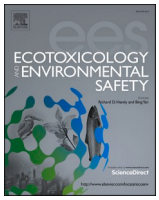
Ecotoxicology and Environmental Safety 233 (2022) 113336

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Changes to the amino acid profile and proteome of the tropical freshwater microalga *Chlorella* sp. in response to copper stress

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ARTICLE INFO

Edited by Paul Sibley

Keywords:
Aquatic toxicology
Proteomics
Biomolecular changes
Dose response
Metabolic pathways
Sublethal toxicity

ABSTRACT

Contamination of freshwaters is increasing globally, with microalgae considered one of the most sensitive taxa to metal pollution. Here, we used 72 h bioassays to explore the biochemical effects of copper (Cu) on the amino acid (AA) profile and proteome of *Chlorella* sp. and advance our understanding of the molecular changes that occur in algal cells during exposure to environmentally realistic Cu concentrations. The Cu concentrations required to inhibit algal growth rate by 10% (EC_{10}) and 50% (EC_{50}) were 1.0 (0.7–1.2) μg L^{-1} and 2.0 (1.9–2.4) μg L^{-1}, respectively. The AA profile of *Chlorella* sp. showed increases in glycine and decreases in isoleucine, leucine, valine, and arginine, with increasing Cu. Proteomic analysis revealed the modulation of several proteins involved in energy production pathways, including: photosynthesis, carbon fixation, glycolysis, and oxidative phosphorylation, which likely assists in meeting increased energy demands under Cu-stressed conditions. Copper exposure also caused up-regulation of cellular processes and signalling proteins, and the down-regulation of proteins related to ribosomal structure and protein translation. These changes in biomolecular pathways have direct effects on the AA profile and total protein content and provide an explanation for the observed changes in amino acid profile, cell growth and morphology. This study shows the complex mode of action of Cu on *Chlorella* under environmentally realistic Cu concentrations and highlights several potential biomarkers for future investigations.

1. Introduction

Metal contamination of freshwater poses a significant global threat to aquatic organisms. Contamination of aquatic ecosystems with copper (Cu) occurs due to mining and its wide use in the construction industry, as an antifouling agent, and in agriculture (Yong et al., 2020). Algae, in general, are one of the most sensitive organisms to metal contamination (Franklin et al., 2000). Copper is an essential micronutrient to algae at low concentrations; however, it becomes toxic when its concentration is elevated, with the sensitivity varying between species (Hamed et al., 2017; Yong et al., 2020). The bioavailability and toxicity of Cu are also controlled by the aquatic chemical environment, including: pH (Franklin et al., 2000), hardness, alkalinity (Riethmuller et al., 2000) and dissolved organic carbon concentration (Macoustra et al., 2019, 2020).

Freshwater microalgae are at the base of the aquatic food web, and therefore, play a key role in aquatic ecosystems. Exposure of microalgae to Cu triggers a wide range of morphological and biological responses such as: impairment of photosynthesis due to reduced chlorophyll synthesis and inhibited photosynthetic efficiency (Pfeiffer et al., 2018), growth inhibition, inhibition of enzyme activity, cell wall thickening, increase in cell size and (ultimately) cell death (Levy et al., 2007; Long et al., 2019; Miazek et al., 2015). Copper is also known to produce reactive oxygen species (ROS) that degrade algal proteins, lipids and nucleic acids (Contreras et al., 2010; Franklin et al., 2002; Hamed et al., 2017). Recent studies have shown that exposure to metals also alters the proteome and abundance of certain AAs within freshwater algae such as *Chlorella* (Khatiwada et al., 2020; León-Vaz et al., 2020; Yong et al., 2020). Amino acids are one of the critical biomolecules transferred up the food chain. Therefore, any changes to the AA profile brought about

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https://doi.org/10.1016/j.ecoenv.2022.113336
Received 21 October 2021; Received in revised form 23 January 2022; Accepted 18 February 2022
Available online 26 February 2022

by exposure to metallic contaminants may also have cascading effects on the overall aquatic food web (Yong et al., 2020; Zhang et al., 2015). Although several studies have reported changes in certain AAs within algae in the presence of metals, the impact on the entire AA profile and associated proteome when exposed to sublethal Cu concentrations has not yet been studied.

In this study, we investigated the toxicity of Cu and its influence on the AA profile and proteome of the green alga *Chlorella* sp. 12 (metrics: growth rate, cell size, cell granularity, chlorophyll content). The results of this study advance our understanding of the molecular changes that occur during exposure to environmentally realistic Cu concentrations ($\leq$ 2 μg L^{-1}; the 95% protection guideline value for Cu in Australia is 1.4 μg L^{-1})(ANZG, 2018; Macoustra et al., 2019) and provides the basic foundation needed for further investigations regarding the molecular regulatory mechanisms underlying the metal stress response in this green algal species.

2. Material and methods

2.1. Algal culture

The culture of the tropical unicellular freshwater green alga *Chlorella* sp. (isolate 12; henceforth called '*Chlorella*') used in this study was obtained from CSIRO Land and Water (Lucas Heights, Australia) and isolated initially from Lake Aesake, Papua New Guinea (Macoustra et al., 2019). The stock culture was maintained in autoclaved Jaworski medium (2/5 strength: Supporting Information (SI) Table S1) at 27 ± 1 °C on a 12 h/12 h light/dark cycle (140 μM photons/m^2/s; daylight fluorescent lighting) (Macoustra et al., 2019), with the alga recultured once per week.

2.2. Growth inhibition bioassays

The toxicity of Cu to *Chlorella* was determined using 72 h growth inhibition bioassays (Franklin et al., 2000; Golding et al., 2018). These tests were conducted in a synthetic softwater medium (80–90 mg $CaCO_3$ L^{-1}), supplemented with nitrate (15 mg NO_3^- L^{-1}; $NaNO_3$) and phosphate (0.15 mg PO_4^{3-} L^{-1}; KH_2PO_4), prepared from analytical grade reagents using a modified USEPA recipe (Weber, 2002) (SI Table S2) in Milli-Q water (18.2 MΩ.cm, Merck Millipore), filtered (0.45 μm pore-size, Millipore MF) and pH adjusted to 7.3 ± 0.1 (Golding et al., 2018). The test medium was stored in a sealed acid washed glass container and refrigerated (4 °C) until use.

