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Comparative study of Cu uptake and early transcriptome responses in the green microalga *Chlamydomonas reinhardtii* and the macrophyte *Elodea nuttallii*

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Abstract

Microalgae are widely used as representative primary producers in ecotoxicology, while macrophytes are much less studied. Here we compared the bioavailability and cellular toxicity pathways of 2h-exposure to $1 \cdot 10^{-6}$ M Cu in the macrophyte *Elodea nuttallii* and the green microalga *Chlamydomonas reinhardtii*.

Toxicokinetics suggested that accumulation is higher in the algae than in the macrophyte, while RNA-Seq revealed a similar number of regulated genes. Early-regulated genes were congruent with expected adverse outcome pathways for Cu with GO terms including gene regulation, energy metabolism, transport, cell processes, stress, RedOx metabolism and development. However, the level of gene regulation was higher in *E. nuttallii* than in *C. reinhardtii* and several categories were more represented in the macrophyte than in the microalga. Moreover, several categories including OPP, nitrate metabolism and metal handling were only found for *E. nuttallii*, while categories such as cell motility, polyamine metabolism, mitochondrial electron transport and TCA were unique to *C. reinhardtii*. These differences were attributed to morphological and metabolic differences and highlighted dissimilarities between a sessile and a mobile species. Our data supports the efficiency of transcriptomics to assess early molecular responses in biota, and the importance of studying more aquatic plants for a better understanding on the impact and fate of environmental contaminants.

Keywords: copper; primary producers; speciation modelling; toxicokinetics; transcriptomics.

Capsule: Cu impact is different for microalgae and macrophytes: accumulation is higher in the algae, but higher transcriptome response occured in the macrophyte

Introduction

Primary producers are key organisms of aquatic ecosystems: phytoplankton sustains the largest ecosystem on the Earth, contributing to about half of the primary production on our planet although accounting for less than 1% of photosynthetic biomass (Bañuelos et al., 1998). Macrophytes, including plants dominate primary production in shallow waters including littorals, rivers, marshes, ponds and lakes (Noges et al., 2010). In addition they are key elements of the aquatic ecosystems by providing support, shelter, food and oxygen to many organisms including epiphytes (Thomaz and Cunha, 2010). Studying primary producers' response to a variation of the concentrations of vital and toxic trace metals is thus an important step to understand and estimate their impact in aquatic ecosystems. Indeed, if primary producers are affected they will also indirectly influence higher trophic levels in an ecosystem through food webs (Fleeger et al., 2003; Daam et al., 2009). Often microalgae are hypothesized as representative primary producers based on the assumption that all organisms respond to stresses similarly (Clemens, 2006). Nevertheless, seldom comparisons, for example of plants with algae (Paz et al., 2007; Beauvais-Fluck et al., 2018a) or mosses (Rother et al., 2006), reveals the existence of different stress and tolerance mechanisms. In such a context, the precise mechanisms of cellular handling (and toxicity) are to further elucidate to better understand the similarities and differences in different primary producers and anticipate and mitigate trace metals effect in the environment.

Copper (Cu) is an essential metal to all plants and animals. It participates in fundamental physiological processes (e.g. photosynthetic electron transport, mitochondrial respiration) and is a cofactor for many enzymes (e.g. superoxide dismutase, cytochrome c oxidase) (Castruita et al., 2011). Due to its high reactivity, Cu concentration is tightly regulated inside cells by a complex homeostasis network (Andres-Colas et al., 2006). This homeostasis network has been studied in

several model species and there are evidences of a high conservation throughout the evolution (Burkhead et al., 2009; Page et al., 2009). However, when in excessive concentrations Cu causes oxidative stress and photosynthesis inhibition due to adverse effects on the same cellular processes where it is needed, such as enzyme activity and photosynthetic electron transport (Monferran et al., 2009; Razinger et al., 2010; Upadhyay et al., 2011). Thus, Cu concentrations, and its biological availability are important parameter for environmental quality in natural environments. Elevated Cu concentrations in aquatic ecosystems are directly related to human activities involving the production of industrial (e.g. pesticide use and agricultural run-off, mine tailings) and domestic wastes (e.g. urbanization, automobile exhausts). Naturally occurring concentrations of Cu range between 10^{-9} M to 10^{-8} M in freshwater systems, but Cu can easily reach 10^{-6} M in locations receiving anthropogenic inputs such as freshwater ecosystems close to vineyards or mining areas (Kupper and Andresen, 2016).

