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Alterations in nitrogen metabolism caused by heavy metals in the acid-tolerant microalga *Coccomyxa onubensis*

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

[a] Laboratory of Biochemistry, Faculty of Experimental Sciences, Marine International Campus of Excellence and RENSMA, University of Huelva, 21071 Huelva, Spain
[b] Plant Biochemistry and Molecular Biology Department, Faculty of Chemistry, Department of Chemistry, University of Seville, 41012 Seville, Spain

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ABSTRACT

The microalga *Coccomyxa onubensis* is an extremophile microorganism with a unique ecosystem (Río Tinto, Huelva, Spain) that contains high amounts of contaminants, including heavy metals, sulphates, and nitrates, in acidic environments (pH 2.5). The current work presents an evaluation of the capacity of *Coccomyxa onubensis* to assimilate different nitrogen sources under Cu^{2+}, Cd^{2+}, AsO_3^{3-}, AsO_4^{3-} and Hg^{2+} stress, and the metabolic implications of these stressors. The results showed that ammonium consumption was less affected than nitrate consumption when microalgae were cultivated with heavy metals (except cadmium). The activities of enzymes involved in nitrogen metabolism, such as nitrite reductase (NiR; EC:1.7.7.1), glutamine synthetase (GS; EC:6.3.2.1) and glutamate dehydrogenase (GDH; EC:1.4.1.2) were characterised to determine the Michaelis-Menten constant (K_m) and optimal temperature and pH values, being 45, 40 and 60 °C and pH values of 7.5, 6.0 and 9.0 for NiR, GS, and GDH, respectively. The effects of different heavy metals on these enzymes were assessed at multiple levels, and the results showed that the enzymatic activity of NiR was downregulated, specially under copper stress, maintaining 23 % of control NiR activity at 2 mM Cu^{2+}. The enzymatic activity of GS was upregulated at low concentrations under cadmium and mercury stress (115–120 % of control cultures GS activity at 25 μM Cd^{2+} and 50 nM Hg^{2+}, respectively) and downregulated at high concentrations of these elements. GDH activity was upregulated in the presence of Cu^{2+}, Cd^{2+}, and Hg^{2+}, with increases up to 192, 155 and 154 % at 1 mM Cu^{2+}, 300 μM Cd^{2+}, and 250 nM Hg^{2+}, respectively. These results provide a better explanation of the effects of heavy metal stress on N metabolism in *Coccomyxa onubensis*, which may be used as a model eukaryotic organism of the Tinto River acidophilic ecosystem.

1. Introduction

The Tinto River (southwest, Spain) is a 92-km-long river located in the Iberian Pyrite Belt (IPB).This area is an unique ecosystem that contains one of the highest deposits of iron and copper sulphides bodies, just as other lead and zinc sulphides [1]. The presence of these compounds has been associated with mining activities for >5000 years [2]. However, recent studies suggest that this area contains an underground bioreactor that creates high amounts of sulphides, heavy metals and an acidic pH (2–3) [2,3]. The special conditions of this environment have attracted the interest of researchers to identify the most representative prokaryotic microorganisms able to survive under extreme conditions. Most of these microorganisms belong to three bacterial genera: *Acidithiobacillus*, *Acidiphilium* and *Leptospirillum* [4]. The IPB ecosystem also contains a surprisingly high number of eukaryotic microorganisms (65 % of the total biomass), despite the extreme pH and heavy metal concentrations. Different species of *Chlamydomonas*, *Dunaliella*, *Chlorella*, *Bacillariphytes* and *Euglenophytes* have been identified in the upper part of the Tinto River, and these communities are more influenced by heavy metal concentrations than pH [5]. Acidophile microorganisms have survival strategies that allow them to live in these very acidic environments: cell membranes more impermeable to H^+; potassium antiport; buffer mechanisms; genes involved in transporters pumping H^+ out of the cell, or in DNA and proteins repair which can be damaged by low pH values [6].

The nitrogen cycle in the Tinto River and its subsurface has been well studied. The presence of nitrate reducers generates reactive nitrogen species that take part in iron oxidation under anaerobic conditions [7]. Microalgae are eukaryotic organisms that participate in the nitrogen cycle in the Tinto River ecosystem. These organisms use ammonium as a

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

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preferred source of nitrogen for their metabolism, but they also assimilate nitrate and nitrite for short periods of time [8,9]. Nitrogen assimilation in microalgae is performed photosynthetically and nitrate is reduced to nitrite in the cytosol via nitrate reductase (NR, EC: 1.6.6.1). Nitrite is transported to the chloroplast and reduced to ammonium by NiR (EC: 1.7.7.1) and ammonium is incorporated via the GS (EC: 6.3.2.1)-glutamate synthase (GOGAT; EC: 1.4.7.1) cycle to produce l-glutamate. The enzyme GDH (EC: 1.4.1.2) can incorporate ammonium under specific circumstances [10].

The presence of heavy metals alters this process and produces different changes in nitrogen consumption, as previously described [11,12]. Phytochelatins are a class of heavy-metal-binding peptides with a general structure of (γ-GluCys)nGly, where $n = 2–11$ [13]. The production of these compounds is related to glutamate and cysteine synthesis, which is dependent on nitrogen and sulphur assimilation [14]. Glu and Cys are also necessary for the production of glutathione, which is an essential metabolite in the antioxidant system of microalgae [15,16]. Although nitrogen metabolism has been widely studied in different microalgae under heavy metal stress, such as the model green microalga *Chlamyodomonas reinhardtii* and the robust microalga *Chlorella sorokiniana* [11,17], the unique conditions of Tinto River ecosystem make that the study of heavy metal-induced alterations in nitrogen assimilation and metabolism in Tinto River eukaryotic microorganisms may be an excellent approach to understand their survival in this extreme environment.

Coccomyxa onubensis (*C. onubensis*) is one of the microalgae isolated in the upper part of the Tinto River [18]. This microalga tolerates low pH (2.5) and high concentrations of heavy metals without significant differences in its growth parameters, and it shows high potential for arsenic bioremediation [15], which could be improved though immobilization methods using surfaces with high efficient capacity to remove pollutants [19,20]. Its ability to grow under heavy metal, salt, and UV stress, and in acidic pH [21], makes this microalga a perfect candidate for the removal of other inorganic or organic compounds. However, none of the studies, as the best of our knowledge, examined the effects of these stressors on *C. onubensis* N metabolism. The current study was conducted to determine how heavy metals, including copper, cadmium and mercury; and the metalloid arsenic, as arsenite and arsenate, altered N metabolism in *C. onubensis*. The capacity to remove nitrogen sources (ammonium or nitrate) was also studied under different heavy metal stresses and acid pH (2.5). An enzymatic characterisation of the nitrogen metabolism enzymes NiR, GS, and GDH was performed to determine how the different heavy metals affect the behaviour of these enzymes.

