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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 2 h-exposure to 10^{-6} mol·L^{-1} Cu in the macrophyte *Elodea nuttallii* and the green microalga *Chlamydomonas reinhardtii*.

Uptake rate was similar but faster in the algae than in the macrophyte, while RNA-Sequencing revealed a similar number of regulated genes. Early-regulated genes were congruent with expected adverse outcome pathways for Cu with Gene Ontology terms including gene regulation, energy metabolism, transport, cell processes, stress, antioxidant metabolism and development. However, the gene regulation level 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 oxidative pentose phosphate pathway (OPP), nitrate metabolism and metal handling were only found for *E. nuttallii*, whereas categories such as cell motility, polyamine metabolism, mitochondrial electron transport and tricarboxylic acid cycle (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 results highlight 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 accumulation is faster in the algae, but greater transcriptome response occurred 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 (Daam et al., 2009; Fleeger et al., 2003). 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 (Beauvais-Fluck et al., 2018a; Paz et al., 2007) or mosses (Rother et al., 2006), revealed 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 various primary producers and anticipate 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 dismutases, cytochrome c oxidases) (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} mol·L^{-1} to 10^{-8} mol·L^{-1} 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* (Planch.) St. John and a green microalga *Chlamydomonas reinhardtii* P.A. Dangeard, 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 (RNAsequencing; 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.

85

86 **Material and methods**

87 *Labware*

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

92

93 *Exposure of algae and macrophytes*

94 Typical growth and exposure conditions were used for each species. *Chlamydomonas reinhardtii*
95 P.A. Dangeard (wild type strain CPCC11, Canadian Phycological Culture Centre) were grown
96 under axenic conditions in an incubator (Multitron Infors HT) at 20.2 ± 0.5 °C with a continuous
97 light cycle (3600 lux) and a rotary shaking (115 rpm). Cells were cultured in a 4× diluted Tris-
98 Acetate-Phosphate medium (TAP) (Rensing et al., 2008). At the mid-exponential growth phase
99 (62 h after inoculation), cells were harvested by centrifugation (10 min, 1300*g*), rinsed and re-
100 suspended in the exposure medium at a final density of $(8.1 \pm 1.1) \cdot 10^5$ cells·mL^{-1}.
101 Shoots of *Elodea nuttallii* (Planch.) St. John were collected in Lake Geneva, and a culture
102 established and maintained in microcosms at 20 ± 1 °C with a 16/8 h light/dark cycle (1000 lux
103 (Regier et al., 2013b). Exposures were initiated 5 h after the start of light on three 10 cm-long
104 shoots without roots.
105 Exposures were conducted in the laboratory under the same controlled conditions than during
106 culture. Both organisms were exposed in triplicates to nominal concentration of $1 \cdot 10^{-6}$ mol·L^{-1}
107 and $2 \cdot 10^{-6}$ mol·L^{-1} Cu added as $CuSO_4$ (Sigma Aldrich) for *E. nuttallii* and *C. reinhardtii*

respectively, in an artificial medium ($8.2 \cdot 10^{-4}$ mol·L^{-1} $CaCl_2$, $3.6 \cdot 10^{-4}$ mol·L^{-1} $MgSO_4$, $2.8 \cdot 10^{-4}$ mol·L^{-1} $NaHCO_3$, $1.0 \cdot 10^{-4}$ mol·L^{-1} KH_2PO_4 and $5.0 \cdot 10^{-6}$ mol·L^{-1} 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 EC20 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 2 h-long exposure (Regier et al., 2015).

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} mol·L^{-1} 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 $2.3 \pm 0.3 \cdot 10^{-9}$ mol·L^{-1} Cu. Measured initial concentration in spiked media were $1.09 \pm 0.20 \cdot 10^{-6}$ mol·L^{-1} Cu ($1.85 \pm 0.47 \cdot 10^{-7}$ mol·L^{-1} Cu^{2+}) and $2.26 \pm 0.2 \cdot 10^{-6}$ mol·L^{-1} Cu ($3.84 \pm 0.51 \cdot 10^{-7}$ mol·L^{-1} 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*):

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

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

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 ($\mu mol_{Cu} \cdot g^{-1} \cdot dw \cdot h^{-1}$), $k_1 \cdot$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 (Gimbert et al., 2008; Martins and Boaventura, 2002). Statistics (t tests) and plots were done in SigmaPlot.

