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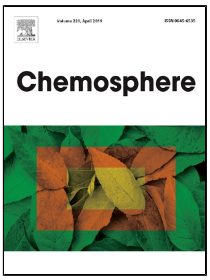

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Cadmium and/or copper excess induce interdependent metal accumulation, DNA methylation, induction of metal chelators and antioxidant defences in the seagrass *Zostera marina*

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Abstract

In this investigation, we assessed the effects of Cu and/or Cd excess on physiological and metabolic processes of the widespread seagrass *Zostera marina*. Adult were exposed to low Cd and Cu (0.89 and 0.8 μM, respectively) and high Cd and Cu (8.9 and 2.4 μM, respectively) for 6 d at: Control conditions; low Cu; high Cu; low Cd; high Cd; low Cd and low Cu; and high Cd and high Cu. Photosynthetic performance decreased under single and combined treatments, although effects were more negative under Cu than Cd. Total Cu accumulation was higher than Cd, under single and combined treatments; however, their accumulation was generally lower when applied together, suggesting competition among them. Levels of glutathione (GSH) and phytochelatins (PCs) followed patterns similar to metal accumulation, with up to PC5, displaying adaptations in tolerance. A metallothionein (*MET*) gene showed upregulation only at high Cd, low Cu, and high Cu. The expression of the enzymes glutathione reductase (*GR*), ascorbate peroxidase (*APX*), and catalase (*CAT*) was greatest at high Cu, and at high Cd and Cu together; the highest expression was under Cu, alone and combined. Both metals induced upregulation of the DNA methyltransferases *CMT3* and *DRM2,* with the highest expression at single Cu. The DNA demethylation *ROS1* was overexpressed in treatments containing high Cu, suggesting epigenetic modifications. The results show that under copper and/or cadmium, *Z. marina* was still biologically viable; certainly based, at least in part, on the induction of metal chelators, antioxidant defences and methylation/demethylation pathways of gene regulation.

Keywords: epigenetics; marine angiosperm; metal tolerance; photosynthesis; mechanism.

1. Introduction

Seagrasses are a small group of monocotyledonous angiosperms that re-colonised the seas, on at least three separate occasions, from around 100 Mya (Dittami et al. 2017). They now occupy soft sedimentary substrata in shallow coastal-waters and estuaries where they can form dense meadows that have important ecological roles and provide a range of ecosystem services (Nordlund et al. 2016). However, they are under threat from biotic and abiotic stresses, including from multiple anthropogenic pressures, that is leading to a global decline in their coverage estimated to be about 7% per annum (Waycott et al. 2009). Proximity to industrial activities and urban development exposes seagrasses to inputs of organic and inorganic chemicals from both point and diffuse sources. While the impacts by organic pollutants may be transitory, the environmental persistence of metals, their accumulation from both water column and sediments and transfer to higher trophic levels are likely contributors to the long-term decline in the health of seagrass meadows (Barwick and Maher 2003, Zheng et al. 2018). Although information on metal toxicity is limited, there are reports of reduced growth rates and impaired photosynthetic performance in a few seagrass species when exposed to metals such as cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) (Macinnis-Ng and Ralph 2002, 2004, Zhao et al. 2006, Ambo-Rappe et al. 2011). Cd and Cu are two of the most widely naturally occurring metals in marine environments, but inputs from anthropogenic sources have altered their natural cycling and, as a consequence, their bioavailability to marine biota has increased (Coelho et al. 2013).

Cu is an essential micronutrient with structural and catalytic roles, as components of proteins and enzymes involved in various metabolic pathways and physiological processes, but can be toxic beyond certain threshold concentrations (Yruela 2005). Cd is a non-essential metal with no known function in plants and animals (Deckert 2005, Park et al.

2008). Toxic concentrations of Cu and Cd can affect several physiological processes and biochemical events such as growth, photosynthesis, cell respiration, biosynthesis of chlorophyll and protein, DNA replication and enzyme activities (Sandalio et al. 2001, Romero-Puertas et al. 2002, Shukla et al. 2003). In this context, it has been proposed that epigenetic modifications that modulate transcriptionally silent or active chromatin by reversible methylation/demethylation processes, may be involved in abiotic stress responses, including metal tolerance in several plant species (Aina et al. 2004, Choi and Sano 2007, Lukens and Zhan 2007, Boyko and Kovalchuk 2008, Greco et al. 2012, 2013, Ding et al. 2014). In plants, cytosine methylation is promoted by three families of DNA methyltransferases: DMT1, DRMs and CMTs (Bartels et al. 2018), while DNA demethylation is addressed by the enzymatic removal of the methylated cytosine initiated by ROS1/DME family (Li et al. 2017). For instance, there is evidence of Cd-induced DNA hypermethylation in radish (Yang et al. 2007), *Arabidopsis thaliana* (Li et al. 2015, Wang et al. 2016), rice (Feng et al. 2016), and also in the seagrass *Posidonia oceanica* (Greco et al. 2012). In contrast, the red seaweed *Gracilaria dura* displayed severe cytosine demethylation under Cd exposure (Kumar et al. 2012). Cu exposure has also led to variations in DNA methylation patterns in red maple (Kalubi et al. 2017), and the aquatic herb *Hydrilla verticillata* (Shi et al. 2017).

The main pathway by which metals such as Cd and Cu induce biological stress is the induction of an oxidative stress condition, mainly caused by the overproduction of reactive oxygen species (ROS) through the disruption of electron transport chains and excess energy transfer to oxygen in chloroplasts and mitochondria (Fryzova et al. 2018). It is widely acknowledged that the "Foyer-Halliwell-Asada" pathway, based on *de novo* synthesis and recycling of the antioxidants glutathione (GSH) and ascorbate (ASC), is the main

mechanism to counteract ROS excess in plants. The process is mediated by the activities of several enzymes, among which are ascorbate peroxidase (APX), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) (Foyer and Noctor 2011). Other enzymes, such as catalase (CAT) and superoxide dismutase (SOD), also contribute in decomposing ROS, specifically hydrogen peroxide (H_2O_2) and superoxide anions ($\bullet O_2^-$), respectively (Foyer and Noctor 2011). For example, with exposure of up to 70 µM Cd for 6 d, GSH concentrations increased in the seagrass *Thalassia testudinum,* and in *Zostera japonica* exposure to 50 µM Cd or Cu for 7 d resulted in increased activities of SOD and CAT (Lin et al. 2016). In response to metal toxicity, plants activate different mechanisms that include detoxification by the binding of metals to ligands (Pal et al. 2018). Metal chelation represents a first line of defence for cells against internalized metals. Free metals are complexed in the cytosol by different chelators to reduce their bioavailability and facilitate their sequestration away from sensitive sites (Seth et al. 2012). These ligands include amino acids, organic acids, the tripeptide glutathione (GSH) and the metal-binding peptides phytochelatins (PCs) and metallothioneins (METs) (Guo et al. 2008, Verbruggen et al. 2009, Foyer and Noctor 2011). PCs are a family of cysteine-rich peptides, with a general structure (γ-Glu-Cys)*n*-Gly) (*n* = 2 to 11), that are synthesised from reduced GSH by the enzyme phytochelatin synthase (PCS) (Cobbett and Goldsbrough 2002). Because of the normally high cytosolic GSH concentrations, an influx of metal ions will generate GSH-metal complexes that are rapidly converted into PCs by the constitutively expressed PCS. Cd is considered the best activator of PCs (Clemens 2006), but other metals (e.g. Cu, Zn, As, Hg, Pb) can also do so (Cobbett 2000). A recent study by Nguyen et al. (2017) reported the occurrence of PC2 and PC3 in the roots of the seagrass *Enhalus acoroides* growing in highly Pb-contaminated sites in Vietnam but, to the best of our knowledge, the presence of

