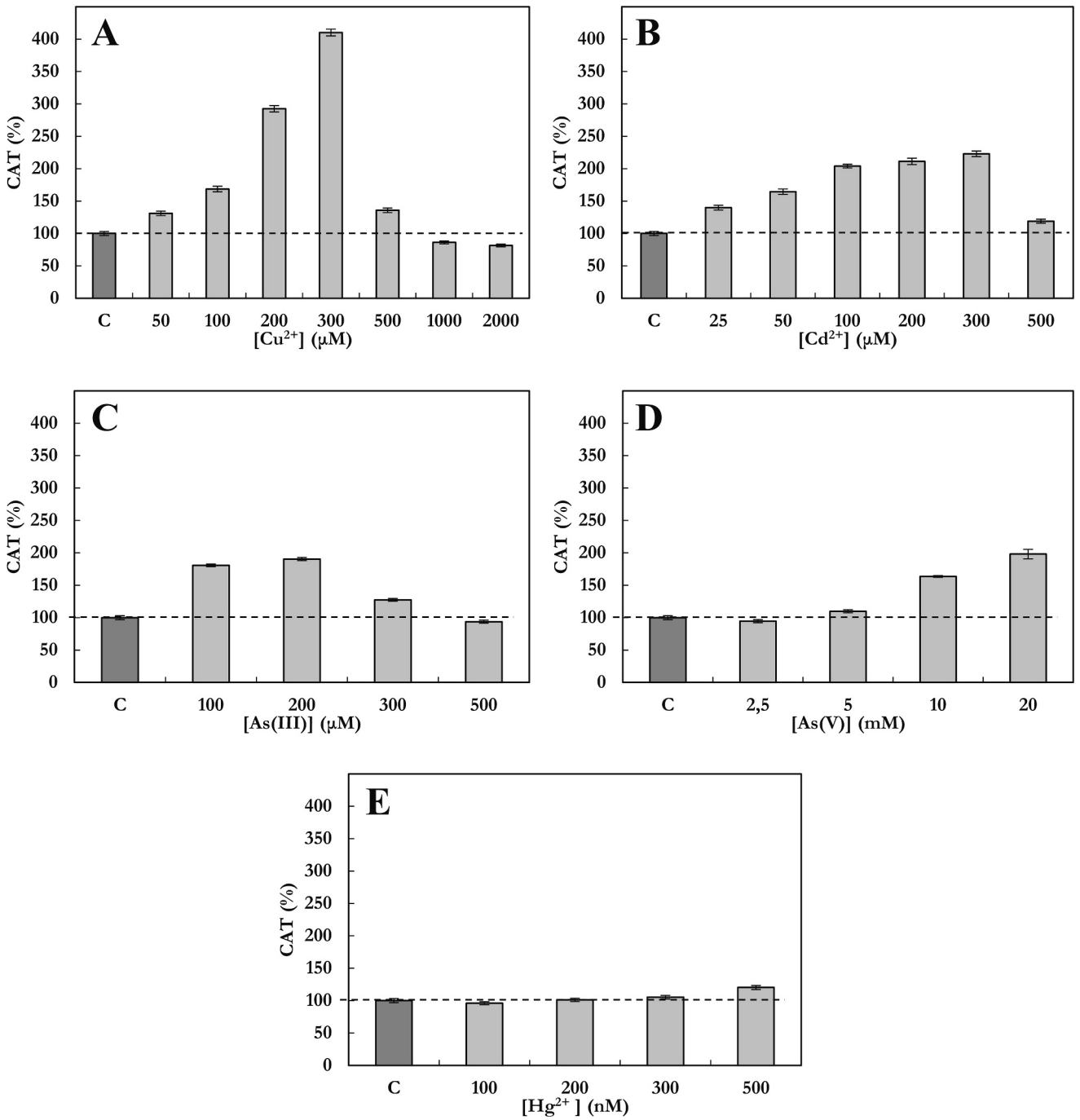

Fig. 5. Effect of different heavy metals, such as copper (A), cadmium (B) and mercury (E), as well as metalloids arsenite (C) and arsenate (D), on *C. onubensis* CAT enzymatic activity after 8 days of growth. The 100 % CAT activity corresponded to 8416.49 ± 95.06 U mg^{-1}.

4. Conclusions

The acidophilic microalga *C. onubensis* is able to tolerate high concentrations of different heavy metals, obtaining better values of cell growth with the presence of moderate-high amounts of Cu^{2+} in its culture medium. Additionally, this microalga can be considered an As hyperaccumulator organism due to its high BCF values. The increase in heavy metal concentrations in the culture medium demonstrated that *C. onubensis* can deal with high amounts of ROS species through the upregulation of antioxidant enzymes, such as ascorbate peroxidase, catalase, or glutathione reductase. Thus, this microalga shows high potential as a model organism for the bioremediation of heavy metals, especially arsenic.