The present study aimed thus to compare Cu toxicokinetic and transcriptomic responses in two aquatic primary producers: a macrophyte *Elodea nuttallii* and a green microalga *Chlamydomonas reinhardtii*, respectively representing aquatic plants and phytoplankton typically found in the benthic environment and the water column. In the present study, we hypothesized that bioavailability and responses to toxic metals were similar in a microalgae and a macrophyte exposed in similar experimental conditions. More in detail, we compared cellular toxicity pathways of Cu in both organisms using transcriptomics (RNAseq) and determining the uptake. This research will thus increase our level of understanding of the functioning of key organisms, and eventually, the data produced will provide a scientific base to conduct sound risk assessment of our freshwater ecosystems to preserve their high socio-economic and environmental value.

Material and methods

Labware

All material was washed in 10 % HNO_3 baths, thoroughly rinsed with ultrapure water (MilliQ Direct system, Merck Millipore) and dried under a laminar flow hood. Material for culture and experiments, including media, were additionally autoclaved (1 bar, 121°C, 20 min) to avoid microbial contamination.

Exposure of algae and macrophytes

Chlamydomonas reinhardtii (wild type strain CPCC11, Canadian Phycological Culture Centre) were grown under axenic conditions in an incubator (Multitron Infors HT) at 20.2 ± 0.5 °C with a 24 h light cycle (110 µmol·phot·m^{-2}·s^{-1}) and a rotary shaking (115 rpm). Cells were cultured in a 4× diluted Tris-Acetate-Phosphate medium (TAP) (Rensing et al., 2008). At the mid-exponential growth phase (62 h after inoculation), cells were harvested by centrifugation (10 min, 1300*g*), rinsed and re-suspended in the exposure medium at a final density of (8.1 ± 1.1)·10^5 cells·mL^{-1}.

Shoots of *E. nuttallii* were collected in Lake Geneva, and a culture established and maintained in microcosms as previously described (Regier et al., 2013b). Exposures were conducted on three 10 cm-long shoots without roots. Cultures and experiments were conducted in the laboratory under the same controlled conditions (16/8 h light/dark; 1000 lux; 20 ± 1 °C).

Both organisms were exposed in triplicates to nominal concentration of 10^{-6} M Cu added as $CuSO_4$ (Sigma Aldrich) in an artificial medium (8.2·10^{-4} M $CaCl_2$, 3.6·10^{-4} M $MgSO_4$, 2.8·10^{-4} M $NaHCO_3$, 1.0·10^{-4} M KH_2PO_4 and 5.0·10^{-6} M NH_4NO_3, pH 6.9 ± 0.1) during 10, 30 min, 1, 4 and

8 h. Organisms exposed in the absence of metal in the medium were used as control. The choice of Cu exposure concentration corresponds to a sublethal concentration in similar experimental conditions, e.g. resulting in the EC10 growth inhibition in *C. reinhardtii* for 24 h-long exposure (Cheloni et al., 2014) and is 4× lower than the concentration resulting in a 15 % decrease of chlorophyll content in *E. nuttallii* after 2h-long exposure.

Cu uptake and modelling

Cu uptake by microalgae and macrophyte was characterized by total and intracellular Cu (Cu_{int}) contents. Half of exposed organisms was rinsed with 10^{-3} M ethylene·diamine·tetraacetic·acid (EDTA; Sigma-Aldrich, Buchs, Switzerland) prepared in the exposure medium, to rinse metal surface-adsorbed or loosely bound to the cell wall and determine Cu_{int}. The other half of exposed organisms was rinsed with medium without Cu (media-rinsed) and therefore represents the sum of adsorbed Cu (Cu_{ads}) and Cu_{int}.

