Assessing Cu impacts on freshwater diatoms: biochemical and metabolomic responses of Tabellaria flocculosa (Roth) Kützing

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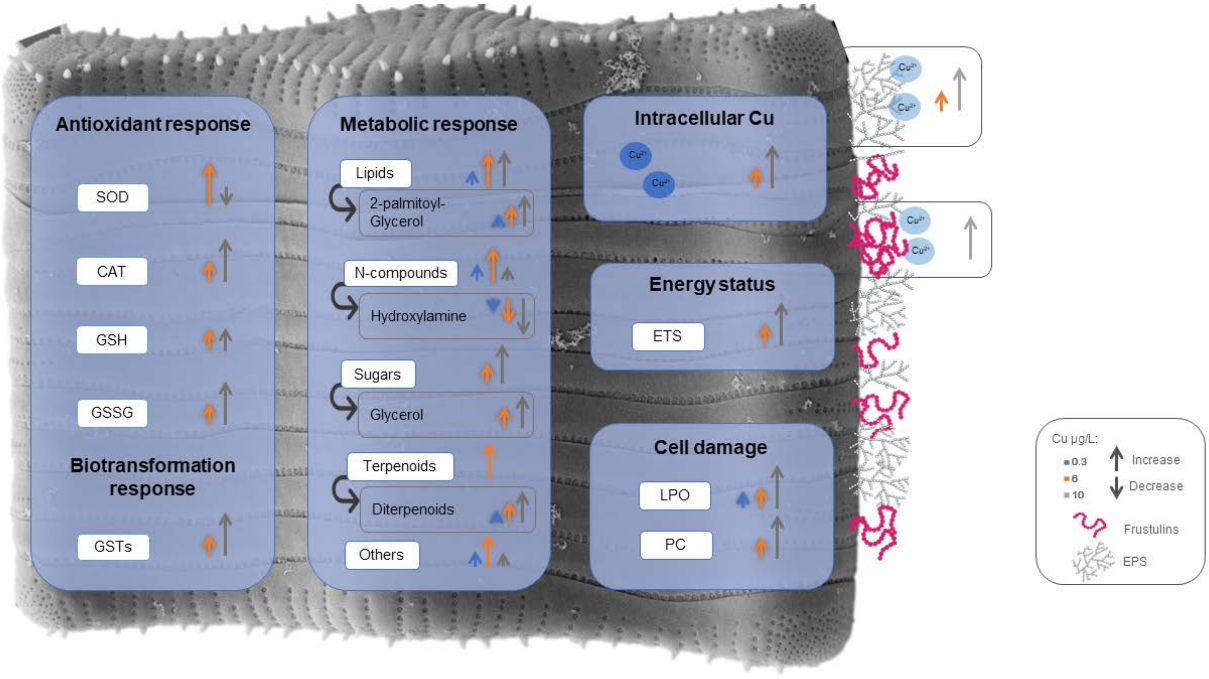

Highlights:

- Cu is toxic to *Tabellaria flocculosa* (*T. flocculosa*) at environmental relevant concentrations

- Low Cu concentration induces oxidative stress, but antioxidant and detoxifying mechanisms are not activated
- Moderate Cu concentration leads to a reconfiguration of the metabolome
- High stress induces the antioxidant response, inactivates enzymes and increases cellular damage
- New specific markers of Cu toxicity are suggested

List of abbreviations:

CAT - Catalase

EPS - Exopolysaccharides

ETS – Electron Transport System in mitochondria

FA – Fatty Acids

FRUST - Frustulins

GC-MS - Gas Chromatography- Mass Spectrometry

GSH – Reduced Glutathione

GSSG – Oxidized Glutathione

GSTs – Glutathione S- transferases

LPO – Lipid Peroxidation

MDA – Malondialdehyde

PC – Protein Carbonylation

ROS – Reactive Oxygen Species

SOD – Superoxide Dismutase

Abstract:

Metals are a recognised threat to aquatic organisms but the impact of metals such as copper (Cu) on benthic freshwater diatoms is poorly understood, even if diatoms are commonly used as water quality indicators. Our study aimed to elucidate the cellular

targets of Cu toxicity and the mechanisms cells resort to counteract toxicity and to increase tolerance to Cu. A concerted approach analysing the biochemical, physiological and metabolome alterations in diatom cells was conducted by exposing the freshwater diatom *Tabellaria flocculosa* to 0.3, 6 and 10 µg Cu/L. Cu was already toxic to *T. flocculosa* at concentrations common in environments and which are not usually considered to be contaminated (0.3 µg Cu/L). Under Cu impact, the metabolome of *T. flocculosa* changed significantly, especially at high concentrations (6 and 10 µg Cu/L). Cu toxicity was counteracted by increasing extracellular immobilization (EPS, frustulins), antioxidant (SOD, CAT) and detoxifying (GSTs) enzymes activity and low molecular weight antioxidants (GSH). These mechanisms were fuelled by higher energy production (increased ETS activity). At the highest Cu concentration (10 µg/L), these processes were specially enhanced in an attempt to restrain the oxidative stress generated by high intracellular Cu concentrations. However, these mechanisms were not able to fully protect cells, and damage in membranes and proteins increased. Moreover, the decrease of hydroxylamine and unsaturated fatty acids and the increase of saturated fatty acids, 2-palmitoylglycerol, glycerol and diterpenoid compounds should be tested as new specific markers of Cu toxicity in future studies. This information can support the prediction of diatom behaviour in different Cu contamination levels, including highly impacted environments, such as mining scenarios, and may assist in environmental risk assessment policies and restoration programs.

Keywords: Copper, freshwater diatoms, *Tabellaria flocculosa,* physiological indicators, metabolomics

1. Introduction:

Metal excess remains one of the most noteworthy anthropogenic-caused stress factor in aquatic ecosystems (Allan and Castillo, 2007; Millennium Ecosystem Assessment, 2005; Ritter et al., 2008). Copper (Cu), in particular is released to the environment by different

sources, mainly mining and agriculture (Hogsden and Harding, 2012; Johnson and Hallberg, 2005). $CuSO_4$ is frequently used to control fungal infections in crops, for example in vineyards, due to its fungicide properties (Viana and Rocha, 2005). Applications of $CuSO_4$ usually end up draining in the surrounding waters, increasing the concentrations of soluble Cu ions, with serious impacts on photosynthetic microorganisms (Jamers et al., 2013a).

Diatoms are one of the most important groups of algae and often dominant in a diverse range of habitats (van den Hoek et al., 1995). Diatoms proved useful as an indicator group for water quality assessment, and are a mandatory group for monitoring under the Water Framework Directive, WFD (Directive 2000/60/EC), (Kelly et al., 2009, 2012). The shift of species composition due to changes in the environment is commonly used to evaluate water quality in monitoring programs (Kelly et al., 1998; Lavoie et al., 2008; Teittinen et al., 2015). However, common diatom-based indices and metrics do not evaluate metal contamination, thus it is necessary to develop tools to help evaluating and monitoring efficiently the stress caused by metals in aquatic ecosystems. It has been shown that diatom cell size and deformities might be good indicators of metal pollution (Ferreira da Silva et al., 2009; Ivorra et al., 2002; Luís et al., 2014, 2009; Morin et al., 2012; Pandey and Bergey, 2016), and several studies reported biochemical and physiological effects of metals on freshwater species and communities (Bonet et al., 2013, 2012; Branco et al., 2010; Corcoll et al., 2012; Leguay et al., 2016; Santos et al., 2013). Nevertheless, most of these studies do not explore the cellular processes occurring under high metal conditions, or explain tolerance levels that certain diatom strains present (Ivorra et al., 2002). Additionally, the tools used in these studies are not specific to assess metal stress.

There is plenty literature evaluating Cu effects on algae including diatoms, but these are mainly marine diatom species (Contreras et al., 2005; Debelius et al., 2009; Florence et al., 1983; Mallick, 2004; Nielsen and Wium-Andersen, 1970; Rijstenbil et al., 1994; Ritter

et al., 2010, 2008; Stauber and Florence, 1987; Torres et al., 2008). Only a few studies report Cu effects on freshwater diatom species (Knauert and Knauer, 2008; Nielsen and Wium-Andersen, 1970; Tripathi et al., 2006). Most of these studies were published in the seventies and eighties, using very high concentrations of Cu (in the range of mg/L to hundreds of µg/mL) (Bringmann and Kühn, 1980; Brown and Rattigan, 1979; Elder and Horne, 1978; Laube et al., 1980; Nielsen and Laursen, 1976; Nielsen and Wium-Andersen, 1970; Walbridge, 1977; Young and Lisk, 1977) and evaluating mostly physiological and biochemical parameters in separate. To our best knowledge, there are few studies that evaluated alterations at the metabolomic level. Recently, Jamers et al. (2013) described the response of the green alga *Chlamydomonas reinhardtii* to Cu toxicity using a transcriptomic and metabolomic integrated approach. Results of that study evidenced changes at the physiological level at low Cu concentrations and effects on growth and metabolome at higher concentrations. However the methodology used (Nuclear magnetic resonance, NMR, spectroscopy) was not sensitive to Cu toxicity (Jamers et al., 2013a).

In European streams, average Cu concentrations vary between 0.23 µg/L to 14.6 µg/L (Salminen et al., 2005). However, in highly polluted sites such as mining areas, the concentrations may reach the mg/L range (Larson, 2010). In this context a question emerges: At what concentration does Cu stops being beneficial and becomes toxic? Based on the Water Framework Directive (Directive 2000/60/EC) and the daughter directive on Priority Substances (Directive 2008/105/EC), each EU member state implemented a national strategy against water pollution. The national legal document that classifies water quality in Sweden (HVMFS, 2016), defines Cu concentrations above 12 µg/L as toxic for freshwater habitats, resulting in a classification of “less than good ecological status” (HVMFS, 2016). If the Cu concentration in the water is less than 12 µg/L, but higher than 0.5 µg/L, a model is used to calculate the bioavailable Cu, and if the bioavailable Cu is higher than 0.5 µg/L the streams are also classified as “less than

good" (HVMFS, 2016). For example, other threshold values for Cu concentration are defined for Portugal (7.8 µg Cu/L)(APA, 2016).

Diatom cells possess mechanisms to protect them from different stresses. Chloroplasts, mitochondria and some enzymes naturally produce reactive oxygen species (ROS) (Mallick, 2004). Since ROS are toxic to cells above threshold levels, cells have mechanisms to scavenge ROS, such as enzymes (SOD and CAT) and low molecular weight molecules (glutathione) with antioxidant activity, and to minimize ROS toxicity, such as biotransformation enzymes (GSTs) (Mallick and Mohn, 2000).

Cu has important biological functions, being a cofactor of several metalloenzymes (Hänsch and Mendel, 2009), such as the Cu/Zn SOD, a component of the photosynthetic electron carrier plastocyanin (Borowitzka et al., 2016; Jamers et al., 2013b; Peers and Price, 2006), a receptor of ethylene or involved in Mo-cofactor biosynthesis (Rodríguez et al., 1999 in Hänsh and Mendel 2009). However, excessive Cu concentrations are toxic and cells possess mechanisms to minimize metal toxicity, as extracellular immobilization (exopolysaccharides and frustulins), restriction of metal ions transport into the cell, intracellular precipitation and chelation (Branco et al., 2010; Figueira et al., 2014; Pinto et al., 2003; Regoli and Giuliani, 2014; Santos et al., 2013). When these mechanisms fail to reduce Cu concentrations, ROS increase and oxidative stress overcomes (Virgilio Perales - Vela et al., 2006), compromising not only membrane integrity (lipid peroxidation) (Mallick, 2004; Mallick and Mohn, 2000), but also interacting with nucleic acids and proteins (protein carbonylation), damaging inflicted cells (Regoli and Giuliani, 2014; Tripathi et al., 2006)

Damage and induction of defense mechanisms can be monitored and used as biomarkers of oxidative stress (Branco et al., 2010; Figueira et al., 2014; Santos et al., 2013). However, these markers are common to all conditions that induce oxidative stress, such as UV light, pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, pharmaceuticals, nanomaterials, or metals. Thus, parameters that specifically mark metal toxicity, and Cu effects in particular, would be important to evaluate the

contribution of each contaminant to the overall impact observed in multi-impacted environments.