97 specified PCs in seagrasses experimentally exposed to elevated concentrations of metals
98 has not been published previously. However, unspecified non-protein thiols (other than
99 glutathione) and phytochelatin-(PC-)like peptides have been identified in leaves of of *P.*
100 *oceanica* and *T. testudinum*, respectively, on exposure to Cd (Maserti et al. 2005).
101 METs are cysteine-rich polypeptides but unlike PCs, MT proteins are encoded by a family
102 of genes. Consequently, a set of MET isoforms can exist that can be species- and metal-
103 specific (Cobbett and Goldsbrough 2002, Leitenmaier and Küpper 2013). METs are found
104 in many groups of organisms including plants, but evidence for their involvement in
105 mediating metal tolerance, distribution and accumulation in plants is limited (e.g. Li et al.
106 2013, Liu et al. 2014). So far, three genomic sequences putatively encoding type-II *METs*
107 *(MET2)* have been isolated from *P. oceanica* (Giordani et al. 2000), and whose expression
108 levels increase under both Cd and Cu excess (Giordani et al. 2000, Cozza et al. 2006).
109 In this study, we investigated the inter-relationship between physiological, metabolic and
110 transcriptomic processes in the seagrass *Zostera marina* (eelgrass) exposed to single and
111 combined Cd and Cu exposure. Specifically, we measured the maximum quantum yield of
112 PSII (*Fv/Fm*), a sensitive indicator of photosynthetic performance and thus of plant health
113 (Maxwell and Johnson 2000), concentrations of the intracellular metal-chelators GSH, PCs
114 and, levels of *MT* transcripts, and modulation of genes involved in antioxidant defence and
115 DNA methylation/demethylation. *Zostera marina* was selected because of its widespread
116 distribution in the temperate northern hemisphere of the Atlantic and Pacific Oceans
117 (Bostrom et al. 2014), and sensitivity to environmental perturbations (Ferrat et al. 2003).

118 **2. Materials and methods**

119 **2.1. Plant materials and sample preparation**

Plants of *Z. marina* were collected from a pristine site in south west England (Salcombe 50°13'30.40"N - 3°46'52.82"W; T°= 14.8°C; salinity= 33 psu; pH = 8.2; O_2 = 8.84 mg L^{-1}), and transported to the laboratory in seawater within 2 h. Plants were rinsed three times with sterilized seawater, all visible epiphytes were removed with a sterile razor blade and then acclimated to laboratory conditions for 2 d in acid-washed 50 L aquaria. Plants were maintained in continuously aerated filtered (0.45 µm) seawater (pH 7.8±0.2), at 15±0.5°C and an irradiance of 45 µmol $m^{-2}s^{-1}$ photosynthetic active radiation (PAR), on a 14/10 h light/dark cycle.

2.2. Metal exposure

Following the acclimation period, 10, similarly sized, adult plants were transferred to 21 individual 2 L aquaria, containing 1.5 L filtered seawater to which Cd and/or Cu was added. The experiment consisted of 7 treatments in triplicate: control (no added metal); Cu ($CuSO_4$) added at nominal concentrations of either 0.8 µM (50 µg L^{-1}) or 2.4 µM (150 µg L^{-1}), Cd ($CdCl_2$) at 0.89 µM (l00 µg L^{-1}) or 8.9 µM (l000 µg L^{-1}), Cu plus Cd at 0.8 µM Cu and 0.89 µM, respectively, Cu plus Cd at 2.4 and 8.9 µM, respectively. Cu and Cd exposure were selected upon environmentally representative concentrations in polluted environments, and also according to recognized chronic levels in different seagrass species, including *Z. marina* (e.g. Barwick and Maher 2003; Macinnis-Ng et al. 2006; Alvarez-Legorreta et al. 2008; Greco et al. 2012; Lin et al. 2016). Plants were exposed to the treatments for 6 d, with growth media replenished on day 3 in order to avoid depletion of nutrients and metals. At the end of the experiment, leaves from all plants were collected, blotted dry, immediately frozen in liquid nitrogen and stored at -80°C until further biochemical and molecular analyses. Biomass for metal analyses was freeze-dried for 24 h and then stored in a desiccator.

144 **2.3. Determination of photosynthetic performance**

145 Measurements of chlorophyll *a* fluorescence (pulse modulated chlorophyll fluorometer
146 FMS-1, Hansatech Instruments Ltd., Norfolk, England) were taken on leaves (n= 10) from
147 3 randomly selected plants from each aquarium prior to the start and at the end of the
148 exposure period. The maximum fluorescence (F_m) of dark adapted leaves (30 min) and
149 minimum fluorescence (F_o) were recorded and the maximum quantum yield of PSII
150 calculated from the ratio of variable to maximum fluorescence (F_v/Fm that is derived from
151 $(F_m - F_o)F_m$).

152 **2.4. Determination of Cd and Cu content in leaves**

153 Between 30-60 mg of freeze-dried (DW) leaves were digested in a microwave oven
154 (MARSX-press) in 2 mL of HNO_3 as described in Roncarati et al. (2015). After digestion,
155 the volume of each sample was adjusted to 10 mL with milli-Q (18 Ω) water. Total
156 concentrations of each metal were determined using ICP-MS (Thermo Scientific, X Series
157 2) as in Roncarati et al. (2015). The same methods were applied to certified reference
158 material (IAEA-140; BCR-279); results showed less than 15% variation according to Cu
159 and Cd reference values.

160 **2.5. Analysis of PCs**

161 PCs were detected with modifications from Lavoie et al. (2009). Briefly, 0.2 g FW of
162 leaves were added to 1.2 mL of 0.1% (w/v) trifluoroacetic acid (TFA), containing 6.3 mM
163 diethylenetriamine-pentaacetic acid (DTPA). The mixture was centrifuged at 7,400 g for 20
164 min at 4°C, and the supernatant recovered. The derivatization of thiol groups with
165 monobromobimane (mBrB) was performed by mixing 250 µL of the clear homogenate, 450
166 µL of 200 mM HEPES pH 8.2, 6.3 mM DTPA, and 10 µL of 25 mM mBrB (Invitrogen,
167 Oregon, USA); incubation was carried out for 30 min at room temperature in the darkness.