EDTA-rinsed and media-rinsed samples were freeze-dried (Beta 1-8 K), digested with 65% HNO_3 (Suprapur Merck KGaA) at 90°C for 1 h and analyzed by inductively coupled plasma mass spectrometry (ICP-MS; 7700x, Agilent Technologies). Concentration in media was measured in acidified samples (0.5% v/v HNO_3 Suprapur) by ICP-MS. Cu concentration in unspiked artificial medium was $2.3 \pm 0.3 \cdot 10^{-9}$ M Cu. Effective initial concentration in spiked media were $1.09 \pm 0.20 \cdot 10^{-6}$ M Cu ($1.85 \pm 0.47 \cdot 10^{-7}$ M Cu^{2+}) and $2.26 \pm 0.21 \cdot 10^{-6}$ M Cu ($3.84 \pm 0.51 \cdot 10^{-7}$ M Cu^{2+}), for *E. nuttallii* and *C. reinhardtii*, respectively.

The Cu uptake was modelled using a first-order mass transfer model following two-compartment system and equations below (*eq 1* and *2*):

$$Ct = Co + \frac{a}{k(1 - e^{-kt})} \quad (eq\ 1)$$

$$a = k1 \times Ce \quad (eq\ 2)$$

where C_t is the metal concentration in cells ($\mu mol_{Cu} \cdot g^{-1} \cdot dw$) at time t (hours), k is the elimination rate constant (h^{-1}) and a is the uptake flux ($\mu mol_{Cu} \cdot g^{-1} \cdot dw \cdot h^{-1}$), k_1 is the uptake rate constant ($\mu mol_{Cu} \cdot g^{-1} \cdot dw \cdot h^{-1}$), Ce is the bioavailable concentration in the medium ($\mu mol_{Cu} \cdot g^{-1}$ dw), and C_0 is the constitutive metal concentration measured in cells at the beginning of the exposure (Martins and Boaventura, 2002; Gimbert et al., 2008).

RNA-sequencing (RNAseq) and quantification of differential gene expression

Transcriptome response of *C. reinhardtii* and *E. nuttallii* exposed 2 h to Cu was assessed though RNASeq (Illumina HiSeq 2500 System). Total RNA was extracted as previously described using TRI Reagent (Sigma-Aldrich, Buchs, Switzerland), and libraries were prepared following manufacturer's protocols (Beauvais-Fluck et al., 2016; Regier et al., 2016; Beauvais-Fluck et al., 2017). For *C. reinhardtii*, reads were aligned with TopHat2 (Kim et al., 2013) to the genome Creinhardtii 236 V.9.0 (Conesa et al., 2005). For *E. nuttallii*, reads were mapped using the Burrows-Wheeler Alignment (BWA v.0.7.10) tool (Li and Durbin, 2010) on the *de novo* transcriptome available for this organism (Regier et al., 2016). For both organisms reads were counted using the Python package HTSeq (Anders et al., 2015). Differential gene expression analysis was performed in the software CLC Main Workbench (Version 7, CLC bio, QIAGEN, Denmark) based on normalized counts and EdgeR package (Robinson et al., 2010). Significant differently expressed transcripts *vs* Control were defined with a threshold of false discovery rate (FDR) <0.1%. Ontology term assignments were done using MapMan (Table S1) (Thimm et al.,

2004; Usadel et al., 2009). Data are available in the Gene Expression Omnibus database (GSE65109).