2. Materials and methods

2.1. Algal strain and cultivation

C. onubensis ACCV1 (SAG2510) was cultivated in modified K-9 media [22], containing (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 a 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 $Mo_7O_{24}(NH_4)_6{\cdot}4H_2O$ under a 24-h photoperiod with a PAR light intensity of 150 μmol m^{-2} s^{-1}, at 25 °C and bubbled with 5 % CO_2-enriched air. The medium was autoclaved at 1.5 atm, at 121 °C for 20 min. After autoclaving, the pH was adjusted to 2.5 to maintain adequate conditions for *C. onubensis* cultivation. pH was controlled along the time to maintain values from 2.5 to 3.0 during the experiments. Heavy metals were added as $CuCl_2$, $CdSO_4$, $NaAsO_2$, Na_2HAsO_4, or $HgCl_2$. Initial concentrations of heavy metals were between 25 μM and 1 mM for Cu^{2+}, 25 μM-1 mM for Cd^{2+} and AsO_3^{3-}, 2.5 mM–20 mM for AsO_4^{3-}, and 50 nM-1 μM for Hg^{2+}, in the consumption experiments; and between 50 μM and 2 mM for Cu^{2+}, 25 μM–500 μM for Cd^{2+}, 100 and 500 μM for AsO_3^{3-}, 2.5 mM–20 mM for AsO_4^{3-}, and 50 to 500 nM for Hg^{2+}, in the activity assay experiments. Additionally, an extra culture without the addition of heavy metals was used as control. When indicated, $NaNO_3$ was substituted for NH_4Cl at the same concentration.

2.2. Analytical determinations

The total ammonium and nitrate in the medium were determined colorimetrically. At each time point, an aliquot of 2 mL of culture was sampled, and centrifuged at 13,400 ×*g*. The supernatant was filtered through 0.45 nylon filters and the samples were stored at −20 °C for subsequent ammonium/nitrate determination.

Ammonium in the culture medium was determined using the phenol-hypochlorite method [23]. One millilitre of sample was mixed with 0.6 mL of water, 0.2 mL of phenol (10 % *w/v*) in ethanol, 0.2 mL of sodium nitroprusside solution (0.5 % w/v), and 0.5 mL of the oxidant solution. The mixture was incubated in the dark and agitated for 2 h. Absorbance was measured at 640 nm.

For nitrate determination 0.5 mL of sample was vigorously mixed with 0.05 mL of sulfamic acid (10 % *w/v*) and incubated for 2 min. Then, 0.2 mL of perchloric acid (20 % *v*/v) was added and the absorbance of the mixture was measured at 210 nm, as described previously [24].

2.3. Preparation of crude extract

C. onubensis cells were harvested by centrifugation for 5 min at 3900 ×*g*., as described previously [15]. The 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 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 mixture was filtered through nylon gauze, washed and centrifuged twice. The supernatant was used as a crude extract source after extraction or stored at −20 °C with the addition of proteases inhibitor cocktail 100× (Merck, Germany). The total protein content of the crude extract was determined using the Bio-Rad Bradford assay with BSA as a standard.

2.4. Enzyme assays

Enzymatic assays were performed using the crude extract prepared as described above. Nitrite reductase assays were performed by adding in 1 mL reaction mixture containing: 150 mM Tris-HCl (pH 7.5), 3 mmol $NaNO_2$, 10 μmol methylviologen and 0.2 mL crude extract. Reaction was started with the addition of sodium dithionite, freshly dissolved in 0.3 M $NaHCO_3$ solution. After 10 min of incubation at 40 °C, nitrite was measured as previously described by Romero et al., [25]. Glutamate dehydrogenase assays were kinetically measuring the NADH content at 340 nm. The measured mixture contained 50 μM Tris-HCl (pH 9,0); 50 μM l-glutamate; 2 μM NAD^+ and 100 μL of crude extract. The enzymatic assay was performed as described by Gronostajski et al., [26]. The glutamine synthetase enzymatic activity was determined colourimetrically at 500 nm by adding to the reaction mixture 60 μM phosphate buffer (pH 6), 120 μM glutamine, 3.2 μM of $MnCl_2$, 1 μM ADP, 240 μM NH_2OH, 60 μM NaOH and 50 μL of crude extract. The reaction was started by the addition of 50 μM Na_2HAsO_4. The samples were incubated at 30 °C for 10 min and the γ-glutamylhydroxamate formed was determined as described in [17].

For the optimal temperature determinations, the experiments were performed in an Ultrospec 3100 Pro spectrophotometer (Amersham Biosciences, Amersham, UK) coupled to a thermostatic bath with a temperature between 0 and 80 °C.

The optimal pH was determined using different buffer solutions: 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 8.0–10.0.

2.5. Statistical analysis

All measurements were performed in triplicate and the results are presented as means ± SD. The significance of values was considered at $*p < 0.05$ and $**p < 0.01$. Statistical analyses were performed using IBM SPSS Statistics 27 software (Armonk, NY, USA) by comparing the mean values using one-way analysis of variance (ANOVA) followed by a post hoc DMRT test.

3. Results and discussion

3.1. Ammonium/nitrate consumption of C. onubensis under heavy metal stress

C. onubensis is a microalga that grows at a low pH (2.5) and under high concentrations of heavy metals, and it even increases its growth rate [15]. However, its capacity to assimilate different nutrients under these stressors has not been studied. The influence of different heavy metals, such as Cu^{2+}, Cd^{2+}, AsO_3^{3-}, AsO_4^{3-}, and Hg^{2+}, on nitrogen assimilation was studied in this microalga. The results showed that *C. onubensis* assimilated better ammonium than nitrate under copper, arsenite and mercury stress (Fig. 1A, C and E), whereas it assimilated faster nitrate than ammonium under cadmium stress (Fig. 1B) and there