The reaction was stopped with the addition of 300 µL of 1 M methanesulfonic acid (MSA). Samples were filtered through 0.45 µm pore size membranes and stored at 4 °C in darkness. PCs were analysed by High Performance Liquid Chromatography (HPLC) using an Agilent 1100 Series system, and data was compiled using Chemstation software. PCs (20 mL extract) were separated on a reversed phase C-18 column (5 µm particle size, 4.6 mm inner diameter, 15 cm length) at 25°C. Elution was performed using solvent A (0.1% TFA in aqueous solution) and solvent B (100% acetonitrile) with linear gradient (10 min from 0 to 20%, 30 min from 20 to 35%, and 10 min from 35 to 100% of solvent B), and a flow rate of 1 mL min^{-1}. PCs were detected by fluorescence at 380 nm excitation and 470 nm emission wavelengths. Pure PCs standards with degrees of polymerization from n=2 to n=6 (AnaSpec Inc., San Jose, CA, USA) were dissolved in filtered water. Retention times of PC2, PC3, PC4, PC5 and PC6 were 4.4, 11.5, 16.9, 19.9 and 21.6 min, respectively.

2.6. Total RNA extraction and reverse transcription

Total RNA was separately extracted from different leaf samples following the protocol of Doyle (1991) with modifications. All solutions were prepared with RNase-free distilled water. Three hundred grams FW ground biomass were mixed with 0.1 g PVP-40. One mL of freshly prepared extraction buffer ([200 mM Tris/HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA, CTAB 3 % [(w/v)]) and thereafter β-mercaptoethanol (final concentration 1.3%) were added to the samples. After 30 min at 60°C, one volume of chloroform/isoamyl alcohol (49:1) was added; the supernatant was recovered after centrifugation at 5,300 g for 15 min and precipitated with isopropanol at –20°C overnight. After centrifugation at 10,000 g for 15 min and washing with 0.2 M sodium acetate in 70% ethanol for 1 h at 4°C, RNA was dried and resuspended in RNase-free water and treated with 30 U of DNase I (Roche) for 15 min at 37°C.

Quality and quantity of RNA was verified using a NanoDrop® spectrophotometer ND-1000; the integrity was checked on agarose 0.8% gel electrophoresis. About 2–3 μg of total RNA was retro-transcribed using a cDNA Synthesis Kit (High Capacity RNA-to-cDNA kit, Life Technologies, Applied Biosystems) according to the kit instructions.

2.7 Quantitative real-time PCR (qPCR)

Different genes associated with a potential detoxification/homeostatic responses were assessed. In relation to metal chelation, the expression levels of METALLOTHIONEIN-LIKE PROTEIN 2A (*MET*), a gene encoded cysteine-rich protein with high metal-metal chelating capacity, was assessed. Associated with antioxidant metabolism, the genes studied were: L-ASCORBATE PEROXIDASE1 (*APX*), which catalyses the conversion of H_2O_2 into H_2O using ascorbate as electron donor, CATALASE (*CAT*), which catalyses the decomposition of H_2O_2, and a chloroplastic GLUTATHIONE REDUCTASE (*GR*), which promotes the reduction of glutathione disulfide (GSSG) to glutathione (GSH). Also, three genes involved in the epigenetic regulation of gene expression were assessed: CHROMOMETHYLASE3 (*CMT3*), involved in cytosine methylation of non-CG sites; DOMAIN REARRANGED METHYLASE2 (*DRM2*), associated to both the maintenance of non-CG methylation and *de novo* methylation in all sequence contexts; and REPRESSOR OF SILENCING 1 (*ROS1*), a 5-methylcytosine DNA glycosylase/lyase important for active DNA demethylation. Specific oligonucleotide primers were designed using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed 11 January 2006), according to Yokoyama and Nishitani (2001) and Applied Biosystem software. Each primer pair used was designed to obtain a final PCR product of approximately 110-170 bp length, and was tested for different parameters including robustness, successful amplification over a range of annealing temperatures, specificity and

the consistency of highly reproducible C_T values within the reactions of a triplicate. Primers for genes of interest were designed considering sequences from the seagrass EST database Dr. Zompo (Wissler et al. 2009) (http://drzompo.uni-muenster.de/). The reference gene *ELONGATION FACTOR* (*ELO_F*) was selected based on previous study by Ransbotyn and Reusch (2006). All primers used are listed in Table 1. qPCR was performed using a QuantStudio 12K Flex provided by Applied Biosystems in a 20 µl total volume containing: 10 µl 2x PowerSYBR Green PCR Master Mix (Applied Biosystems, Italy); 400 nM of each primer; and 30 ng cDNA. Reactions were performed in triplicate with the following cycles: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. To test primer specificity, melting curve analysis (from 60°C to 95°C with an increasing heat rate of 0.5°C s^{-1}) was performed after amplifications. The calculations for determining the relative level of gene expression were made using the cycle threshold (C_T) value, according the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

2.8. Statistical analysis

The data were tested for homogeneity of variance and normality and then subjected to one-way Analysis of Variance (ANOVA). Differences between individual means were determined from Tukey's *post hoc* multiple range test at 95% confidence interval using the software Origin Pro 8 (OriginLab).

3. Results

3.1 *Photosynthetic performance*

Compared to controls, there was a significant ($p < 0.05$) decrease in Fv/Fm under all Cd and Cu treatments, with exposure to Cu having a greater effect than Cd (Figure 1).

3.2 Metal accumulation

239 The concentrations of Cd and Cu in leaves increased significantly with increasing metal
240 exposure; however, patterns differed when metals were applied singly or in combination
241 (Figure 2). Typically, significantly more Cu than Cd was accumulated and significantly
242 higher concentrations of both were accumulated when applied singly. For example, under
243 high Cd or high Cu, the concentrations of the two metals were 59 and 360 nmol g^{-1}(DW),
244 respectively. In contrast, under a combination of high Cd and Cu, only 47 and 293 nmol g^{-1}
245 (DW), respectively, were accumulated.

246 **3.3 Metal chelators**

247 The production of all thiols (GSH and PC2, PC3, PC4 and PC5) was similarly dependent
248 on the metal, the concentration and whether in combination or single exposure (Figure 3).
249 For all thiols, their concentrations significantly increased with greater single and combined
250 metal exposure, although when Cd and Cu were applied together, levels of all thiols were
251 significantly higher than when exposing to the same concentrations of Cu or Cd alone. The
252 only exception to the latter was PC2 at high Cd and low Cu, which presented no significant
253 differences compared with high Cu (see Figure 3B). Moreover, levels of GSH and PCs
254 were generally significantly higher when *Z. marina* was exposed to Cu than Cd, under the
255 two concentrations of exposure for both metals, although in PC3 and PC5 levels of PCs
256 were not significantly different between low Cu and low Cd alone (Figure 3C, 3E). Finally,
257 it was observed that the different thiols decreased their concentration as their level of
258 polymerization increased. For instance, the highest concentrations of GSH, PC2, PC3, PC4
259 and PC5 were detected at high Cd and Cu together, with concentrations of 1375, 280, 204,
260 63 and 30 nmol g^{-1} FW (Figure 3).

261 Level of transcripts encoding *MET* were only significantly higher than the controls at high
262 Cd, and at low and high Cu; these metal treatments also did not present significant

differences with each other (Figure 4). The expression of *MET* was not significantly different between controls compared with the treatments at low Cd and low Cd and Cu together. The only downregulation observed in *MET* was recorded under the combination of high Cd and Cu (Figure 4).