Results and discussion

Cu uptake by primary producers

Accumulation of Cu was measured in media-rinsed and EDTA-rinsed *E. nuttallii* and *C. reinhardtii* over time. Exposure to Cu in *C. reinhardtii* resulted in higher and faster accumulation than in *E. nuttallii* (Figure 1, Table 1). Concentrations measured in media-rinsed *C. reinhardtii* reached a plateau in 2h, and resulted in the highest measured bioaccumulation (up to 40 $\mu mol \cdot g^{-1}$ dw). The uptake of *C. reinhardtii* was of the same order of magnitude than reported in previous studies with *C. reinhardtii* and other green freshwater algae (Stoiber et al., 2012). For comparison, media-rinsed *E. nuttallii* showed a bioaccumulation of close to 8 $\mu mol \cdot g^{-1}$ dw at 8h, and no obvious evidence of a plateau. Moreover, after 2h exposure *E. nuttallii* internalized (EDTA-washed) 1.33 ± 0.31 $\mu mol \cdot g^{-1}$ dw (1.04 ± 0.24 $\mu mol \cdot g^{-1}$ dw in control), while *C. reinhardtii* internalized 4.69 ± 0.18 $\mu mol \cdot g^{-1}$ dw (1.44 ± 0.06 $\mu mol \cdot g^{-1}$ dw in control). Data suggested that internalization is higher and faster in the algae than in the macrophyte. This difference can be attributed to the fact that the full surface of the unicellular algae is in contact with the media, whereas in the macrophyte only the external layer of cells is directly exposed, most certainly resulting in a gradient of metal concentrations between cells. Besides, the surface-to-volume ratio is much higher in an unicellular organism and thus is expected to result in higher uptake (Lindemann et al., 2016). However, proportion of Cu accumulated in cell walls was higher in *C. reinhardtii* than in *E. nuttallii*, suggesting that adsorption of Cu was predominant in *C. reinhardtii* and/ or EDTA-washing procedure was more efficient. Cell walls are known to

play a central role in plant and microalgal tolerance to metals: for example, 50 % of Cu was accumulated in the cell walls in *Cystoseira tamariscifolia* (Celis-Pla et al., 2018), 20% was adsorbed (or EDTA-extractable) for *Chlorella kessleri* (Lamelas et al., 2009). In the charophyte, *Nitellopsis obtusa* exposed 3h to both Cu-nanoparticles or $CuSO_4$, the major part of Cu accumulated in cell walls (Manusadzianas et al., 2017). Similarly, a previous study in *E. nuttallii* measured an increased proportion over time of cadmium and mercury in cell walls, concomitantly with an increased lignification of cell walls after 7 d exposure (Larras et al., 2013).

The one-compartment model well fitted Cu accumulation in *E. nuttallii* and *C. reinhardtii*. In both organisms significant and similar (*a*) and (*k*) were estimated by the model normalized by the effective concentration in media. Modelling further allowed estimating that the steady state was approached in less than 2h for *C. reinhardtii*. Similar, fast uptake and plateau has been observed in *C. reinhardtii* exposed to increasing concentrations of Cu^{65} (Jamers et al., 2013).

Transcriptomic response

In total 1397 and 1258 genes were regulated by 2h exposure to $1 \cdot 10^{-6}$ M Cu in *C. reinhardtii* and *E. nuttallii* respectively. The similar number of regulated genes, used as a proxy of stress, suggested that both *E. nuttalli* and *C. reinhardtii* faced a similar level of stress (Dranguet et al., 2017). However, among those, 841 (67%) and 624 (44%) genes were upregulated, while 417 and 773 were down-regulated in *E. nuttalli* and *C. reinhardtii*, respectively. Besides, the level of gene regulation was higher in *E. nuttallii* (log2FCrange= 17.9) than in *C. reinhardtii* (log2FCrange= 8.8), suggesting a higher impact of Cu in the macrophyte than the microalgae, in

line with similar observations made for Hg in controlled and in the field exposure comparing the same species (Dranguet et al., 2017; Beauvais-Fluck et al., 2018a).

In term of abundance of GO terms for both species a predominant part of regulated genes had unknown function (62% for *C. reinhardtii* and 33 % for *E. nuttalli*; Figure 3) indicating considerable potential for new discovery in the biology of Cu. Enriched pathway analysis revealed that Cu exposure regulated genes involved in gene regulation (i.e. RNA, protein, signaling) and energy metabolism (Figure 3). Other categories included genes involved in transport, cell processes, hormone metabolism, stress, RedOx metabolism and development (Figure 3). Overall, results support a response of both species to avoid stress (e.g. oxidative stress) and effects on development/growth and nutrition with a significant modification of the energy metabolism. Regulated genes were thus in line with expected adverse outcome pathways for Cu, i.e. impact on photosynthesis, RedOx, growth and nutrition, although only 2h exposure was performed. This confirms the potential of transcriptomics to reveal early-responses at environmental concentrations (Regier et al., 2013a; Dranguet et al., 2017; Beauvais-Fluck et al., 2018b). Not surprisingly this short exposure resulted in few physiological endpoints significantly different *vs* control (Table S2 and S3) (Jamers et al., 2013; Jiang et al., 2016). More in detail, here photosynthesis efficiency is reduced in *C. reinhardtii* by 7% (Table S3) and class III peroxidase activity (POD) is reduced 50× in *E. nuttallii* (Table S2). In the same line, a previous study in *C. reinhardtii* revealed that exposure to a similar free ion concentration 10^{-7} M Cu^{2+}, induced Glutathione Peroxidase genes after 2h and reduced growth after 24h, although no cellular impact was measured including membrane permeability, reactive oxygen species production and lipid peroxidation (Cheloni et al., 2014).

