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*Journal of Proteomics* 2015, 120, 75-94.

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DOI link to article:

http://dx.doi.org/10.1016/j.jprot.2015.03.004

Date deposited:

29/06/2016

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Effects of heavy metals on Cyanothecce sp. CCY 0110 growth, extracellular polymeric substances (EPS) production, ultrastructure and protein profiles

Rita Mota\textsuperscript{a,b,c,1}, Sara B. Pereira\textsuperscript{a,b,1}, Marianna Meazzini\textsuperscript{d}, Rui Fernandes\textsuperscript{a,b}, Arlete Santos\textsuperscript{a,b,c}, Caroline A. Evans\textsuperscript{e}, Roberto De Philippis\textsuperscript{d,f}, Phillip C. Wright\textsuperscript{e}, Paula Tamagnini\textsuperscript{a,b,c,⁎}

\textsuperscript{a}Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal
\textsuperscript{b}IBMC — Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal
\textsuperscript{c}Faculdade de Ciências, Departamento de Biologia, Universidade do Porto, Porto, Portugal
\textsuperscript{d}Department of Agrifood Production and Environmental Sciences, University of Florence, Florence, Italy
\textsuperscript{e}ChELSI Institute, Department of Chemical and Biological Engineering, University of Sheffield, Sheffield, United Kingdom
\textsuperscript{f}Institute of Ecosystem Study (ISE), National Research Council (CNR), Sesto Fiorentino (FI), Italy

ARTICLE INFO

Article history:
Received 29 October 2014
Accepted 7 March 2015
Available online 14 March 2015

Keywords:
Cyanobacteria
Cyanothecce
Extracellular polymeric substances (EPS)
Heavy metals
iTRAQ
Proteome

ABSTRACT

The effects of several heavy metals on the growth/survival, EPS production, ultrastructure and protein profiles of the highly efficient extracellular polymeric substances (EPS)-producer cyanobacterium Cyanothecce sp. CCY 0110 were evaluated. Our results clearly show that each heavy metal affects the cells in a particular manner, triggering distinctive responses. Concerning chronic exposure, cells were more affected by Cu\textsuperscript{2+} followed by Pb\textsuperscript{2+}, Cd\textsuperscript{2+}, and Li\textsuperscript{+}. The presence of metal leads to remarkable ultrastructural changes, mainly at the thylakoid level. The comparison of the proteomes (iTRAQ) allowed to follow the stress responses and to distinguish specific effects related to the time of exposure and/or the concentration of an essential (Cu\textsuperscript{2+}) and a non-essential (Cd\textsuperscript{2+}) metal. The majority of the proteins identified and with fold changes were associated with photosynthesis, CO\textsubscript{2} fixation and carbohydrate metabolism, translation, and nitrogen and amino acid metabolism. Moreover, our results indicate that during chronic exposure to sub-lethal concentrations of Cu\textsuperscript{2+}, the cells tune down their metabolic rate to invest energy in the activation of detoxification mechanisms, which eventually result in a remarkable recovery. In contrast, the toxic effects of Cd\textsuperscript{2+} are cumulative. Unexpectedly, the amount of released polysaccharides (RPS) was not enhanced by the presence of heavy metals.

Biological significance

This work shows the holistic effects of different heavy metals on the cells of the highly efficient EPS-producer the cyanobacterium Cyanothecce sp. CCY 0110. The growth/survival,

⁎ Corresponding author at: IBMC — Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal. Tel.: +351 226074900; fax: +351 226099157.
E-mail address: pmtamagn@ibmc.up.pt (P. Tamagnini).

\textsuperscript{1} Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jprot.2015.03.004
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1. Introduction

Cyanobacteria are photoautotrophic prokaryotes capable of producing extracellular polymeric substances (EPS), which can remain associated with the cell surface as sheaths, capsules and/or slimes, or be released into the surrounding environment as released polysaccharides (RPS). Several roles are attributed to EPS, such as protection of the cells against desiccation, predators and UV radiation, contribution to the formation of biofilms, and/or the sequestration/immobilization of nutrients and metal ions [1,2].

Heavy metals are one of the worldwide most common causes of pollution, being a serious hazard to the environment and public health [3,4]. EPS-producing cyanobacteria or their isolated EPS can be an advantageous alternative to conventional physicochemical methods to remove heavy metals from polluted waters, since these microorganisms have minimal nutritional requirements, are easy to cultivate and exhibit rapid metal removal kinetics [5–7]. Moreover, the particular characteristics of cyanobacterial EPS make them very attractive for bioremediation. The overall negative charge of the polymer confers a high affinity towards metal cations, and the high number of possible conformations facilitating the interactions between metal ions and the EPS binding sites [2,8]. However, a successful implementation of biotechnological systems based on cyanobacteria/cyanobacterial-EPS will depend on the characteristics of the pollutant(s), the strain and/or the EPS utilized. Depending on the metal(s)/metal(s) concentration(s) and its toxic effects, it may be more advantageous to use whole cultures or the isolated EPS. Therefore, it is necessary to assess the interactions between the cells and the metal ions, e.g. if it exists an active uptake of the metal by the cells and/or a passive biosorption of the cations [9,10].

Some metals are essential to many physiological processes (e.g. copper and iron), while others are non-essential to the cell life (e.g. cadmium and lead). However, in excess, all metals are deleterious to the cells [11–13]. Several protection mechanisms have been described for cyanobacterial cells to avoid/minimize the toxicity of heavy metals. These strategies can occur extracellularly as, for example, the sequestration of metal ions on the outer cell surface or the release of ligands. However, these strategies usually need to be combined with other cellular mechanisms to achieve homeostasis, such as exporting P-type ATPases to detoxify heavy metal cations by efflux and/or the binding of these cations to metallothioneins, preventing free toxic metals [14,15]. Nevertheless, these mechanisms may not be enough to cope with the toxic effects, leading to an unbalanced oxidative state mainly by the formation of reactive oxygen species (ROS), which can result in cell death [16].

This work aimed at evaluating the effects of the presence/concentration of several heavy metals commonly found in polluted water bodies (copper, lead, cadmium and lithium) in the growth/survival, ultrastructure and EPS production by the unicellular cyanobacterium *Cyanothece* sp. CCY 0110 (hereafter referred as *Cyanothece*). This marine *N*₂-fixing cyanobacterium has its genome fully sequenced and is among the most efficient RPS producers [17]. In addition, the proteomes of *Cyanothece* grown in the absence/presence of copper or cadmium were compared using iTRAQ isobaric tagging technology. The results presented here will contribute to understand how the cyanobacterial cells cope with the presence and different concentrations of metals and, in the future, will help to implement metal removal systems based on cyanobacteria/cyanobacterial EPS.