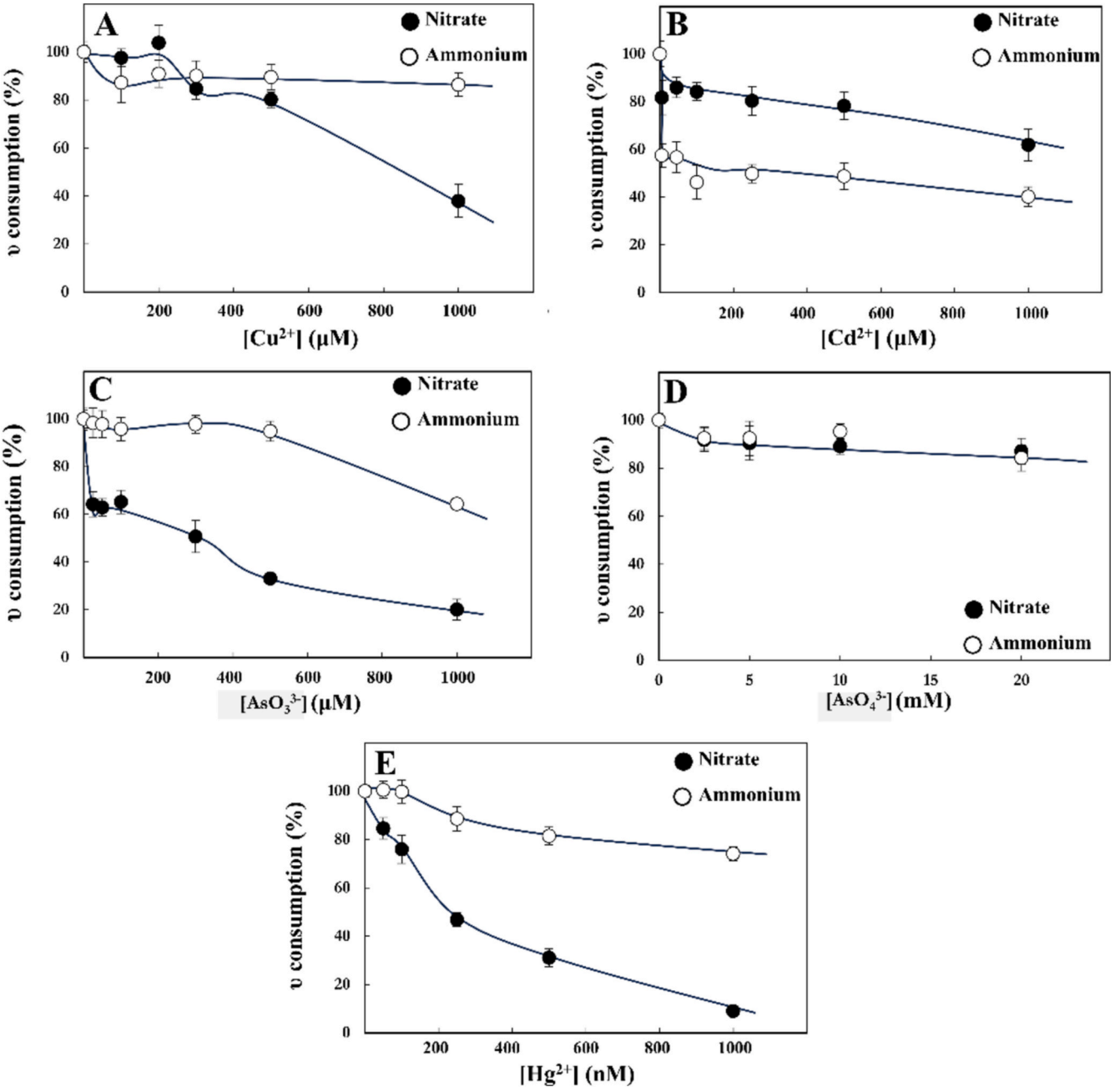

Fig. 1. Nitrate (black) and ammonium (white) consumption of *C. onubensis* cultured with different concentrations of Cu^{2+} (A), Cd^{2+} (B), AsO_3^{3-} (C), AsO_4^{3-} (D), and Hg^{2+} (E). *C. onubensis* was cultured in modified K9 media, as described in the Materials and Methods, using NH_4Cl or $NaNO_3$ as the nitrogen source. The of consumption rates were 2.93 ± 0.08 and 3.85 ± 0.19 μmol h^{-1} mg chl^{-1} for nitrate and ammonium, respectively.

were no differences between nitrogen sources under arsenate stress (Fig. 1D). Microalgae use ammonium as a preferred nitrogen source under standard conditions [10]. However, there were some differences under stress conditions. The N consumption rate was greater with nitrate than ammonium as shown in cadmium cultures (Fig. 1B).

For Cu^{2+} cultures, there was a slight increase in nitrate consumption at concentrations until 0.3 mM (Fig. 1A). These results correlated with the growth data reported for *C. onubensis* in our previous studies, which showed an increase in growth rates when it was cultivated with high concentrations of Cu^{2+} [15]. However, there was a significant ($p < 0.01$) decrease in the consumption rate of nitrate at concentrations 0.3 mM which did not occur with ammonium, where significant differences ($p < 0.05$) only appeared at 1 mM of Cu^{2+} (Fig. 1A). These data are consistent with Leon-Vaz et al., [11], where ammonium consumption speed was not affected at these concentrations of copper in *Chlorella sorokiniana*.

C. onubensis cultures under cadmium stress were more affected than the other heavy metals, even at low concentrations (Fig. 1B). At 50 μM Cd^{2+}, nitrate and ammonium consumption were significantly ($p < 0.01$) inhibited 20 % and 42 %, respectively, compared to the control cultures. These data may be explained by the high toxicity of this heavy metal. A high inhibition of ammonium assimilation speed by cadmium was also reported for *Chlorella sorokiniana*, which was the most toxic heavy metal for this metabolic route [11].

Arsenic cultures exhibited two different situations in *C. onubensis*. For arsenite, there was a slight decrease in ammonium consumption and a significant ($p < 0.01$) decrease in nitrate consumption (Fig. 1C). However, the presence of arsenate did not affect the consumption of the two nitrogen sources (Fig. 1D) and significant differences ($p < 0.05$) only appeared at 20 mM of AsO_4^{3-}. These results with arsenate were attributed to the capacity of *C. onubensis* to accumulate this metalloid [15], being also reported that accumulation rates were faster under N deprivation in *Chlamydomonas reinhardtii* [27]. Moreover, *C. onubensis* was isolated from an ecosystem with high concentrations of this metalloid, and likely has developed different mechanisms, such as oxidation or methylation of the metalloid, to avoid arsenic-induced stress [28].