3.4 Antioxidant metabolism

There were different patterns in the expression of the studied antioxidant enzymes. For *GR*, there was observed downregulation compared to the controls under treatments low Cd, high Cd and low Cu; between these single metal treatments, the lowest expression was recorded at high Cd, although without significant differences with low Cd (Figure 5A). The highest upregulation of *GR* was observed at the high Cu treatment, followed by the treatment at high Cd and Cu together. Transcript levels of *GR* were not significantly different in relation to the controls at low Cd and Cu together. For *APX*, trends showed a concomitant increase in the expression, from low Cd, high Cd, low Cu to high Cu; the latter treatments showed the highest levels of expression (Figure 5B). Transcripts of APX displayed no significant changes for low Cd and Cu together, in relation to the controls (Figure 5B). At high Cd and Cu together, there was upregulation of *APX*, although with no significant differences with treatments at single low or high Cu (Figure 5B). The expression of *CAT* was not significantly different between treatments at low levels of Cd or Cu, with respect to controls (Figure 5C). There was significant upregulation of *CAT* at high Cd or high Cu, and even higher at low Cd and Cu together; however, the latter treatment did not show significant differences with at low Cd (Figure 5C). The highest levels of expression were observed at high Cd and Cu together (Figure 5C).

3.5 Epigenetic regulation of gene expression

Distinct trends in the expression of DNA methylation/demethylation-related genes were detected for different treatments (Fig. 6). Both *CMT3* and *DRM2* were significantly upregulated in all metal treatments, but the relative levels of expression did not follow the same patterns. For *CMT3*, the highest levels of expression were when exposed to Cu only, and then to Cd only; the lowest expression was observed when Cu and Cd were combined (Figure 6A). For *DRM2*, the highest level of expression was under high Cu, with intermediate overexpression in the combined treatments and lowest under low Cu and low and high Cd (Figure 6B). In contrast, there was downregulation of *ROS1* with exposure to Cd and to low Cu (Figure 6C). Only exposure to high Cu and to a combination of high Cd and Cu resulted in significant upregulation of this gene (Figure 6C).

4. Discussion

In this study, for the first time, we provide information on physiological and metabolic modifications in a seagrass species under both single and combined metal (Cd and Cu) exposure. More specifically, through the identification of treatment-specific patterns in photosynthetic performance, metal accumulation, thiols (GSH and PCs) production, and the expression of genes responsible for induction of metallothionein (MTs), antioxidant enzymes, as well as involved in DNA methylation/demethylation for modulating gene expression. We have gained a better understanding of the potential mechanisms involved in cellular detoxification and homeostasis that provides a degree of tolerance in this ecologically important seagrass species.

Under all single and combined metal treatments, levels of photosynthesis maximum quantum yield of PSII (Fv/Fm) decreased, although those containing Cu displayed more detrimental effects than when exposed only to Cd (except at low Cd and Cu together). It is known that excess Cd and Cu induce ROS over-production, especially in the chloroplast

(Fryzova et al. 2018), and it is very likely oxidative damage is responsible for the observed reduced photoinhibition of PSII under metal treatments. Since total accumulation of Cu was generally higher than that observed for Cd under all single and combined treatments; thus, it is reasonable that potentially less Cd bioavailability intracellularly caused smaller photoinhibition. Although it is known that Cd and Cu can target different components of PSII (Burda et al. 2003, Gonzalez-Mendoza et al. 2007), given the concentrations of exposure used in this study, metal-mediated photoinhibition seems to be principally induced upon concentrations of exposure and uptake.

It has been observed that *Z. marina*, as well as other seagrasses (e.g. *Cymodocea nodosa*, *P. oceanica*), preferentially accumulate Cd in leaves, whereas Cu accumulates in both leaves and roots (Lyngby and Brix 1984, Llagostera et al. 2011, Sanz-Lázaro et al. 2012). In our investigation, both Cd and Cu accumulation in the leaves of exposed plants increased with exposure dose, suggesting a good translocation to the aerial organs and/or increased direct uptake by leaves. The uptake of Cu was greater than Cd even if the levels of exposure to Cu, compared with those of Cd, were lower; the latter considering single and combined Cu and Cd treatments. In particular, while the accumulation of Cu increased proportionally to the Cu applied, in case of Cd it only increased 2.5 times when Cd exposure was 10 times greater (23 and 58 nmol g^{-1} DW at 0.89 and 8.9 μM Cd, respectively). These results are in line with previous studies, which showed that Cu uptake compared with Cd in different tissues of *Z. marina* (leaves, rhizomes and roots) treated with increasing concentrations of these metals together of up to 50 μM (Lyngby and Brix 1982, 1984). A similar accumulation ratio between metals has been observed in the leaves of the seagrasses *C. nodosa* (Llagostera et al. 2011), *Thalassia hemprichii, Enhalus acoroides* and *Cymodocea rotundata* (Li et al. 2012). Interestingly, the concentrations of Cd and Cu accumulated

334 under combined metal treatments were lower than that under single metals treatment; a
335 probable mechanism of metal competition for the binding site occurred (Foster et al. 2014).
336 Taking into consideration the essential and non-essential role for plants of Cu and Cd,
337 respectively, it is not surprising that their accumulation differed. Indeed, plants have several
338 Cu-specific transporters (e.g. Atx1, Atox1, CUER, COPZ) (Ducic and Polle 2005), whereas
339 Cd has complex uptake mechanisms though unspecific transporters (e.g. Fe transporters
340 *IRT1* and *IRT2)* (Connolly et al. 2002, Nakanishi et al. 2006, Vert et al. 2009).
341 In this study the metal-complexing peptides GSH and PCs were detected in *Z. Marina,*
342 using PCs with molecular structure $(\gamma\text{-Glu-Cys})_n$gly of up to n=5 (PC5). *Z. marina* respond
343 to Cd, Cu, and to a combination of these metals excess by inducing the synthesis of both,
344 short (PC2-PC3) and long (PC4-PC5) chain PCs; however, the longer the PCs the less
345 produced. In spite of the latter, it was generally shown that PCs induction was higher under
346 Cd and Cu combined if compared with single metal treatments. It has been demonstrated
347 that the level of polymerization of PCs has an important influence on metal tolerance;
348 indeed, the longer the PC, the higher capacity will the species have to chelate and detoxify
349 bioavailable metals (Clemens 2006). To the extent of our knowledge, records of PCs in
350 seagrasses are restricted to the species *T. testudinum* and *E. acoroides.* While in *T.*
351 *testudinum* PCs were detected as long as PC2 under Cd excess of up to 70 µM (Alvarez-
352 Legorreta et al. 2008), in *E. acoroides* PCs were recorded with length of up to PC3 in Pb
353 polluted sites (Nguyen et al. 2017). In spite of the scarce information that is available in this
354 regard for seagrasses, records on other aquatic plants can provide insights on PCs-related
355 induction under metal excess. For instance, Török et al. (2015) exposed the aquatic plants
356 *Elodea canadensis, Salvinia natans* and *Lemna minor* to single Cd exposure of 818 mM, or

the latter combined with 260 mM Cu and 280 mM Zn, for 6 d. These authors observed that either under single Cd or Cd combined with Cu and Zn, the highest accumulation of these metals was observed in *L. minor*. Interestingly, only *L. minor* displayed PCs of up to PC7 under metal treatments, whereas *E. canadensis* and *S. natans* showed PCs only as long as PC3 (Török et al. 2015); it was also demonstrated that the activity of PCS was the highest in *L. minor* under control and metal treatments. Their data supports *L. minor* as metal, in particular Cd, Cu and Zn, tolerant species, also evidencing that PCs may have an important role in its metal tolerance strategies. Our results demonstrate, with up to PC5, that PCs are a relevant mechanism for the detoxification of bioavailable metals in *Z. marina*, also considering that levels of metal exposure in our study were considerable lower compared with those used, for instance, by Alvarez-Legorreta et al. (2008) and Török et al. (2015). It is also important to mention that our records constitute, for the first time, evidence of a seagrass species capable of synthetizing highly polymerized PCs of up to PC5 under metal excess.