2. Material and methods

2.1. Organism and culture conditions

The unicellular cyanobacterium *Cyanothece* sp. CCY 0110 (Culture Collection of Yerseke, The Netherlands) was grown in 100 ml Erlenmeyer flasks containing ASNIII medium [18] supplemented with 1 M MOPS buffer (pH 7.0), at 30 °C under a 12 h light (50 μE/m²/s)/12 h dark regimen with magnetic stirring (150 rpm). These conditions were previously identified as maximizing *Cyanothece’s* growth and EPS production [17].

2.2. Growth curves

*Cyanothece* was grown during 28 days (in the case of chronic exposure) under the conditions mentioned above (control) or with the medium supplemented with different concentrations of metals: 40, 50, 60 and 70 mg/l (5.79, 7.20, 8.64 and 10.10 μM) of lithium (Li⁺, stock solution 1000 mg/l in 2% HNO₃, PerkinElmer, MA, USA); 0.1, 0.2 and 0.3 mg/l (1.57, 3.15 and 4.72 μM) of copper (Cu²⁺, stock solution 10,000 mg/l in 1% HNO₃, Sigma-Aldrich Co., MO, USA); 1, 5, 10 and 15 mg/l (4.83, 24.10, 48.30 and 72.30 μM) of lead (Pb²⁺, stock solution 10,000 mg/l in 5% HNO₃, Sigma-Aldrich); or 1, 3, 5, 7 and 10 mg/l (8.89, 26.70, 44.50, 62.30 and 88.90 μM) of cadmium (Cd²⁺, stock solution 10,000 mg/l in 5% HNO₃, Sigma-Aldrich). For acute exposure, *Cyanothece* cells were grown in control conditions during 9 days, followed by the addition of 1 mg/l of Cu²⁺ or 50 mg/l of Cd²⁺ and the samples were collected after 24 h. Growth measurements were performed by monitoring the Optical Density (OD) at 730 nm [19] using a SmartSpec 3000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and chlorophyll a content was determined as described by Meeks and Castenholtz [20]. The total carbohydrate content and the amount of carbohydrates released to the culture medium (RPS) were measured using the phenol–sulfuric acid assay [21], as described previously [17]. All experiments were performed with three biological replicates and all experiments were performed with three biological replicates and all
measurements were performed in triplicate. Results are expressed as mean values ± standard deviation.

2.3. Transmission electron microscopy (TEM)—energy dispersive X-ray (EDX) spectroscopy

For TEM—EDX the Cyanothece cells were collected after 10, 20 and 30 days of culture, either in the absence of heavy metals (control) or in the presence of 0.1 mg/l Cu²⁺; 1 mg/l Pb²⁺; 5 mg/l Cd²⁺ or 60 mg/l Li⁺, and after 24 h of culture in the presence of 1 mg/l Cu²⁺ or 50 mg/l Cd²⁺. The cells were centrifuged and processed as described previously [22], except that samples were embedded in EMBed-812 resin and sections were examined using a JEM-1400Plus (Jeol Ltd., Inc., MA, USA). For EDX analysis, 100 nm thick sections were mounted on nickel grids and a beryllium holder (EM-21150, Jeol Ltd.) was used. An X-Max 80 mm² (Oxford Instruments, Bucks, England) operated at 80 kV was coupled to the microscope.

2.4. Atomic absorption spectroscopy

Cells were grown in ASNIII buffered medium or buffered medium supplemented with 0.1 mg/l of Cu²⁺ or 5 mg/l of Cd²⁺ (for 10 or 20 days, chronic exposure) or 1 mg/l of Cu²⁺ or 50 mg/l of Cd²⁺ (24 h, acute exposure). To measure the concentration of copper and cadmium in the supernatant, the cultures were centrifuged at 3850 g for 20 min at room temperature, the cells were discarded, and the concentration of the metals was determined using a flame atomic absorption spectrophotometer (PU 9200X, Philips Scientific, New Jersey, USA) operating at wavelengths of 232.00 nm for Cu²⁺ and 228.80 nm for Cd²⁺ quantification.

2.5. iTRAQ experimental design

The experiments comprised two biological replicates for each 8-plex iTRAQ independent experiment. Two iTRAQ studies were performed (see Fig. 1), namely the comparison of the proteomes of Cyanothece grown in the absence or presence of copper (iTRAQ study 1) or cadmium (iTRAQ study 2). The biological replicates used as control were common to the two studies (Fig. 1). Both studies comprised four phenotypes of cells grown with a 12 h light (50 μE/m²/s) and temperature, the cells were discarded, and the concentration of the metals was determined using a flame atomic absorption spectrophotometer (PU 9200X, Philips Scientific, New Jersey, USA) operating at wavelengths of 232.00 nm for Cu²⁺ and 228.80 nm for Cd²⁺ quantification.

2.6. Protein extraction and quantification

The cells were harvested by centrifugation (3850 g for 15 min at room temperature), washed with buffer (50 mM Tris, pH 7.4, 100 mM EDTA, pH 8.0, and 25% (w/v) sucrose) and re-suspended in phosphate buffer (50 mM K₂HPO₄, 50 mM KH₂PO₄, pH 6.8). The proteins were extracted using the FastPrep®-24 cell disruptor, output 6.5 m/s, 5 cycles of 30 s (MP Biomedicals, LCC, CA, USA) and glass beads (425–600 μm, Sigma-Aldrich) for mechanical cell disruption, followed by centrifugation at 16,000 g for 15 min at 4 °C. The supernatant containing the soluble proteins was recovered and stored at −80 °C. The protein concentration was measured using the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc., IL, USA) and iMark Microplate Absorbance Reader (Bio-Rad Laboratories), according to the manufacturer’s instructions.

2.7. Protein sample processing and peptide labeling with Isobaric tags for relative and absolute quantification (iTRAQ) peptide labeling reagents

Proteins were precipitated by adding 6 volumes of ice-cold acetone to 150 μg of the protein extract, resuspended in 20 μl of TEAB (triethylammonium bicarbonate, 1 M, pH 8.5) and denatured by adding 1 μl of 2% SDS. Cysteines were reduced with 2 μl of tris(2-carboxyethyl)phosphine (TCEP, 50 mM) and alkylated with 1 μl MMTS (s-methyl methanethiosulphonate, 200 mM). Subsequently, the proteins were digested with trypsin as previously described [23]. The quality and amount of proteins and the efficiency of the trypsin digestion were controlled by analyzing 20 μg of protein extract in a 10% acrylamide gels. The iTRAQ labeling of the digests, and the combining of the labeled digests into one sample mixture was performed using the manufacturer’s protocols (iTRAQ® Reagents — 8plex, AB SCIEX™, Framingham, MA, USA). iTRAQ labeling efficiency was 95.1% for iTRAQ study 1 (Cu²⁺) and 95.9% for iTRAQ study 2 (Cd²⁺). Combined samples were concentrated by vacuum (Eppendorf, Hamburg, Germany).