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, 2017; Regier et al., 2016). 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 entailing 181'663 contigs with an average length of 880 bp (Regier et al., 2016). We selected contigs showing a minimum coverage of 20 raw counts in all samples,

154 resulting in 99'030 sequences analyzed for differential gene expression in CLC Main Workbench
155 (Version 7, CLC bio, QIAGEN, Denmark). 50% of contigs are covered by the reads of at least in
156 one sample.
157 For both organisms reads were counted using the Python package HTSeq (Anders et al., 2015).
158 Differential gene expression analysis was performed in the software CLC Main Workbench
159 (Version 7, CLC bio, QIAGEN, Denmark) based on normalized counts and EdgeR package
160 (Robinson et al., 2010). Significant differently expressed transcripts *vs* Control were defined
161 with a threshold of false discovery rate (FDR) <0.1%. Ontology term assignments were done
162 using MapMan (Table S1) (Thimm et al., 2004; Usadel et al., 2009). Data are available in the
163 Gene Expression Omnibus database (GSE65109).
164
165 **Results and discussion**
166 *Cu uptake by primary producers*
167 Accumulation of Cu was measured in media-rinsed and EDTA-rinsed *E. nuttallii* and *C.*
168 *reinhardtii* over time (Figure 1). The one-compartment model well fitted Cu accumulation in *E.*
169 *nuttallii* and *C. reinhardtii*. In both organisms significant and similar (*a*) and (*k*) were estimated
170 by the model normalized by the effective concentration in media (Table 1). Modelling further
171 allowed estimating that the steady state was approached in less than 2 h for *C. reinhardtii*.
172 Concentrations measured in media-rinsed *C. reinhardtii* (up to 40 $\mu mol \cdot g^{-1}$ dw) reached a plateau
173 in 2 h. Similar, fast uptake and plateau has been observed in *C. reinhardtii* exposed to increasing
174 concentrations of ^{65}Cu (Jamers et al., 2013). The cellular concentrations of *C. reinhardtii* were in
175 the same order of magnitude as in previous studies with *C. reinhardtii* and other green freshwater
176 algae (Stoiber et al., 2012). For comparison, media-rinsed *E. nuttallii* (8 $\mu mol \cdot g^{-1}$ dw at 8h)

showed no obvious evidence of a plateau. Moreover, after 2 h exposure *E. nuttallii* internalized (EDTA-washed) 1.33 ± 0.31 µmol·g^{-1} dw (1.04 ± 0.24 µmol·g^{-1} dw in control), while *C. reinhardtii* internalized 4.69 ± 0.18 µmol·g^{-1} dw (1.44 ± 0.06 µmol·g^{-1} dw in control). Data showed that internalization is similar when normalized by the effective concentration in media, differences reflecting exposure condition, but 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, that is not observed in our experimental conditions, but could also result in a faster 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 3 h 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).

Circadian clock and light are also known to be central for nutrient acquisition in plants because nutrient demands of a plant change according to the time of day, e.g. to drive photosynthesis in

chloroplasts and daily rhythms in transpiration rates (Haydon et al., 2015). Light intensity and spectral composition also affected Cu uptake to *C. reinhardtii* (Cheloni et al., 2014). Here the differences in light cycle applied to the microalgae (continuous) and the macrophyte (16 h light) might also affect uptake because here plants have a synchronized circadian rhythm, while microalgae show an average of all circadian stages. In *Arabidopsis thaliana*, cytosolic Cu was shown to increase during light period and decrease during dark period (Penarrubia et al., 2009). It is unclear if this is also the case here, but if this is the case, the different light regimes used for both species could also explain in part the higher Cu concentrations reached in the microalgae than the macrophyte. However, in similar experimental conditions including light regime, *E. nuttallii* exposed to Hg and Cd showed a plateau around 48 h-long exposure (Larras et al., 2013), supporting that metal uptake in this species takes longer to reach equilibrium than in *C. reinhardtii*. Nonetheless, because experimental settings can affect uptake and typical experimental conditions are in general different for microalgae and plants, future research should test organisms in completely identical conditions to allow a detailed comparison of bioaccumulation data.