METs are cysteine-rich proteins first described as metal-chelators, although it has been proven that their cysteine residues have also high ROS scavenging capacity to counteract oxidative stress and damage (Kumari et al. 1998). In seagrasses, the induction of METs under metal excess has been only assessed in *P. oceanica*, which showed the expression of 10 different *METs* under 1 µM Cu or 10 µM Cd for 2 d (Cozza et al. 2006), although the levels of transcripts were not quantified. Despite this information, MET coding genes have been also previously detected in *Z. marina* under high temperatures (Reusch et al. 2008) and increased salinities (Kong et al. 2013), likely to be induced to counteract oxidative stress. Our results demonstrate upregulation of *MET* only under single treatments of high Cd, and at low and high Cu. Interestingly, when metals were combined the levels of

expression were no different from the controls, in case of at low Cd and Cu, or down regulated, for high Cd and Cu. Even though the expression of this *MET* suggest its participation in Cd and Cu detoxification/homeostasis at least under single treatments, further research is necessary to address for its role as metal chelator and/or ROS scavenger. Moreover, considering that *Z. marina* encodes 10 different *MET* isoforms (see genome at http://bioinformatics.psb.ugent.be/orcae/overview/Zosma), it is very likely that other METs play different roles in detoxification and homeostatic control of metal excess, but this requires further investigation.

With regard to the reactive oxygen metabolism, *Z. marina* under Cd and/or Cu excess displayed enhanced antioxidant defences, manifested in increased production of GSH and higher expression levels of *GR*, *APX* and *CAT*, albeit to different extents depending on the metal, their concentration and whether single or combined exposure. However, there are common patterns showing that enhanced antioxidant defences were activated under high Cu, when supplied alone but also when combined with high Cd, and to lesser extent on exposure to only Cd. GSH trends are in agreement with those observed in *T. testudinum*, which displayed higher GSH content under Cd of up to 70 µM for 6 d (Alvarez-Legorreta et al. 2008). As far as we are aware, there are no published data on the expression of *GR*, *APX* and *CAT* under metal stress in seagrasses, although the activity of CAT has been shown to increase proportionally with Cu exposure of up to 50 µM, but not under Cd concentrations also of up to 50 µM (Lin et al. 2016). Also, from research on terrestrial plants species there is evidence for the expression and activities of enzymes associated with antioxidant metabolism (e.g. GR, APX and CAT), to change under Cd and/or Cu exposure (e.g. Shah et al. 2013, Shahabivand et al. 2016, Kisa 2017, Yadav et al. 2018). An interesting feature is that the expression of *GR* in *Z. marina* did not follow the same

patterns as GSH production, especially under single Cd or Cu treatments; thus, the information suggests that other *GR* isoforms may be acting, part of GR activity is not transcriptionally regulated, and/or the *de novo* pathway ending in the activity of glutathione synthase (GS) is also participating in the restoration of GSH in *Z. marina*. Further investigation considering also *de novo* GSH synthesis under Cd and/or Cu excess may help disclosing these assumptions.

DNA hypermethylation plays a major role in modulating gene expression and it is considered an efficient protective mechanism to maintain genome integrity against homologue recombination and unwanted transposition that could be enhanced by abiotic stressors (Bender 1998, Bilichak et al. 2012). In *Z. marina*, both metals induced the overexpression of the DNA methyltransferases *CMT3* and *DRM2*, but to different magnitudes, which could suggest metal-specific methylation strategies. In line with these results, it has been demonstrated that *de novo* DNA hypermethylation is directly correlated with the upregulation of *CMT1* in *P. oceanica* up to 50 µM Cd for as long as 4 d (Greco et al. 2012). Similar results were obtained when analysing the expression of several DNA methyltransferases, including *DMT1-2* and *CMT3-2,* in rice plants after exposure to 1 µM Cu and 10 µM Cd for 7 d, a feature inherited in subsequent progeny (Ou et al. 2012). Furthermore, in *H. verticillata* treated with 0.16 µM Cu for 5 d there was increased production of four proteins with DNA methyltransferase activity, which was reflected later with hypermethylation of genomic DNA (Shi et al. 2017). However, in the same study, Cu in excess of 1.6 µM triggered DNA demethylation as a consequence of Cu-mediated oxidative stress (Shi et al. 2017). Interestingly, in our study, the 5-methylcytosine DNA glycosylase *ROS1* involved in DNA demethylation was downregulated after treatments at low and high Cd, and at low Cu, whereas it was overexpressed in treatments containing

high Cu, even when combined with Cd; thus, similar to the observations on *H. verticillata*, up-regulation of *ROS1* could be a direct consequence of an oxidative stress condition induced by these metal treatments. In addition, the overexpression of *ROS1* and consequently DNA hypomethylation could allow the selective expression and activation of genes involved in stress response and tolerance, as it was observed with *GR*, *APX*, and *CAT*.

5. Conclusion

The seagrass *Z. marina* exposed to Cd and/or Cu excess demonstrated interdependent physiological, metabolic and transcriptomic responses. The seagrass showed to be biologically viable within the range of Cd and Cu concentrations applied in this study, when exposed singly and in combination treatments, which was observed in terms of their photosynthetic performance. Metal accumulation and the activation of intracellular defences demonstrated increased intracellular metal concentrations in *Z. marina*, under single and combined treatments. Specifically, intracellular metal homeostasis and detoxification of the metals involved the induction of GSH, PCs and METs, the expression of antioxidant enzymes and the activation of methylation/demethylation pathways of gene regulation. This represents the first investigation at different levels of biological organization on seagrasses under combined metal exposure, providing insights of their physiological and metabolic strategies in order to cope with metal-mediated stress in polluted environments.

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Figure 1: The maximum quantum yield (Fv/Fm) of leaves of *Zostera marina* exposed to one of 6 treatments for 6 d: control (no metals added), 0.89 µM Cd (0.89 Cd); 8.9 µM Cd (8.9 Cd); 0.8 µM Cu (0.8 Cu); 2.4 µM Cu (2.4 Cu); 0.89 µM Cd + 0.8 µM Cu (0.89 Cd + 0.8 Cu); and 8.9 µM Cd + 2.4 µM Cu (8.9 Cd + 2.4 Cu). Bars represent means ± SD (n = 3). Different letters denote significant differences at 95% confidence interval ($p < 0.05$).