2.8. High-resolution hydrophilic interaction chromatography (HILIC) fractionation

Samples were resuspended in HILIC buffer A (10 mM NH₄HCO₃, 80% ACN, pH 3.0) and fractionated by HILIC using a PolyHydroxyethyl™ A column (PolyLC, Columbia, MD, USA) with 5 μm particle size, 20 cm length × 2.1 mm diameter and 200 Å pore size on a Ultimate 3000 HPLC (Thermo Scientific, formerly Dionex, Amsterdam, The Netherlands) controlled by Chromelon Software, version 6.5 (Thermo Scientific). A set of binary gradient buffers was used for liquid chromatography: buffer A (see above) and buffer B (10 mM NH₄HCO₃, 5% ACN, pH 4.0). The binary gradient began with 0% B for 10 min, followed by a linear ramp from 0 to 60% B for 30 min, an extended ramp from 60 to 100% B for 5 min, a further isocratic wash 100% B for 10 min, and column re-equilibration at 0% B for 1 min, in a total of 66 min. Injection volume was set at 20 μl with a constant chromatographic flow rate of 0.5 ml/min. Fractions were collected using a Foxy Jr. Fraction Collector (Dionex, Sunnyvale, CA, USA) in 30 s intervals across 60 min, while the chromatogram was monitored at a wavelength of 280 nm. The fractions were cleaned using C18 UltraMicroSpin Columns (The Nest Group Inc., Southborough, MA, USA) according to the manufacturer’s guidelines, prior to vacuum centrifugation (Eppendorf).
2.9. Reverse phase liquid chromatography (RPLC)–MS analysis

RPLC analysis was performed using an Acclaim® PepMap100 C18 column (Thermo Scientific) with 3 µm particle size of 15 cm length × 75 µm diameter and 100 Å pore size on a Ultimate 3000 HPLC ( Dionex), and the MS analysis was performed using QStar XL Hybrid ESI Quadrupole Time-of-Flight Mass Spectrometer, ESI-qQ-TOF-MS/MS (AB SCIEX™; MDS-SCIEX, Concord, Ontario, Canada). Samples were resuspended in RPLC buffer C (3% ACN and 0.1% TFA), injected and captured onto a 0.3 × 5 mm pre-analytical trap cartridge (5 µm C18 columns) (Thermo Scientific). Peptides were subsequently eluted using an automated gradient with a flow rate of 0.3 µl/min. Online nLC was achieved using a 150 min binary gradient with RPLC buffer A (0.1% formic acid and 3% ACN), and RPLC buffer B (0.1% formic acid and 97% ACN). A programmed gradient started with a 20 min linear ramp from 0 to 3% buffer B, 95 min ramp from 3 to 35% buffer B, a 30 s rapid ramp up to 90% buffer B, 6.5 min isotropic wash 90% buffer B, 30 s rapid ramp down to 3% buffer B, followed by 27.5 min isotropic wash 3% buffer B. Data acquisition in the mass spectrometer was set to acquire in the positive ion mode, with the precursor ion scan performed within a range of 330–2000 m/z and a selected mass detector range of 400–1250 m/z, on a predefined accumulation time of 1 s ( AnalystQS Software, AB SCIEX™). During the TOF-MS scan, two dynamically selected precursors with a +2 or +3 charge state were isolated for CID fragmentation. Samples were reanalyzed on a second LC–MS injection with identical parameters to increase sample coverage [24].

2.10. MS data analysis

Peak list conversion was performed using the mascot.dll embedded script (V1.6) coupled with Analyst QS 1.1.1 (AB SCIEX™) with MS/MS group summations and the iTRAQ region deisotoping removed. Protein identification and quantification was carried out in Phenyx v2.6 (GeneBio S.A., Geneva, Switzerland), using a database comprising all Cyanobacteria sp. CCY 0110 protein sequences obtained from UniProt (6413 entries retrieved, March 2014). General search parameters allowed for MS and MS/MS tolerance up to 0.1 Da and one missed cleavage. Fixed protein modifications included iTRAQ lysine and iTRAQ N-terminus (+304 Da) and methyl-thiol of cysteins (+46 Da), and the oxidation of methionine (+16 Da) was defined as variable modification. Acceptance threshold for peptide identification was set at peptide length ≥6, z-score ≥5 and p-value ≤1 e – 4. False discovery rate (FDR) was calculated using a decoy database automatically created by reversing the sequences from the target database, and only proteins satisfying a 1% FDR and identified with at least two peptides unique were considered for further quantitative analysis. iTRAQ
labeling efficiency was calculated using peptide data where iTRAQ lysine and iTRAQ N-terminus (+304 Da) modifications were set as variable instead of fixed, and was 95.1% and 95.9% for the copper and cadmium data sets respectively. Since iTRAQ ratios and determination of proteins altered between samples, it was carried out an in-house data analysis pipeline [25] by which protein quantifications were obtained by computing the geometric means of the reporters’ intensities. Median correction was subsequently applied to every reporter in order to compensate for systematic errors. These factors, estimated at the protein level, are used in subsequent analysis. The reporters’ intensities, in each individual MS/MS scan, were then themselves median corrected using the same factors. Since two replicates are available for each condition, a change is reported only if it is significant regardless of which replicate is chosen to perform the t test comparison. Proteins were subsequently organized into functional groups according to their Gene Ontology information available in Uniprot (http://www.uniprot.org/).

2.11. Quantification of RbcL and PsaC by Western blot analysis

The relative abundances of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) — RbcL and the photosystem I (PSI) protein PsaC were determined by Western blot analysis as described previously [26] using 3 biological replicates and 3 technical replicates, and using the same protein extracts as for the iTRAQ assays. The primary antibodies used were rabbit polyclonal antibody anti-RbcL at a 1:5000 dilution (RuBisCo Quantification Kit, Agrisera Antibodies, Vännäs, Sweden) and anti-PsaC at a 1:1000 dilution (Agrisera Antibodies), and the secondary antibody was goat anti-rat immunoglobulin G linked to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK) at a 1:5000 dilution. Blot images were acquired using the ChemiDoc™ XRS® System and analyzed using the Image Lab™ software (Bio-Rad Laboratories).

2.12. In vivo detection of ROS production

4 ml of cultures (3 biological replicates) grown in the same conditions as the ones used for iTRAQ assays, were incubated with 4 μL of 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA; Molecular Probes, Inc., Eugene, OR, USA) for 1 h in the dark at 30 °C. Stock solution contains 5 mM (w/v) of H2DCF-DA in 100% DMSO and was kept at −20 °C. The fluorescence of the samples was measured by a spectrofluorometer (FluoroMax-4®, Horiba Scientific, Japan) with an excitation wavelength of 485 nm and emission wavelengths between 500 nm and 600 nm, at 30 °C.