Transcriptomic response

In total 1397 and 1258 genes were regulated by 2 h exposure to 10^{-6} mol·L^{-1} Cu in *C. reinhardtii* and *E. nuttallii* respectively. The similar number of regulated genes, used as a proxy of stress, suggested that both *E. nuttallii* 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. nuttallii* and *C. reinhardtii*, respectively (Table S2 and S3). 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

223 microalgae (Figure 2), in line with similar observations made for Hg in controlled and in the
224 field exposure comparing the same species in identical conditions (Beauvais-Fluck et al., 2018a;
225 Dranguet et al., 2017).
226 Among the 20 most highly regulated genes, 13 and 14 had an unknown function in in *C.*
227 *reinhardtii* and *E. nuttallii*, respectively (Table 2). The 7 most highly regulated genes with
228 known function were homologous to genes involved in reduction-oxidation (RedOx)
229 metabolism, in gene regulation and nutrient transport, as well as multigenic families with
230 numerous biochemical functions which precise function is therefore difficult to establish based
231 on sequence homologies. More globally, in term of abundance of GO terms for genes
232 significantly regulated in both species, a predominant part of regulated genes had unknown
233 function (62% for *C. reinhardtii* and 33% for *E. nuttalli*; Figure 3) indicating considerable
234 potential for new discovery in the biology of Cu. In *C. reinhardtii*, the most abundant GO
235 categories regulated by Cu exposure were involved in "gene regulation" (44%, i.e. RNA, protein,
236 signaling) and "cell processes" (10%; i.e. cell organization and cell motility) suggesting an
237 adaptation of the cell metabolism and structure (Figure 3). Transport and photosynthesis both
238 represented 8% of regulated genes. In *E. nuttallii*, the most abundant GO categories regulated by
239 Cu exposure were involved in "gene regulation" (34%, i.e. RNA, protein, signaling) and
240 "transport" (10%). The GO category "stress" represented 8% of regulated genes, while "cell
241 processes" represented 6% of regulated genes. Other GO categories including hormone
242 metabolism, stress, RedOx metabolism and development were less than 5% of regulated genes.
243 Enriched pathway analysis revealed a response of both species to avoid stress (e.g. oxidative
244 stress) and effects on development/growth and nutrition with a significant modification of the
245 energy metabolism. Regulated genes were thus in line with expected adverse outcome pathways

246 for Cu, i.e. impact on photosynthesis, RedOx, growth and nutrition, although only 2 h exposure
247 was performed. This confirms the potential of transcriptomics to reveal early-responses at
248 environmental concentrations (Beauvais-Fluck et al., 2018b; Dranguet et al., 2017; Regier et al.,
249 2013a). Not surprisingly this short exposure resulted in few physiological endpoints significantly
250 different *vs* control (Table S4 and S5) (Jamers et al., 2013; Jiang et al., 2016). More in detail,
251 here photosynthesis efficiency is reduced in *C. reinhardtii* by 7% (Table S5) and class III
252 peroxidase activity (POD) is reduced 50× in *E. nuttallii* (Table S2). In the same line, another
253 study with *C. reinhardtii* revealed that exposure to a similar free ion concentration 10^{-7} mol·L^{-1}
254 Cu^{2+}, induced Glutathione Peroxidase genes after 2 h and reduced growth after 24 h, although no
255 cellular impact was measured including membrane permeability, reactive oxygen species
256 production and lipid peroxidation (Cheloni et al., 2014).
257 A previous study showed that exposure of *E. nuttallii* to 10^{-6} mol·L^{-1} Cu reduced superoxide
258 dismutases activity after 1 h and reduced root growth after 24 h, but had no significant effect on
259 chlorophyll content, photosynthesis efficiency and class III peroxidase activity (Regier et al.,
260 2015). Similar observation has been obtained with Cu toxicity in *C. reinhardtii*: exposure to
261 excess Cu induced ROS production and antioxidative response in *C. reinhardtii* (Cheloni et al.,
262 2019; Jamers et al., 2006; Jiang et al., 2016; Stoiber et al., 2013). In the same line, a study on the
263 rootless submerged shoots of *Ceratophyllum demersum* exposed 6 weeks to a range of
264 concentrations between 10^{-9}-10^{-7} mol·L^{-1} Cu, showed that nutrient uptake/distribution,
265 photosynthesis efficiency and chlorophyll content were affected by Cu (Thomas et al., 2013).
266 Nutrition is impacted because an excess Cu competes with the various essential metals according
267 to the Irving–William series and induces deficiency of essential ions (Mg^{2+}, Zn^{2+}, etc.) (Mosulen
268 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; Simon et al., 2008; Weber et al., 2006). Nonetheless, data have rarely been compared between species exposed in similar experimental settings (Beauvais-Fluck et al., 2018a; Dranguet et al., 2017).