A previous study showed that exposure of *E. nuttallii* to 10^{-6} M Cu reduced superoxide dismutases activity after 1h and reduced root growth after 24h, but had no significant effect on chlorophyll content, photosynthesis efficiency and class III peroxidase activity (Regier et al., 2015). Similar observation has been obtained with Cu toxicity in *C. reinhardtii*: exposure to excess Cu induced ROS production and antioxidative response in *C. reinhardtii* (Jamers et al., 2006; Stoiber et al., 2013; Jiang et al., 2016). In the same line, a study on the rootless submerged shoots of *Ceratophyllum demersum* exposed 6 weeks to a range of concentrations between 10^{-9}-10^{-7} M Cu, showed that nutrient uptake/distribution, photosynthesis efficiency and chlorophyll content were affected by Cu (Thomas et al., 2013). Nutrition is impacted because an excess Cu competes with the various essential metals according to the Irving–William series and induces deficiency of essential ions (Mg^{2+}, Zn^{2+}, etc.) (Mosulen et al., 2003) and impairment of metalloprotein functioning. However, although all toxic metals might induce the same core stress related changes on genes, transcriptome analysis has resulted in the identification of genes specific to each metal (Kovalchuk et al., 2005; Weber et al., 2006; Simon et al., 2008). Nonetheless, data have rarely been compared between species exposed in similar experimental settings (Dranguet et al., 2017; Beauvais-Fluck et al., 2018a).

Here, several categories were more represented in the macrophyte than in the microalga (Figure 2), including stress (abiotic), development, cell vesicle transport, hormone metabolism (abscisic acid, ethylene, jasmonate), cell wall (cellulose, hemicellulose and pectin synthesis), secondary metabolism (phenylpropanoid, wax, flavonoids), and transport (transport P- and V-ATPases, Major Intrinsic Proteins, nitrate). The present data for *E. nuttallii* were in agreement with a microarray analysis in roots of rice exposed 3h to $5 \cdot 10^{-6}$ M Cu, notably concerning dysregulation of genes involved in vesicle transport, flavonoids metabolism and jasmonate (Lin et al., 2013).

242 Authors further showed by knockout of genes necessary for this vesicle transport and exposure
243 of roots to vesicle trafficking inhibitors, that Cu interacts with vesicle transport and that this
244 vesicle transport is essential for signaling via ROS for activating defenses (Lin et al., 2013).
245 Results of the present study allowed to propose a possible model of cellular mechanisms
246 involved in Cu detoxification and protection in *E. nuttallii*: Cu increases intracellular transport,
247 e.g. vesicle trafficking and ABC transport, and induces a flavonoid-mediated detoxification
248 pathway. In addition, the toxicity mechanisms such as JA biosynthesis and cellular component
249 biogenesis were regulated in response to Cu exposure. In comparison, the categories of OPP,
250 nitrate metabolism and metal handling were absent in Cu regulated genes in *C. reinhardtii*.
251 Conversely, cell motility, DNA, polyamine metabolism, mitochondrial electron transport, and
252 TCA categories were found in *C. reinhardtii*, while absent in *E. nuttallii*. Moreover, the level of
253 regulation of the categories found in common in both species was higher in *E. nuttallii* than in *C.*
254 *reinhardtii*. These differences certainly highlight the dissimilarities between basal and
255 background metabolism in two different species, as well as between a sessile and a mobile
256 organism (Dranguet et al., 2017). Besides, genome sequencing has revealed that *C. reinhardtii*
257 possesses numerous genes derived from the last plant-animal common ancestor that have been
258 lost in angiosperms, including transporters and the possibility of extensive metabolic flexibility
259 (Merchant et al., 2007). Taken together, our divergent observations on how an unicellular and a
260 multicellular organism take up and are impacted by Cu may imply that homeostasis networks are
261 more species-specific than generally thought.
262 We further found several differences at the level of subcategories. For example, in the
263 'Photosynthesis' category, genes of *C. reinhardtii* were mainly involved in the light reaction, in
264 particular photosystem I (PSI), while in *E. nuttallii* genes were involved both in PS I and PS II,