2.13. Superoxide dismutase (SOD) activity measurements

Proteins extracts (3 biological replicates) from cell grown in the same conditions as for iTRAQ assays were obtained using 50 mM phosphate buffer pH 6.8 supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH, Penzberg, Germany). Extraction and quantification were performed as described above. SOD gel zymography was performed as described previously [23]. Gel images were acquired using the GS-800™ Calibrated densitometer and analyzed using the Quantity One® 1-D analysis software (Bio-Rad Laboratories).

2.14. O2 evolution measurements

For the O2 evolution measurements the cells were grown in the same conditions as for the iTRAQ experiments and O2 evolution was measured using a Clark type O2 electrode (Oxygraph, Hansatech Ltd., Norfolk, UK). The calibration was performed using sodium bisulfite and air saturated water at 30 °C. Assays were carried out using 1 ml of culture under a continuous irradiance of 50 μE/m2/s. A magnetic stirrer operating at 100 rpm was used to obtain a homogeneous suspension and the temperature of the chamber was kept at 30 °C. Protein content was determined using the Lowry method [27], and the rates of O2 evolution were expressed as nmol O2/ min/ mg protein.

2.15. Statistical analysis

Data obtained in Western blots, in vivo detection of ROS production, gel zymography and O2 evolution measurements were statistically analyzed in GraphPad Prism v6 (GraphPad Software, Inc., San Diego, CA, USA) using a one-way analysis of variance (ANOVA), followed by a Tukey’s multiple comparison with a confidence level of 95% (α = 0.05). To investigate the groups of proteins with similar variation of its relative levels in the different phenotypes, a hierarchical cluster analysis was performed. For that, protein ratios were transformed into ordinal/ranked variables according to their values, namely: 0 — significant fold change < 1, 1 — no significant fold change, 2 — significant fold change > 1 and clustered using the “Centroid Linkage” method and the “Squared Euclidean Distance” measure. The cluster analysis was performed using the IBM® SPSS® Statistics 20.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Effects of different metals/metal concentrations in growth and EPS production by Cyanothece cells

To obtain a holistic perspective of the strategies (including short- and long-term mechanisms) triggered by the cells to decrease the toxic effects of different metals, different concentrations and exposure times were assessed. For this purpose, the effects of mono- (Li+) and divalent cations (Cu2+, Pb2+, Cd2+) on the growth/survival of Cyanothece cells and EPS production were evaluated. Standard high purity metal solutions were used and the cultures were buffered to maintain neutral pH throughout the experiments (for details see Material and methods). The growth was monitored measuring the OD and chlorophyll a content, and the data obtained revealed linear growth patterns. As anticipated, cell growth was negatively affected by the presence of the metals, with increasing concentrations leading to a corresponding decrease in growth rate and maximum OD obtained. Concerning the chronic exposure (up to 1 month), cells were more affected by Cu2+ (with 0.2 mg/l leading to cell death), followed by Pb2+ (10 mg/l), Cd2+ (10 mg/l) and Li+ (70 mg/l); see Fig. 2 and Supplementary Fig. 1. When the cells were exposed to 0.1 mg/l of Cu2+, the decrease in growth was more...
pronounced in first 10 days of culture with a clear recovery observed afterwards (this pattern was always observed for this sub-lethal concentration). For practical reasons, the effects of acute exposure were only assessed for one essential metal (Cu²⁺) and one non-essential metal (Cd²⁺). For this purpose, Cyanothece cells were grown for 9 days in ASNIII buffered medium, before adding 10 times the sub-lethal metal concentrations: 1 mg/l of Cu²⁺ or 50 mg/l of Cd²⁺. After 24 h, the decrease in growth (chlorophyll a content) for the cultures supplemented with Cu²⁺ and Cd²⁺ respectively, was 6.4% and 13.7% compared to the control, continuing to decline progressively after that (data not shown). In general, total carbohydrates and RPS production followed the pattern of growth (data not shown), with the RPS production reaching a maximum of 258 mg/l in the control conditions (without added metal) and constituting in average 58 ± 6% of the total carbohydrates in all cultures tested (with or without metal).

3.2. Ultrastructural changes and elemental composition analysis

TEM–EDX analyses were performed in order to observe ultrastructural changes and to identify possible sites of metal accumulation inside the cells. In the presence of sub-lethal concentrations of any of the four metals tested, significant ultrastructural changes were observed at 10, 20 and 30 days of culture, particularly in the presence of Cd²⁺ and Li⁺. For parsimony reasons, only images of cells collected after 30 days are shown in Fig. 3A–E (other results in Supplementary Fig. 2). The ultrastructural changes are mainly related to the thylakoids, with disorganization and disintegration of thylakoid membranes and increase of the intrathylakoidal space. In addition, the occurrence of inclusions was also observed. After 24 h acute exposure, the effects of Cd²⁺ are similar to those observed for chronic exposure (Fig. 3G), while the ultrastructure of the cells exposed to 1 mg/l of Cu²⁺ is altered overall (Fig. 3F). No obvious intracellular sequestration of the metals was detected by EDX spectroscopy. However, several differences can be observed by comparison of the elemental composition of the cells grown in the absence or in the presence of each metal (Fig. 4). In general, these differences were consistent in the four different areas of the cells chosen to perform this analysis, namely outer membrane, cytoplasm, thylakoids and inclusions. Carbon, oxygen and nitrogen were, as expected, the main elements observed, but also nine micro-elements (Na, Mg, Al, P, S, Cl, Ca, Fe and Cu) were detected. Overall, a higher percentage of micro-elements was detected in the cells grown in the presence of metal ions. In particular, in those grown in the presence of Li⁺ increased percentages of magnesium, aluminum, sulfur and calcium were observed (Li⁺ itself could not be detected by the technique used due to its single electron valence).

Furthermore, the amount of metals in the supernatant was evaluated by atomic absorption spectroscopy (data not shown). The results obtained showed that for chronic exposure and Cd²⁺ acute exposure most of the metals are found in the supernatant, while for Cu²⁺ acute exposure only half of the amount is found in the supernatant.
3.3. Proteomes of *Cyanothece* grown in the absence/presence of metals

To evaluate, at the molecular level, the putative effects of chronic (sub-lethal metal concentrations for 10 or 20 days) and acute (10 times the sub-lethal metal concentration for 24 h) exposure to an essential and a non-essential metal, two independent 8-plex iTRAQ studies were performed, one for Cu²⁺ (iTRAQ study 1) and another for Cd²⁺ (iTRAQ study 2). Using the stringent criteria defined in the Material and methods, 202 (98 with two or more peptides) and 268 (130 with two or more peptides) proteins were identified and quantified for studies 1 and 2, respectively. Detailed information is provided in Mota et al. [28].