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 3 h to $5 \cdot 10^{-6}$ mol·L^{-1} Cu, notably concerning dysregulation of genes involved in vesicle transport, flavonoids metabolism and jasmonate (Lin et al., 2013). Authors further showed by knockout of genes necessary for this vesicle transport and exposure of roots to vesicle trafficking inhibitors, that Cu interacts with vesicle transport and that this vesicle transport is essential for signaling via ROS for activating defenses (Lin et al., 2013). Results of the present study allowed to propose a possible model of cellular mechanisms involved in Cu detoxification and protection in *E. nuttallii*: Cu increases intracellular transport, e.g. vesicle trafficking and ABC transport, and induces a flavonoid-mediated detoxification pathway. In addition, the toxicity mechanisms such as JA biosynthesis and cellular component biogenesis were regulated in response to Cu exposure. In comparison, the categories of OPP, nitrate metabolism and metal handling were absent in Cu regulated genes in *C. reinhardtii*. Conversely, cell motility, DNA, polyamine metabolism, mitochondrial electron transport, and TCA categories were found in *C. reinhardtii*, while absent in *E. nuttallii*. Moreover, the level of regulation of the categories found in common in both species was higher in *E. nuttallii* than in *C.*

292 *reinhardtii*. These differences certainly highlight the dissimilarities between basal and
293 background metabolism in two different species, as well as between a sessile and a mobile
294 organism (Dranguet et al., 2017). Besides, genome sequencing has revealed that *C. reinhardtii*
295 possesses numerous genes derived from the last plant-animal common ancestor that have been
296 lost in angiosperms, including transporters and the possibility of extensive metabolic flexibility
297 (Merchant et al., 2007). Taken together, our divergent observations on how an unicellular and a
298 multicellular organism take up and are impacted by Cu may imply that homeostasis networks are
299 more species-specific than generally thought.
300 We further found several differences at the level of subcategories. For example, in the
301 'Photosynthesis' category, genes of *C. reinhardtii* were mainly involved in the light reaction, in
302 particular photosystem I (PSI), while in *E. nuttallii* genes were involved both in PS I and PS II,
303 as well as photorespiration, suggesting that the photosynthesis was impacted more widely by Cu
304 toxicity in the macrophyte. Generally, Cu has been reported to impact more PS II than PS I in
305 plants. In PS II, the reaction center and LHC II by substitution of Mg^{2+} in its chlorophyll have
306 been shown to be targets of Cu toxicity (Kupper and Andresen, 2016; Kupper et al., 1996). In the
307 macrophyte *C. demersum* nanomolar concentrations of Cu affected the PS II reaction center
308 (Thomas et al., 2013). In this regard, our finding of Cu impact on PSI in *C. reinhardtii* is striking
309 and might point to structural differences between photosystems as well as background defense
310 pools such as metallothioneins, phytochelatins and redox enzymes in the studied species
311 (Castruita et al., 2011).
312
313 **Conclusion**

Overall, the exposure to 10^{-6} mol·L^{-1} 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. Nonetheless, because experimental settings are known to affect responses and experimental conditions are in general different for microalgae and plants, special attention has to be put in future research in testing organisms in completely identical conditions. Defining ecological thresholds of adverse outcomes for environmental contaminants represents a critical component of chemical assessment and management programs. Our data also call for including more species of aquatic plants for determining ecological thresholds for environmental contaminants, e.g. more tests using representative species of plants in the laboratory and *in situ* will be necessary in future studies (Beauvais-Fluck et al., 2018a; Dranguet et al., 2017). However, transcriptomics is confirmed as a useful tool to assess early responses at environmental concentrations and is promising for contaminated sites.