Metabolite profiling analysis has until now focused mainly on the identification the metabolites of algae with economic value (Jamers et al., 2009), while the environmental perspectives have been poorly explored (Johnson et al., 2017;). The metabolite profiling approach is a powerful tool to characterize organisms' cellular response to environmental stress stimuli. The identification of the changes in specific metabolites may elucidate the metabolic pathways involved in the response to a specific stress (Borowitzka et al., 2016; Heydarizadeh et al., 2013; Jamers et al., 2009; Serra-Compte et al., 2017; Viant, 2009) and may help find biomarkers to specific contaminants such as Cu.

The combination of biochemical, physiological and Omics analysis may evaluate more accurately the impacts of stressors, such as Cu in freshwater ecosystems than each approach alone (Branco et al., 2010; Dyhrman et al., 2012; Jamers et al., 2013a, 2009; Morelli and Scarano, 2001; Pinto et al., 2003; Rijstenbil et al., 1994). Thus, this study aims to explore the responses of the freshwater diatom *Tabellaria flocculosa* (Roth) Kützing to environmentally relevant Cu concentrations, using a multidimensional (biochemical, physiological and metabolomic) approach. Experiments were carried out using *T. flocculosa* isolated from a stream with 6.2 µg Cu/L and pH 5.0 and exposing diatom cells to Cu concentrations representing environments with low (0.3 µg/L) to relatively high Cu contamination (10 µg/L). The use of a diatom isolated from a contaminated site was intentional, as we aimed at understanding which mechanisms underlie Cu tolerance, and which markers can reflect Cu impact. The parameters evaluated included growth, cell damage (LPO, and PC), physiological alterations (proteins, EPS, frustulins, ETS), antioxidant (SOD, CAT GSH and GSSG) and biotransformation (GSTs) response. Metabolite profiling obtained through Gas Chromatography- Mass Spectrometry (GC-MS) was also used. To our knowledge, such an integrated approach for studying Cu effects on a freshwater benthic diatom was never carried out before, and results might deliver new and specific markers of Cu toxicity.

2. Material & Methods:

2.1 Region characterization, diatom isolation, cultivation and growth:

Dalarna, a geographical region in Sweden, is known for metal exploitation (Larson, 2010). The region contains old (approx. 1000 years) and new mines and an history of mine effluents draining into the surrounding waters (Larson, 2010). According to the report from Dalarna's Administration County Board (2010), in some river sites Cu concentration in water may be above 580 µg/L, with a measured maximum of 1800 µg/L. The species used in this study, *Tabellaria flocculosa,* was isolated from a stream in this region with 6.2 µg Cu/L and pH~5 (water parameters summarized in supplementary table S1). The isolation of a single cell was performed by the micropipette technique, isolated cells were grown on agar plates (Andersen, 2005; Round et al., 1990), followed by transfer to WC (Wright's cryptophyte) liquid medium (Guillard and Lorenzen, 1972) and acclimatized during 6 months. *T. flocculosa* culture is now part of the Swedish University of Agricultural Sciences diatom culture collection (reference number D9).

The strain was pre-cultured in WC medium at pH 5, until exponential growth was reached. Cells at exponential growth were used for Cu experiments by transferring approximately 250 000 cells to new sterile flasks containing 250 ml of WC modified medium (1000 cells/mL), supplemented with different Cu concentrations. The experiments were performed at 20 ± 2 °C with a 12 h/12 h light/dark cycle at 75 µmol/m^2/s in WC medium, added nominal Cu concentrations of 0, 0.3, 6 and 10 µg/L (added as $CuSO_4$) during 96h. The WC medium was modified by using 1/10 of the EDTA/trace metal solution to ensure that the added Cu would be present mainly as free ions instead of chelated by EDTA (calculations done by Visual MINTEQ ver. 3.0). Cu concentration in WC medium alone (control) was 1 µg/L, thus, final nominal concentrations were 1, 1.3, 7 and 11 µg Cu/L. Along the document, we refer to the nominal concentrations. Twelve independent experiments were carried out with 3 to 5 replicates per condition. At the end

of exposure time, 1 mL of each Erlenmeyer was preserved with Lugol's solution for cell counting. Cell density was measured by direct counting in a Neubauer chamber using Nikon Eclipse 80i microscope.

The concentrations for Cu exposure were chosen considering modelled values of average Cu concentrations common in Europe (Salminen et al., 2005), concentrations in the Swedish national monitoring system of reference sites (HVMFS, 2016), concentrations in mining impacted areas (e.g. Larson 2010) and recommendations for threshold values for risk assessment of Cu (HVMFS, 2016). Measured concentrations in the Swedish national monitoring system of reference sites are on average 0.5 µg/L, values up to 3 µg/L in non-impacted streams are not uncommon (HVMFS, 2016), which fits quite well with our nominal control concentration of Cu in the WC medium (1 µg/L). Cu concentration of 0.3 µg/L was chosen as a median concentration occurring in European streams, and 6 and 10 µg Cu/L as concentrations commonly recorded in metal contaminated streams (Salminen et al., 2005). Selection of 10 µg Cu/L ensured sufficient biomass for analyses, and not a total growth inhibition of TFLO cells.

2.2 Cu quantification:

Cell cultures were centrifuged at 5000 rpm for 15 min. Cell pellets were re-suspended in deionized water, vortexed and centrifuged again. The washing procedure was repeated two more times to ensure the removal of all the Cu from the culture medium. Pelleted cells were re-suspended in 3 ml EDTA (0.1 M pH 7.8) and incubated overnight, at 4ºC under agitation (150 rpm). Incubated cells were centrifuged (9000xg, 2 min, at 4ºC), the supernatant discarded and the cell pellets re-suspended in deionized water, vortexed and centrifuged again as described by Santos et al. (2013). Cell pellets were re-suspended in 1ml of potassium phosphate buffer (50 mM pH 7.0, 1mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) PVP, and 1mM DTT) and ultra-sonicated for 20 s at 0.5 cycles. The suspension was centrifuged at 10,000×g at 4 ºC for 10 min and supernatant

collected. HNO_3 65% (100µl) was added to the supernatant and deionized water was added until a final volume of 5 ml was reached. Cu intracellular concentrations were quantified by ICP-MS.

2.3 Lipid peroxidation (LPO)

LPO was measured by quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described by Buege and Aust (1978). *T. flocculosa* cells were harvested and washed as described in section 2.2. The pellet was suspended in TCA (20%), sonicated and centrifuged as in section 2.2. Absorbance was measured at 535 nm ($\varepsilon = 1.56 \times 10^5 M$ /cm). Results were expressed in µmol of MDA equivalents per million cells (µmol MDAeq /M cells).

2.4 Proteins

Intracellular proteins (Prot)

Protein concentration was measured following the method described by Bradford (1976) and adapted by Spector (1978). *T. flocculosa* cells were harvested, washed and supernatant extracted as described in section 2.2. Protein quantification took place at 595 nm with a microplate reader, using bovine serum albumin (BSA) as standard. Results were expressed in milligram protein per million cells (mg protein /M cells).

Frustulins (Frust)

Frutulins' extraction was prepared as described by Kröger et al., (1994) and adapted by Santos et al. (2013). Harvested and washed cells (section 2.2) were re-suspended in 3 ml EDTA (0.1 M pH 7.8) and incubated overnight, at 4ºC under agitation (150 rpm). Incubated cells were centrifuged (9000xg, 2 min, at 4ºC) and the supernatant absorbance read at 280nm (Noble and Bailey, 2009). Results were expressed in µg protein per million cells (µg protein /M cells).

Protein carbonylation (PC)

Carbonyl groups (CG) in proteins were quantified based on the method described by (Mesquita et al., 2014). In the same cell extracts used for intracellular protein determination, the amount of CG was quantified spectrophotometrically at 450 nm (Ɛ = 22 308M /cm) and results were expressed in nmol of CG per million cells (nmol CG /M cells).

2.5 Electron chain transport system activity (ETS)

ETS activity was measured based on King and Packard (1975) method with modifications described by De Coen and Janssen (1997). Cells were harvested and washed as in section 2.2. Pelleted cells were sonicated and centrifuged also as in section 2.2. The absorbance was read at 490 nm. The amount of formazan formed was calculated using the molar extinction coefficient of formazan (15900 M /cm) and the results were expressed in µmol of formazan per million cells (µmol /M cells).

2.6 Attached and non-attached exopolysaccharides (EPS)

Undisturbed culture medium (non-attached) and cell attached EPS were determined using Staats et al. (2000) procedure adapted by Santos et al. (2013). Attached and non-attached EPS were determined by the phenol-sulphuric acid colorimetric method (DuBois et al., 1956). The absorbance was measured at 490 nm using sucrose as standard. Results were expressed in µg saccharides per million cells (µg /M cells).

2.7 Antioxidant response

Superoxide dismutase activity (SOD)

Harvested and washed cells (section 2.2) were re-suspended in extraction buffer (50 mM potassium phosphate, pH 7.0, 1mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) PVP, and 1mM DTT) and sonicated for 20 s at 0.5 cycles. The extract was centrifuged at 10,000×g

at 4ºC for 10 min. The method of Beauchamp and Fridovich (1971) was followed with slight modifications (Branco et al., 2010). The absorbance was measured at 560 nm using a standard curve with SOD standards (0-1.5 U/mL). Results were expressed in enzyme units per million cells (U /M cells).

Catalase activity (CAT)

Cell extract was obtained using the procedure described for SOD. CAT activity was determined by the method of Johansson and Håkan Borg (1988) adapted by Branco et al. (2010). The absorbance was measured at 540 nm using formaldehyde as standards (0-60 µM). Results were expressed in milliunits per million cells (mU /M cells).

Glutathione S-transferase activity (GSTs)

Cell extract was obtained using the procedure described for SOD. GSTs activity was determined by the method of Habig and Jakoby (1981) using CDNB (2,4-dinitrochlorobenzene) as substrate. The thioether (Ɛ =9.6 mM /cm) can be monitored by the increase of absorbance at 340nm. Results were expressed in milliunits per million cells (mU /M cells).

Glutathione content

Cell extract was obtained using the procedure described for SOD, but pelleted cells were suspended in a different extraction buffer (0.1% Triton X-100, 0.6% sulphosalicylic acid in KPE buffer (0.1M phosphate buffer, 5Mm EDTA, pH= 7.5)). Glutathione content was quantified as described by Rahman et al. (2006). GSH and total GSH were determined by measuring at 412 nm the TNB (2-nitro-5-thiobenzoate) formed. Oxidized glutathione was estimated as the difference between total and reduced glutathione (GSSG=2(GSHT-GSH)), since one molecule of GSSG is formed by the oxidation of two GSH. Results were expressed in nmol GSH or GSSG per million cells (nmol /M cells).

2.8 Metabolite profiling by GC-MS

Sample preparation

Sample preparation was performed according to Gullberg et al. (2004) using 300 µL of extraction buffer (20/20/60 v/v chloroform:water:methanol) including internal standards which were added to 1 million *T. flocculosa* cells. At the end of the procedure, 250 µL of supernatant was transferred to a microvial and solvents were evaporated.