To identify relatively homogeneous groups of proteins (clusters) with similar variation patterns, hierarchical cluster analyses were performed. The strength of the analyses performed was improved by including all the possible comparisons/ratios (Fig. 1), which minimizes the effects of over- or underestimated ratios and increases confidence. For each iTRAQ study, 6 statistically supported protein clusters (A–F) were formed (Fig. 5; [28]). Regarding study 1, 80% of proteins was included in cluster A1 (no significant change in any of the conditions tested), cluster B1 (no significant change...
Fig. 4 – Energy dispersive X-ray (EDX) spectroscopy of *Cyanothece* sp. CCY 0110. Weight percentage of micro-elements detected in four different areas of *Cyanothece* cells: outer membrane (A), cytoplasm (B), thylakoids (C) and inclusions (D). Cells were grown 30 days in ASNIII buffered medium (control) or the same medium supplemented with sub-lethal concentrations of copper (0.1 mg/l), lead (1 mg/l), cadmium (5 mg/l) or lithium (60 mg/l), with a 12 h light (50 $\mu$E/m$^2$/s)/12 h dark regimen, 30 °C and orbital shaking at 150 rpm.

Fig. 5 – Hierarchical cluster analysis of the proteins quantified in iTRAQ study 1 (Cu$^{2+}$ exposure) and iTRAQ study 2 (Cd$^{2+}$ exposure). Six (A–F) clusters of proteins were defined according to the variation of their relative levels in *Cyanothece* cells grown in ASNIII buffered medium supplemented with 0.1 mg/l of Cu$^{2+}$ or 5 mg/l of Cd$^{2+}$ (for 10 or 20 days, chronic exposure) or 1 mg/l of Cu$^{2+}$ or 50 mg/l of Cd$^{2+}$ (24 h, acute exposure). The number of proteins in each cluster is indicated in the right columns. Clusters were calculated using all ratios to minimize over- or underestimations. Data were converted into ordinal/ranked variables and clustered using the “centroid linkage” method and the “squared Euclidean distance” measure.
in 10 and 20 days chronic exposure, and higher abundance in acute exposure) and cluster C1 (no significant change in 10 and 20 days chronic exposure, and lower abundance in acute exposure). Overall, the acute exposure was the condition that promoted more quantitative proteome changes — 19%. Concerning study 2, 87% of the proteins were found in cluster A2 (no change in any of the conditions) and cluster B2 (lower abundance in 10 and 20 days chronic exposure). A number of proteins were also grouped in cluster C2 (higher abundance in 10 and 20 days chronic exposure). In contrast with what was observed for Cu$^{2+}$, in study 2 — Cd$^{2+}$, the 10 and 20 days chronic exposure were the conditions that caused more differential protein expression, 12% and 13% respectively. 

To gain insight into the biological significance of the changes observed, the proteins were grouped according to their annotated function and the Gene Ontology information. The majority of the proteins with known functions were associated with photosynthesis, CO$_2$ fixation and carbohydrate metabolism, translation, and nitrogen and amino acid metabolism (Figs. 6A and 7A, Table 1). Nevertheless, 27% and 24% of the proteins identified/quantified for studies 1 and 2 respectively, remain uncharacterized. It was also observed that, within each functional category, the number of differentially expressed proteins in metal-exposed cells compared to the control were different in iTRAQ study 1 — Cu$^{2+}$ exposure and iTRAQ study 2 — Cd$^{2+}$ exposure (Figs. 6B and 7B).

Several proteins involved in photosynthesis were reliably identified and quantified (Table 1; [28]). The levels of PSI and PSII components were differentially affected by the metals tested, generally increasing in cells after acute Cu$^{2+}$ exposure.

**Fig. 6** — Distribution and fold changes of proteins identified and quantified in iTRAQ study 1 (Cu$^{2+}$ exposure). (A) Distribution by functional groups — the total number of proteins and the number in each group is indicated. (B) Percentage of proteins with significant fold changes in each functional category compared to the control (no metal supplementation).
cluster B1) and remaining constant in all Cd^{2+}-exposed phenotypes (cluster A2). Likewise, the levels of the components of the phycobilisomes (PBS) were also differentially affected, but in this case did not change significantly when the cells were exposed to Cu^{2+} (most grouped in cluster A1, even though the levels of some components were lower after 10 and 20 days chronic exposure, clusters C1 and D1), and generally decreased in Cd^{2+} chronic exposure conditions (cluster B2). Interestingly, for ATP synthase, the levels of the alpha and beta subunits responded to Cu^{2+} metal stress, increasing in acute exposure. In contrast, the ferredoxin-NADP reductase (FNR) followed a converse pattern, decreasing in Cu^{2+} exposure and Cd^{2+} at 20 days chronic exposure. Concerning plastocyanin, the levels increased in acute Cu^{2+} exposure and decreased in all Cd^{2+} stress conditions.

In agreement with that observed for the photosynthesis related proteins, the levels of enzymes involved in CO₂ fixation and carbohydrate metabolism were also differentially affected by Cu^{2+} and Cd^{2+}. The levels of the large subunit of RuBisCo (RbcL) decreased in Cu^{2+}-exposed cells, as well as in 20 days chronic Cd^{2+} stress. Considering the glycolytic enzymes, one glyceraldehyde-3-phosphate dehydrogenase and the phosphoglycerate kinase decreased in acute Cu^{2+}-exposed cells, whereas the phosphoglucomutase levels increased in chronic conditions. The levels of the later enzyme also decreased in Cd^{2+}-exposed cultures. On the other hand, the levels of fructose-bisphosphate aldolase and sodium-dependent bicarbonate transporter decreased in all Cd^{2+} stress conditions.

Fig. 7 – Distribution and fold changes of proteins identified and quantified in iTRAQ study 2 (Cd^{2+} exposure). (A) Distribution by functional groups — the total number of proteins and the number in each group is indicated. (B) Percentage of proteins with significant fold changes in each functional category compared to the control (no metal supplementation).
### Table 1 – List of quantified proteins obtained for iTRAQ studies 1 and 2 and discussed in this work.

<table>
<thead>
<tr>
<th>Gene name/locus tag</th>
<th>Uniprot ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein (gene product)&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>iTRAQ study 1 (Cu&lt;sup&gt;2+&lt;/sup&gt; exposure)</th>
<th>iTRAQ study 2 (Cd&lt;sup&gt;2+&lt;/sup&gt; exposure)</th>
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<td></td>
<td></td>
<td>10d/ control 20d/ control Acute/ control Cluster&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10d/ control 20d/ control Acute/ control Cluster&lt;sup&gt;d&lt;/sup&gt;</td>
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<th>iTRAQ study 2 (Cd^{2+} exposure)</th>
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</table>
The relative levels of glutamine synthetase (GS), the key enzyme involved in amino acid/nitrogen metabolism, increased after chronic Cd\(^{2+}\) exposure.

Although only four proteins involved in nucleotide sugar metabolism were identified, their levels were unchanged in all metal-exposed conditions. These results are consistent with the constant rate of RPS production observed for metal-supplemented cultures of *Cyanothece*.