The presence of Hg^{2+} also significantly ($p < 0.01$) inhibited nitrate consumption speed by *C. onubensis* at all the concentrations tested and ammonium consumption speed at concentrations up to 250 nM (Fig. 1E). This inhibition was likely due to the high toxicity of mercury, which was used at nM concentrations instead of μM. Similar studies performed with the cyanobacteria *Microcystis aeruginosa* showed that it did not tolerate concentrations up to 100 nM of Hg^{2+}, after 4 days of cultivation [29]. However, *C. onubensis* not only tolerated 100 nM of this heavy metal, but also nitrogen consumption was unaffected when the microalga was cultivated with ammonium and had a slight decrease of nitrate consumption speed (20 % of control cultures) after 8 days of cultivation (Fig. 1). Indeed, *C. onubensis* was a robust microalga cultivated under Hg^{2+} stress.

3.2. Characterisation of nitrogen metabolism enzymes in C. onubensis

Heavy metals cause oxidative stress in *C. onubensis* cells [15]. Therefore, the presence of reactive oxygen species (ROS) modifies other metabolic routes, such as nutrient assimilation, via nitrogen metabolism enzymes. Prior to the study of abiotic stress, the kinetic parameters of NiR, GS and GDH were characterised as this investigation represents the first study of these enzymes in *C. onubensis*.

3.2.1. Nitrite reductase

NiR is a chloroplastic enzyme that reduces nitrite to ammonium using reduced ferredoxin [10]. The characterisation of this enzyme in *C. onubensis* cells was performed to determine the optimal values of pH, temperature and K_m of the different substrates, such as nitrite, ferredoxin (Fd) or methyl viologen (MV); (Table 1). The K_m values were 1.9 ± 0.2 mM, 16.9.0 ± 2.3 mM, and 7.2 ± 0.8 μM for nitrite, MV and Fd, respectively. These results demonstrated that *C. onubensis* NiR had higher affinity for Fd and lower affinity for nitrite and MV than other microalgal species, such as *Chlamydomonas reinhardtii* or *Monoraphidium braunii* [25,30] (Table 1).

Table 1
Physicochemical properties of the nitrite reductase (NiR), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) enzymes reported in previous literature for different microalgae.

Organism	Enzyme	K_m (mM)	Temperature (°C)	pH	Reference
C. onubensis	NiR	1.9 NO_2^- 16.9 MV 0.0072 Fd	45	7.5	This study
Chlamydomonas reinhardtii	NiR	0.38 NO_2^- 0.91 MV 0.0024 Fd	40	7.5	[25]
Monoraphidium braunii	NiR	0.70 NO_2^- 0.26 MV 0.0010 Fd	40	8.0	[30]
C. onubensis	GS	19.3 Gln 2.0 NH_2OH 0.0006 ADP 0.0597 Mn^{2+}	40	6.0	This study
Chlamydomonas reinhardtii	GS1	2.5 Gln 2.1 NH_2OH nd ADP 0.21 Mn^{2+}	nd	6.0	[31]
Chlamydomonas reinhardtii	GS2	10.0 Gln 2.0 NH_2OH nd ADP 0.20 Mn^{2+}	nd	5.5	[31]
C. onubensis	GDH	1.1 2-KG 29.0 NH_4^+ 0.054 NADH	60	9.0	This study
Chlamydomonas reinhardtii	GDH1	0.36 2-KG 30.0 NH_4^+ 0.070 NADH	60	8.5	[32]
Chlorella sorokiniana	GDH	2.0 2-KG 40.0 NH_4^+ 0.150 NADH	nd	8.0	[26]

The influence of temperature and pH on NiR enzymatic activity was also determined in *C. onubensis*. The highest activity values occurred at 45 °C and pH 7.5 in 50 mM potassium phosphate buffer (Fig. S1 A and B, respectively). These optimal temperature and pH values were similar to other microalgal species (Table 1) [25,30]. However, this enzyme in *C. onubensis* showed low stability at different temperatures and pH values and only showed >50 % activity at temperatures between 40 and 50 °C or pH values from 7 to 7.5 (Fig. S1A and B). Therefore, further experiments with this enzyme were performed at 45 °C and pH 7.5.

3.2.2. Glutamine synthetase

GS produces l-glutamine from l-glutamate and ammonium using ATP [10]. This enzyme also produces γ-glutamylhydroxamate from l-glutamine and hydroxyl amine, as shown by the assay performed by Devriese

et al., [17]. The results of the characterisation of GS in *C. onubensis* were shown in Table 1 and Fig. S1C and D. The K_m values for the different substrates were 19.3 ± 1.6 mM for glutamine, 2.0 ± 0.13 mM for NH_2OH, 0.6 ± 0.04 μM for ADP and 59.7 ± 3.9 μM for Mn^{2+} (Table 1). *C. onubensis* GS had similar K_m values for hydroxyl amine to the two isoforms of *Chlamydomonas reinhardtii*. However, the affinity of *C. onubensis* GS for Mn^{2+} was greater and its affinity for glutamine was lower than this model microalga [31]. The optimal temperature and pH values of GS were also determined for *C. onubensis*. The optimal temperature for this enzyme was 40 °C and the optimal pH was 6.0 using phosphate buffer (Table 1; Fig. S1C and D). Notably, this enzyme tolerated a greater range of temperatures and pH values than NiR, and it showed activity values >40 % of the optimal values in the range 25–55 °C and 5–7.5 pH values (Fig. S1C and D). Therefore, further experiments were performed at 40 °C and pH 6.0.

3.2.3. Glutamate dehydrogenase

GDH participates in the reaction of 2-ketoglutarate (2-KG) and ammonium to produce l-glutamate [10]. The characterisation of this enzyme in *C. onubensis* showed a high affinity for NADH and ammonium as substrates, with K_m values of 54.0 ± 4.1 μM and 29.0 ± 1.8 mM, respectively, and a low affinity for 2-KG, with a K_m value of 1.1 ± 0.22 mM (Table 1). The ammonium K_m values were consistent to the previous reported for other microalgae, such as *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* (Table 1), but the K_m values for NADH showed higher affinity in *C. onubensis* GDH than other microalgae (Table 1); [26,32].