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519 **Table 1**: Modelled parameters of Cu uptake in ethylenediaminetetraacetic acid (EDTA)-rinsed *E.*
520 *nuttallii* or *C. reinhardtii* exposed to 10^{-6} mol·L^{-1} Cu. Uptake flux (*a*) and elimination rate
521 constant (*k*) of Cu were divided by the effective concentration of metal at beginning of the test to
522 allow inter species comparison and are thus presented as *a'* and *k'*.

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

523

524 Table 2: List of the 10 most up-regulated and 10 most down-regulaed genes in *C. reindhardtii* and *E.nuttallii* exposed 2 h to Cu.
525 Identification, differential expression analysis (log2FC, FDR) and GO annotation are shown (NA = not assigned; Table S2 and S3
526 show complete analysis).

ID	log2FC	FDR	GO
C. reinhardtii			
g9712	5.64	6.05E-79	alpha/beta-Hydrolases superfamily protein
Cre07.g321800	4.63	1.68E-69	--NA--
Cre08.g360200	3.73	1.32E-09	solute:sodium symporters;urea transmembrane transporters
Cre12.g494600	3.55	3.89E-06	--NA--
Cre03.g173100	3.51	7.93E-83	--NA--
Cre02.g143900	3.22	2.01E-10	GDSL-like Lipase/Acylhydrolase superfamily protein
Cre12.g497550	2.90	1.05E-17	NAD(P)-binding Rossmann-fold superfamily protein
Cre10.g431050	2.88	4.50E-29	--NA--
Cre17.g737300	2.81	1.18E-69	--NA--
Cre12.g525450	-2.02	8.26E-23	--NA--
Cre13.g587350	-2.20	5.70E-17	--NA--
Cre12.g540100	-2.21	2.11E-12	--NA--
Cre06.g305100	-2.22	3.32E-18	--NA--
g5945	-2.22	2.34E-04	Histone superfamily protein
Cre12.g493100	-2.25	6.29E-07	--NA--
Cre16.g681350	-2.44	6.14E-06	--NA--
Cre16.g668850	-2.64	7.29E-44	--NA--
Cre16.g651050	-2.79	1.89E-10	Cytochrome c
Cre06.g253000	-3.12	7.92E-49	--NA--
E. nuttallii			
Locus_106988_Transcript_1_1_Confidence_1.000_Length_217	9.26	2.63E-12	---NA---
Locus_74420_Transcript_1_1_Confidence_1.000_Length_486	6.24	8.01E-67	---NA---
Locus_50101_Transcript_1_1_Confidence_1.000_Length_289	6.13	1.37E-10	probable e3 ubiquitin-protein ligase bah1-like 1

Locus_46396_Transcript_2_2_Confidence_0.750_Length_415	5.65	2.67E-08	---NA---
Locus_99585_Transcript_1_1_Confidence_1.000_Length_553	5.63	2.45E-34	---NA---
Locus_31732_Transcript_1_2_Confidence_0.667_Length_327	5.47	2.79E-10	---NA---
Locus_56031_Transcript_1_1_Confidence_1.000_Length_615	5.12	1.01E-36	---NA---
Locus_26963_Transcript_1_1_Confidence_1.000_Length_256	5.06	2.98E-13	---NA---
Locus_22115_Transcript_2_4_Confidence_0.375_Length_528	5.01	1.75E-06	Uncharacterized protein TCM_030494
Locus_25722_Transcript_4_7_Confidence_0.389_Length_809	4.91	1.84E-12	---NA---
Locus_5098_Transcript_3_4_Confidence_0.625_Length_746	-3.41	7.18E-05	octicosapeptide phox bem1p
Locus_155_Transcript_2_8_Confidence_0.474_Length_2350	-3.46	9.23E-29	nitrate reductase
Locus_6174_Transcript_8_8_Confidence_0.524_Length_1224	-3.52	1.18E-08	fe(2+) transport protein 1-like
Locus_6580_Transcript_4_11_Confidence_0.433_Length_1496	-3.58	4.77E-10	hpp family expressed
Locus_1727_Transcript_3_9_Confidence_0.450_Length_2249	-3.88	7.64E-12	uroporphyrinogen-iii c-methyltransferase-like
Locus_97468_Transcript_1_2_Confidence_0.667_Length_399	-4.40	9.24E-05	---NA---
Locus_97468_Transcript_2_2_Confidence_0.667_Length_399	-5.26	5.69E-11	---NA---
Locus_104442_Transcript_1_1_Confidence_1.000_Length_777	-5.40	1.72E-17	---NA---
Locus_7125_Transcript_1_3_Confidence_0.600_Length_453	-8.54	5.50E-08	---NA---
Locus_71727_Transcript_1_1_Confidence_1.000_Length_499	-8.65	6.76E-05	---NA---