The abundance of most of the chaperones identified did not change in Cd\(^{2+}\) stress conditions, with the exception of one of the GroEL isoforms, which was present in higher levels in 20 days and acute Cd\(^{2+}\) exposure and the DnaK which was present in higher levels in acute Cd\(^{2+}\) exposure. The abundance of these proteins and GroES was also affected by Cu\(^{2+}\), with increased levels at 10 days and different fold changes in acute exposure.

Regarding the cell’s anti-oxidative defenses, the levels of two ferritin and Dps proteins were differentially altered in 20 days Cd\(^{2+}\) exposure, while the abundance of rehydrin decreased in all metal-exposed cells. SOD and a thioredoxin increased in Cu\(^{2+}\) acute stress conditions.

A couple of proteins involved in the acquisition and metabolism of inorganic nutrients such as sulfur and iron increased in Cu\(^{2+}\) acute stress, while four proteins involved in translation were differentially expressed. Moreover, two ribosomal proteins decrease and a long-chain-fatty-acid CoA ligase increased in Cd\(^{2+}\) chronic exposed cells.

It is worth to notice that the levels of 22 uncharacterized proteins were also affected by metal stress.

---

### Table 1 (continued)

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<thead>
<tr>
<th>Gene name/locus tag</th>
<th>Uniprot ID(^{b})</th>
<th>Protein (gene product)(^{c})</th>
<th>iTRAQ study 1 (Cu(^{2+}) exposure)</th>
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<td>A3IVT9</td>
<td>Rhodanese-like protein</td>
<td>1.00 1.00 7.37</td>
<td>1.00 1.00 1.00</td>
</tr>
</tbody>
</table>

\(^{a}\) Proteins with ratio \(\neq 1.00\) are considered differently expressed according to the stringent criteria defined in this work (see Material and methods) [25]. For easier interpretation of the data, the fold-change values of proteins that did not passed the significance threshold criteria were set to 1.00.

\(^{b}\) Accession number according to UniProt database.

\(^{c}\) Protein name according to UniProt database.

\(^{d}\) Cluster membership according to the hierarchical cluster analysis performed with all ratios (i.e. not only metal-exposed/control, but also metal-exposed 1/ metal-exposed 2). The number of unique peptides and peptides spectral matches is provided in Mota et al. [28].
3.4 iTRAQ validation studies: PsaC and RbcL relative levels, ROS content, SOD activity and O2 evolution measurements

Western blot analyses were performed to further confirm the fold change directions observed for PsaC and RbcL in the iTRAQ studies. In general, the data obtained are in agreement with that of the iTRAQ; the levels of the PSI component PsaC were shown to increase significantly in Cu2+ acute stress (Fig. 8), whereas the levels of RuBisCo decreased in acute Cu2+ exposure, as well as in 20 days chronic Cd2+ stress (Fig. 8). The decrease in RuBisCo observed for Cu2+ chronic exposure in iTRAQ study 1 is also observed in the Western blot although not statistically significant.

Given the effects of the metals in the abundance of several proteins involved in cell redox homeostasis, the levels of intracellular ROS and the activity of SOD were also assessed (Fig. 9). The amount of the fluorescent probe, and thus of ROS, was significantly higher in Cd2+ 20 days chronic exposure (Fig. 9). Concerning SOD, gel zymography assays revealed that the activity of this enzyme increases in Cu2+ acute stress and in 20 days and acute Cd2+ exposure. However, these results are only supporting iTRAQ study 1 since no variation was observed for the levels of SOD in iTRAQ study 2.

The levels of O2 evolution drastically decreases when the cells are exposed to Cu2+ for 10 days but it is possible to observe a recovery after 20 days (Fig. 10). The effects of Cu2+
acute exposure were much more severe with no net O\textsubscript{2} evolution observed, similar to what was observed for Cd\textsuperscript{2+} (Fig. 10).

### 4. Discussion

The different tolerances exhibited by Cyanothece cells grown in the presence of the metals studied (Cu\textsuperscript{2+} < Pb\textsuperscript{2+} < Cd\textsuperscript{2+} < Li\textsuperscript{+}) are probably related to the specific nutritional requirements/mechanisms of metal uptake and accumulation in the cells [14,29].

The addition of 0.1 mg of copper per l of culture medium affected negatively the growth of Cyanothece cells. Similar concentrations had also toxic effects in Spirulina platensis-S5 [30] Nostoc punctiforme PCC 73120 cells [31], and Gloeothecae sp. PCC 6909 [32]. Cu\textsuperscript{2+} is an essential micronutrient that acts as a cofactor for several proteins/enzymes, such as plastocyanin and cytochrome c oxidase, involved in the oxygenic photosynthetic electron transfer chain [33,34]. Therefore, in contrast with other bacteria in which most of Cu\textsuperscript{2+}-containing proteins are located in the plasma membrane or the periplasm, in cyanobacteria this metal is required at the thylakoid level, which imposes further complexity to its homeostasis [33–36]. In cyanobacteria, Cu\textsuperscript{2+} is imported into the cell by a P\textsubscript{1}-type ATPase (CtaA), then chaperoned by Atx1 until the thylakoids, and finally transported into the thylakoids lumen by a second P\textsubscript{1}-type ATPase (PacS) [33,35–37]. However, it is well established that an excess of Cu\textsuperscript{2+} can be toxic to the cells by promoting the generation of ROS and/or competing for the binding sites of superoxide dismutase (SOD) activity measurements.
metallothioneins harboring other metals, impairing cell function and eventually causing cell death [29,38]. To cope with the excess of Cu^{2+}, bacterial cells developed several efflux mechanisms to regulate its intracellular concentration [39]. In cyanobacteria, one family of cation-transporting CPx-type ATPases is known to be involved in the export of Cu^{2+} [15]. Moreover, in Synechocystis sp. PCC 6803, it was shown that a two-component system (CopRS) is essential for copper resistance by regulating the expression of a Cu^{2+} efflux system (HME-RND: heavy-metal resistance-nodulation-cell division) [35].

The addition of 1 mg/l of lead resulted in growth impairment of Cyanothece cells. The effect of this sub-lethal concentration was previously observed in Anabaena flos-aquae [40], and in Spirulina platensis-S5 concentrations down to 0.2 mg/l also affected growth [30]. In contrast, for Gloeothecae sp. PCC 6909 and its sheathless mutant CCY 9612 the effects of the presence of the metal were only observed for concentrations of 10 mg/l and 20 mg/l, respectively [32]. The authors also demonstrated that the RPS of these strains have a high affinity to Pb^{2+} what could explain the higher tolerance. Pb^{2+} has no known biological function and its toxicity has been linked to the inactivation of the cellular antioxidant pool and disruption of metabolic balance leading to inhibition of photosynthetic activity [30,32,40-42]. However, the mechanisms by which the metal enters the cells are not fully characterized, but one possible explanation is that the uptake of Pb^{2+} is performed by unspecific binding to importing ATPases [39]. In bacteria, two DNA-binding Cd^{2+}/Pb^{2+}-sensing transcriptional repressors (CadC and CmtR) are described to trigger the de-repression of genes encoding proteins involved in the export of these ions [36,39]. In cyanobacteria, another family of CPx-type ATPases, than the one mentioned above, is known to be involved in the export of divalent cations such as Pb^{2+} and Cd^{2+} [15].