Growth inhibition and amino acid bioassay experiments were conducted in 250 mL Erlenmeyer flasks pre-coated with coatasil silanising solution (APS Ajex Finechem) to reduce metal adsorption and acid-washed with 10% concentrated HNO_3 (Sigma-Aldrich) before use. A stock solution of Cu (II) (5 mg Cu L^{-1}) was prepared from copper sulfate ($CuSO_4$.5 H_2O, Merck) and acidified with HCl (Sigma-Aldrich) (pH < 2) to ensure that Cu ions remain in solution in dissolved state. Bioassay experiments consisted of 7 different concentrations of Cu (1, 2, 3, 4, 5, 8 and 12 μg L^{-1}; 5 replicates each) together with controls, prepared by diluting Cu stock with synthetic softwater (Franklin et al., 2000). Samples for Cu analysis were taken at the beginning and end of experiment, filtered through 0.45 μm pore-size glass fibre filters (Wattman) and preserved with 0.2% ultrapure HNO_3 before analysis.

Chlorella in exponential growth was harvested from a 5–7 day old stock culture by centrifugation (170 × *g*; 7 min; Spintron GT-175BR; 3 × wash in synthetic softwater). Each test flask was inoculated to give an initial cell density of 2–4 × 10^3 cells mL^{-1} and incubated in a growth cabinet (27 °C; 12 h/12 h light/dark cycle; 140 mol photons $m^{-2}s^{-1}$) for 72 h. Flasks were swirled and subsampled every 24 h for algal cell density counts.

Algal cell density was measured by a Beckman Coulter Cytoflex Flow Cytometer (Beckman Coulter, US). A bead tube (3 drops of CytoFLEX Daily QC Fluorospheres + 1 mL of Milli-Q water) was measured daily and used as quality assurance. Algal cells were counted by drawing a polygon based on side scatter (SSC-A) vs forward scatter (FSC-A) around a healthy *Chlorella* population, excluding any non-algal and dead cells (Franklin et al., 2005). Cell density was obtained by counting for 120 s, using a flow rate of 10 μL min^{-1}. The population doubling rate (doublings per day) was determined from the slope of $\log_{10}$ cell density vs. time (h) regression for each sample (Franklin et al., 2002). Population doubling rate, expressed as a percent of control, was used as the test endpoint (Franklin et al., 2000). Pellets of the remaining algal cells were collected at the end of the 72 h growth bioassays by centrifugation (170 × *g*; 7 min; 3 × wash in Milli-Q water) and frozen at - 80 °C for analysis of AA profile. Samples treated with 8 μg L^{-1} and above were not used for AA analysis.

2.3. Copper analysis

Dissolved Cu was determined by graphite furnace atomic absorption spectrometry (GFAAS) using a GTA 120 Agilent Graphite tube Atomizer coupled to a 240 FS Atomic Absorption Spectrometer (Agilent, Australia) (Cu detection limit = 0.5 μg L^{-1}). Each Cu analysis batch included multi-point calibrations using certified standards (Quality Control Standard 21, PerkinElmer Pure), blanks and quality control samples having concentration of 12 μg Cu L^{-1} for quality assurance (QA) purposes.

2.4. Amino acid (AA) analysis

The determination of AAs: alanine (Ala), aspartic acid (Asp) + asparagine (Asn) expressed as Asx, arginine (Arg), glycine (Gly), glutamic acid (Glu) + glutamine (Gln) expressed as Glx, histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), proline (Pro), cystine (Cys-Cys), phenylalanine (Phe), serine (Ser), threonine (Thr), tyrosine (Tyr), valine (Val) and tryptophan (Trp) were based on sample processing and analysis techniques previously described in Dwyer et al. (2018). One mL of algal sample with known cell density was freeze-dried in pyrolysed tubes (550 °C) before hydrolysis with 6 N HCl containing 0.02% phenol at 110 °C for 24 h under an argon atmosphere (Fountoulakis and Lahm, 1998). The acid was removed using a rotary vacuum concentrator (40 °C; 4.5 h; RVC 2–18 CDplus; Martin Christ, Germany), the AA mixture was reconstituted with 80 μL 3:1 borate buffer: Milli-Q solution (pH 9, Merck Centripur, Germany) and derivatized with 20 μL 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC; 3 mg mL^{-1}). Derivatized samples were heated at 55 °C for 10 mins and diluted (2 ×) with 0.1% formic acid prior to chromatographic analysis.

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) consisted of a Shimadzu Nexera X2 UPLC coupled to a Shimadzu 8045 triple quadrupole mass spectrometer operated in positive ion electrospray ionisation (ESI) mode with multiple reaction monitoring (MRM) (Shimadzu corporation Kyoto, Japan). Separations of AAs was achieved using gradient elution (0.55 mL min^{-1}) through a Waters Aquity UPLC BEH C18 column (2.1 ×150 mm; pore size 1.7 μm) maintained at 50 °C (2 μL injection volume; 10 min run time). For the mass spectrometer, neutralizer gas flow, the gas temperature, drying gas flow and interface voltage were set at 3.0 L min^{-1}, 275 °C, 17.0 L min^{-1}, 2.5 kV, respectively. LabSolutions software (Shimadzu, Tokyo, Japan) was used for system control and data analysis.

Calibration standards were prepared from an AA standard H (Waters Corporation) spiked with Glu, Asp and Trp, prepared at final concentrations of 0.01–2 pmol μL^{-1}. Reagent blanks (20 μL of 0.1% formic acid, 60 μL borate buffer and 20 μL AQC), 0.1% formic acid solution and sample blanks (Milli-Q water treated as samples) were also prepared for QA purposes, along with quality control (QC) samples with selected AAs (His, Arg, Glu, Lys and Ile) to check peak positions and drift. Bovine Serum Albumin (BSA; Sigma-Aldrich, St Louis, USA) was used as a QC for the hydrolysis procedure with recoveries of each AA reported in Supplementary Information (SI) Table S3.

2.5. Proteomic analysis

A separate algal bioassay experiment was conducted in 2 L silanised Schott bottles to obtain sufficient material for proteomic analysis. Treatments consisted of control and two Cu concentrations (5 replicates each) corresponding to the effective concentration determined to cause a 10% (EC_{10} (1 μg L^{-1})) and 50% (EC_{50} (2 μg L^{-1})) reduction in growth rate compared to control; all procedures were identical to that described for the growth inhibition and AA bioassay experiments outlined above. After 72 h, algal cells were collected by centrifuging (170 × *g*; 15 min, 3 × wash in Milli-Q water) and stored at − 80 °C.

During the analysis, proteins were identified and quantified using the label-free quantification (**LFQ**) method. Details of protein extractions (Rappsilber et al., 2007), identification and quantification, database searches and bioinformatic analyses are provided in SI. Samples were also analysed for AA following the methods outlined in Section 2.4 to ensure the upscaling of the bioassay (from 250 mL to 2 L) did not result in different AA profile changes.