Derivatization

Derivatization of the previous samples was performed according to Gullberg et al. (2004) using a volume of 7.5 µL of methoxyamine, MSTFA (N-methyl-N-trimethylsilytrifluoroacetamide) and methyl stearate solution instead of 30 µL.

Analytical procedure

Gas chromatography with mass spectrometry analysis was performed as described previously (Gullberg et al., 2004; Nordström and Lewensohn, 2010). In detail, 1 µL of the derivatized sample was injected splitless (or split 1:20) by an CTC Combi Pal Xt Duo (CTC Analytics AG, Switzerland) autosampler/robot into an Agilent 7890A gas chromatograph equipped with a 30 m × 0.25 mm i.d. fused-silica capillary column with a chemically bonded 0.25 µm DB 5-MS UI stationary phase (J&W Scientific, Folsom, CA). The injector temperature was 260 °C, the purge flow rate was 20 mL/min, and the purge was turned on after 75 seconds. The gas flow rate through the column was 1 mL /min, and the column temperature was held at 70 °C for 2 min, followed by an increase of 20 °C /min until 320 °C, and held at 320ºC for 8 min. The column effluent was introduced into the ion source of a Pegasus HT time-of-flight mass spectrometer, GC/ToFMS (Leco Corp., St Joseph, MI). The transfer line and the ion source temperatures were 250 and 200 °C, respectively. Ions were generated by a 70 eV electron beam at an ionization current of 2.0 mA, and 20 spectra /s were recorded in the mass range m/z 50−800. The acceleration voltage was turned on after a solvent delay of 290 seconds. The detector voltage was 1500-2000V.

Data analysis

MS-files from the obtained metabolic analysis were exported from the ChromaToF software in NetCDF format to MATLABÔ R2011b (Mathworks, Natick, MA, USA), where

all data pre-treatment procedures, such as base-line correction, chromatogram alignment, data compression and Hierarchical Multivariate Curve Resolution (H-MCR), were performed using custom scripts according to Jonsson et al. (2006). The extracted mass spectra were identified by comparisons of their retention index and mass spectra with libraries of retention time indices and mass spectra (Schauer et al., 2005). All multivariate statistical investigations (PCA- Principal Component Analysis, OPLS-DA- Orthogonal Projections to Latent Structures Discriminant Analysis) were performed using the software package SIMCA version 13.0.2 (Umetrics, Umeå, Sweden). Identification of compounds was based on comparison with mass spectra libraries (in-house database) as well as retention index.

2.9 Statistical analysis

Hypothesis testing was performed by Permutation Multivariate Analysis of Variance (PERMANOVA) (Anderson et al., 2005). All data analysis was performed with the software PRIMERv6 (Clarke and Gorley, 2006) with the add-on PERMANOVA+ (Anderson et al., 2008). To run the PERMANOVA tests we considered 9999 Monte Carlo permutations and pairwise comparisons between Cu conditions. Our null hypothesis was: different Cu concentrations (0.3, 6 and 10 µg /L) do not cause differences in T. *flocculosa* relatively to control condition. This hypothesis was tested for all parameters described in section 2, including metabolomic analysis. Values of $p \leq 0.05$ revealed that conditions differed significantly, indicated in figures by different lowercase letters.

Matrix gathering the biomarker responses (Growth, LPO, Prot, Frust, PC, ETS, EPS, SOD, CAT, GSTs, GSSG and GSH) was used to calculate the Euclidean distance similarity matrix. The data used to calculate the matrix was previously normalized and transformed (square root). The matrix was then simplified through the calculation of the distance among centroid based on the condition and then submitted to ordination analysis, performed by Principal Coordinates (PCO). Spearman correlation vectors (r>

0.90) of *T. flocculosa* cells' biochemical responses were superimposed on the top of the PCO graph.

3. Results

3.1 Growth

Exposure to Cu for 96h induced significant growth differences at the highest concentrations (6 and 10 µg/L), with approximately 50% and 70% growth inhibition, respectively. Although not significant ($p>0.05$), at 0.3 µg/L, cells showed a 25% growth decrease compared to the control (Figure 1A).

3.2 Cu concentration

In the cytosol Cu was below detection limit (<0.04 µg/L) for the control (0) and 0.3 µg/L conditions. However, when growth medium was supplemented with 6 or 10 µg /L, cytosolic Cu concentrations were 0.04 and 0.18 µg/M cells, respectively (Figure 1B). Thus, in cells grown at 10 µg/L intracellular Cu increased 4.5-fold compared to the 6 µg/L condition.

3.3 Cell damage:

LPO tended to increase as cells were exposed to higher Cu concentrations (Figure 1C). Moreover, differences were significant ($p<0.05$) between the two lowest concentrations (0.3 and 6 µg/L) and the control. The highest levels of LPO were recorded at 10 µg/L, which were significantly higher compared to control (4.5-fold) and to 0.3 and 6 µg/L (~2-fold).

The increasing trend was also observed for PC as Cu concentrations increased (Figure 1D). At 0.3 µg Cu/L, no significant differences were observed compared to the control (0). However, PC increased 2-fold at 6 µg/L, compared to 0 and 0.3 µg/L, and 3-fold at 10 µg/L (Figure 1D).

3.4 Physiological parameters:

T. flocculosa cells did not show significant differences in protein content among all tested conditions. However, cells grown at 6 µg/L had 3-fold more protein than the remaining three conditions (Figure 1E).

Control and 0.3 µg/L conditions presented similar levels of ETS activity (Figure 1F). Increases were observed at higher Cu concentrations. At 6 and 10 µg Cu/L, significant increases compared with 0 and 0.3 µg/L were observed. ETS activity was highest at 10 µg Cu/L, with a 4-fold increase compared to 0 and 0.3 µg/L.

The production of EPS attached and non-attached to the frustule showed that non-attached represented less than 1% of total EPS, therefore they were considered not important and were not presented here. Attached EPS increased significantly in all Cu exposures compared to the control. At 0.3 and 6 µg/L, EPS increased 3 and 9-fold, respectively. The highest increase was observed at 10 µg/L (33 to 98-fold) (Figure 1G).

Frustulins showed also an increasing trend as Cu concentrations in the medium increased, but changes were not significant among 0, 0.3 and 6 µg Cu/L (Figure 1H). At 10 µg/L, a significant increase compared with the remaining conditions was found.

3.5 Antioxidant and Biotransformation Response

The highest SOD activity was recorded at 6 µg/L. No significant differences were found between 0 and 0.3 µg/L. Activity changed significantly compared to the control at 6 µg/L (4-fold increase) and 10 µg Cu/L (2-fold decrease) (Figure 2A).

CAT activity increased with increasing Cu concentrations. Between 0 and 0.3 µg/L and between 6 and 10 µg/L no significant differences were observed. However, a 2 to 3-fold increase separates the lowest (0 and 0.3 µg/L) from the highest (6 and 10 µg/L) Cu concentrations.

GSTs activity (biotransformation response) also showed and increasing trend as Cu concentrations increased, with significant differences at 6 (2-fold) and 10 µg/L (3.5-fold) compared to the control and 0.3 µg Cu/L (Figure 2C).

Glutathione synthesis (GSH and GSSG) was induced by Cu. However, the increase at the lowest Cu concentration (0.3 µg/L) was not significantly different from the control both for GSH and GSSG (Figures 2D and 2E). Increases in GSH and GSSG were observed at higher Cu concentrations (6 and 10 µg/L) compared to the control for GSH (Figure 2D) and for GSSG (Figure 2E).

3.6 PCO biochemistry analysis

Principal Coordinates (PCO) analysis resulting from applying a multivariate analysis to the biochemical and physiological determinants for each condition tested (0, 0.3, 6 and 10 µg Cu/L) evidenced PCO_1 as the main axis (85,8%) explaining the variation obtained between conditions (Figure 3). Along PCO_1, three groups were clearly separated, 0 and 0.3 µg/L on the negative side, 6 µg/L close to the origin of PCO_1 axis and 10 µg/L on the positive side of the axis. PCO_2, explained 12,9% of total variation, separating 6 µg/L in the positive side from the other three conditions on the negative side of the axis. From

PCO analysis it is possible to observe that SOD activity was strongly correlated (r=0.92) with PCO_2 and therefore more related with 6 µg/L. On the other hand, PC (r=0.94), ETS (r=0.96), GSSG (r=0.97) and LPO (r=0.97) were more correlated with PCO_1 and 10 µg/L. Indeed, these four parameters increased at 10 µg Cu/L compared to the remaining conditions (0, 0.3 and 6 µg Cu/L).

3.7 Metabolomics analysis

Metabolomics' analysis was able to separate 123 compounds (Supplementary table S2), from these, 102 compounds were found to differ significantly between Cu exposures, and thus those were further analysed. The sum of the peak area of all the compounds (123) showed a slight but not significant increase from 0 to 0.3 µg/L. At 6 µg/L, the total peak area was significantly higher compared to other conditions. At 10 µg/L, total peak area was not significantly different from 0 and 0.3 µg/L conditions (Figure 4A).

The analysis of the compounds with significantly different peak areas between two conditions (Figure 4B) showed that the number of compounds varying between conditions was lower at 0 - 0.3 µg/L, 0.3 - 10 µg/L and 6 - 10 µg/L (<40). On the other hand, higher differences were observed at 0 - 6 µg/L (87), 0 - 10 µg/L (73) and 0.3 - 6 µg/L (72). These results evidenced that the 6 µg Cu/L condition included many compounds changing peak areas relatively to the conditions with lower Cu concentrations (0 and 0.3 µg/L). Although the total peak area at 10 µg/L condition was similar to 0 and 0.3 µg/L, a higher number of compounds differed between 0 - 10 µg/L than between 0.3 – 10 µg/L.

From the 102 compounds showing differences between conditions, 13 were not considered for the analysis of the chemical families (Figure 4C) since their identification was not possible. The remaining 89 were divided by chemical families. Lipids, N-compounds and terpenoids were the most representative families (Figure 4C). Lipid

percentage was similar among 0, 0.3 and 6 µg/L conditions, increasing about 20 % at 10 µg/L. Sugar percentage increased at the highest Cu concentration compared with other conditions. The percentage of N- compounds decreased between the control and the remaining conditions. Terpenoid compounds increased from 0 to 6 µg/L and decreased steadily between 6 and 10 µg/L (Figure 4C).

The total peak area for each chemical family of compounds is presented in Figure 5. The variation at each Cu concentration compared to the control of the most biologically relevant compounds is also presented (c.f. Figure 5).

The total peak area of lipids increased steadily with Cu concentrations (Figure 5A). Differences in the total peak areas were not significant between 6 and 10 µg/L, but these two conditions had significant higher lipid areas than 0.3 µg/L and especially 0 µg/L. Several types of lipids, such as: saturated, unsaturated, cyclic, branched and glycerol derivated were identified. Twenty-one saturated and 12 unsaturated where identified but the total area of both classes was similar at 0, 0.3 and 6 µg/L (Supplementary table S2). At 10 µg/L, the unsaturated decreased 30% relatively to the saturated lipids. The lipid compounds presented are fatty acids (FA) with 10 to 22 carbons. These lipids displayed two variation trends: some, in most cases unsaturated FAs, increased their total area at 0.3 and 6 µg/L followed by a decrease at 10 µg/L. The other FAs increased with Cu exposure, especially at 6 and 10 µg/L, and most of them are saturated lipids, but Myristoleic acid (an unsaturated FA) also increased. Myristoleic acid, 1-Hexadecanol, Eicosanoic acid and 2-palmitoylglycerol displayed the most expressive increases at high Cu concentrations. Indeed, these compounds showed a 12.5, 5.9, 2.7 and 22.9-fold increase, compared to the control, respectively (c.f. Figure 5A).