In our study, levels of cadmium higher than 3 mg/l influenced negatively Cyanothece growth, while for Nostoc punctiforme PCC 73120 [31], Anabaena flos-aquae [40] and Microcystis aeruginosa Kütz 854 [43] one order of magnitude lower concentrations were found to be lethal. Cd^{2+} is a non-essential metal with an uptake system barely understood. In bacteria and plants, it is described that Cd^{2+} can enter the cell by uptake systems used by other cations, such as magnesium, manganese and calcium [29]. The cells tolerance to Cd^{2+} can be due to mechanisms already discussed above for other metals, but also be achieved by binding to metallothioneins. In bacteria, the first metallothionein to be characterized was a Cd^{2+} binding protein found in the unicellular cyanobacterium Synechococcus sp. [29,44].

Cyanothece growth was barely affected by the presence of lithium, only concentrations as high as 70 mg/l resulted in cell dead. There is not much information about Li^{+} biological function and/or uptake and efflux in cyanobacterial cells. One study in Synechocystis sp. PCC 6803 hypothesized that Li^{+}, and other chemically similar monovalent cations, could be uptaken via a potassium transport system [45].

Overall, Cyanothece cells are able to cope with different metals/different metal concentrations. Most probably, the high amount of RPS present in the medium contributes to this tolerance, since a positive correlation between EPS production and metal tolerance has been observed for other cyanobacteria [46,47]. It is worthwhile to mention that Cyanothece is one of most efficient RPS producers [17]. These polymers, with their overall anionic charge constitute an effective sink decreasing the amount of metal in solution [8,32]. In addition, a recent work has demonstrated that EPS can also protect the cells from metal toxicity by an indirect mechanism that involves Fe homeostasis [48].

The ultrastructural changes observed by TEM in Cyanothece cells grown in the presence of metals, namely the increase of the intrathylakoidal space and the formation of thylakoid membrane vesicles (Fig. 3), are similar to the ones observed for several cyanobacterial strains exposed to other stress factors such as high light intensities or changes in the wavelength [49]. Indeed, as observed for high light intensities, the presence of heavy metals is known to induce a change in the redox status of the electron transport chain, which influences the structure and functional organization of the thylakoids [14]. In addition, a higher number of inclusions were also observed. Ultrathin sections of other cyanobacterial strains grown in the presence of Pb^{2+} or Cu^{2+} also showed expanded thylakoids and an increase in inclusions/polyporphosphate bodies [32,50]. Polyporphosphate, which has a negative surface charge can provide binding sites for heavy metals, helping in the detoxification of metals [9]. No intracellular sites of metal accumulation were detected by EDX spectroscopy in Cyanothece cells (Fig. 4), most probably due to the reduced sub-lethal metal concentrations added to the cultures. In agreement, Maldonado et al. only observed intracellular accumulation of Pb^{2+} in different
cyanobacteria by increasing the concentration of the metal up to 10 mM [50]. Another plausible reason is the existence of efficient metal efflux systems, that allow the cells to cope with metal toxicity at the concentrations/time-points tested. The results from atomic absorption spectroscopy corroborate this hypothesis.

The comparison of the proteomes of cells grown in the absence or presence of an essential metal (Cu²⁺) and a non-essential metal (Cd²⁺) was performed by iTRAQ. The inclusion of the same control replicates in study 1 (Cu²⁺ exposure) and study 2 (Cd²⁺ exposure) allows the direct comparison of the data between studies (see Fig. 1).

Regarding study 1, the acute exposure was the condition that promoted more quantitative changes and stronger O₂ evolution impairment, despite the modest growth inhibition observed 24 h after adding the Cu²⁺ to the culture. This strongly suggests that the cells are already coping with the metal, as supported by TEM and atomic absorption spectroscopy results. Conversely, the higher number of proteins with unchanged levels after 10 and 20 days chronic Cu²⁺ exposure suggests that those conditions are less severe to the cells, allowing the growth recovery observed at day 20. Similar growth recovery has been observed for Anabaena dolium exposed to Cu²⁺, and this recovery was correlated with a decrease of the intracellular concentration of the metal [51]. Metal efflux is recognized as the main bacterial resistance mechanism to overcome the toxicity of transition metal ions [52]. After 24 h of acute Cu²⁺ stress, the levels of the majority of PBS components were unchanged, whereas the levels of several PSI and PSII proteins increased. On the other hand, the O₂ evolution rate ceases as previously observed in other cyanobacteria [51,53]. Thus, it is possible that the increased levels of PSI and PSII proteins arise from the need to replace inactivated PS components. The increase of plastocyanin levels is one of the strategies used by the cells to reduce the free intracellular Cu²⁺ concentration, with this Cu²⁺-containing protein replacing cytochrome C₅₆ in the electron transfer [36,54-56]. The concomitant increase in the levels of the alpha and beta subunits of ATP synthase and decrease of the FNR abundance strongly points towards an imbalance of the ATP:NADPH ratios. This assumption is consistent with the lower abundance of the RuBisCO large subunit. Likewise, the lower levels of glycolysis-related proteins suggest a decrease in the metabolic flux through this pathway, which results in lower amounts of phosphoenolpyruvate for the tricarboxylic acid (TCA) cycle, and consequently less 2-oxoglutarate (2-OG) [57]. This compound was identified as metabolic signal for controlling the carbon:nitrogen balance of the cells, linking carbohydrate metabolism to nitrogen assimilation [58]. Therefore, it is likely that nitrogen fixation is somehow impaired, even if the levels of the GS did not change according to the stringent criteria of significance imposed in this work [25]. The metabolic rearrangements discussed above may result in an energy investment in metal extrusion and are in agreement with the previous reported low intracellular levels of ATP in Cu²⁺ exposed cells [51]. By comparing the results obtained for Cu²⁺ acute and chronic exposure, it is possible to predict a recovery of photosynthetic activity during chronic exposure, as supported by the unchanged levels of PSI and PSII components and an increase in O₂ evolution from 10 to 20 days. Most probably, the lower abundance of PBS also contributes to this recovery, by allowing the cells to reduce the amount of energy that reaches the photosynthetic apparatus, thus preventing and/or minimizing ROS-induced damages [16,55]. This prediction is also supported by the reduction in the number of proteins involved in carbohydrate metabolism in Cu²⁺ chronic exposure compared to the control, although the levels of RbcL are still lower. The degradation of PBS also releases cellular components, which can be recycled and used in the production of proteins necessary for Cu²⁺ detoxification.