2.6. Statistical analyses

The EC_{10} and EC_{50} values were calculated within the R environment (Version 4.02) using a three-parameter log-logistic model in the "Dose-Response Curve" (drc) package (Ritz et al., 2015) and visualized using ggplot2 (Wickham, 2009). The significance of differences in total amino acid (TAA) content and relative abundance of each AAs between treatments was tested using one-way analysis of variance (ANOVA) followed by Tukey-HSD tests after confirming the normal distribution of data using the Shapiro test and homogeneity of variance by the Bartlett test in R. Principal component analysis (PCA) was done in R using the FactoMineR package (Lê et al., 2008) to visualise differences in the AA profile expressed as the relative abundance of each AA (mol%) among treatment groups. Permutational Multivariate analysis of variance (PERMANOVA) was carried out to determine significant variations in the AA profile between treatment groups (Primer 7 and PERMANOVA+ software; (Anderson et al., 2008)).

Quantitative proteomic data were normalised using the quantile method before all statistical analyses. Principal component analysis (PCA) was carried out in R using the FactoMineR package (Lê et al., 2008) to visualize the grouping among the treatments. Differential abundance analysis of proteins was applied to determine log2-fold changes between control and Cu treated groups (EC_{10} vs control; EC_{50} vs control). Volcano plots were produced by plotting $\log_{10}$(adjusted *p*-value) vs. $\log_2$ fold-change ($\log_2$FC) as per convention. To obtain the quantitative changes due to Cu stress, 2σ in $\log_2$FC was set as a cut off with *p*-values < 0.05 to determine significantly up-regulated and down-regulated proteins.

3. Results and Discussion

3.1. Effect of Cu on growth and amino acid content of Chlorella

Increasing Cu concentrations showed clear growth inhibition in *Chlorella* after 72 h exposure (Fig. 1**a**). Estimated EC_{10} and EC_{50} values for Cu were found to be 1 μg L^{-1} (0.7–1.2) and 2 μg L^{-1} (1.9–2.4), respectively, indicating that this alga is very sensitive to Cu. The EC_{10} and EC_{50} values determined for this study are similar to those previously reported for this test organism (EC_{10}: 0.83 μg L^{-1}, EC_{50}: 1.8 μg L^{-1}) and show strong support for *Chlorella* as an ideal test species to determine the effects of environmentally realistic Cu concentrations on the biomolecular makeup of aquatic organisms (Macoustra et al., 2019). A significant increase in algal cell size and granularity was also shown in the presence of Cu (SI Fig. S1) and has also been shown in previous studies for *Chlorella* exposed to Cu (Franklin et al., 2002; Wilde et al., 2006).

Total amino acid (TAA) content within control algae was 41.0 ± 5.1% of dry weight (Fig. 1**b**), similar to values previously reported for

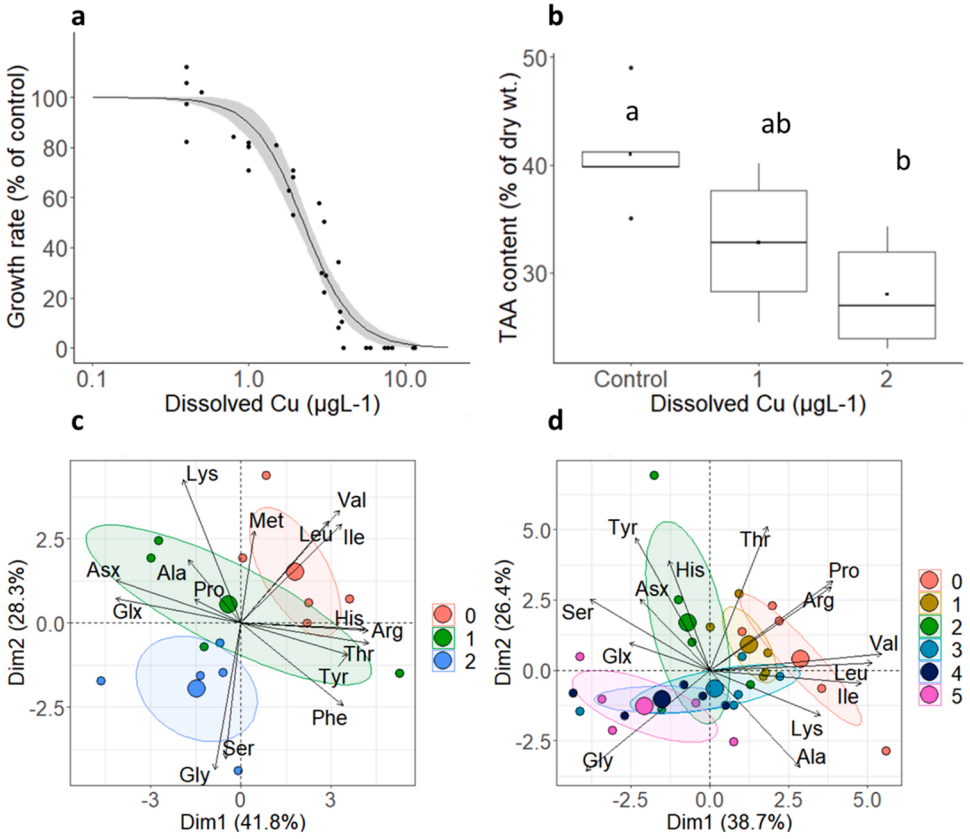

Fig. 1. Response of *Chlorella* sp. to Copper (Cu) toxicity. a. Dose-response curve for *Chlorella* exposed to Cu over 72 h in synthetic softwater at pH 7.3, normalized against the control responses (n = 5), shaded areas represent the 95% confidence envelop; b. The total amino acid (TAA) content in *Chlorella* exposed to 1 and 2 μg L^{-1} Cu treatments after 72 h exposure. Different letters represent a significant difference ($p < 0.05$) c; Principal component analysis (PCA) plot based on the amino acid profile (mol %) of *Chlorella* exposed to 0 (n = 5), 1 (n = 4, one of the replicates was an outlier based on the proteomic analysis hence the data point was removed) and 2 μg L^{-1} Cu (n = 5); d. PCA plot based on the amino acid profile of *Chlorella* exposed to Cu concentration of 0–5 μg L^{-1} .0 = Control; 1 = 1 μg L^{-1}, 2 = 2 μg L^{-1},3 = 3 μg L^{-1}, 4 = 4 μg L^{-1}, 5 = 5 μg L^{-1}.

Chlorella vulgaris (Chia et al., 2015; Tibbetts et al., 2015). Exposure to the Cu concentration of 1 μg L^{-1} did not significantly change the TAA content (33.0 ± 6.2% of dry weight), however, at 2 μg L^{-1} Cu, the TAA content (28.0 ± 5% of dry weight) was significantly lower than the control (ANOVA, $df = 2$, $F = 7.32$, $p = 0.008$).