Notably GDH activity in *C. onubensis* exhibited high tolerance to temperature and pH alterations (Fig. S1E and F). Although its optimal pH value was 9.0 using Tris-HCl buffer, GDH activity was maintained at up to 90 % of the total activity in a pH range from 8.5 to 12.0 (Fig. S1F). Similar results were reported for the temperature assay, with an optimal temperature of 60 °C, although GDH activity did not decrease by >20 % in the range of 45 to 75 °C (Fig. S1E). These results are consistent with previous studies and showed the high potential of this enzyme for nitrogen assimilation, especially under extreme conditions [32]. Thus,

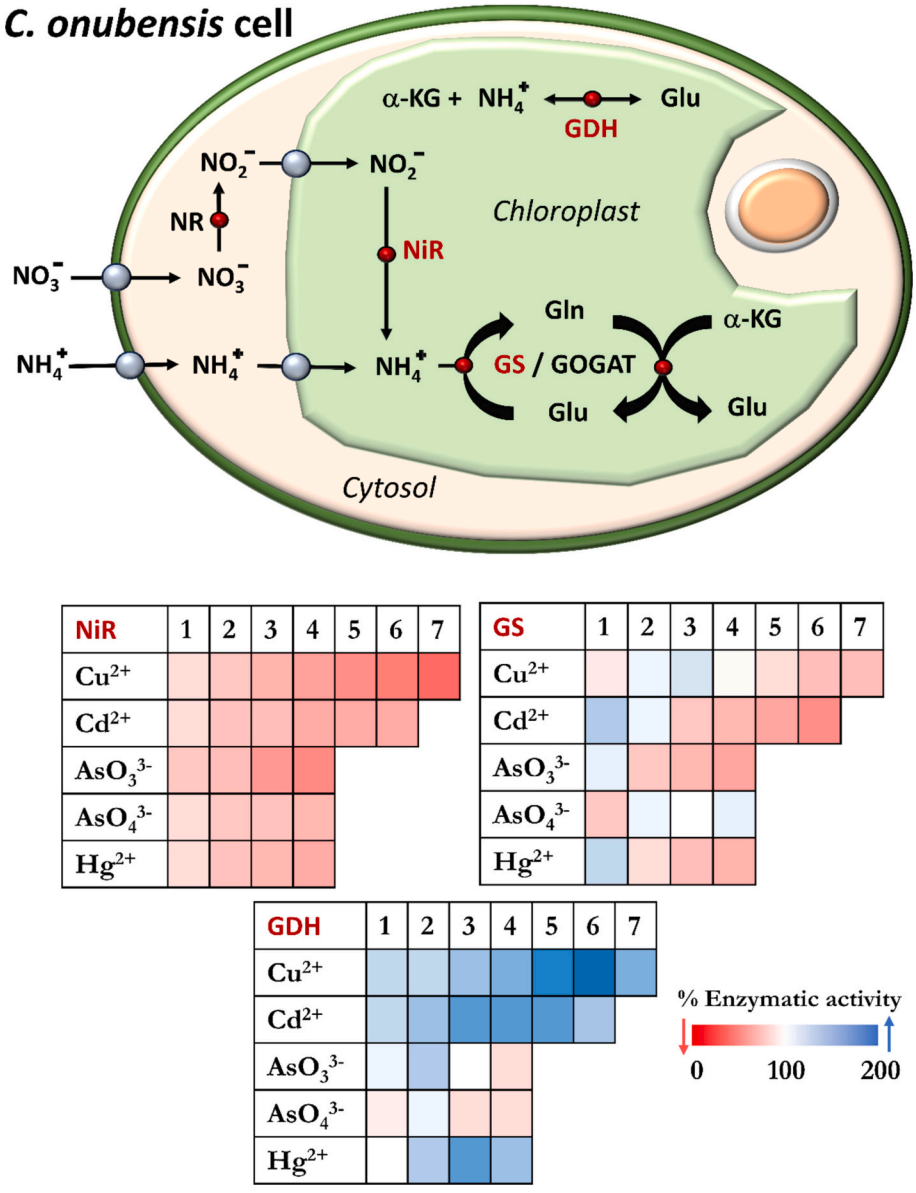

Fig. 2. Effect of heavy metals tested at different concentrations in *C. onubensis* nitrogen enzymes. Concentrations tested were 50, 100, 200, 300, 500, 1000 and 2000 μM of Cu^{2+} (1–7); 25, 50, 100, 200, 300 and 500 μM of Cd^{2+} (1–6), 100, 200, 300 and 500 μM of AsO_3^{3-} (1–4), 2.5, 5, 10 and 20 mM of AsO_4^{3-} (1–4); and 50, 100, 250 and 500 nM Hg^{2+} (1–4).

subsequent GDH experiments were carried out at 60 °C and pH 9.0.

3.3. Effect of heavy metals on the nitrogen metabolism enzymes of *C. onubensis*

After the characterisation of these enzymes and using the optimal parameters obtained, the effects of copper, cadmium, arsenite, arsenate and mercury on the enzymatic activities of NiR, GS and GDH were studied for *C. onubensis*, after 8 days of cultivation. A summary of the results is represented in Fig. 2.

3.3.1. Nitrite reductase

The effect of heavy metal stress on NiR activity at pH 2.5 was tested using similar concentrations of these elements as for nitrogen consumption studies (Fig. 3). NiR enzymatic activity generally decreased due to the presence of different heavy metals in the culture medium. However, the results showed a significantly larger decrease under copper, arsenite and mercury stress than under cadmium or arsenate treatment (Fig. 3). These results are consistent with the nitrate consumption data shown in Fig. 1, which also pointed a prominent decrease when *C. onubensis* was cultured with these elements. For Cu^{2+}, concentrations up to 200 μM caused a significant ($p < 0.01$) inhibition of NiR enzymatic activity, maintaining 44, 32 and 23 % of control enzymatic activity at concentrations of 500, 1000 and 2000 μM (Figs. 2 and 3A). However, at those concentrations, the growth rates were greater than the control cultures, as reported by Romero-Cruz et al., [15]. Thus, *C. onubensis* could have developed an alternative nitrogen assimilation mechanism under copper stress. It should be noted that Tinto River ecosystem contains high amounts of this element and, consequently, its extremophile organisms are acclimated to high copper concentrations [2]. NiR activity was significantly ($p < 0.05$) affected at concentrations up to 50 μM of Cd^{2+}, maintaining at 50–100 μM Cd^{2+} 80 % of control cultures activity and, between 200 and 500 μM Cd^{2+}, 60 % of control enzymatic activity (Figs. 2 and 3B), However, nitrogen consumption rates did not show decreases >20 % at these concentrations (Fig. 1). Previous studies reported that *Chlamydomonas reinhardtii* NiR activity was not affected at concentrations of 100 μM Cd^{2+} [17]. However, its exposure time was until 48 h, which is less time than *C. onubensis* treatment (192 h). Thus, cadmium could alter nitrogen metabolism after long term exposure due to bioaccumulation processes [33].