The levels of chaperones during Cu²⁺ exposure are also affected. Chaperones are known to mediate the correct assembly of several polypeptides [59]. In cyanobacteria, the heat-shock proteins GroEL and GroES are the most prominent chaperones, being up-regulated in a need-based manner [60]. The increased levels of GroES in Cu²⁺ acute stress strongly suggest that this protein is involved in the mitigation of the metal toxic effects. Conversely, the lower levels of both GroEL isoforms are in contrast with previous works that report increased GroEL levels under this stress condition [61]. It is possible that under metal stress these proteins might undergo a different time-dependent induction, with GroES being induced prior to GroEL. The fold changes observed for these proteins in cells exposed to 10 days chronic Cu²⁺ stress further support this assumption. Regarding DnaK, the unchanged levels observed after acute Cu²⁺ stress and higher abundance after 10 days chronic exposure may be an indication that this protein is involved in long-term Cu²⁺ resistance. Noteworthy, the increased levels of a yet uncharacterized chaperon in acute Cu²⁺ conditions may unveil a new player in the mitigation of the effects resulting from metal stress.

As expected, Cu²⁺ exposure also lead to an unbalance of ROS. The increased levels and activity of Cyanothece FeSOD in Cu²⁺ acute conditions emphasize the stress imposed by the metal to PS machinery, and is in agreement with that described for other cyanobacteria grown in contact with heavy metals [30,51,62-65].

Ten days chronic and acute Cu²⁺ exposure also affected the metabolism of other inorganic elements, such as sulfur, phosphate and iron. The changes in the levels of sulfur- and iron-related proteins may be related with an increased demand for these two elements, in order to synthesize or repair damaged Fe-S clusters, including those present in PS components [55,66]. However, further analysis needs to be carried out to elucidate these interactions.

Concerning Cd²⁺ exposure, the results differ substantially of those obtained for Cu²⁺. During the chronic exposure the cells seem to accumulate damages and a recovery is not observed as for Cu²⁺. In contrast, after acute exposure the levels of most proteins remained unchanged. TEM results showed that after 24 h acute metal exposure cells have already ultrastructural changes at the thylakoid level, as supported by the impairment in O₂ evolution, although the modest growth inhibition and the proteome data suggest that these effects are still insufficient to trigger a significant activation of metal detoxification mechanisms and impair growth. The decreased levels of PBS components in Cd²⁺ chronic exposure are consistent with the need to recycle protein components, as previously suggested for similar conditions [43,55,62]. The unchanged levels of PSI and PSII
components in all Cd\(^{2+}\)-exposed conditions, together with the impairment in O\(_2\) evolution, are in agreement with reports that indicate that the site for Cd\(^{2+}\)-induced inhibition of photosynthesis is beyond PSI [43,67]. Despite that, the key target is controversial, with some studies suggesting that the metal interacts with the sulfhydryl groups of FNR, while others rule it out as primary target [43,67]. Our data is consistent with a time-dependent degradation of FNR, consequently decreasing NADPH availability. The decrease of plastocyanin levels strongly suggests this protein is not the main electron carrier between cytochrome b\(_6\)f complex and PSI in Cd\(^{2+}\) exposure conditions. Regarding carbohydrate metabolism, the lower levels of RcbI, after 20 days chronic Cd\(^{2+}\) exposure are consistent with a long term negative effect in CO\(_2\) fixation. This decrease in carbon assimilation seems to be a widespread response of cyanobacteria to Cd\(^{2+}\) exposure [52,62,67,68]. On the other hand, the effects of this metal on other carbohydrate metabolic pathways are more difficult to predict. The decreased levels of fructose-biphosphatase aldolase and phosphoglycerate kinase, in all Cd\(^{2+}\) conditions, seem to point out to a metal-induced reorganization of the carbon flux. Regarding the first enzyme, it is possible that Cd\(^{2+}\) replaces Zn\(^{2+}\) in the enzyme active center, as previously observed for Escherichia coli [69]. This overall shift in the route followed by carbon compounds seems to be more accentuated after 20 days chronic stress, as suggested by the higher levels of glucose-6-phosphate isomerase. Previous works on Cd\(^{2+}\)-induced toxicity in cyanobacteria indicate a decrease in carbohydrate metabolism, which extends to nitrogen assimilation to save energy and reducing power and prevent the poisoning incorporation of Cd\(^{2+}\) in metalloenzymes [52,62,68]. On the contrary, in this work, the levels of GS were shown to increase after chronic exposure to Cd\(^{2+}\), remaining unchanged after acute stress. Nonetheless, it is important to point out that those studies were performed in other cyanobacterial strains and that the period of metal-exposure was always considerably shorter, usually up to 48 h. Overall, the data obtained here for Cyanothece are consistent with a time-dependent increase in nitrogen assimilation.

As observed for Cu\(^{2+}\)-exposure conditions, the levels of one GroEL and DnaK changed after Cd\(^{2+}\) exposure, suggesting that these chaperones are also involved in the response to Cd\(^{2+}\). However, the higher abundance of these proteins in acute stress conditions, points out to a rapid activation of their regulatory mechanisms, rather than the time-independent induction observed for Cu\(^{2+}\). Although the levels of the majority of the proteins involved in ROS detoxification was unchanged after chronic exposure to Cd\(^{2+}\) (including SOD), the increased ROS levels after 20 days chronic stress and higher SOD activity in both 20 days chronic and 24 h acute exposure, unequivocally show that Cd\(^{2+}\) affects ROS homeostasis. The high extent of the cellular damages under these conditions is also evident in the high levels of a long-chain-fatty acid CoA ligase, which is involved in the activation of long-chain fatty acids for synthesis and degradation of cellular lipids, including the activation of endogenous free fatty acids released from membrane lipids [70].

5. Conclusions

Our study demonstrates that different heavy metals affect Cyanothece sp. CCY 0110 cells differently, and the response triggered to cope with these metals is also quite distinctive. Independently of the metal tested, sub-lethal concentrations affect the photosynthetic-driven ATP synthesis) contributes to the lower efficiency of the mechanisms of Cd\(^{2+}\) detoxification compared to those of Cu\(^{2+}\). However, one should also bear in mind that Cd\(^{2+}\) is a non-essential metal and therefore the cells might not have the same capacity to deal with it. Despite the different effects of the different metals/metals concentrations and physiological responses of the cells, the amount of EPS produced still follows the growth pattern, suggesting that the relationship between central carbon metabolic pathways and EPS production is not straightforward.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.03.004.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

This work was funded by FEDER Funds through the Operational Competitiveness Programme — COMPETE and by national funds through FCT — Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-028314 (PTDC/BIA-MIC/2889/2012) and the scholarships SFHR/BD/84914/2012 and SFRH/BDP/72400/2010. We thank Professor Lucas Stal for providing Cyanothece sp. CCY 0110. Sheffield acknowledges the EPSRC (EP/E036252/1).

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