Among the sixteen AAs detected in *Chlorella,* dominant AAs were (Table S4): Ala and Gly, which accounted for about 26–28% of the AA pool, while Tyr, His and Met were only present in small amounts (< 2% each). Green algae like *Chlamydomonas reinhardtii*, *Volvox aureus*, and *Volvox globator* were previously reported to have a higher content of Ala and Gly in their cell wall (Voigt et al., 1994). Gly rich glycoproteins also have been reported as the main constituent of the cell wall in other green algae (Domozych et al., 2012). Sulfur rich Cys-Cys was not detected as reported in other algae (Kakinuma et al., 2001) and Met (0.1% of total AA recovered) was detected only in control. Exposure to Cu concentrations of 1 μg L^{-1} and 2 μg L^{-1} caused a significant change in the AA profile (mol%) of *Chlorella* (PERMANOVA, Pseudo-$F_{2,12}$ = 3.52, $p = 0.003$)(Fig. 1**c**). Pairwise *t*-tests revealed that while 1 μg L^{-1} of Cu did not significantly affect the AA profile relative to controls, at 2 μg L^{-1} Cu, there was a significant difference in the AA profile ($t = 3.15$, $p < 0.05$). Overall, Cu significantly increased Gly and decreased the branched chain amino acids (BCAAs) (Val, Ile, Leu) and Arg (Fig. 1c, d and SI Table S4). Similar changes in AA profile from the samples collected after the growth inhibition bioassay and the proteome bioassay confirmed that Cu interferes with AA biosynthesis of this alga. (Fig. 1c,d; SI Table S5).

3.2. Effect of Cu on the proteome of Chlorella

A total of 765 proteins were obtained after the removal of decoy matched and contaminant proteins. After removing decoy matched and contaminants, box plot and correlation plots (SI Fig. S2a and b) constructed using the LFQ intensity of identified proteins obtained for all 15 samples (5 replicates for each of control, 1 μg L^{-1} and 2 μg L^{-1}) showed LFQ intensities recorded for L5 were in lower range compared to other 14 samples with lowest correlation coefficient recorded among the replicates of low Cu treatment group (1 μg L^{-1}) that indicates L5 was an outlier. 598 proteins were identified in L5 whereas the number of proteins identified for L1, L2, L3 and L4 were 473, 510, 472 and 499, respectively. Furthermore, the principal component analysis confirmed that one 1 μg L^{-1} sample (L5) was an outlier. Specifically, some proteins present in L5 were missing from the other (4) replicates (SI Fig. S2c) however these proteins were not identified. PCA on the reduced data set (L5 removed) showed clear separation of the treatment groups (SI Fig. S2d) and clustering within the groups, demonstrating that Cu exposure altered the proteome of *Chlorella.*

While differential abundance analysis of proteins was performed using a linear modeling approach, only those proteins present in all samples and all replicates within a sample were used. Overall, 39 proteins showed a significant response to Cu exposure. The abundance of 24 and 30 proteins were significantly different in the EC_{10} (1 μg L^{-1}) and EC_{50} (2 μg L^{-1}) treatments, respectively, relative to controls (Fig. S3a and b) with some common proteins responding to both treatments (Fig. S3c and d). Of the 39 differentially abundant proteins, 30 could be grouped into 3 functional categories: i) cellular processes and signalling (8) ii) information storage and processing (10) and iii) metabolism (12), based on the EuKaryotic Orthologous Groups (KOG) classification tool; the remaining 9 proteins were not in the KOG classification scheme and are referred to as other in Fig. 2 and SI Table S6. Cu exposure caused the up-regulation of proteins involved in cellular processing and signalling and the down-regulation of proteins involved in information storage and processing, with mixed results shown for metabolism and other proteins (Fig. 2). Previous studies have also reported proteomic changes in green algae due to metal stress (Andrade et al., 2021; Khatiwada et al., 2020; Li et al., 2013). For example, Andrade et al. (2021) reported changes to fatty acid biosynthesis and carbon fixation in *C. vulgaris* when exposed to 900 μg L^{-1} Cu. Khatiwada et al., 2020 reported increased abundance of thio-rich proteins and proteins involved in cellular stress response in *Euglena gracilis* during exposure to Cd. According to Li et al., 2013, proteins related to carbohydrate metabolism, carbon fixation, TCA cycle, lipid metabolism, protein biosynthesis, ATP and RNA biosynthesis were differentially expressed in *C. proththecoids* when exposed to Cu (II) concentration of 31.4 mg L^{-1}. These studies have reported biomolecular changes in algae when exposed to substantially higher concentrations of Cu than what we have used in our study. The Cu concentrations used in these studies are also extremely high compared to the 95% protection guideline value of 1.4 μg L^{-1} in Australia and New Zealand (ANZG, 2018). Our study, however, is the first to report such changes at Cu concentrations as low as 1 and 2 μg L^{-1}, representative of systems without obvious Cu contamination.

3.3. Alteration of metabolic pathways

Proteins involved in AA metabolism, energy metabolism and carbohydrate metabolism were found to be differentially regulated in response to Cu stress (SI Table S7) and were found to be interconnected with one another (Fig. 3). AA metabolism pathways affected were Arg and Pro metabolism, Glu metabolism; Cys metabolism; and branched-chain amino acids (BCAAs) degradation (Fig. 3). A range of energy and carbohydrate metabolic pathways were also affected, including: glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, carbon fixation and sulfur metabolism (Fig. 3).

3.3.1. Glutamate metabolism differed at low and high Cu stress

Glutamate metabolism (Fig. 3a) in *Chlorella* was affected in the presence of Cu with the conversion of Glu into carbamoyl phosphate (*via* Gln) up-regulated due to the up-regulation of carbamoyl-phosphate synthase (glutamine-hydrolyzing) (ec: 6.3.5.5). Carbamoyl phosphate is converted to Arg, suggesting that algae convert Gln into carbamoyl phosphate during Cu stress to increase Arg biosynthesis. Arg acts as a precursor for Pro biosynthesis, which has been suggested to play a protective role during Cu stress (Winter et al., 2015; Wu et al., 1998; Zhang et al., 2008). During exposure to 2 μg L^{-1} Cu, Gln derived from Glu further acted as a precursor for amino sugar metabolism, which is shown by the up-regulation of glutamine–fructose-6-phosphate transaminase (ec: 2.6.1.16). However, such changes to glutamate metabolism did not significantly change the relative abundance of Glx (Glu + Gln) in *Chlorella*.