The enzymatic activity of *C. onubensis* NiR was significantly inhibited ($p < 0.05$) at all concentrations of arsenite tested, and at concentrations up to 2.5 mM of arsenate (Fig. 2, 3C and D). *C. onubensis* NiR activity and N consumption rates under AsO_4^{3-} stress did not show inhibition rates >15–20 % of the control cultures at all the concentrations tested (Fig. 1),

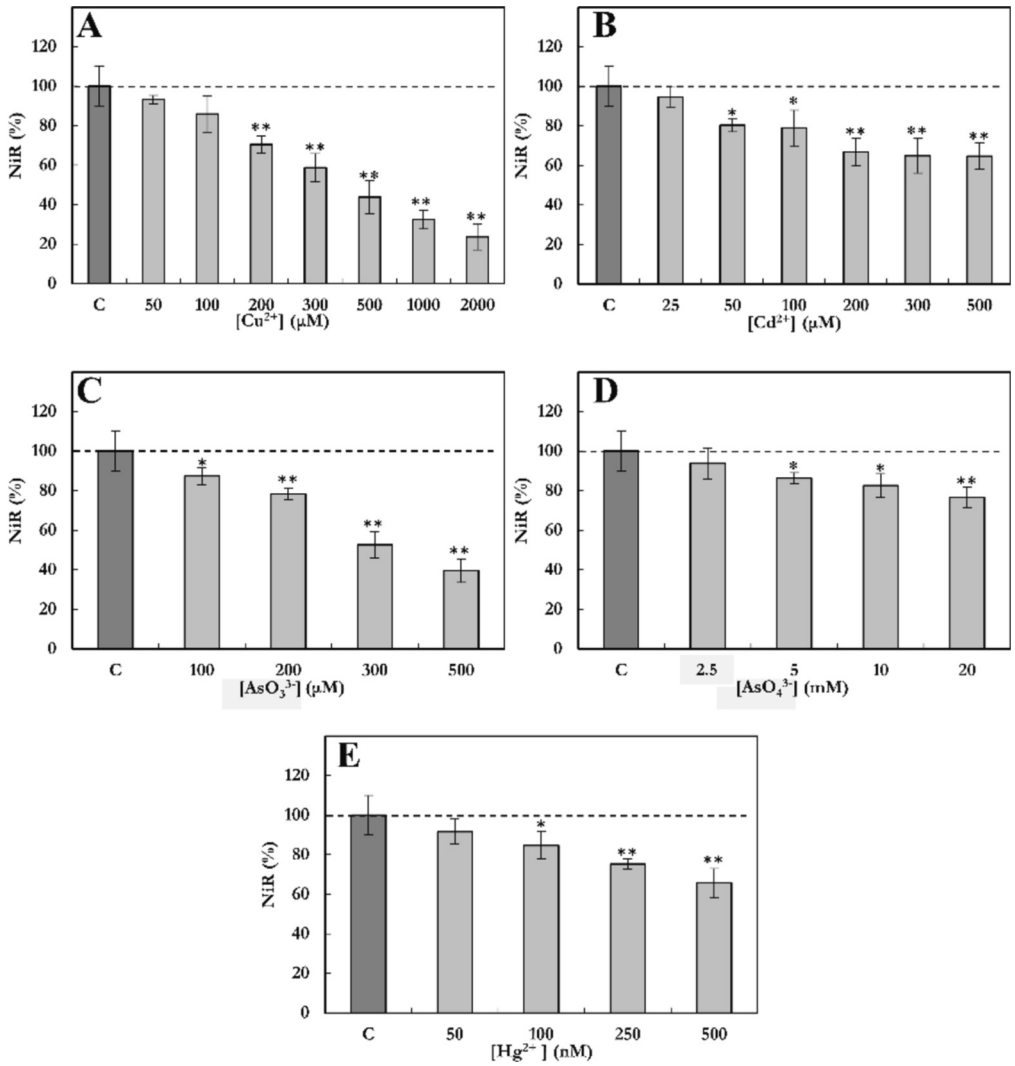

Fig. 3. Effects of different heavy metals, including copper (A), cadmium (B) and mercury (E), just like the metalloids arsenite (C) and arsenate (D), on the enzymatic activity of NiR in *C. onubensis* after 8 days of growth. The 100 % NiR activity corresponded to 568.69 ± 5.37 U mg^{-1}. * Significant differences in biomass between control and heavy metal treatment at $p < 0.05$ and ** at $p < 0.01$.

demonstrating the low toxicity of arsenate to this microalga. Previous studies of our research group demonstrated the capacity of *C. onubensis* to accumulate high amounts of intracellular arsenic [15], being a mechanism to reduce the toxicity of these pollutants [34]. Indeed, it could explain the high tolerance to arsenic and the slight alterations produced by arsenic in *C. onubensis* metabolism. The decrease in NiR enzymatic activity under Hg^{2+} stress was similar to the other heavy metals, with activity values between 60 and 80 % of the control cultures (Figs. 2 and 3E). At these mercury concentrations, the decrease of nitrogen consumption rates was higher than NiR activity when *C. onubensis* was cultured using nitrate as N source, maintaining values between 30 and 80 % of control cultures (Fig. 1E). However, in the presence of ammonium as N source, *C. onubensis* nitrogen consumption was not as affected as with nitrate. Consumption data, together with NiR enzymatic activity results, suggest that the microalga has another limitation to assimilate nitrate that is not arising in ammonium assimilation route. Previous studies demonstrated that NR was highly altered by the presence of mercury in *Hizikia fusiformis*, where a concentration of 200 nM of Hg^{2+} produced a considerable decrease after 7 days of exposure [35]. Thus, the limitation in nitrate assimilation may be related with NR instead of NiR.

3.3.2. Glutamine synthetase

The enzymatic activity of GS in *C. onubensis* was also altered by the presence of heavy metals in the culture medium at pH 2.5. There was a slight increase at low concentrations of heavy metals, which was significant ($p < 0.05$) at 25 μM of Cd^{2+} and 50 nM of Hg^{2+}. However, this increase was inhibited at high concentrations of these elements (Fig. 2). *C. onubensis* cells cultured under copper stress exhibited a slight increase in GS enzymatic activity until 200 μM Cu^{2+} treatment. Then, the results were similar to control cultures until 1 mM Cu^{2+}, when there was a significant decrease ($p < 0.05$), likely caused by the toxicity of this metal, although GS activity was around 80 % of control cultures (Fig. 4A). The same behaviour was reported for Cd^{2+}, which exhibited a significant ($p < 0.01$) increase in GS activity at 25 μM Cd^{2+} (120 % of control cultures), followed by a significant decrease ($p < 0.05$) at concentrations up to 100 μM Cd^{2+}, remaining only 50 % of control GS activity at 500 μM Cd^{2+}(Fig. 2 and 4B). This significant decrease in GS activity under copper and cadmium stress was also reported in other microalgae, such as *Chlamydomonas reinhardtii* at concentrations of 100 μM of Cd^{2+} after 7 h; and *Chlorella sorokiniana* at 500 μM of Cu^{2+} and 250 μM of Cd^{2+}, after 42 h of culture [11,17,36]. Thus, *C. onubensis* N metabolism had higher tolerance to copper stress than other microalgae probably caused by the high concentrations of this metal in its environment. However, the results under cadmium stress showed that the mechanisms to cope with this cation in the extremophile microalga were not as adequate as *Chlorella sorokiniana* mechanisms, which includes high accumulation capacity, upregulation of antioxidant enzymes and alterations in C, N and S assimilation routes [37].