as well as photorespiration, suggesting that the photosynthesis was impacted more widely by Cu toxicity in the macrophyte. Generally, Cu has been reported to impact more PS II than PS I in plants. In PS II, the reaction center and LHC II by substitution of Mg^{2+} in its chlorophyll have been shown to be targets of Cu toxicity (Kupper et al., 1996; Kupper and Andresen, 2016). In the macrophyte *C. demersum* nanomolar concentrations of Cu affected the PS II reaction center (Thomas et al., 2013). In this regard, our finding of Cu impact on PSI in *C. reinhardtii* is striking and might point to structural differences between photosystems as well as background defense pools in the studied species (Castruita et al., 2011).

Conclusion

Overall, the exposure to 10^{-6} M Cu resulted in different cellular toxicity pathways in a microalga and a macrophyte. This fact together with the distinct exposure routes of the benthic macrophyte and lentic microalgae suggest that similar Cu concentrations might affect differently both species in the ecosystem. Defining ecological thresholds of adverse outcomes for environmental contaminants represents a critical component of chemical assessment and management programs. Our data call for including more species of aquatic plants for determining ecological thresholds for environmental contaminants. In this context, transcriptomics is confirmed as a useful tool to assess early responses at environmental concentrations.

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291

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476

477 **Table 1**: Modelled parameters of Cu uptake in EDTA-rinsed *E. nuttallii* or *C. reinhardtii*
478 exposed to $1 \cdot 10^{-6}$ M Cu. Uptake flux (*a*) and elimination rate constant (*k*) of Cu were divided by
479 the effective concentration of metal at beginning of the test to allow inter species comparison and
480 are thus presented as *a'* and *k'*.

	E. nuttallii	*C. reinhardtii*
a' ($L \cdot g^{-1} \cdot h^{-1}$)	0.74 ± 0.87	0.32 ± 0.05
k' ($\mu mol \cdot L^{-1} \cdot h^{-1}$)	0.06 ± 0.08	0.07 ± 0.04
R^2	0.93	0.85