3.3.2. Amino acid metabolism helps in metal detoxification

Changes in the proteins involved in Pro (Fig. 3a) and Cys metabolism (Fig. 3b) were also observed in response to Cu. Significant reduction in Arg (Fig. 3a) in the 2 μg L^{-1} Cu treatment may be explained by changes to Arg and Pro metabolism. Our result showed a significant up-regulation of ornithine aminotransferases (ec: 2.6.1.13) and down-regulation of arginine decarboxylase (ec: 4.1.1.19) in both Cu treatment groups. The up-regulation of ornithine aminotransferases and down-regulation of arginine decarboxylase (ec: 4.1.1.19) likely increased conversion of Arg to Pro through the ornithine pathway and limited conversion of Arg into agmatine (Fig. 3). Over-expression of ornithine aminotransferases has previously been shown to increase free Pro in rice, enhancing tolerance to Cu (Chen et al., 2001). However, free Pro was not measured in this study. Our results showed no significant difference in Pro concentrations (free + protein-bound) between control and Cu treatments. Previous studies on other species of *Chlorella* show that free Pro accumulation is triggered within a few hours of metal treatment and that its accumulation reduces Cu uptake (Mehta and Gaur, 1999; Wu et al., 1998; Zhang et al., 2008). Agmatine is a part of the polyamines synthesis pathway in algae. Its down-regulation may impact the production of polyamines like putrescine, spermidine and spermine found in green algae (Lin and Lin, 2019). Disruption of polyamine homeostasis in green algae has affected cell division and growth

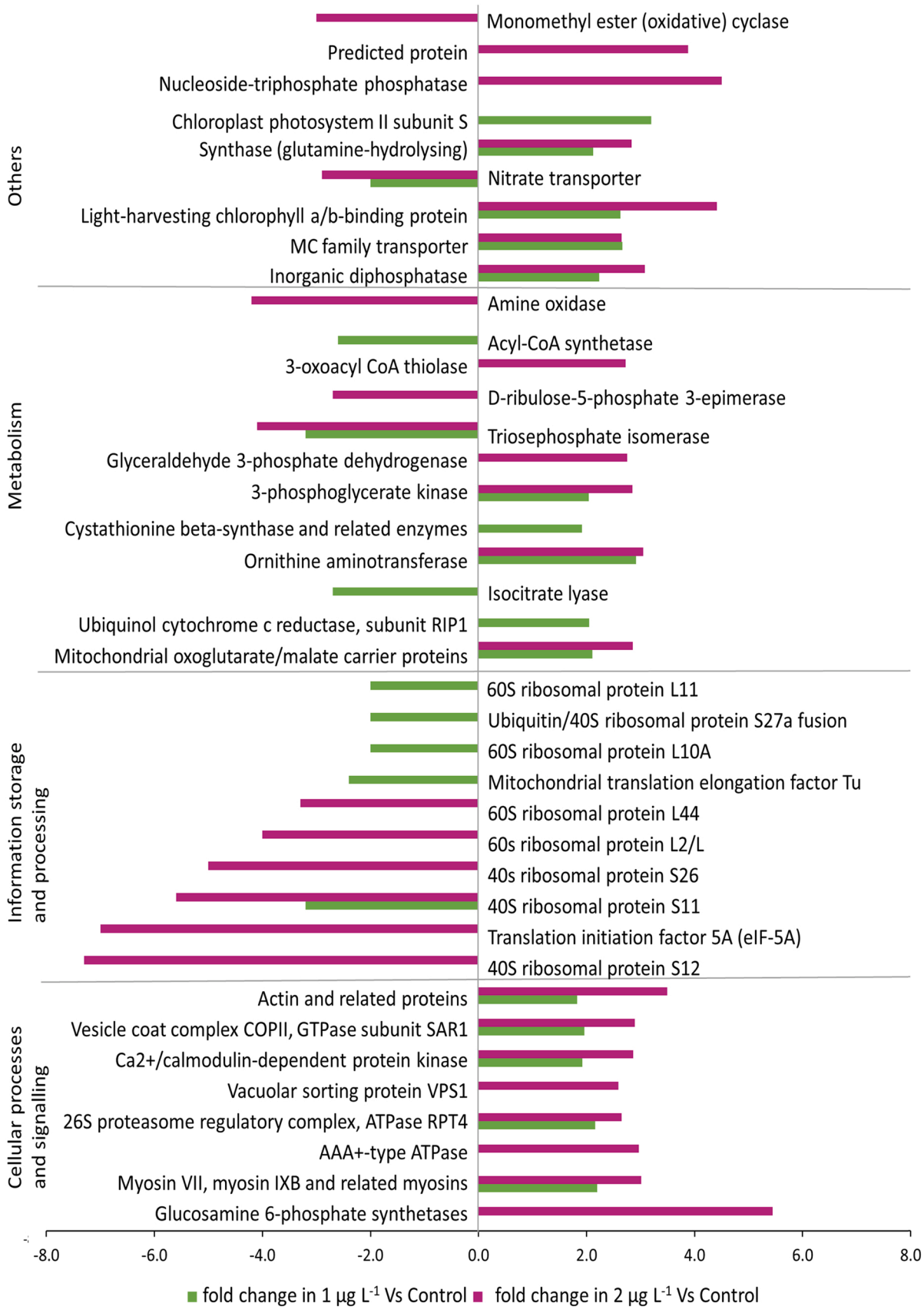

Fig. 2. Response of 39 proteins (up or down-regulated) relative to controls in response to Cu exposure (EC_{10}: 1 µg L^{-1} (green) and EC_{50}: 2 µg L^{-1} (purple); 72 h). Proteins are aggregated into 4 groups based on EuKaryotic Orthologous Groups (KOG) functional classification. Negative values indicate down-regulation and positive up-regulation of proteins compared to control.