In contrast, the enzymatic activity of *C. onubensis* GS in the presence of As differed depending on the oxidation state of the metalloid. Arsenite slightly increased GS activity at 100 μM and significantly ($p < 0.05$)

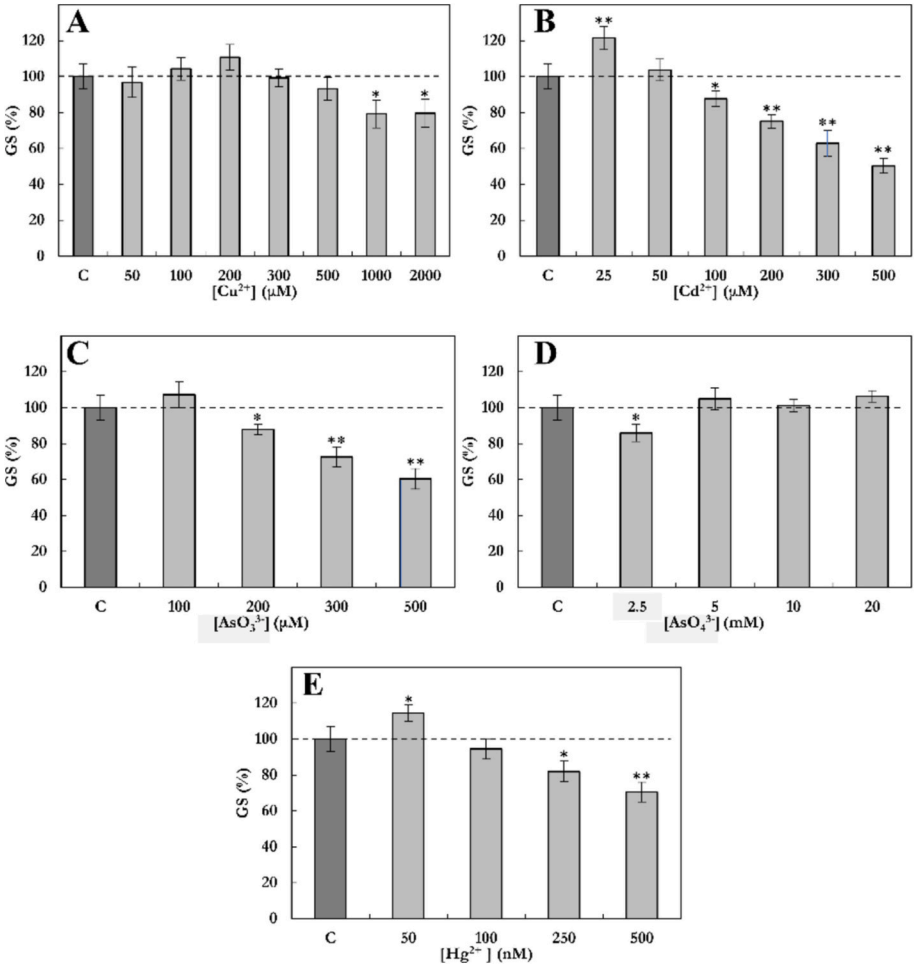

Fig. 4. Effects of copper (A), cadmium (B) and mercury (E), and the metalloids arsenite (C) and arsenate (D), on *C. onubensis* GS enzymatic activity after 8 days of growth. The 100 % GS activity corresponded to 1.52 ± 0.15 U mg^{-1}. * Significant differences in biomass between control and heavy metal treatment at $p < 0.05$ and ** at $p < 0.01$.

decreased GS activity at concentrations up to that, maintaining between 60 and 90 % of the control GS activity (Figs. 2 and 4C). However, arsenate did not significantly change GS activity at the highest concentrations tested, showing only a significant decrease ($p < 0.05$) at 2.5 mM of arsenate (Figs. 2 and 4D). This stable GS activity under arsenate stress contrasts with the results of *Chlorella sorokiniana* [11], which showed a significant ($p < 0.01$) decrease of GS gene expression at 10 mM of AsO_4^{3-}. Indeed, the different responses between microalgal species can be produced by an adaptation mechanism for this metalloid developed by *C. onubensis* due to the concentration of this metalloid in Tinto River ecosystem. One of the suggested mechanisms for arsenic tolerance in *C. onubensis* compared with *Chlorella sorokiniana* is the accumulation capacity. While *C. onubensis* is an arsenic hyperaccumulator organism [15], *Chlorella sorokiniana* did not accumulate arsenic after 72 h of culture [11]. The presence of Hg^{2+} in the culture medium provoked a significant increase ($p < 0.05$) only at 50 nM Hg^{2+}, and a significant decrease ($p < 0.05$) at concentrations up to 100 nM Hg^{2+}, remaining 81 and 70 % of control GS activity at concentrations of 250 and 500 nM of Hg^{2+}, respectively (Figs. 2 and 4E). It has been reported that inorganic mercury inhibits glutamine metabolism in plants and animals [38], However, further studies are needed in microorganisms to understand how this element works in their nitrogen metabolism.