Figure 2: The total concentration of Cd and Cu accumulated in leaves of *Zostera marina* exposed for 6 d to: control (no metals added), 0.89 µM Cd (0.89 Cd); 8.9 µM Cd (8.9 Cd); 0.8 µM Cu (0.8 Cu); 2.4 µM Cu (2.4 Cu); 0.89 µM Cd + 0.8 µM Cu (0.89 Cd + 0.8 Cu); and 8.9 µM Cd + 2.4 µM Cu (8.9 Cd + 2.4 Cu). Bars represent means ± SD (n = 3). Different letters denote significant differences at 95% confidence interval ($p < 0.05$); lower and uppercase letters represent differences in total Cd and Cu accumulation, respectively.

Figure 3: The concentrations of levels of glutathione (GSH. A), phytochelatins 2 (PC2, B), PC3 (C), PC4 (D) and PC5 (E) in *Zostera marina* exposed for 6d to: control (no metals added), 0.89 µM Cd (0.89 Cd); 8.9 µM Cd (8.9 Cd); 0.8 µM Cu (0.8 Cu); 2.4 µM Cu (2.4 Cu); 0.89 µM Cd + 0.8 µM Cu (0.89 Cd + 0.8 Cu); and 8.9 µM Cd + 2.4 µM Cu (8.9 Cd + 2.4 Cu). Bars represent means ± SD (n = 3). Different letters denote significant differences at 95% confidence interval ($p < 0.05$).

Figure 4: Levels of expression of METALLOTHIONEINS (*MET*) in *Zostera marina* exposed for 6 d to: control (no metals added), 0.89 µM Cd (0.89 Cd); 8.9 µM Cd (8.9 Cd); 0.8 µM Cu (0.8 Cu); 2.4 µM Cu (2.4 Cu); 0.89 µM Cd + 0.8 µM Cu (0.89 Cd + 0.8 Cu);

and 8.9 µM Cd + 2.4 µM Cu (8.9 Cd + 2.4 Cu). Bars represent means ± SD (n = 3). Different letters denote significant differences at 95% confidence interval ($p < 0.05$).

Figure 5: Levels of expression of chloropastic GLUTATHIONE REDUCTASE (*GR*; A), ASCORBATE PEROXIDASE1 (*APX*; B) and CATALASE (*CAT*; C) in *Zostera marina* exposed for 6 d to: control (no metals added), 0.89 µM Cd (0.89 Cd); 8.9 µM Cd (8.9 Cd); 0.8 µM Cu (0.8 Cu); 2.4 µM Cu (2.4 Cu); 0.89 µM Cd + 0.8 µM Cu (0.89 Cd + 0.8 Cu); and 8.9 µM Cd + 2.4 µM Cu (8.9 Cd + 2.4 Cu). Bars represent means ± SD (n = 3). Different letters denote significant differences at 95% confidence interval ($p < 0.05$).

Figure 6: Levels of expression of CHROMOMETHYLASE3 (*CMT3*; A), DOMAIN REARRANGED METHYLASE2 (*DRM2*; B) and REPRESSOR OF SILENCING 1 (*ROS1*; C) in *Zostera marina* exposed for 6 d to: control (no metals added), 0.89 µM Cd (0.89 Cd); 8.9 µM Cd (8.9 Cd); 0.8 µM Cu (0.8 Cu); 2.4 µM Cu (2.4 Cu); 0.89 µM Cd + 0.8 µM Cu (0.89 Cd + 0.8 Cu); and 8.9 µM Cd + 2.4 µM Cu (8.9 Cd + 2.4 Cu). Bars represent means ± SD (n = 3). Different letters denote significant differences at 95% confidence interval ($p < 0.05$).