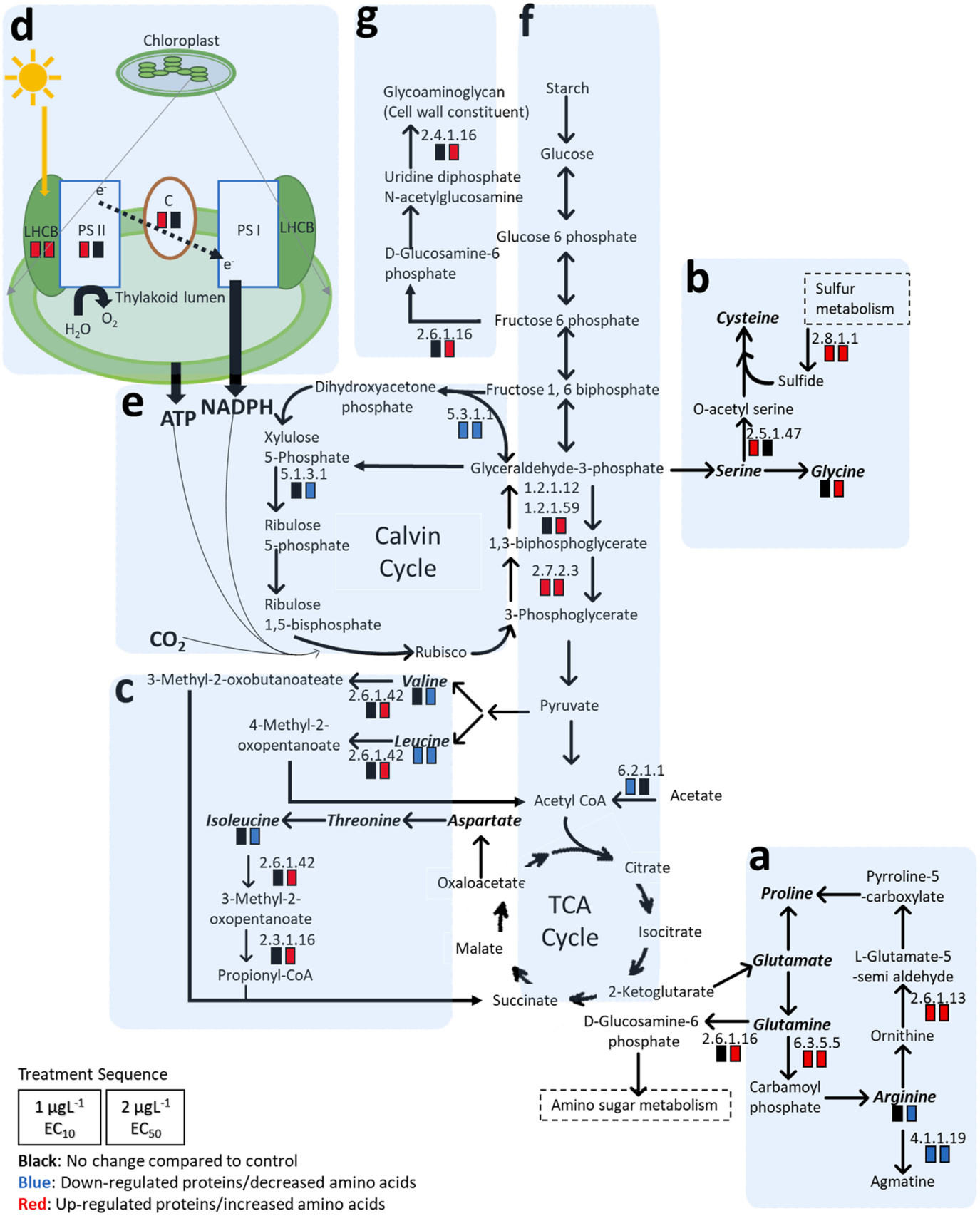

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Fig. 3. Pathway mapping indicating the impact of 1 μg L^{-1} and 2 μg L^{-1} Cu on the amino acid (AAs), energy and carbohydrate metabolism in *Chlorella* sp. Functional annotation of differentially regulated proteins was conducted using the Blast2Go program against the nonredundant protein database (nr; NCBI). The GO (Gene Ontology) enrichment analysis was carried out in Blast2GO for these proteins to determine their description. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to analyse different metabolic pathways affected by the responsive proteins and their functions using Blast2GO. Differentially regulated enzymes are denoted by their EC number based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Amino acids are indicated in bold. Red bar below EC number, AAs, or protein indicate significantly increased levels in Cu treated samples relative to control; blue bars indicate significantly decreased levels and black bars indicate no significant differences between treated and control samples. For the quantitation of change and name of the protein, see Supporting Information Table S7. LHCB = light-harvesting chlorophyll a/b binding protein, PS II = Chloroplast photosystem II, PS I = Chloroplast photosystem I, C = Cytochrome, e^- = electron, dotted black arrow indicates electron transport chain. Letters **a** – **g** correspond to discrete pathway sections discussed in the main text.

(Lin and Lin, 2019). The decline in algal growth rate may be associated with the disruption of this pathway in Cu treated algae in this study.

Cysteine metabolism (Fig. 3b) was also up-regulated in Cu treated algae. Up-regulation of Cys metabolism would lead to metal-induced Cys accumulation in the algae (not measured here). Cysteine is a sulfur-containing AA with a critical role in regulating tolerance to metals (Khatiwada et al., 2020). We observed an increase in cysteine synthase (ec 2.5.1.47) in *Chlorella* exposed to 1 μg L^{-1} Cu as well as thiosulfate sulfurtransferase (ec: 2.8.1.1) in algae exposed to both Cu treatments (Fig. 3b); these enzymes catalyse the metabolism of sulfur into Cys. Cysteine is also a precursor for glutathione and phytochelatins (glutathione-derived peptide) biosynthesis. Phytochelatins have been reported to play an important role in metal detoxification in algae (Levy et al., 2007; Mendoza-Cozatl et al., 2005). Glutathione synthesis also requires Gly as a precursor for its biosynthesis (Kelly and Pearce, 2020; León-Vaz et al., 2020). A significant increase in Gly was shown in the 2 μg L^{-1} Cu treatment, suggesting Gly might be playing a role in the production of glutathione and phytochelatins in Cu exposed algae.

3.3.3. *Amino acids acting as energy metabolites during metal stress*

The effect of Cu on BCAAs degradation pathways is consistent with the changes we detected in the AA profile of *Chlorella* (Fig. 1c, d; SI Fig. S4). The reduction in the abundance of BCAAs in the 2 μg L^{-1} Cu treatment can be explained by the up-regulation of branched-chain-amino-acid transaminase (BCAAT, ec: 2.6.1.42) and acetyl-CoA C-acyltransferase (ec: 2.3.1.16) (Fig. 3c). These enzymes take part in the biodegradation pathways of BCAAs, converting BCAAs to acetyl-CoA and succinyl-CoA and contribute to the tricarboxylic acid (TCA cycle), thus acting as an energy source for the cell during stressed conditions (Nie et al., 2018). Down-regulation of acetyl-CoA synthetase (ACS) (ec: 6.2.1.1) was observed in 1 μg L^{-1} Cu treated algae, limiting carbon supply via acetate.