Although the relative percentage of N-compounds decreased with Cu (Figure 4C), the total peak areas were similar between 0 and 0.3 µg/L, increased at 6 µg/L and decreased at 10 µg/L (Figure 5B). About 58% of the N-compounds showing variations were amino acids. All the amino acids presented a maximum area at 6 µg/L and some decreased from 6 to 10 µg Cu/L to lower values than the control. Serine was the only amino acid

increasing for all the Cu exposures compared to the control. Arginine and Lysine (both positively charged) and Glutamine (polar amino acid) evidenced the most expressive decreases at 10 µg Cu/L compared to the control (c.f. Figure 5B). The non-amino acid N-compound hydroxylamine decreased steadily with increasing Cu concentrations and pyroglutamic acid showed a modest increase at 6 and 10 µg/L compared to the control (c.f. Figure 5B).

The total peak area of sugar related compounds showed an increasing trend with Cu concentrations, not significant at 0.3 µg/L but significant at 6 and 10 µg/L compared to the control (Figure 5C). However, in this group distinct variations were observed. Exposure to Cu increased glycerol compared to the control (1.2 to 7.1-fold). Sucrose showed a slight decrease at 0.3 µg/L compared to the control and increased 2.5 to 3.3-fold at 6 and 10 µg/L, respectively. Other disaccharides and trisaccharides increased at 0.3 and 6 µg/L and decreased at 10 µg/L. The disaccharide cellobiose showed a 20-fold increase at 6 and 10 µg/L conditions compared to the control.

Identified terpenoids included diterpenoids, steroids and sterols (Figure 5D). The total peak area of terpenoids followed a similar trend of N-compounds, increasing until 6 µg Cu/L and decreasing from 6 to 10 µg/L, with the peak area at 10 µg/L not being significantly different from the control condition (Figure 5D). Several sterols followed this trend. The most abundant terpenoids (highest peak areas) were diterpenoids, all showing small increases at 0.3 µg/L (2-fold) and a 5 to 26-fold increase at 6 and 10 µg/L compared to the control. Phytol, a linear terpenoid and a component of chlorophylls, increased at 0.3 and 6 µg/L relatively to the control but decreased at 10 µg/L (c.f. Figure 5D).

Some identified compounds did not belong to any of the mentioned chemical families (Figure 5E). The overall trend of these compounds was similar to N-compounds and terpenoids increasing at 0.3 and 6 µg/L and decreasing at 10 µg/L. For tris(2,4-di-tert-butylphenyl) phosphate, an increasing trend was recorded for all Cu concentrations compared to the control. Glutaric acid also increased with Cu gradient (1.4 to 2.9-fold)

but, for GABA, increases were noticed for 0.3 and 6 µg/L (1.2 and 2.4) followed by a decrease at 10 µg/L compared to the control (c.f. Figure 5E).

4. Discussion

In this study we assessed the impact of Cu on the freshwater diatom *Tabellaria flocculosa* by analysing biochemical, physiological and metabolomic alterations, as approaches to detect the effects of Cu in freshwater ecosystems. Tolerance of *T. flocculosa* to Cu was relatively high compared to earlier assumptions that it would be sensitive to metals (Morin et al., 2008). Our strain must have developed efficient tolerance mechanisms, recorded in this laboratory study, allowing its survival at relatively high Cu concentrations, such as the concentrations found in the original site (6.2 µg Cu/L).

Responses of *T. flocculosa* to Cu impact differed with the level of stress imposed. Cu induced toxic effects on *T. flocculosa* at low concentrations (0.3 µg Cu/L). However, not all the tolerance mechanisms were activated. At this concentration its full tolerance mechanisms are not yet activated. Growth was inhibited and LPO increased. Enzymatic antioxidant systems (SOD, CAT and GSTs) and soluble antioxidant molecules (GSH) were not activated. However, other antioxidants (diterpenoids) and extracellular immobilization (EPS) increased, counteracting Cu toxicity at this concentration.

At 6 µg Cu/L tolerance mechanisms were fully activated. At this concentration the cellular metabolism (ETS) increased, probably providing energy to mitigate Cu toxicity. Enzymatic (SOD and CAT) and non-enzymatic (glutathione) antioxidant response was activated, diterpenoids, lipids and N-compounds continued to increase. All together these alterations were efficient enough to control oxidative stress, since LPO did not increase significantly relatively to 0.3 µg Cu/L.

Finally, under the highest Cu treatment (10 µg Cu/L), tolerance mechanisms were not sufficient to maintain a redox balance in the cell. We observed that *T. flocculosa* cells were under extreme stress, reflected by profound physiological (ETS, protein levels), biochemical and metabolic (generally decrease of amino acids and terpenoids) alterations, high membrane (LPO) and protein (PC) damage, inhibition of enzymatic activity (SOD), as shown by PCO analysis, and possibly interference in frustule peptides involved with diatom biosilification (decrease in hydroxylamine). Other mechanisms were maintained (CAT and glutathione), or even increased (GSTs, EPS, frustulins, diterpenoids), contributing to minimize the deleterious effects of Cu to *T. flocculosa* cells.

1- Tolerance to Cu

Previous field and mesocosm studies showed a decrease in algal abundance with Cu concentrations between 5-10 µg Cu/L(Leland and Carter, 1984) or a reduction in diatom species diversity at 30 µg Cu/L (Kaufman, 1982). Several studies assessed the effects of Cu exposure in algae but most did not use realistic Cu concentrations (mg/L range) for European streams (Viana and Rocha, 2005). Additionally, most of the species used *Nitzschia closterium* and *Asterionella glacialis* (Stauber and Florence, 1987), *Phaeodactylum tricornutum* (Morelli and Scarano, 2004; Smith et al., 2014) and *Ceratoneis closterium* (Smith et al., 2014) are marine and will hardly reflect the conditions experienced by benthic diatom communities in freshwater streams. Steeman-Nielsen & Wlum-Anderson (1970) were the exception, showing complete growth inhibition of the freshwater diatom *N. palea* exposed to 5 µg Cu/L. Most of the studies evaluated Cu effects by EC50 values, growth inhibition or photosynthetic activity (reviewed in Viana and Rocha, 2005). In fact, all these studies evaluated the negative effects of Cu in algae. However, the use of different Cu concentrations, species from different environments (marine/freshwater), different exposure periods, different experimental set-ups and different endpoints makes it difficult to clarify the Cu impact on freshwater algae communities. .

2- Physiological and biochemical response

Extracellular polysaccharides (EPS) was the only biochemical parameter significantly increasing at low Cu concentrations (0.3 µg/L). At 6 µg Cu/L both EPS and frustulins, showed an increasing trend, but it was at 10 µg Cu/L that a significant increase was observed. Both parameters were described in other studies as mechanisms of metals tolerance. Florence et al. (1983) reported the increase of EPS production in the marine diatom *Nitzschia closterium* in response to increasing Cu concentrations. The excretion of protective extracellular products, such as polysaccharides, by biofilms, seems to be responsible for reducing the sensitivity to Cu exposure (Admiraal et al., 1999; Real et al., 2003; Sabatini et al., 2009). Frustulins increased more than 6-fold in *N. palea* exposed to Cd (Santos et al., 2013). However, studies evaluating the response of frustulins in diatoms exposed to Cu was not yet presented. In our study, EPS and frustulins increase appears as a tolerance strategy to decrease the entrance of Cu into *T. flocculosa* cells, protecting them especially at high Cu concentrations.

GSH and GSSG contents also increased at the highest Cu concentrations (6 and 10 µg/L) in *T. flocculosa,*.These results are not supported by previous studies. GSH decreased under Cu impact in the green algae *Chlamydomonas reinhardtii* (Jamers et al., 2013a), *Chlorella vulgaris* (Mallick, 2004), *Chlorella pyrenoidosa (*Stauber and Florence, 1987)*,* and in the marine diatoms *Nitzschia closterium* and *Asterionella glacialis* (Stauber and Florence, 1987). Moreover, Smith et al. (2014), compared the glutathione content between *Phaeodactylum tricornutum* and *Ceratoneis closterium,* exposed to Cu (5 to 24 µg Cu/L) during 72h. Total glutathione increased in *P. tricornutum* but decreased in *C. closterium* and the GSH (reduced) content did not vary in *P. tricornutum* but decreased in *C. closterium.* Given the antioxidant properties of GSH and their involvement in both antioxidant (GPx) and biotranformation (GSTs) enzymes, the authors hypothesized that differences in the sensitivity to Cu observed between the two species could be related to differences in intracellular detoxification strategies, such as

glutathione synthesis and oxidation status. These results confirmed the importance of glutathione in the diatom tolerance, helping to support the high tolerance of *T. flocculosa* to Cu.

ROS scavenging mechanisms were not activated at 0.3 µg Cu/L, and although Cu was not detected intracellularly at this concentration, oxidative stress was higher than in the control and significant membrane damage (LPO increase) was observed. The activation of the antioxidant response (SOD, CAT, GSTs, glutathione) at 6 µg Cu/L was enough to maintain similar levels of LPO compared to 0.3 µg/L. Even though at 6 µg Cu/L protein damage (PC) increased significantly (2-fold) the total protein content also increased compensating the higher damage (Santos et al., 2016). GSTs activity can help to minimize the effects of LPO products in cells. Higher glutathione concentrations allow higher efficiency of cytosolic metal complexation and higher availability of substrate for the transformation of aldehydes into less toxic compounds, reducing metal deleterious effects and contributing for this species tolerance. The effort to synthesize proteins was supported by the increase of amino acids showed by the metabolomics analysis.

At 10 µg/L, CAT activity was maintained but SOD was severely inhibited, evidencing the higher vulnerability of SOD to oxidative stress. Previous studies using biochemical approaches to assess Cu toxicity showed a general increase of the antioxidant activity and LPO levels (Mallick, 2004; Rijstenbil et al., 1994). Morelli and Scarano, (2004) used 10 µM Cu (635 µg/L) in *Phaeodactylum tricornutum* (during 7h, 24h and 48h) and observed an increase in LPO and in SOD and CAT activity. Sabatini et al. (2009), exposing two green algae during one week to Cu concentrations ranging from 6.2 to 414 µM (394 µg/L to 26 mg/L) reported increases in protein and GSH content and in SOD and CAT activity.

3- Metabolomic response

At 0.3 µg Cu/L, an increase in the metabolite profiling was noticed. However, significant alterations were only observed in lipids, evidencing lipids as the most sensitive metabolites to low Cu stress. Real et al. (2003), used concentrations of 44 µg Cu/L in a simple aquatic food chain. The biofilm lipid content increased, in agreement with what is shown in our study, also evidencing lipids importance in Cu toxicity. At higher Cu stress, our results showed that lipids unsaturation decreased, which may conduct to a decrease in membrane fluidity, but also rendering membranes more tolerant to oxidation, and thus allowing the preservation of membrane integrity under oxidative conditions (Upchurch, 2008). The oxidation of fatty acids (FA) is generally dependent on the unsaturation level, the higher the unsaturation the higher the susceptibility to oxidation (Richard et al., 2008). The increase in the content of docosahexaenoic acid (DHA) and the decrease of hexadecanoic acid (palmitic acid), which are the most and the least susceptible FAs to oxidation, respectively (Richard et al., 2008), was observed in our study and support that Cu induced a mechanism preserving membrane integrity. Eicosapentanoic acid (EPA) is an effective O_2^- scavenger and increased at high Cu concentrations compared to the control. Both, EPA and DHA, where described to scavenge ROS (Richard et al., 2008), and may indicate the attempt of *T. flocculosa* cells to control lipid peroxidation and decrease the vulnerability to Cu toxicity.