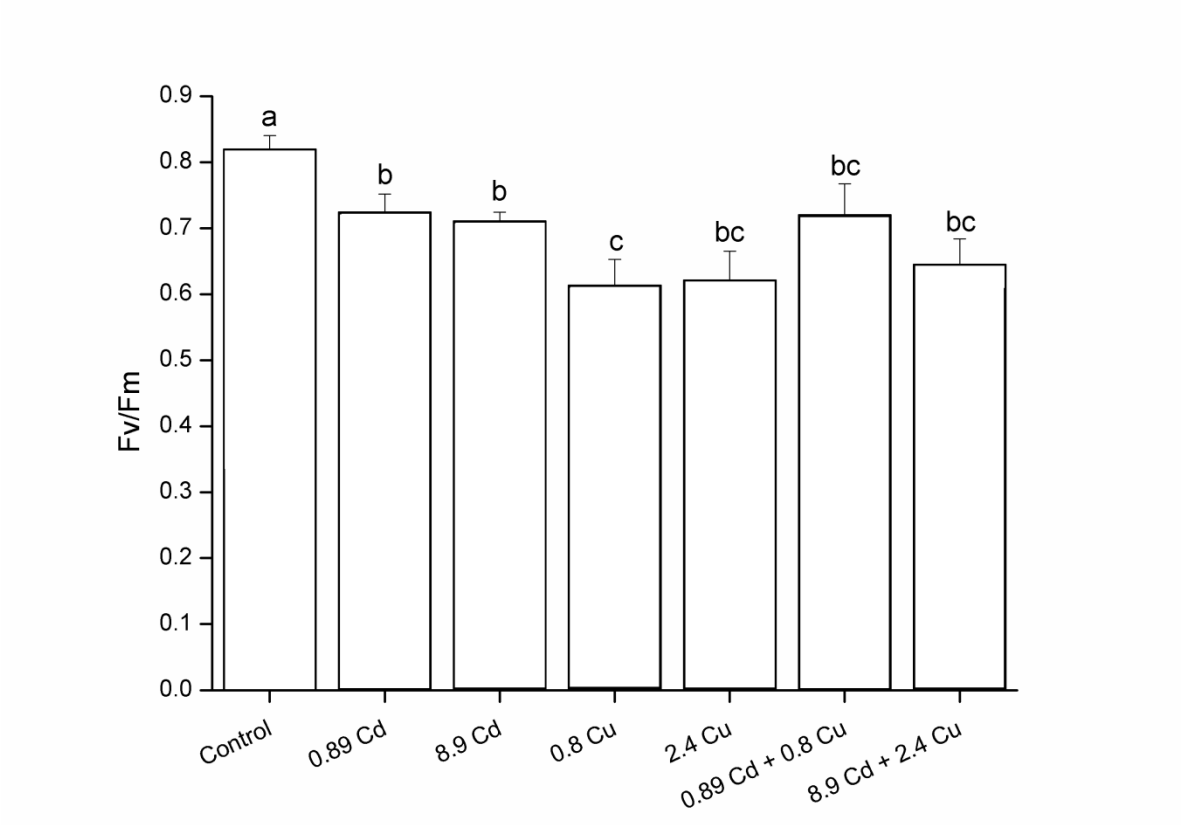

Figure 1

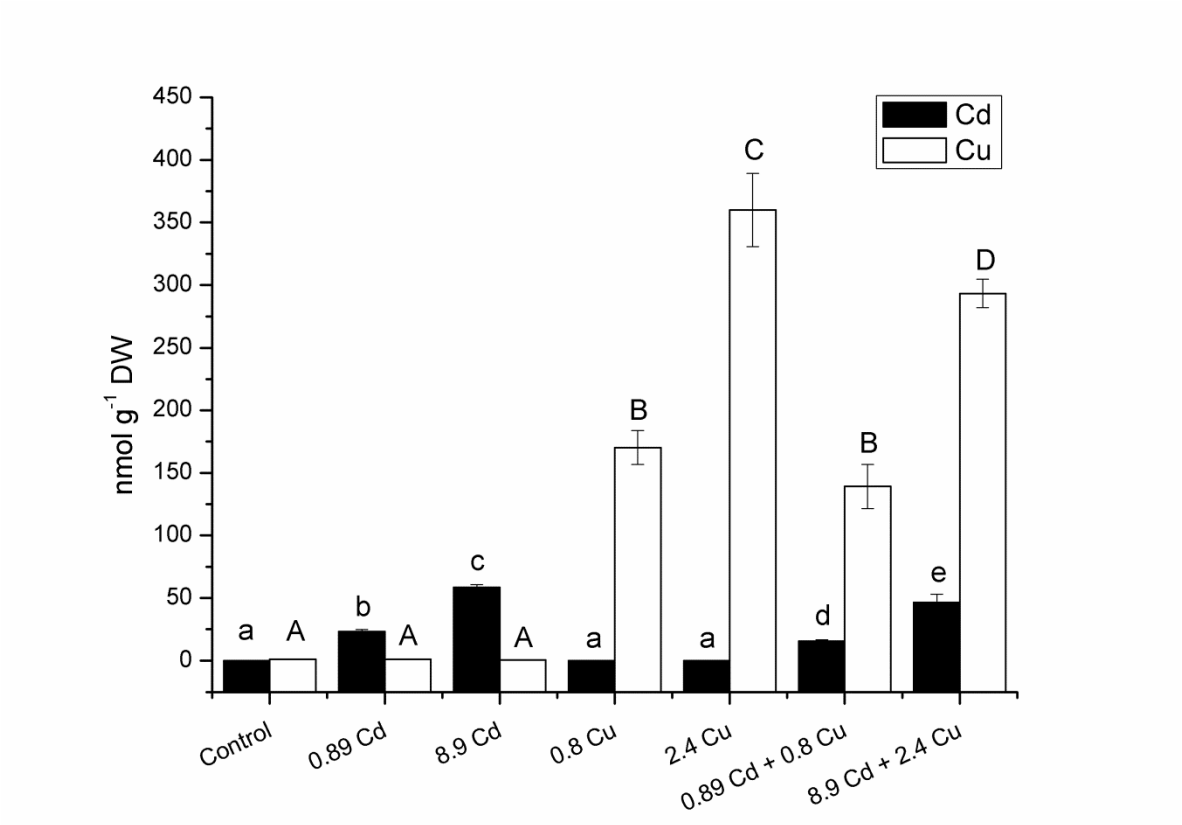

Figure 2

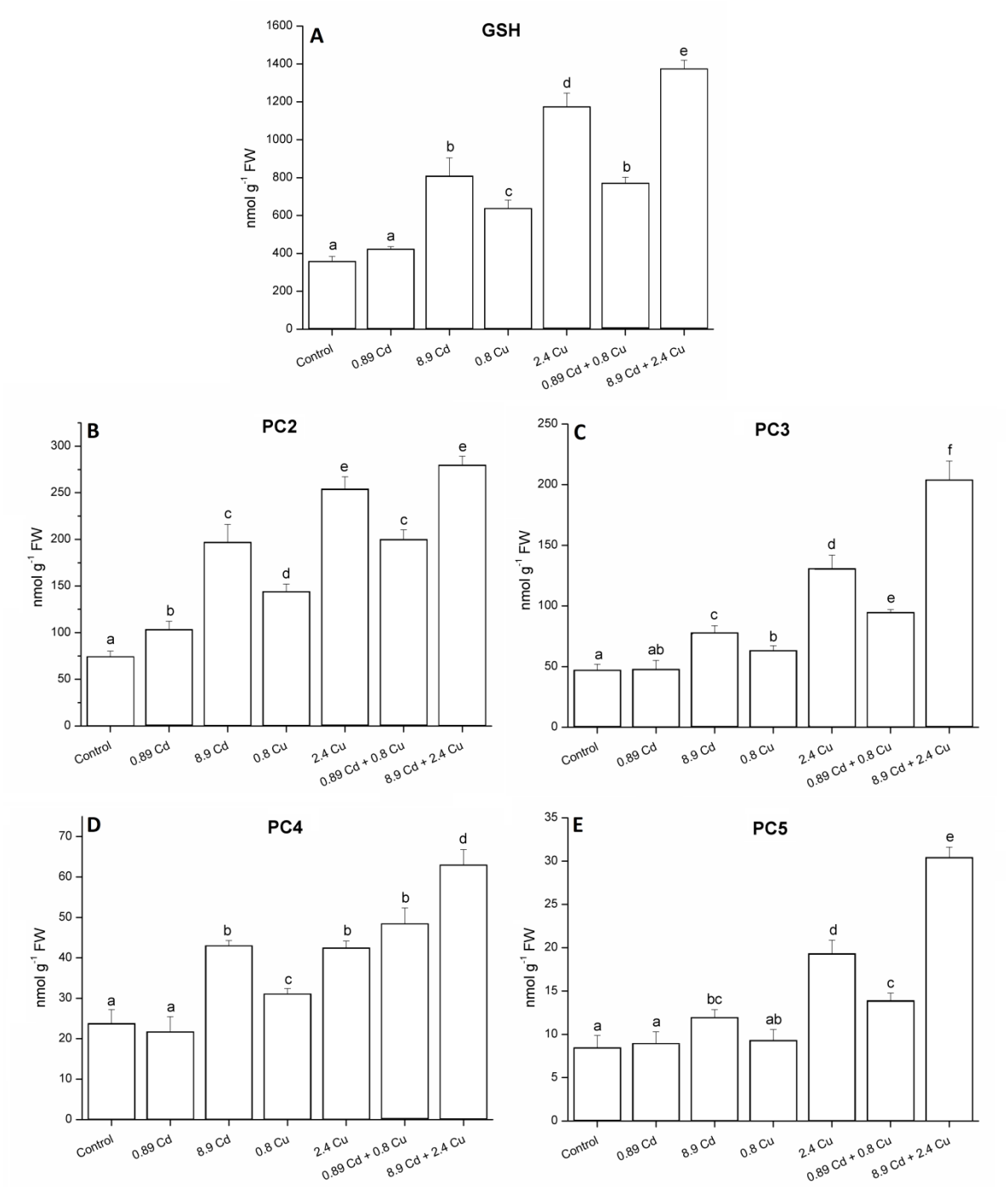
A
GSH
nmol g-1 FW
B
PC2
C
PC3
D
PC4
E
PC5
Control
0.89 Cd
8.9 Cd
0.8 Cu
2.4 Cu
0.89 Cd + 0.8 Cu
8.9 Cd + 2.4 Cu