3.3.4. *Up-regulation of energy production*

The primary source of energy to green algae during autotrophic growth is light. There was an up-regulation of the light-harvesting chlorophyll a/b-binding (LHCB) protein (2.6 fold in 1 μg L^{-1} and 4.4 fold in 2 μg L^{-1}), the chloroplast photosystem II (PS II) subunit S (3.2 fold in 1 μg L^{-1}) and ubiquinol cytochrome c reductase (C), subunit RIP1 (2 fold in 1 μg L^{-1}) in algae during exposure to Cu (Table S6. Fig. 3d). LHCB is a part of the light-harvesting complex of PS II and usually are associated with chlorophyll serving as the antenna complex (Xu et al., 2012). These antenna complexes absorb sunlight and transfer the excitation energy to the core complexes of PS II in order to increase energy production through generation of ATP via photophosphorylation (Xu et al., 2012). Ubiquinol cytochrome c reductase(C), subunit RIP1 is involved in the photosynthetic electron transport in chloroplasts in algae (Berry et al., 1991). The up-regulation of these photosynthesis-related proteins would help increase the production of ATP and nicotinamide adenine dinucleotide phosphate (NADPH) to meet growing energy demand during the stressed condition. ATP and NADPH are then used to fix CO_2 from the atmosphere to ribulose 1,5-bisphosphate (RuBP) to produce Rubisco within the Calvin cycle (Fig. 3e) (Bonora et al., 2012). Rubisco then enters the reduction phase of the Calvin cycle and is converted into Glyceraldehyde-3-phosphate (G3P). Under normal conditions, most of the G3P produced inside the chloroplast will enter the regeneration phase of the Calvin cycle, where it combines with ATP for the production of RuBP. However, during Cu stressed conditions, we observed down-regulation of triose-phosphate isomerase (ec: 5.3.1.1) and ribulose-5-phosphate 3-epimerase (Rpe) (ec: 5.1.3.1) involved in the regeneration of RuBP from G3P, suggesting a down-regulation of G3P in the regeneration phase of the Calvin cycle. Instead, exposure to Cu caused the up-regulation of glyceraldehyde-3-phosphate dehydrogenase (NAD(P)+) (phosphorylating) (GAPDH, ec: 1.2.1.12) and phosphoglycerate kinase (ec: 2.7.2.3), suggesting a redirection of the majority of carbon flux (G3P) toward glycolysis, gluconeogenesis and the TCA cycle for energy production within the mitochondria (Fig. 3f).

The inner mitochondrial membrane is not permeable (Griffin et al., 2000). Special mitochondrial carrier proteins assist in the transportation of intermediates of the TCA cycle across the membrane. One such protein is the mitochondrial oxoglutarate/malate carrier protein. This protein catalyzes the transport of 2-oxoglutarate across the inner mitochondrial membrane and helps to regulate the malate - Asp shuttle (UNIPROT, 2021). Up-regulation of mitochondrial oxoglutarate/malate carrier proteins in both Cu treatments (Table S6) might have helped actively transport TCA intermediates (oxoglutarate and malate) across the inner mitochondrial membrane for the biosynthesis of Asp (Fig. 3f). Recent research has also suggested that the malate shuttle plays a vital role in ROS production and programmed cell death (PCD) (Zhao et al., 2018). Up-regulation of mitochondrial oxoglutarate/malate carrier proteins may also have been associated with ROS production and PCD within Cu treated algae.

Environmental stressors strongly modulate the phosphorylation of ATP to regulate fundamental cellular function during stressed conditions (Bonora et al., 2012). Consistent with this, proteins related to oxidative phosphorylation were also found to be up-regulated in Cu stressed algae. Inorganic diphosphatase (ec: 3.6.1.1) catalyses oxidative phosphorylation in cells and plays an essential role in phosphate metabolism (Satoh et al., 1998). Inorganic diphosphatases also play a role in stress adaptive responses (Gutiérrez-Luna et al., 2018). Inorganic diphosphatase is involved in the regulation of intracellular levels of pyrophosphate via the hydrolysis of inorganic pyrophosphate (PPi), which is generated through the hydrolysis of ATP in cells. The hydrolysis of PPi into two phosphate ions is a highly exergonic reaction that can under certain conditions substitute for ATP-derived energy and thus may act as an alternative energy source in algae exposed to Cu.

Overall, Cu-exposed *Chlorella* requires a high amount of energy to cope with the adverse effects of Cu, with Cu causing the up-regulation of several proteins involved in energy production via photosynthesis, carbon fixation, glycolysis and oxidative phosphorylation. The increased energy demand also directly affected AA metabolism with the BCAA degradation pathway up-regulated to increase production of acetyl CoA and generation of electron carriers via the TCA cycle for use during oxidative phosphorylation to generate ATP.

3.3.5. *Cu down-regulated proteins related to information storage and processing*

Ten proteins related to information storage and processing (subgroup: translation, ribosomal structure and biogenesis) were all down-regulated in Cu-treated algae with only one common protein (40 S ribosomal protein S11) down-regulated in both Cu treatments (Fig. 2). The differential expression of these ribosomal proteins in *Chlorella* when

exposured to 1 and 2 μg L^{-1} Cu concentrations suggests that such a low Cu stress substantially affects the character of the proteome in *Chlorella*. This result also suggested that a reduction in ribosome protein production might be indicative of metal exposure not just Cu, as this has been previously reported in different yeast strains exposed to arsenic (Guerra-Moreno et al., 2015).

Previous studies have shown responses of algal ribosomal proteins to metal exposure (León-Vaz et al., 2020; Luo et al., 2014). Ribosomal proteins are known to play a role in cellular growth and regulation (Guo and Yang, 2015). The production of cytoplasmic 60 S and 40 S subunits of ribosomes are energetically costly to the cells (Guo and Yang, 2015). Hence, algal cells may down-regulate the ribosome biogenesis in order to cope with the high energy demand during stressed conditions. For instance, the abundance of 60 S ribosomal protein L5 was reported to be down-regulated by 94 fold in *Chlorella protothecoides* when exposed to Cu (Li et al., 2013). Several ribosomal proteins were also down-regulated in *Chlorella sorokiniana* when exposed to cadmium (León-Vaz et al., 2020) and *Synechocystis 6803* (Angeleri et al., 2019) when exposed to Cu. Down-regulation of the mitochondrial translation elongation factor Tu and translation initiation factor 5 A (eIF-5A) was also found in Cu treated algae in this study, supporting previous findings of Angeleri et al. (2019). The down-regulations of the ribosomal proteins and regulators involved in translation indicate that the efficiency of protein synthesis was decreased in *Chlorella* due to Cu exposure (Luo et al., 2014; Wase et al., 2014). Metals have also been reported to cause oxidative damage to biomolecules such as proteins (Wood et al., 2012). During metal exposure, cells are thought to limit the synthesis of new proteins by down-regulating ribosomal proteins, allowing protein degradation pathways to deal with damaged proteins (Guerra-Moreno et al., 2015). This interpretation is further supported by the decreased TAA content in Cu treatments compared to the control in this study.