At high Cu stress, unsaturated FA are oxidized by ROS, increasing FA peroxidation and their degradation products. Lipid peroxidation products are toxic to cells, interfering with molecules such as proteins (PC) and inactivating enzymes (Birben et al., 2012). These products are converted into less toxic compounds, such as the alcohol 1- Hexadecanol by biotransformation enzymes, such as GSTs to minimize damage (Regoli and Giuliani, 2014).

Monoglycerols, as 2-palmitoyl glycerol, are derivates from triacyclglycerols β-oxidation (Du and Benning, 2016; Guschina and Harwood, 2006; Sato et al., 1986; van den Berg et al., 2006)The increase of monoglycerols and glycerol, observed in this study, evidence

the use of storage lipids as energy source in severe stress situations, as elevated Cu concentrations, evidencing the low energy status of cells.. As diatoms accumulate large amounts of storage lipids and lipids are very energetic, their oxidation constitutes an efficient way to provide the energy needed to fuel mechanisms conferring tolerance to Cu.

The severe decrease in amino acids observed at high Cu concentrations was consistent with the decreasing trend of proteins. Specially Alanine, Valine, Glutamine, Arginine and Lysine, which are main constituents and precursors of proteins synthesis decreased steadily. Real et al. (2003), Prat et al. (1987) and Luderitz and Nicklisch (1989) showed decreases in protein content in stream biofilms exposed to metals. Jamers et al. (2013) also found changes in the content of amino acids (for example decrease of serine) when *Chlamydomonas reinhardtii* was exposed to different Cu concentrations. In our study, serine evidenced an opposite trend, increasing as Cu concentrations increased. Serine provides methyl groups for DNA methylation, thus controlling gene transcription (Maddocks et al., 2016) and it is also important in proteins with serine residues that can be phosphorylated, as kinases, which are involved in cell signalling. Thus, the increase in serine at high Cu concentrations, evidences the cells attempt to fight the stress induced by Cu. In turn, Glutamine is deaminated to glutamate and further to glutaric acid for energy production in Krebs cycle, explaining the increase in glutaric acid observed in our work at higher Cu concentrations. Valine and Arginine feed the biosynthesis of secondary metabolites as terpenoids, which increased at higher Cu concentrations (Hildebrandt et al., 2015).

Diterpenoids, some of which have antioxidant properties (Ayyad et al., 2011; Foti et al., 1994; Kabouche et al., 2007), increased as Cu concentrations increased. Due to their lipophilic nature, diterpenoids may be able to protect membranes from peroxidation and render cells more tolerant to Cu toxicity. The linear increase of diterpenoids to Cu concentrations, evidences these compounds as excellent markers of Cu exposure. The

decrease of phytol, a constituent of chlorophylls, at high Cu concentrations may be related to the inability of cells to maintain chlorophyll synthesis (Hildebrandt et al., 2015). Phytol was also reported to feed the biosynthesis of tocopherols, which are well known lipid antioxidants and are able to protect membranes from peroxidation (Mach, 2015; Pinto et al., 2003; Tripathi et al., 2006).

Hydroxylamine (an analogue of hydroxyproline) is a constituent of a fibrous protein in the diatom silicified wall (Round et al., 1990). Its decrease may thus be related to alterations in the frustule formation and to teratologies. In a previous study, it was shown that exposure to Cd affected the silicate ordering during frustule synthesis, probably by interfering with frustule peptides involved with diatom biosilification (Heredia et al., 2012).

Tris 2,4 di-tert-butylphenyl phosphate increased as *T. flocculosa* was exposed to increasing Cu concentrations. This compound was isolated from *Vitex negundo* leaves and was also reported to have antioxidant properties (Vinuchakkaravarthy et al., 2011). Thus, the increase of this compound may be also related with the effort of *T. flocculosa* cells to fight the oxidative stress generated by Cu.

Alterations in metabolic pathways synthetizing compounds involved in stress tolerance, enzymes activity or extracellular metal ions immobilization are processes that require energy. And thus, at high Cu concentrations *T. flocculosa* cells decrease the storage reserves (lipids) and increased the electron transport system activity (ETS) to supply the energy demand to trigger defence mechanisms and render cells more tolerant to Cu.

5. Conclusions:

This study generated fundamental knowledge that may help to assess the effects of environmental relevant concentrations of Cu using responses at the biochemical, physiological and metabolomics levels. The three studied Cu concentrations caused different responses in *T. flocculosa*, ranging from a relatively low stress response, to an active response that can control damages but at the cost of an increased energy demand

and increased metabolism to an extreme stress response where several important cell pathways are affected, leading to cellular damages and growth inhibition. Based on this study new research should be conducted to test the new proposed markers in other Cu concentrations and in other organisms individually (different algae species) or in assemblages (biofilms).

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References:

Admiraal, W., Blanck, H., Buckert-De Jong, M., Guasch, H., Ivorra, N., Lehmann, V., Nystr??m, B.A.H., Paulsson, M., Sabater, S., 1999. Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream. Water Res. 33, 1989–1996. https://doi.org/10.1016/S0043-1354(98)00426-6

Allan, J.D., Castillo, M.M., 2007. Stream Ecology : Struture and functions of running waters, second. ed. Springer US. https://doi.org/10.1007/978-1-4020-5583-6

Andersen, R.A., 2005. Algal culturing techniques. Elsevier Academic Press.

Anderson, M., Gorley, R., Clarke, R., 2008. Permanova+ for Primer: Guide to Software and Statisticl Methods.

Anderson, M., Gorley, R., Clarke, R., 2005. Permanova. Multivar. Anal. variance, a Comput. ….

APA, 2016. – *Environmental Portuguese Agency* Plano de Gestão de Região Hidrográfica 2016/2021.

Ayyad, S.-E.N., Makki, M.S., Al-kayal, N.S., Basaif, S.A., El-Foty, K.O., Asiri, A.M., Alarif, W.M., Badria, F.A., 2011. Cytotoxic and protective DNA damage of three new diterpenoids from the brown alga Dictyota dichotoma. Eur. J. Med. Chem. 46, 175–182. https://doi.org/10.1016/j.ejmech.2010.10.033

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287. https://doi.org/10.1016/0003-2697(71)90370-8

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organ. J. 5, 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Bonet, B., Corcoll, N., Acuña, V., Sigg, L., Behra, R., Guasch, H., 2013. Seasonal changes in antioxidant enzyme activities of freshwater biofilms in a metal polluted Mediterranean stream. Sci. Total Environ. 444, 1–10. https://doi.org/10.1016/j.scitotenv.2012.11.036

Bonet, B., Corcoll, N., Guasch, H., 2012. Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms. Ecotoxicol. Environ. Saf. 80, 172–178. https://doi.org/10.1016/j.ecoenv.2012.02.024

Borowitzka, M.A., Beardall, J., Raven, J.A., 2016. The physiology of microalgae.

Branco, D., Lima, A., Almeida, S.F.P., Figueira, E., 2010. Sensitivity of biochemical markers to evaluate cadmium stress in the freshwater diatom Nitzschia palea (K??tzing) W. Smith. Aquat. Toxicol. 99, 109–117. https://doi.org/10.1016/j.aquatox.2010.04.010

Bringmann, G., Kühn, R., 1980. Comparison of the toxicity thresholds of water pollutants to bacteria, algae, and protozoa in the cell multiplication inhibition test. Water Res. 14, 231–241. https://doi.org/10.1016/0043-1354(80)90093-7

Brown, B.T., Rattigan, B.M., 1979. Toxicity of soluble copper and other metal ions to Elodea canadensis. Environ. Pollut. 20, 303–314. https://doi.org/10.1016/0013-9327(79)90153-8

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

Clarke, K., Gorley, R., 2006. PRIMER V6: user manual-tutorial.

Contreras, L., Moenne, A., Correa, J.A., 2005. Antioxidant responses in Scytosiphon lomentaria (Phaeophyceae) inhabiting copper-enriched coastal environments. J. Phycol. 41, 1184–1195. https://doi.org/10.1111/j.1529-8817.2005.00151.x

Corcoll, N., Bonet, B., Morin, S., Tlili, A., Leira, M., Guasch, H., 2012. The effect of metals on photosynthesis processes and diatom metrics of biofilm from a metal-contaminated river: A translocation experiment. Ecol. Indic. 18, 620–631. https://doi.org/10.1016/j.ecolind.2012.01.026

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recover. 6, 43–55. https://doi.org/10.1023/A:1008228517955

Debelius, B., Forja, J.M., DelValls, Á., Lubián, L.M., 2009. Toxicity and bioaccumulation of copper and lead in five marine microalgae. Ecotoxicol. Environ. Saf. 72, 1503–1513. https://doi.org/10.1016/j.ecoenv.2009.04.006

Du, Z.-Y., Benning, C., 2016. Triacylglycerol Accumulation in Photosynthetic Cells in Plants and Algae. Springer, Cham, pp. 179–205. https://doi.org/10.1007/978-3-319-25979-6_8

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350–356. https://doi.org/10.1021/ac60111a017

Dyhrman, S.T., Jenkins, B.D., Rynearson, T.A., Saito, M.A., Mercier, M.L., Alexander, H., Whitney, L.P., Drzewianowski, A., Bulygin, V. V., Bertrand, E.M., Wu, Z., Benitez-Nelson, C., Heithoff, A., 2012. The Transcriptome and Proteome of the Diatom Thalassiosira pseudonana Reveal a Diverse Phosphorus Stress Response. PLoS One 7, e33768. https://doi.org/10.1371/journal.pone.0033768

Elder, J.F., Horne, A.J., 1978. Copper cycles and CuSO4 algicidal capacity in two California lakes. Environ. Manage. 2, 17–30. https://doi.org/10.1007/BF01866443

Ferreira da Silva, E., Almeida, S.F.P., Nunes, M.L., Lu??s, A.T., Borg, F., Hedlund, M., de S??, C.M.,

Patinha, C., Teixeira, P., 2009. Heavy metal pollution downstream the abandoned Coval da M?? mine (Portugal) and associated effects on epilithic diatom communities. Sci. Total Environ. 407, 5620–5636. https://doi.org/10.1016/j.scitotenv.2009.06.047

Figueira, E., Freitas, R., Guasch, H., Almeida, S.F.P., 2014. Efficiency of cadmium chelation by phytochelatins in Nitzschia palea (K??tzing) W. Smith. Ecotoxicology 23, 285–292. https://doi.org/10.1007/s10646-013-1172-8

Florence, T.M., Lumdens, B.G., Fardy, J.J., 1983. EVALUATION OF SOME PHYSICO-CHEMICAL TECHNIQUES FOR THE DETERMINATION OF THE FRACTION OF DISSOLVED COPPER TOXIC TO THE MARINE DIATOM NITZSCHIA CLOSTERIUM. Anal. Chim. Acta 151, 281–295.

Foti, M., Piattelli, M., Amico, V., Ruberto, G., 1994. Antioxidant activity of phenolic meroditerpenoids from marine algae. J. Photochem. Photobiol. B Biol. 26, 159–164. https://doi.org/10.1016/1011-1344(94)07038-5

Guillard, R.R.L., Lorenzen, C.J., 1972. Yellow-green algae with chlorophyllide C. J. Phycol. 8, 10–14. https://doi.org/10.1111/j.1529-8817.1972.tb03995.x

Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M., Moritz, T., 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomic studies with gas chromatography/mass spectrometry. Anal. Biochem. 331, 283–295. https://doi.org/10.1016/j.ab.2004.04.037

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45, 160–186. https://doi.org/10.1016/j.plipres.2006.01.001

Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Methods Enzymol. 77, 398–405.