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482

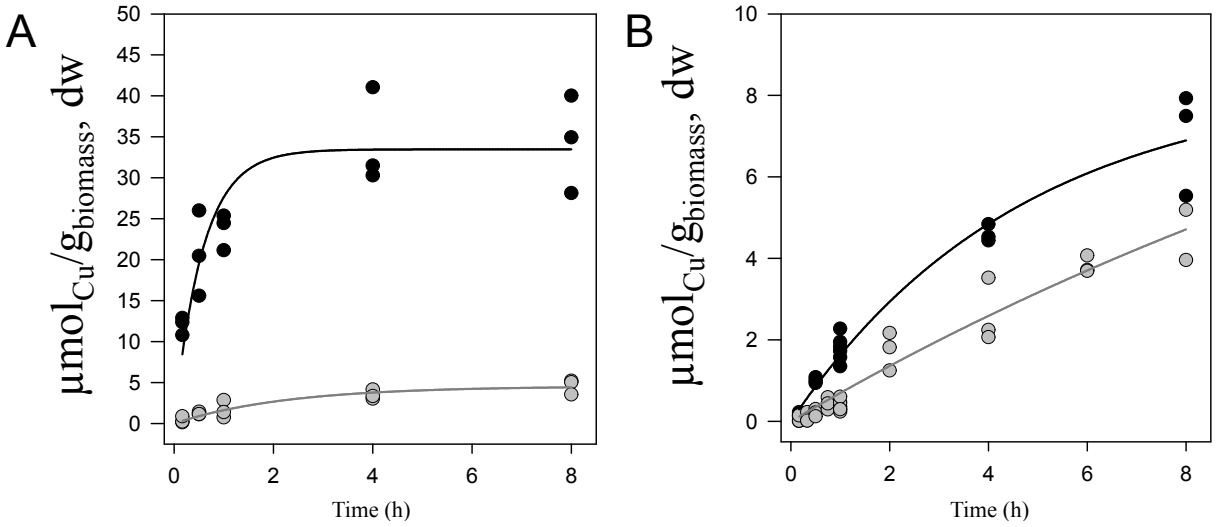

Figure 1: Cu toxicokinetics in *C. reinhardtii* (A) and *E. nuttallii* (B) exposed to $1 \cdot 10^{-6}$ M Cu. Organisms were EDTA-rinsed (grey) or media-rinsed (black) to differentiate between adsorbed and internalized metal.

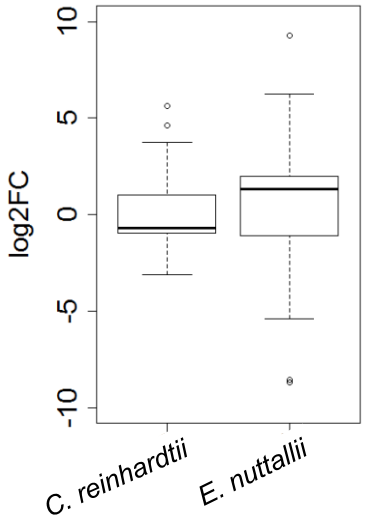

Figure 2: Fold-changes (log2FC) of significant regulated genes in *C. reinhardtii* and *E. nuttallii* exposed 2h to $1 \cdot 10^{-6}$ M Cu.