The two Cu treatments affected two distinct sets of proteins related to translation, ribosomal structure, and biogenesis. This result suggests that even though we detected a continuous dose-response based on the growth rate of algae on exposure to different Cu concentrations, the influence of Cu on proteins is concentration dependent. This decrease in ribosomal proteins may be a significant contributing factor to the inhibition of cell growth in *Chlorella* when exposed to Cu.

3.3.6. *Up-regulation of cellular processes and signalling related proteins during Cu stress*

Proteins belonging to cellular processes and signalling were up-regulated within algae exposed to both Cu treatments in this study. The number of up-regulated proteins and fold change increased with increasing Cu concentrations (Fig. 2), suggesting that an increasing number of pathways and mechanisms are affected in response to increasing Cu. To illustrate, proteins, such as Myosin VII, Myosin IXB and related Myosins; 26 S proteasome regulatory complex, ATPase RPT4; Ca^{2+}/calmodulin-dependent protein kinase; Vesicle coat complex COPII, GTPase subunit SAR1, Actin and related proteins showed higher fold changes in 2 μg L^{-1} treated algae compared to 1 μg L^{-1}.

Among these, 26 S proteasome regulatory complex (26 SP), ATPase RPT4 and AAA+ -type ATPase are related to post-translational modification, protein turnover and chaperones, which function together to maintain cell stability through proteolysis (Coll-Martínez and Crosas, 2019; Gemperline et al., 2019). These proteolytic mechanisms also help maintain adequate AA levels for protein homeostasis (Suraweera et al., 2012). The up-regulation of 26SP in Cu-treated algae in this study might help remove damaged proteins and help the alga accommodate changing environmental conditions. Previous studies have explored the beneficial role of 26SP during moderate oxidative stress in the green alga *C. reinhardtii* against selenite toxicity (Vallentine et al., 2014) and higher plants like maize when exposed to Cd (Pena et al., 2007) and methyl viologen (Kurepa et al., 2008). The synthesis of 26S proteasomes is energetically costly to the cells (Marshall and Vierstra, 2019), and its synthesis might be associated with the increased energy demand of Cu exposed algae.

Another group of proteins up-regulated: Glucosamine 6-phosphate synthetases and actin, play a role in cell wall biogenesis and the cytoskeleton (Fig. 3g). Glucosamine 6-phosphate synthase (ec: 2.4.1.16/2.6.1.16; the highest fold change (5.4 fold) out of all proteins at 2 μg L^{-1} Cu) aids in the production of glycosaminoglycan present in the cell wall (Zeroual et al., 2020), and actin plays a role in maintaining the integrity of the algal cell (Kulikova et al., 2009). Up-regulation of proteins related to the cell wall and cytoskeleton constituents might have increased cell size, and granularity in Cu stressed algae, as shown in our results (SI Fig. S1). Furthermore, Gly is one of the major AAs found in the cell wall of green algae. A significant increase in the proportion of Gly in 2 μg L^{-1} Cu treated algae is consistent with the observed cell wall thickening in Cu stressed algae. Cu has previously been shown to alter the cellular structure of *Chlorella* (Franklin et al., 2002; Wilde et al., 2006). The cell wall contains negatively charged carboxyl and phosphatic functional groups that can bind metals through ion exchange and protect against metal toxicity by reducing the transport of metals into the cytoplasm (Mellado et al., 2012). Hence our results suggest *Chlorella* actively thickens its cell wall as a prominent adaptive feature to cope with metal exposure.

Finally, this study, has shown the dose dependent toxic effects of environmentally realistic low Cu concentrations on different levels of biological organization within the microalgae *Chlorella,* including cell division, alteration to cell physiology, AA profile and proteome. This suggests that AA profiling and proteomics may be used to detect biomolecular changes due to exposure to low Cu concentrations before changes to traditional endpoints such as cell division are shown. The use of such techniques should be used to complement traditional chronic toxicity endpoints, improving management and regulatory decision making. For example, there is a global effort from organizations such as the Organisation for Economic Co-operation and Development (OECD) and US Environmental Protection Agency (USEPA) to develop Adverse Outcome Pathway models (Coady et al., 2019) that integrate available toxicological knowledge and describe the causal linkage between the first interaction of a chemical with a biological macromolecule and subsequent measurable responses across biological levels of organisation. Data such as that provided in this study can go towards developing such models to help predict the effect of metals and improve chemical risk assessment and management activities.

4. Conclusions

Cu as low as 1 μg L^{-1} caused toxic effects to *Chlorella*, with a decline in cell division, alteration to cell physiology, synthesis of AAs and changes to the proteome detected. Sublethal Cu stress of 2 μg L^{-1} caused increases in glycine and decreases in isoleucine, leucine, valine, and arginine, in *Chlorella*. Furthermore, modulation of several proteins involved in energy production pathways, cellular processes and signalling proteins, and proteins related to ribosomal structure and protein translation were observed. Observed impacts on the AA profile and proteome were shown to be dose-dependent. This study shows that AA profiling and proteomic studies can detect sublethal Cu toxicity in unicellular organisms like *Chlorella* when exposed to very low Cu concentrations of 1 and 2 μg L^{-1} and even before substantial decreases in cell division are observed. Amino acids like Gly, Arg and BCAAs; and proteins involved in metabolism of these AAs such as branched-chain-amino-acid transaminase, carbamoyl-phosphate synthase (glutamine-hydrolyzing), ornithine aminotransferase and thiosulfate sulfurtransferase show potential as biomarkers of toxicity, however, further research on the impact of Cu and other metals on proteome and AA profiles of different test organisms is needed to better understand their potential as biomarkers.

CRediT authorship contribution statement

Manisha Shakya: Conceptualization, Methodology design, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Ewen Silvester**: Supervision, Conceptualization, Methodology design, Resources, Visualization, Writing – review & editing. **Gavin N. Rees**: Supervision, Writing – review & editing, **Kolin Harinda Rajapaksha:** Methodology design and Investigation for proteomic analysis, bioinformatics, **Pierre Faou:** Methodology design and Investigation for proteomic analysis, bioinformatics **Aleicia Holland**: Supervision, Conceptualization, Methodology design, Resources, Visualization, Writing – review & editing.

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.

Acknowledgements

This work was supported by La Trobe University Postgraduate Research Scholarship (LTUPRS), a La Trobe University Full Fee Research Scholarship (LTUFFRS) and Ph.D. Top-Up scholarship supported by the Murray Darling Basin Joint Governments in association with the Murray-Darling Freshwater Research Centre (MDFRC/CFE). The assistance of Shimadzu Australasia (Chris Bowen and Rudi Hollander) in developing the LCMS analytical methods used here is greatly appreciated.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113336.

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