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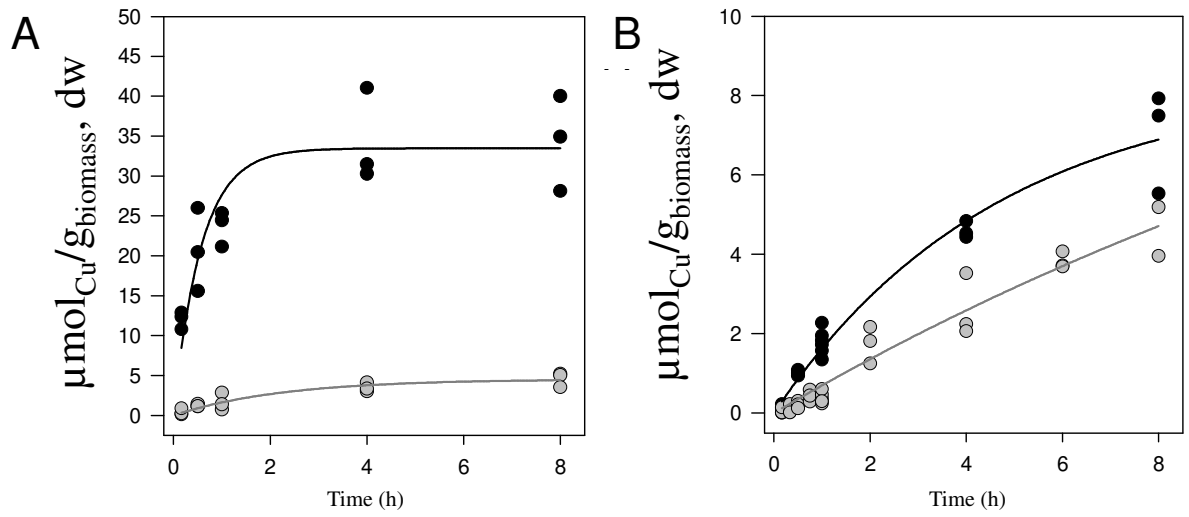

530

531 **Figure 1**: Cu toxicokinetics in *C. reinhardtii* (A) and *E. nuttallii* (B) exposed to 10^{-6} mol·L^{-1} Cu.
532 Organisms were ethylenediaminetetraacetic acid (EDTA)-rinsed (grey) or media-rinsed (black)
533 to differentiate between adsorbed and internalized metal.

534

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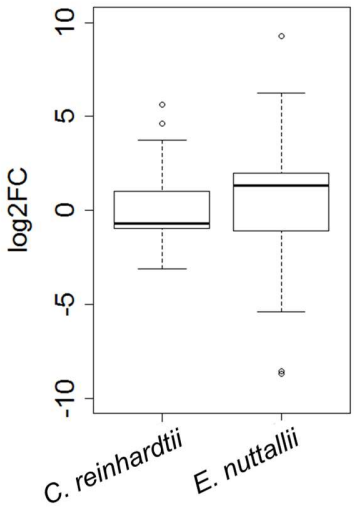

537

538 **Figure 2**: Fold-changes (log2FC) of significant regulated genes in *C. reinhardtii* and *E. nuttallii*

539 exposed 2 h to 10^{-6} mol·L^{-1} Cu.