Hänsch, R., Mendel, R.R., 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr. Opin. Plant Biol. 12, 259–266. https://doi.org/10.1016/j.pbi.2009.05.006

Heredia, A., Figueira, E., Rodrigues, C.T., Rodríguez-Galván, A., Basiuk, V.A., Vrieling, E.G., Almeida, S.F.P., 2012. Cd2+ affects the growth, hierarchical structure and peptide composition of the biosilica of the freshwater diatom Nitzschia palea (Kützing) W. Smith. Phycol. Res. 60, 229–240. https://doi.org/10.1111/j.1440-1835.2012.00652.x

Heydarizadeh, P., Marchand, J., Chenais, B., Sabzalian, M.R., Zahedi, M., Moreau, B., Schoefs, B., 2013. Functional investigations in diatoms need more than a transcriptomic approach. Diatom Res. 29, 75–89. https://doi.org/10.1080/0269249X.2014.883727

Hildebrandt, T.M., Nunes Nesi, A., Araújo, W.L., Braun, H.-P., 2015. Amino Acid Catabolism in Plants. Mol. Plant 8, 1563–1579. https://doi.org/10.1016/j.molp.2015.09.005

Hoek, C. van den (Christiaan), Mann, D.G., Jahns, H.M., 1995. Algae : an introduction to phycology. Cambridge University Press.

Hogsden, K.L., Harding, J.S., 2012. Anthropogenic and natural sources of acidity and metals and their influence on the structure of stream food webs. Environ. Pollut. 162, 466–74. https://doi.org/10.1016/j.envpol.2011.10.024

HVMFS, 2016. Miljögifter i vatten – klassificering av ytvattenstatus Vägledning för tillämpning av HVMFS *Havs-och VattenMyndighetens FöreSkrifter - Legal regulation of the Swedish Agency for Marine and Water Management* 2013:19. Göteborg.

Ivorra, N., Barranguet, C., Jonker, M., Kraak, M.H.S., Admiraal, W., 2002. Metal-induced tolerance in the freshwater microbenthic diatom Gomphonema parvulum. Environ. Pollut. 116, 147–157. https://doi.org/10.1016/S0269-7491(01)00152-X

Jamers, A., Blust, R., De Coen, W., 2009. Omics in algae: Paving the way for a systems biological understanding of algal stress phenomena? Aquat. Toxicol. 92, 114–121. https://doi.org/10.1016/j.aquatox.2009.02.012

Jamers, A., Blust, R., De Coen, W., Griffin, J.L., Jones, O.A.H., 2013a. Copper toxicity in the microalga Chlamydomonas reinhardtii: An integrated approach. BioMetals 26, 731–740. https://doi.org/10.1007/s10534-013-9648-9

Jamers, A., Blust, R., De Coen, W., Griffin, J.L., Jones, O.A.H., 2013b. An omics based assessment of cadmium toxicity in the green alga Chlamydomonas reinhardtii. Aquat. Toxicol. 126, 355–364. https://doi.org/10.1016/j.aquatox.2012.09.007

Johansson, L.H., Håkan Borg, L.A., 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem. 174, 331–336. https://doi.org/10.1016/0003-2697(88)90554-4

Johnson, D.B., Hallberg, K.B., 2005. Acid mine drainage remediation options: A review. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2004.09.002

Johnson, W.M., Kido Soule, M.C., Kujawinski, E.B., 2017. Extraction efficiency and quantification of dissolved metabolites in targeted marine metabolomics. Limnol. Oceanogr. Methods 15, 417–428. https://doi.org/10.1002/lom3.10181

Jonsson, P., Johansson, E.S., Wuolikainen, A., Lindberg, J., Schuppe-Koistinen, I., Kusano, M., Sjöström, M., Trygg, J., Moritz, T., Antti, H., 2006. Predictive Metabolite Profiling Applying Hierarchical Multivariate Curve Resolution to GC−MS DataA Potential Tool for Multi-parametric Diagnosis. J. Proteome Res. 5, 1407–1414. https://doi.org/10.1021/pr0600071

Kabouche, A., Kabouche, Z., Öztürk, M., Kolak, U., Topçu, G., 2007. Antioxidant abietane diterpenoids from Salvia barrelieri. Food Chem. 102, 1281–1287. https://doi.org/10.1016/j.foodchem.2006.07.021

Kaufman, L.H., 1982. Stream aufwuchs accumulation: Disturbance frequency and stress resistance and resilience. Oecologia 52, 57–63. https://doi.org/10.1007/BF00349012

Kelly, M., Bennett, C., Coste, M., Delgado, C., Delmas, F., Denys, L., Ector, L., Fauville, C., Ferréol, M., Golub, M., Jarlman, A., Kahlert, M., Lucey, J., Ní Chatháin, B., Pardo, I., Pfister, P., Picinska-Faltynowicz, J., Rosebery, J., Schranz, C., Schaumburg, J., van Dam, H., Vilbaste, S., 2009. A comparison of national approaches to setting ecological status boundaries in phytobenthos assessment for the European Water Framework Directive: results of an intercalibration exercise. Hydrobiologia 621, 169–182. https://doi.org/10.1007/s10750-008-9641-4

Kelly, M.G., Cazaubon, A., Coring, E., Dell'Uomo, A., Ector, L., Goldsmith, B., Guasch, H., Hürlimann, J., Jarlman, A., Kawecka, B., Kwandrans, J., Laugaste, R., Lindstrøm, E.-A., Leitao, M., Marvan, P., Padisák, J., Pipp, E., Prygiel, J., Rott, E., Sabater, S., van Dam, H., Vizinet, J., 1998. Recommendations for the routine sampling of diatoms for water quality assessments in Europe. J. Appl. Phycol. 10, 215–224. https://doi.org/10.1023/A:1008033201227

Kelly, M.G., Gómez-Rodríguez, C., Kahlert, M., Almeida, S.F.P., Bennett, C., Bottin, M., Delmas, F., Descy, J.-P., Dörflinger, G., Kennedy, B., Marvan, P., Opatrilova, L., Pardo, I., Pfister, P., Rosebery, J., Schneider, S., Vilbaste, S., 2012. Establishing expectations for pan-European diatom based ecological status assessments. Ecol. Indic. 20, 177–186. https://doi.org/10.1016/j.ecolind.2012.02.020

King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton1. Limnol. Oceanogr. 20, 849–854. https://doi.org/10.4319/lo.1975.20.5.0849

Knauert, S., Knauer, K., 2008. The role of reactive oxygen species in copper toxicity to two freshwater green algae. J. Phycol. 44, 311–319. https://doi.org/10.1111/j.1529-8817.2008.00471.x

Kröger, N., Bergsdorf, C., Sumper, M., 1994. A new calcium binding glycoprotein family constitutes a major diatom cell wall component. EMBO J. 13, 4676–83.

Larson, D., 2010. Metallpåverkade sjöar och vattendrag i Dalarna. Konsekvenser av en tusenårig gruvhistoria. Rapporter från Länsstyrelsen i Dalarnas län. Falun: Miljöenheten Länsstyrelsen Dalarna.

Laube, V.M., McKenzie, C.N., Kushner, D.J., 1980. Strategies of response to copper, cadmium, and lead by a blue-green and a green alga. Can. J. Microbiol. 26, 1300–1311. https://doi.org/10.1139/m80-217

Lavoie, I., Campeau, S., Darchambeau, F., Cabana, G., Dillon, P.J., 2008. Are diatoms good integrators of temporal variability in stream water quality? Freshw. Biol. 53, 827–841. https://doi.org/10.1111/j.1365-2427.2007.01935.x

Leguay, S., Lavoie, I., Levy, J.L., Fortin, C., 2016. Using biofilms for monitoring metal contamination in lotic ecosystems: The protective effects of hardness and pH on metal bioaccumulation. Environ. Toxicol. Chem. 35, 1489–1501. https://doi.org/10.1002/etc.3292

LELAND, H. V., CARTER, J.L., 1984. Effects of copper on species composition of periphyton in a Sierra Nevada, California, stream. Freshw. Biol. 14, 281–296. https://doi.org/10.1111/j.1365-2427.1984.tb00041.x

Luís, A.T., Bonet, B., Corcoll, N., Almeida, S.F.P., Da Silva, E.F., Figueira, E., Guasch, H., 2014. Experimental evaluation of the contribution of acidic pH and Fe concentration to the structure, function and tolerance to metals (Cu and Zn) exposure in fluvial biofilms. Ecotoxicology 23, 1270–1282. https://doi.org/10.1007/s10646-014-1270-2

Luís, A.T., Teixeira, P., Almeida, S.F.P., Ector, L., Matos, J.X., Ferreira Da Silva, E.A., 2009. Impact of acid mine drainage (AMD) on water quality, stream sediments and periphytic diatom communities in the surrounding streams of aljustrel mining area (Portugal). Water. Air. Soil Pollut. 200, 147–167. https://doi.org/10.1007/s11270-008-9900-z

Mach, J., 2015. Phytol from Degradation of Chlorophyll Feeds Biosynthesis of Tocopherols. Plant Cell 27, tpc.15.00860. https://doi.org/10.1105/tpc.15.00860

Maddocks, O.D.K., Labuschagne, C.F., Adams, P.D., Vousden, K.H., 2016. Serine Metabolism Supports the Methionine Cycle and DNA/RNA Methylation through De Novo ATP Synthesis in Cancer Cells. Mol. Cell 61, 210–221. https://doi.org/10.1016/j.molcel.2015.12.014

Mallick, N., 2004. Copper-induced oxidative stress in the chlorophycean microalga Chlorella vulgaris: response of the antioxidant system. J. Plant Physiol. 161, 591–7. https://doi.org/10.1078/0176-1617-01230

Mallick, N., Mohn, F.H., 2000. Reactive oxygen species: response of algal cells. J. Plant Physiol. 157, 183–193. https://doi.org/10.1016/S0176-1617(00)80189-3

Mesquita, C.S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J., Marcos, J., 2014. Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. https://doi.org/10.1016/j.ab.2014.04.034

Millennium Ecosystem Assessment, 2005. Ecosystems and Human Well-being: Synthes. Island Press, Washington, DC.