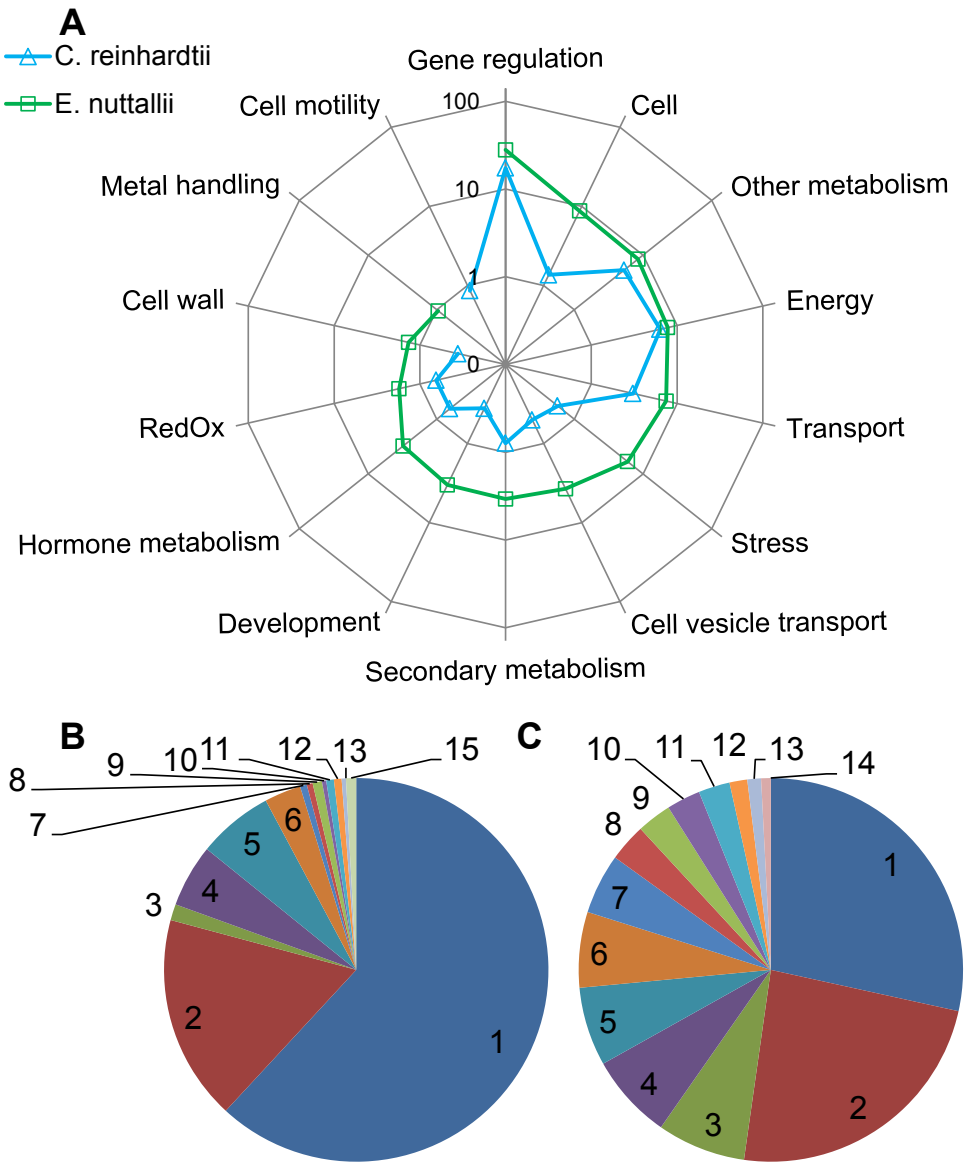

Figure 3: Number of significant dysregulated genes (DG) annotated in the respective enriched biological pathways (MapMan) in *C. reinhardtii* (triangles) and *E. nuttallii* (square) exposed 2h to Cu (A). Proportion (%) of main functional GO categories (MapMan) of dysregulated genes in *C. reinhardtii* (B) and *E. nuttallii* (C) exposed 2 h to Cu (1: Unknown, 2: Gene regulation, 3: Cell process, 4: Other metabolism, 5: Energy metabolism, 6: Transport, 7: Stress, 8: Cell vesicle transport, 9: Secondary metabolism, 10: Development, 11: Hormone metabolism, 12: RedOx, 13: Cell wall, 14: Metal handling, 15: Cell Motility).

SUPPORTING INFORMATIONS

Comparative study of Cu uptake and early transcriptome responses in the green microalga *Chlamydomonas reinhardtii* and the macrophyte *Elodea nuttallii*

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Table S1: MapMan ontology enrichment analysis for significantly dysregulated transcripts measured in *Chlamydomonas reindhardtii* and *Elodea nuttallii* exposed 2 h to Cu vs control (XLS file in attachment).

Table S2: Effect on pigments content (chlorophyll; anthocyanin) and oxidative stress enzymes activity (POD, class III peroxidase; SOD, superoxide dismutase) in *E. nuttallii* exposed 2 h to Cu or not (control) (results are presented as % of control; bold characters indicate significant difference with control, n = 3 ± SD; T-test *p*-value < 5%; fw, fresh weight).

E. nuttallii	Chlorophyll (mg/g fw)	Anthocyanin (µg/g fw)	POD activity (nkat/$mg_{protein}$)	SOD activity (U/$µg_{protein}$)
Control	100.0 ± 15.2	100.0 ± 15.1	100 ± 20.1	100.0 ± 7.9
Cu	87.0 ± 16.5	110.7 ± 7.1	**2.4 ± 0.3**	108.9 ± 5.9

Table S3: Effect on chlorophyll content and efficiency, and oxidative stress biomarkers (membrane integrity, ROS) in *C. reinhardtii* exposed 2 hours to Cu or not (control) (results are presented as % of control; bold characters indicate significant difference with control, n = 3 ± SD; T-test *p*-value < 5%).

C. reinhardtii	chlorophyll a	Fv/Fm	membrane integrity (% cell affected)	ROS generation (% cell stained)
Control	100 ± 6.5	100 ± 1.7	10 ± 1.5	10.0 ± 1.5
Cu	91.9 ± 3.3	**93.2 ± 1.0**	12.5 ± 1.0	12.5 ± 1.0