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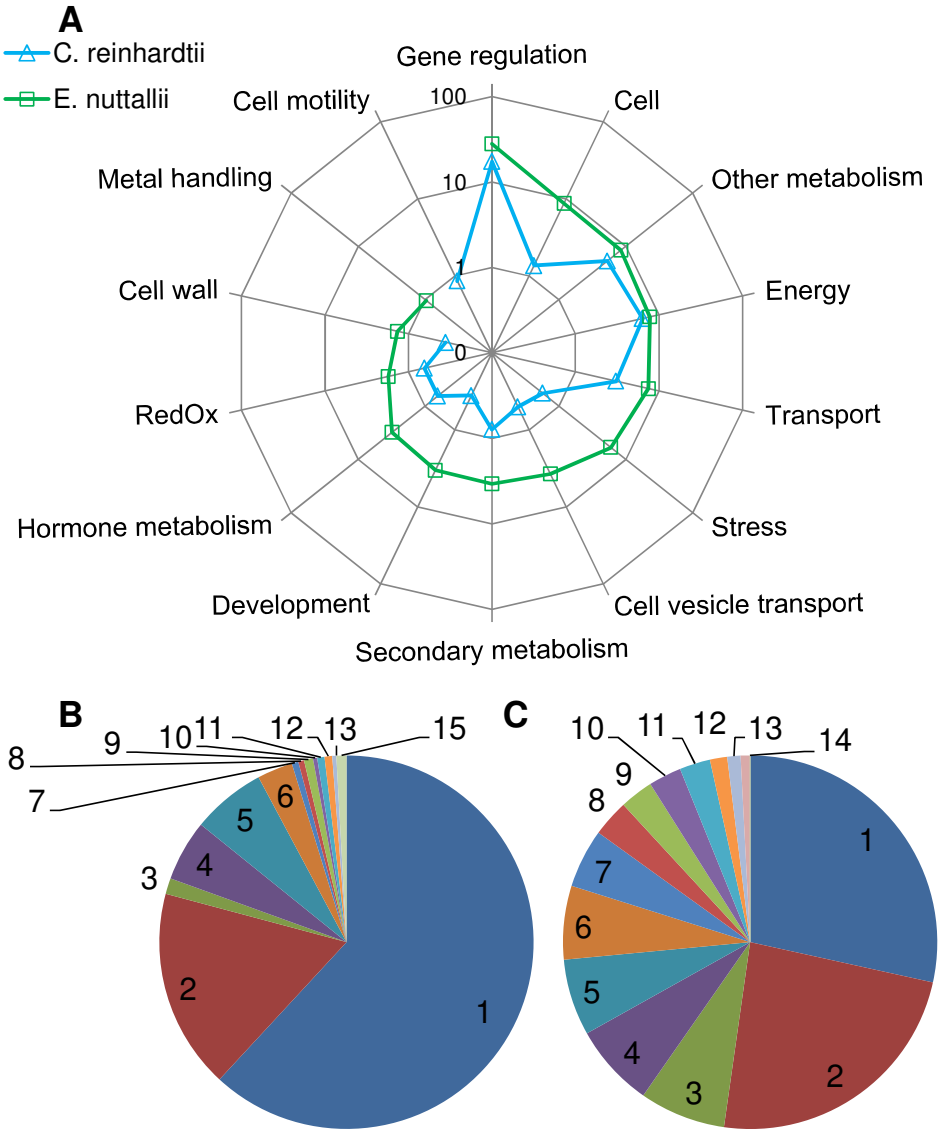

542 **Figure 3:** Genes regulated in *C. reinhardtii* and *E. nuttallii* exposed 2 h to 10^{-6} mol·L^{-1} Cu. The
543 total number of significant dysregulated genes (DG) in gene ontology (GO) categories
544 (MapMan) in *C. reinhardtii* (triangles) and *E. nuttallii* (square) exposed 2 h to Cu (A). The
545 proportion (%) of main functional GO categories (MapMan) of dysregulated genes in *C.*
546 *reinhardtii* (B) and *E. nuttallii* (C) (GO categories legend is 1: Unknown, 2: Gene regulation, 3:
547 Cell process, 4: Other metabolism, 5: Energy metabolism, 6: Transport, 7: Stress, 8: Cell vesicle

548 transport, 9: Secondary metabolism, 10: Development, 11: Hormone metabolism, 12: Oxidative
549 stress, 13: Cell wall, 14: Metal handling, 15: Cell Motility).