Morelli, E., Scarano, G., 2004. Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga Phaeodactylum tricornutum. Plant Sci. 167, 289–296. https://doi.org/10.1016/j.plantsci.2004.04.001

Morelli, E., Scarano, G., 2001. Synthesis and stability of phytochelatins induced by cadmium and lead in the marine diatom Phaeodactylum tricornutum. Mar. Environ. Res. 52, 383–395. https://doi.org/10.1016/S0141-1136(01)00093-9

Morin, S., Cordonier, A., Lavoie, I., Arini, A., Blanco, S., Bonet, B., Duong, T.T., Torne, E., Becares, E., Coste, M., Faggiano, L., Laviale, M., Pe, F., Fortin, C., Guasch, H., Sabater, S., 2012. Consistency in diatom response to metal contaminated environments, in: Emerging and Priority Pollutants in Rivers. pp. 117–146. https://doi.org/10.1007/978-3-642-25722-3

Morin, S., Coste, M., Hamilton, P.B., 2008. Scanning electron microscopy observations of deformities in small pennate diatoms exposed to high cadmium concentrations. J. Phycol. 44, 1512–1518. https://doi.org/10.1111/j.1529-8817.2008.00587.x

Nielsen, E.S., Laursen, H.B., 1976. Effect of CuSO 4 on the Photosynthetic Rate of Phytoplankton in Four Danish Lakes. Oikos 27, 239. https://doi.org/10.2307/3543901

Nielsen, E.S., Wium-Andersen, S., 1970. Copper ions as poison in the sea and in freshwater. Mar. Biol. 6, 93–97. https://doi.org/10.1007/BF00347237

Noble, J.E., Bailey, M.J.A., 2009. Quantitation of Protein, in: Methods in Enzymology. pp. 73–95. https://doi.org/10.1016/S0076-6879(09)63008-1

Nordström, A., Lewensohn, R., 2010. Metabolomics: Moving to the Clinic. J. Neuroimmune Pharmacol. 5, 4–17. https://doi.org/10.1007/s11481-009-9156-4

Pandey, L.K., Bergey, E.A., 2016. Exploring the status of motility, lipid bodies, deformities and size reduction in periphytic diatom community from chronically metal (Cu, Zn) polluted waterbodies as a biomonitoring tool. Sci. Total Environ. 550, 372–381. https://doi.org/10.1016/j.scitotenv.2015.11.151

Peers, G., Price, N.M., 2006. Copper-containing plastocyanin used for electron transport by an oceanic diatom. Nature 441, 341–344. https://doi.org/10.1038/nature04630

Pinto, E., Sigaud- Kutner, T.C.S., Leitao, M.A.S., Okamoto, O.K., 2003. REVIEW HEAVY METAL – INDUCED OXIDATIVE STRESS IN ALGAE 1 Ernani Pinto Teresa C . S . Sigaud-Kutner , Maria A . S . Leita. J. Phycol. 39, 1008–1018. https://doi.org/10.1111/j.0022-3646.2003.02-193.x

Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat. Protoc. 1, 3159–65. https://doi.org/10.1038/nprot.2006.378

Real, M., Mu??oz, I., Guasch, H., Navarro, E., Sabater, S., 2003. The effect of copper exposure on a simple aquatic food chain. Aquat. Toxicol. 63, 283–291. https://doi.org/10.1016/S0166-445X(02)00187-X

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117. https://doi.org/10.1016/j.marenvres.2013.07.006

Richard, D., Kefi, K., Barbe, U., Bausero, P., Visioli, F., 2008. Polyunsaturated fatty acids as antioxidants. Pharmacol. Res. 57, 451–455. https://doi.org/10.1016/j.phrs.2008.05.002

Rijstenbil, J.W., Derksen, J.W.M., Gerringa, L.J.A., Poortvliet, T.C.W., Sandee, A., Van den Berg, M., Van Drie, J., Wijnholds, J.A., 1994. Oxidative stress induced by copper: defense and damage in the marine planktonic diatom Ditylum brightwellii, grown in continuous cultures with high and low zinc levels. Mar. Biol. 119, 583–590.

Ritter, A., Goulitquer, S., Salaün, J.P., Tonon, T., Correa, J.A., Potin, P., 2008. Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp Laminaria digitata. New Phytol. 180, 809–821. https://doi.org/10.1111/j.1469-8137.2008.02626.x

Ritter, A., Ubertini, M., Romac, S., Gaillard, F., Delage, L., Mann, A., Cock, J.M., Tonon, T., Correa, J.A., Potin, P., 2010. Copper stress proteomics highlights local adaptation of two strains of the model brown alga Ectocarpus siliculosus. Proteomics 10, 2074–2088. https://doi.org/10.1002/pmic.200900004

Rodríguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E., Bleecker, A.B., 1999. A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science 283, 996–8.

Round, F.E., Crawford, R.M., Mann, D.G., 1990. Diatoms: Biology and Morphology of the Genera - F. E. Round, R. M. Crawford, D. G. Mann - Google Livros, 2nd ed. Cambridge University Press.

Sabatini, S.E., Juárez, Á.B., Eppis, M.R., Bianchi, L., Luquet, C.M., Ríos de Molina, M. del C., 2009. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. Ecotoxicol. Environ. Saf. 72, 1200–1206. https://doi.org/10.1016/j.ecoenv.2009.01.003

Salminen, R., Batista, M.J., Bidovec, M., Demetriades, A., De Vivo, B., De Vos, W., Duris, M., Gilucis, A., Gregorauskiene, V., Halamic, J., Heitzmann, P., Lima, A., Jordan, G., Klaver, G., Klein, P., Lis, J., Locutura, J., Marsina, K., Ma, T., 2005. Geochemical Atlas of Europe. Part 1: Background Information, Methodology and Maps. Salminen, R. (Chief-editor), Batista, M. J., Bidovec, M., Demetriades, A., De Vivo, B., De Vos, W., Duris, M., Gilucis, A., Gregorauskiene, V., Halamic, J., Heitzmann, P., Lima, A., Jordan, G., Klaver, G., Klein, P., Lis, J., Locutura, J., Marsina, K., Ma.

Santos, J., Almeida, S.F.P., Figueira, E., 2013. Cadmium chelation by frustulins: A novel metal tolerance mechanism in Nitzschia palea (Kützing) W. Smith. Ecotoxicology 22, 166–173. https://doi.org/10.1007/s10646-012-1013-1

Santos, J., Almeida, S.F.P., Freitas, R., Velez, C., Esteves, S., Figueira, E., 2016. Intraspecific differences in cadmium tolerance of Nitzschia palea (K??tzing) W. Smith: a biochemical approach. Ecotoxicology 25, 1305–1317. https://doi.org/10.1007/s10646-016-1683-1

Sato, N., Seyama, Y., Murata, N., 1986. Lipid-Linked Desaturation of Palmitic Acid in Monogalactosyl Diacylglycerol in the Blue-Green Alga (Cyanobacterium) Anabaena variabilis Studied in Vivo. Plant Cell Physiol. 27, 819–835. https://doi.org/10.1093/oxfordjournals.pcp.a077168

Schauer, N., Steinhauser, D., Strelkov, S., Schomburg, D., Allison, G., Moritz, T., Lundgren, K., Roessner-Tunali, U., Forbes, M.G., Willmitzer, L., Fernie, A.R., Kopka, J., 2005. GC-MS libraries for the rapid identification of metabolites in complex biological samples. FEBS Lett. 579, 1332–1337. https://doi.org/10.1016/j.febslet.2005.01.029

Serra-Compte, A., Corcoll, N., Huerta, B., Rodríguez-Mozaz, S., Sabater, S., Barceló, D., Álvarez-Muñoz, D., 2017. Fluvial biofilms exposed to desiccation and pharmaceutical pollution: New insights using metabolomics. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2017.09.258

Smith, C.L., Steele, J.E., Stauber, J.L., Jolley, D.F., 2014. Copper-induced changes in intracellular thiols in two marine diatoms: Phaeodactylum tricornutum and Ceratoneis closterium. Aquat. Toxicol. 156, 211–220. https://doi.org/10.1016/j.aquatox.2014.08.010

Spector, T., 1978. Refinement of the Coomassie blue method of protein quantitation. Anal. Biochem. 86, 142–146. https://doi.org/10.1016/0003-2697(78)90327-5

Staats, N., Stal, L.J., Winder, B., Mur, L.R., 2000. Oxygenic photosynthesis as driving process in exopolysaccharide production of benthic diatoms. Mar Ecol Prog Ser 193, 261–269.

Stauber, J.L., Florence, T.M., 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. Mar. Biol. 94, 511–519. https://doi.org/10.1007/BF00431397

Teittinen, A., Taka, M., Ruth, O., Soininen, J., 2015. Variation in stream diatom communities in relation to water quality and catchment variables in a boreal, urbanized region. Sci. Total Environ. 530–531, 279–289. https://doi.org/10.1016/j.scitotenv.2015.05.101

Torres, M.A., Barros, M.P., Campos, S.C.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P., 2008. Biochemical biomarkers in algae and marine pollution: A review. Ecotoxicol. Environ. Saf. 71, 1–15. https://doi.org/10.1016/j.ecoenv.2008.05.009

Tripathi, B.N., Mehta, S.K., Amar, A., Gaur, J.P., 2006. Oxidative stress in Scenedesmus sp. during short- and long-term exposure to Cu2+ and Zn2+. Chemosphere 62, 538–544. https://doi.org/10.1016/j.chemosphere.2005.06.031

Upchurch, R.G., 2008. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnol. Lett. 30, 967–977. https://doi.org/10.1007/s10529-008-9639-z

van den Berg, R.A., Hoefsloot, H.C., Westerhuis, J.A., Smilde, A.K., van der Werf, M.J., Botte, P., Gallo, C., Fontana, A., 2006. Potential of lipid metabolism in marine diatoms for biofuel production. BMC Genomics 7, 142. https://doi.org/10.1186/1471-2164-7-142

Viana, S.M., Rocha, O., 2005. The toxicity of Copper Sulphate and Atrazine to the diatom Aulacoseira Granulata (Ehrenberg) Simmons . Ecologia 17, 291–300.

Viant, M.R., 2009. Applications of metabolomics to the environmental sciences. Metabolomics 5, 1–2. https://doi.org/10.1007/s11306-009-0157-3

Vinuchakkaravarthy, T., Kumaravel, K.P., Ravichandran, S., Velmurugan, D., 2011. Active compound from the leaves of Vitex negundo L. shows anti-inflammatory activity with evidence of inhibition for secretory Phospholipase A(2) through molecular docking. Bioinformation 7, 199–206.

Virgilio Perales - Vela, H., Mario Peñ - Castro, J., Olivia Cañ izares - Villanueva, R., 2006. Heavy metal detoxification in eukaryotic microalgae. https://doi.org/10.1016/j.chemosphere.2005.11.024

Walbridge, C., 1977. A Flow-Through Testing Procedure with Duckweed(Lemna Minor L.).

Young, R.G., Lisk, D.J., 1977. Effect of Copper and Silver Ions on Algae. J. (Water Pollut. Control Fed. https://doi.org/10.2307/25037583

Figures:

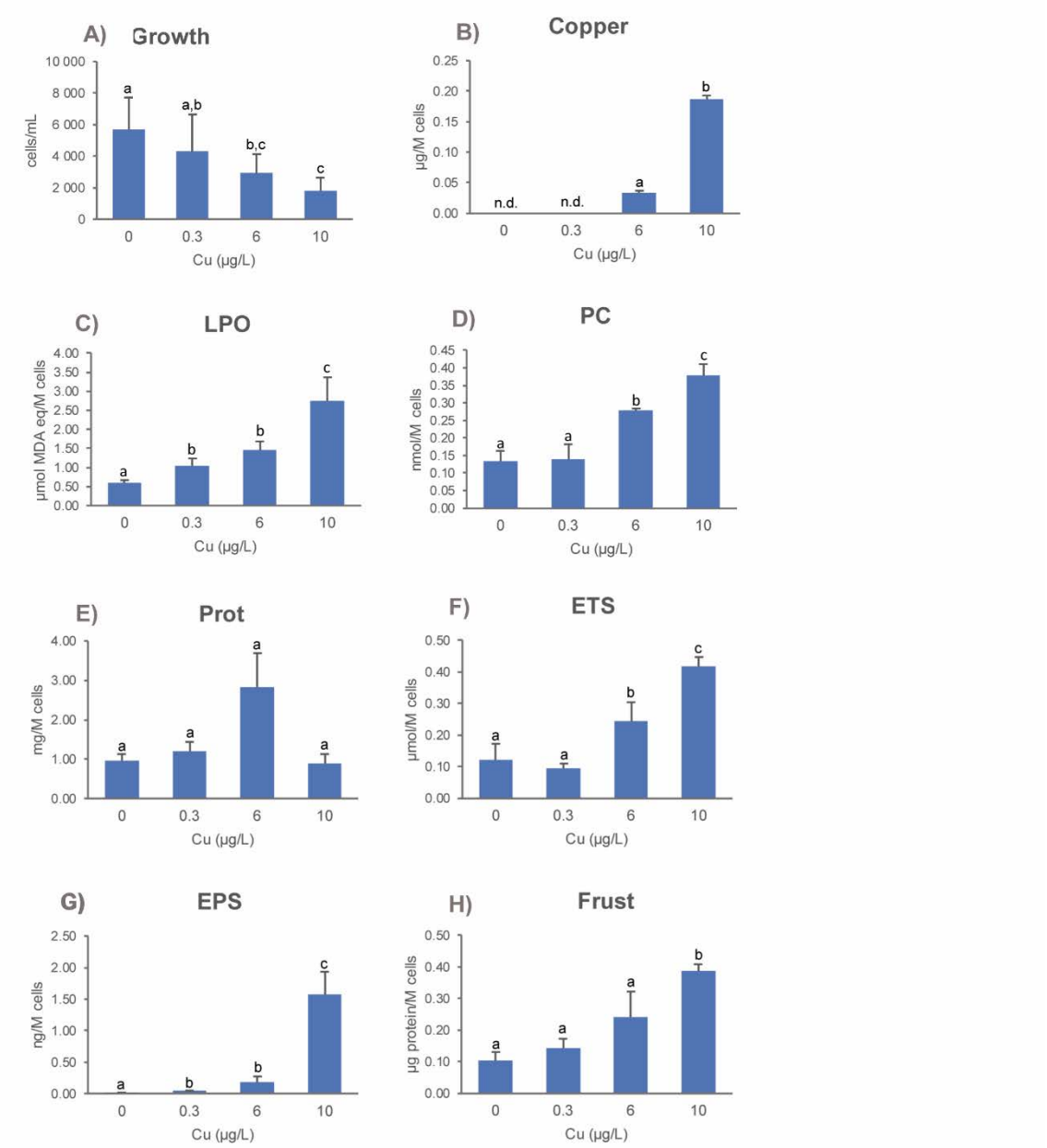

Figure 1 – Physiological responses of *Tabellaria flocculosa* cells exposed for 96h to different Cu concentrations (0, 0.3, 6 and 10 µg/L). A) Growth of TFLO. B) Intracellular Cu concentrations (n.d.- not detected). C) Lipid peroxidation (LPO). D) Protein carbonylation (PC). E) Total protein, (Prot). F) Mitochondrial electron chain activity (ETS). G) Attached exopolysaccharides (EPS) and H) Frustulins (Frust). For growth (A) values are means (+ standard deviation) of 36 replicates from 12 independent experiments; for intracellular Cu (B), LPO (C), PC (D), Prot (E) and ETS (F) values are means (+ standard deviation) of 6 replicates from 2 independent experiments; and for EPS (G) and Frust (H) values are means (+ standard deviation) of 12 replicates of 4 independent experiments. For each parameter, lowercase letters indicate significant differences among Cu exposures $p<0.05$.

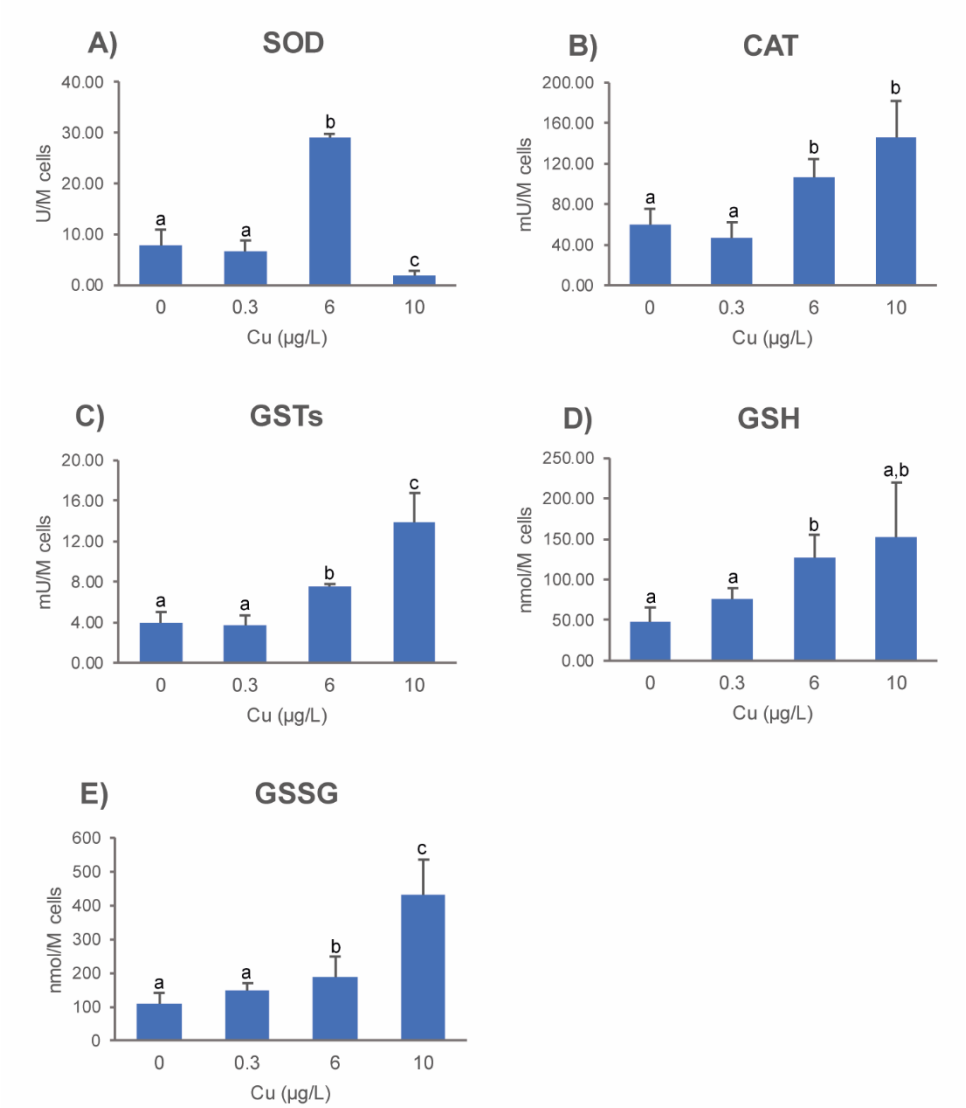

Figure 2 – Antioxidant response of Tabellaria flocculosa (TFLO) cells exposed for 96h to different Cu concentrations (0, 0.3, 6 and 10 µg/L). A) Superoxide dismutase (SOD). B) Catalase (CAT). C) Glutathione S-transferases activity (GSTs). D) Reduced (GSH) glutathione. E) Oxidized (GSSG) glutathione. Values are means (+ standard deviation) of 6 replicates from 2 independent experiments. For each parameter lowercase letters indicate significant differences among Cu exposures $p<0.05$

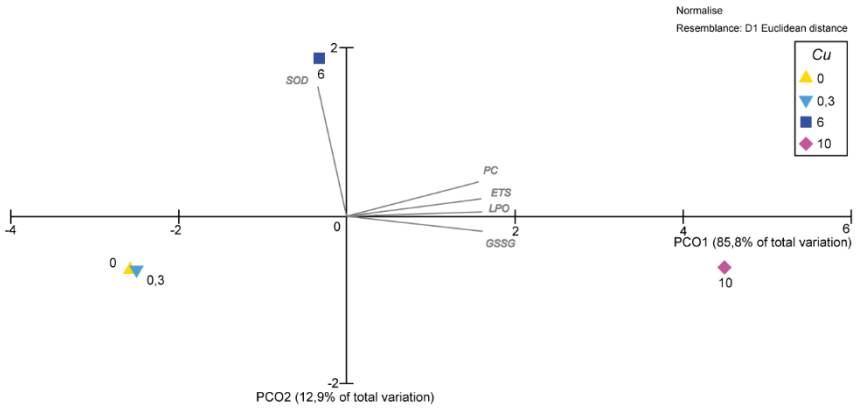

Figure 3 – Principal Coordinates (PCO) with Centroids ordination of *Tabellaria flocculosa* cells exposed for 96h to different Cu concentrations (0, 0.3, 6 and 10 µg/L). Pearson correlation vector imposed lipid peroxidation (LPO), protein carbonylation (PC), electron transport chain (ETS), oxidized glutathione (GSSG) and superoxide dismutase (SOD) concentrations (r>0.90).

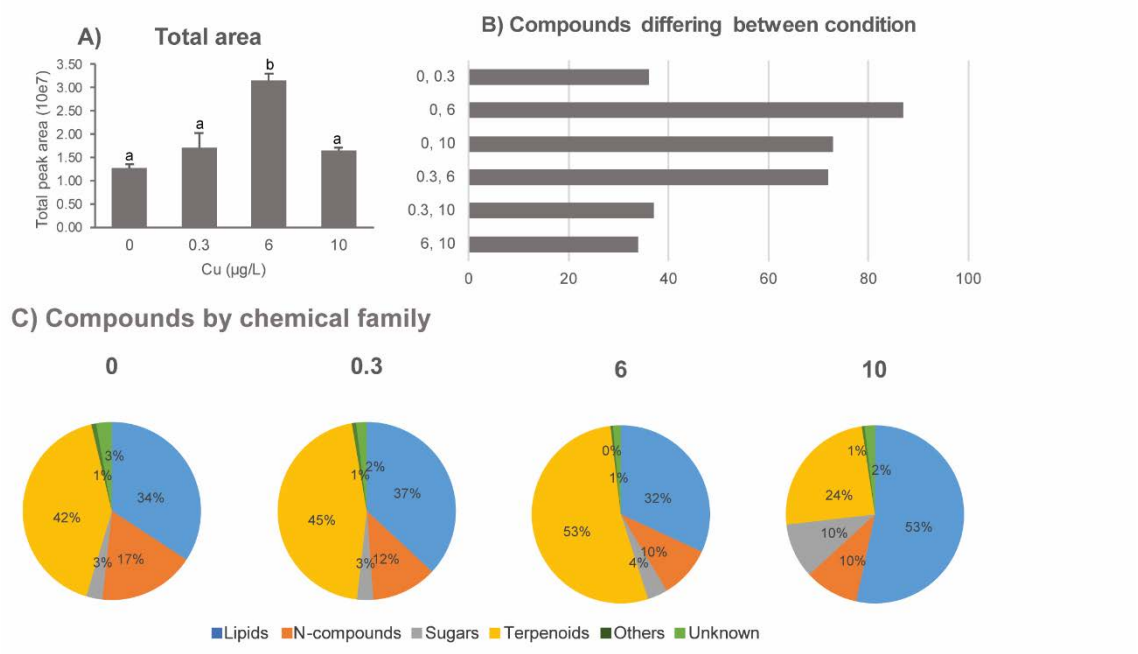

Figure 4- Metabolomic analysis of *Tabellaria flocculosa* cells exposed for 96h to different Cu concentrations (0, 0.3, 6 and 10 µg/L). A) Total peak area from 123 compounds. B) Number of compounds differing significantly ($p<0.05$) between two conditions. C) Distribution of compounds by chemical family (based on peak areas). Values are means (+ standard deviation) of 3 replicates from 1 independent experiment.

Figure 5- Metabolomic profiling (total peak area) of *Tabellaria flocculosa* (TFLO) cells exposed for 96h to different Cu concentrations (0, 0.3, 6 and 10 µg/L) and ratio (control/ Cu condition) of biological relevant compounds for each family of compounds. A) Lipids. B) N-compounds. C) Sugar related compounds. D)

Terpenoids; E) Others. Values are means (+ standard deviation) of 3 replicates from 1 independent experiment. Lowercase letters indicate significant differences among Cu exposures $p<0.05$.