CRediT authorship contribution statement

Conceptualization, MC.R-C., A.L-V. and J.V.; methodology, MC.R-C. and I.G; resources, J.V.; writing—original draft preparation, A.L-V., and MC.R-C.; writing—review and editing, J.V., JM.V, and A.L-V; supervision, J.V., and JM.V.

All authors have read and agreed to the published version of the manuscript.

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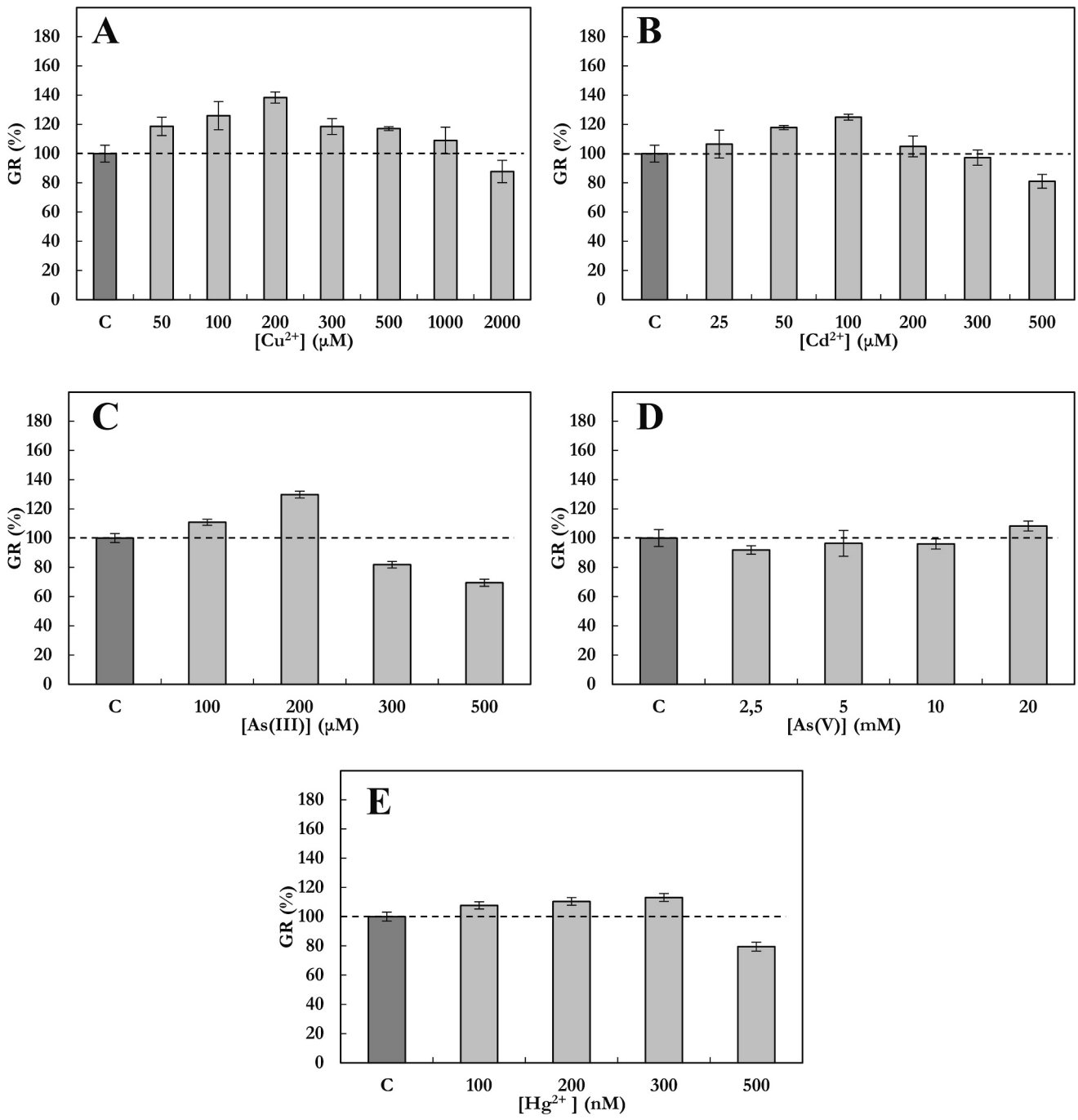

Fig. 6. Effect of different heavy metals, such as copper (A), cadmium (B) and mercury (E), as well as metalloids arsenite (C) and arsenate (D), on *C. onubensis* GR enzymatic activity after 8 days of growth. The 100 % GR activity corresponded to 17.77 ± 2.04 U mg^{-1}.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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