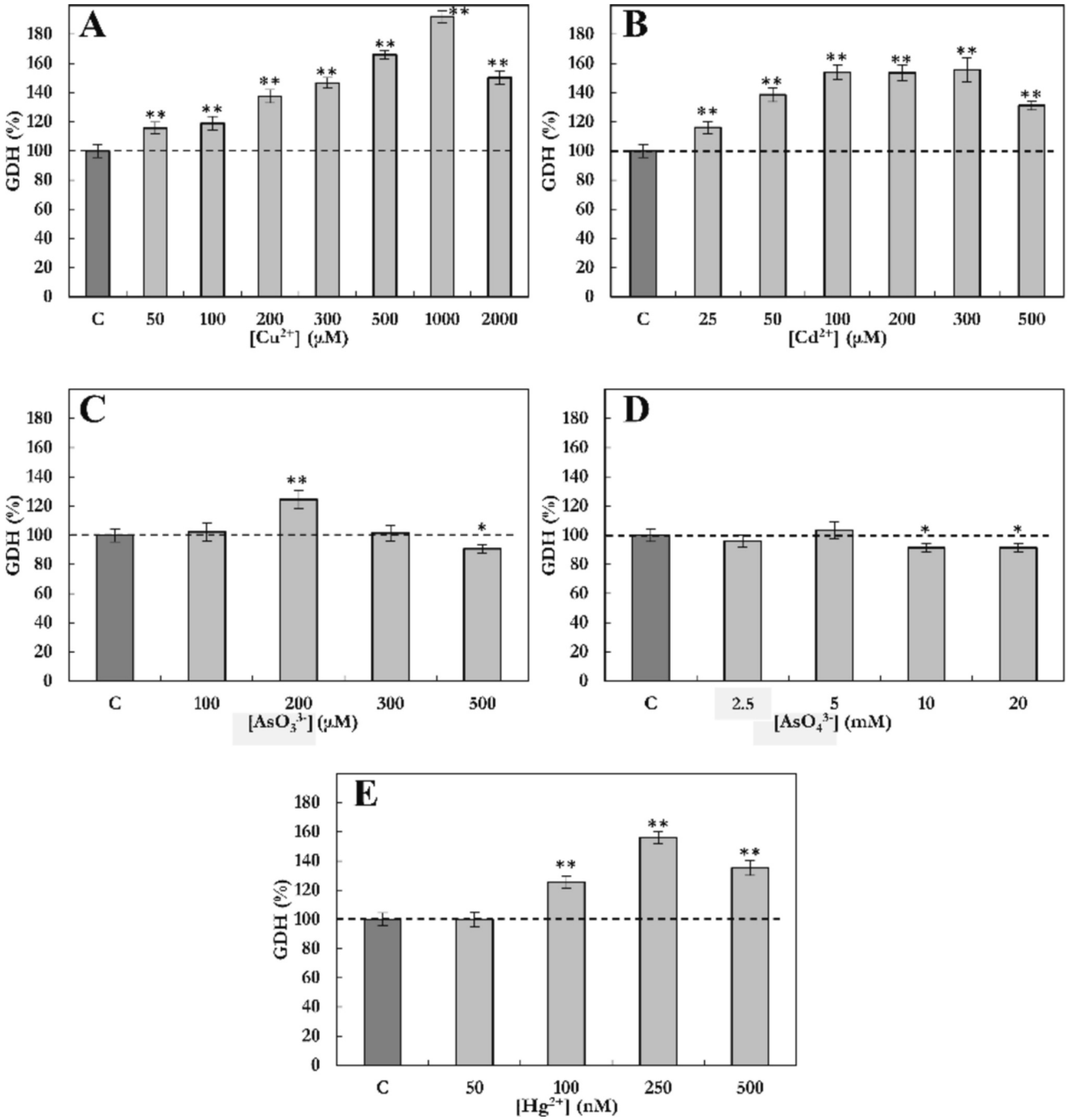

Fig. 5. Effects of the heavy metals copper (A), cadmium (B) and mercury (E), and the metalloids arsenite (C) and arsenate (D), on *C. onubensis* GDH enzymatic activity after 8 days of growth. The 100 % GDH activity corresponded to 13.59 ± 1.44 U mg^{-1}. * Significant differences in biomass between control and heavy metal treatment at $p < 0.05$ and ** at $p < 0.01$.

3.3.3. Glutamate dehydrogenase

The effects of heavy metals on GDH enzymatic activity are considerable in *C. onubensis* cells (Fig. 2). GDH activity in *C. onubensis* was significantly upregulated ($p < 0.01$) when cultured with all concentrations of copper, cadmium or mercury, except 50 nM Hg^{2+} (Fig. 5A, B and E, respectively). However, under arsenic stress (both arsenite and arsenate), there were no significant increases in GDH activity between the *C. onubensis* treated and the control group, except for the 200 μM arsenite cultures (Figs. 2, 5C and D). In the heavy metal cultures, GDH enzymatic activity increased until 192 % of control cultures at 1 mM of Cu^{2+}, 155 % at 100–300 μM of Cd^{2+}, and 154 % at 250 nM of Hg^{2+} (Fig. 5A, B and E). These increases demonstrated that GDH enzymatic activity can be upregulated under different stresses in microalgae. Other examples are *Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, and *Dunaliella* sp., whose GDH enzymatic activity increase until 195 % and 160 % of control cultures under cadmium stress [11,39]. It has been described that GDH shunt to return the carbon in amino acids back into reactions of carbon metabolism and the tri-carboxylic acid cycle [40]. Additionally, it is the responsible to maintenance the internal l-glutamate concentration when GS-GOGAT cycle is not fully operative in green microalgae [41]. Therefore, the upregulation of GDH (Fig. 5), and the downregulation of GS (Fig. 4) under heavy metal stress may be an alternative approach for *C. onubensis* cells to maintain the production of l-glutamate as a detoxification mechanism, because l-glutamate is a precursor of phytochelatins involved in heavy metals detoxification [11].

These results demonstrated that *C. onubensis* has an alternative method to produce l-glutamate and to obtain nitrogen for its development under heavy metals stress. However, further research, such as omics studies, is necessary to deep in the full mechanism of nutrient assimilation that this extremophile microalga has developed to cope with low pH values and high concentrations of heavy metals in their environment.

4. Conclusions

The extremophile microalga *C. onubensis* better assimilated ammonium than nitrate as an N source when cultivated under copper, arsenic or mercury stress at pH 2.5. However, the presence of cadmium in the culture medium provoked a greater decrease in ammonium consumption than nitrate consumption. The increase in heavy metals in the culture medium involved a decrease in nitrite reductase activity and, a general increase in glutamate dehydrogenase activity under copper, cadmium and mercury stress, which was likely for the maintenance of l-glutamate levels in cells. The presence of heavy metals in *C. onubensis* culture medium promoted the upregulation of glutamine synthetase activity only at low concentrations and the downregulation of this enzyme at high concentrations of heavy metals. These data provide a better understanding of the alterations in N metabolism in eukaryotes due to the extreme conditions of acidic pH and heavy metal concentrations in the Tinto River ecosystem.

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CRediT authorship contribution statement

María del Carmen Romero-Cruz: Methodology, Investigation, Conceptualization. **Antonio Leon-Vaz:** Writing – review & editing, Writing – original draft, Formal analysis. **José María Vega:** Writing – review & editing, Supervision. **Javier Vigara:** Writing – review & editing, Supervision, Investigation.

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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