Figure 3

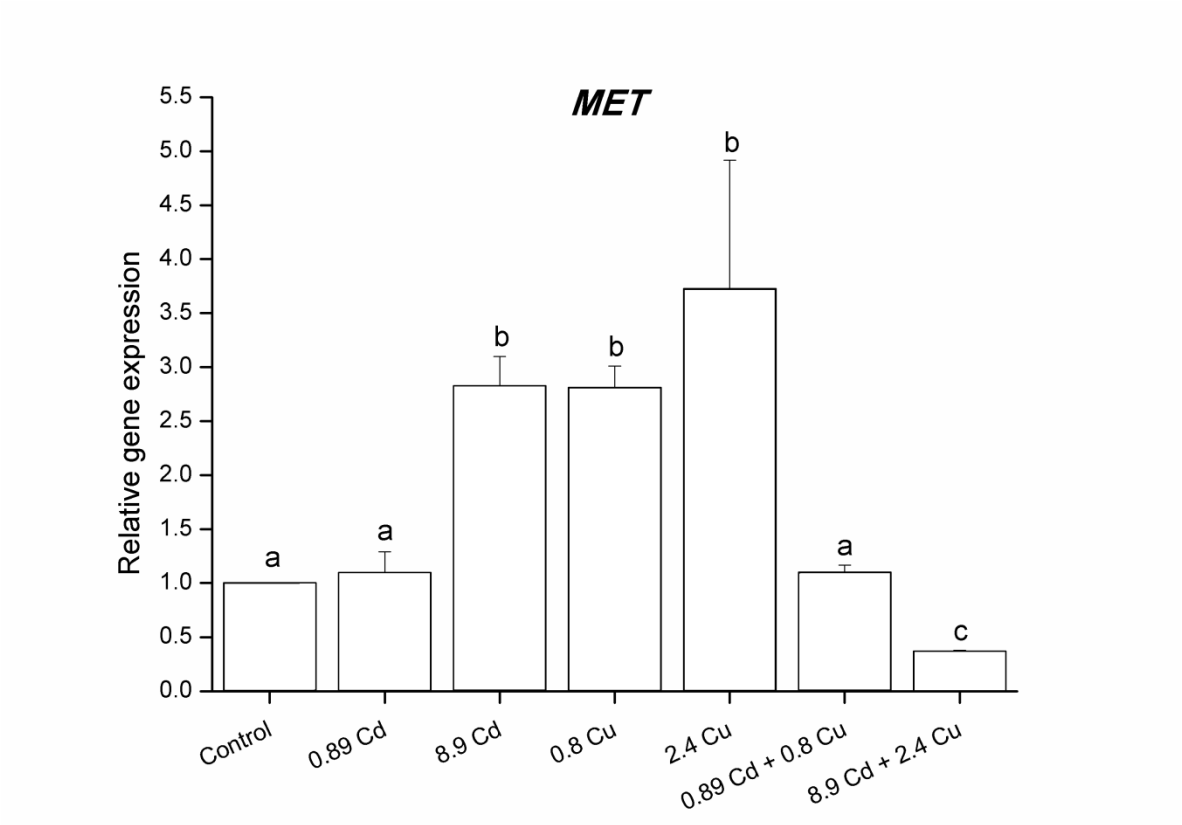

Figure 4

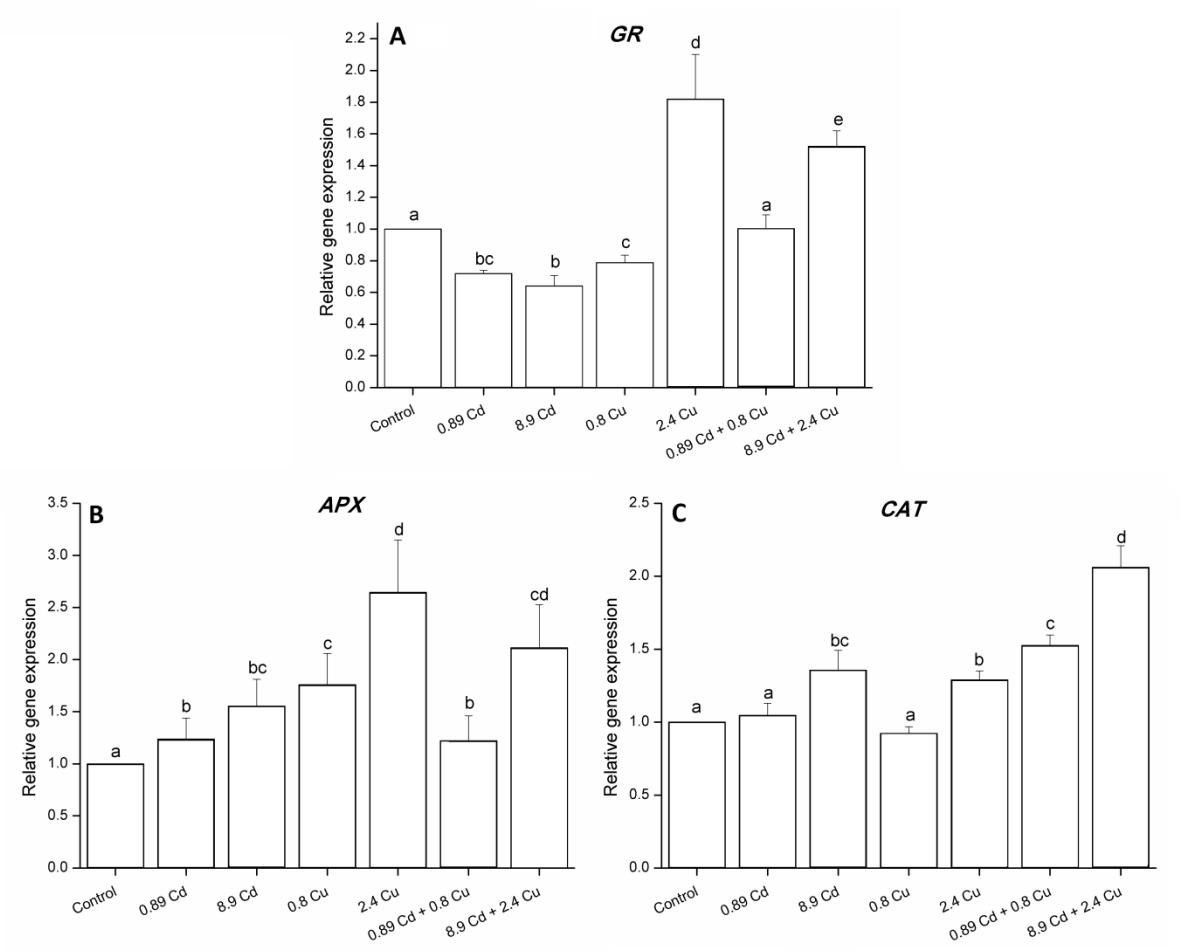

Figure 5

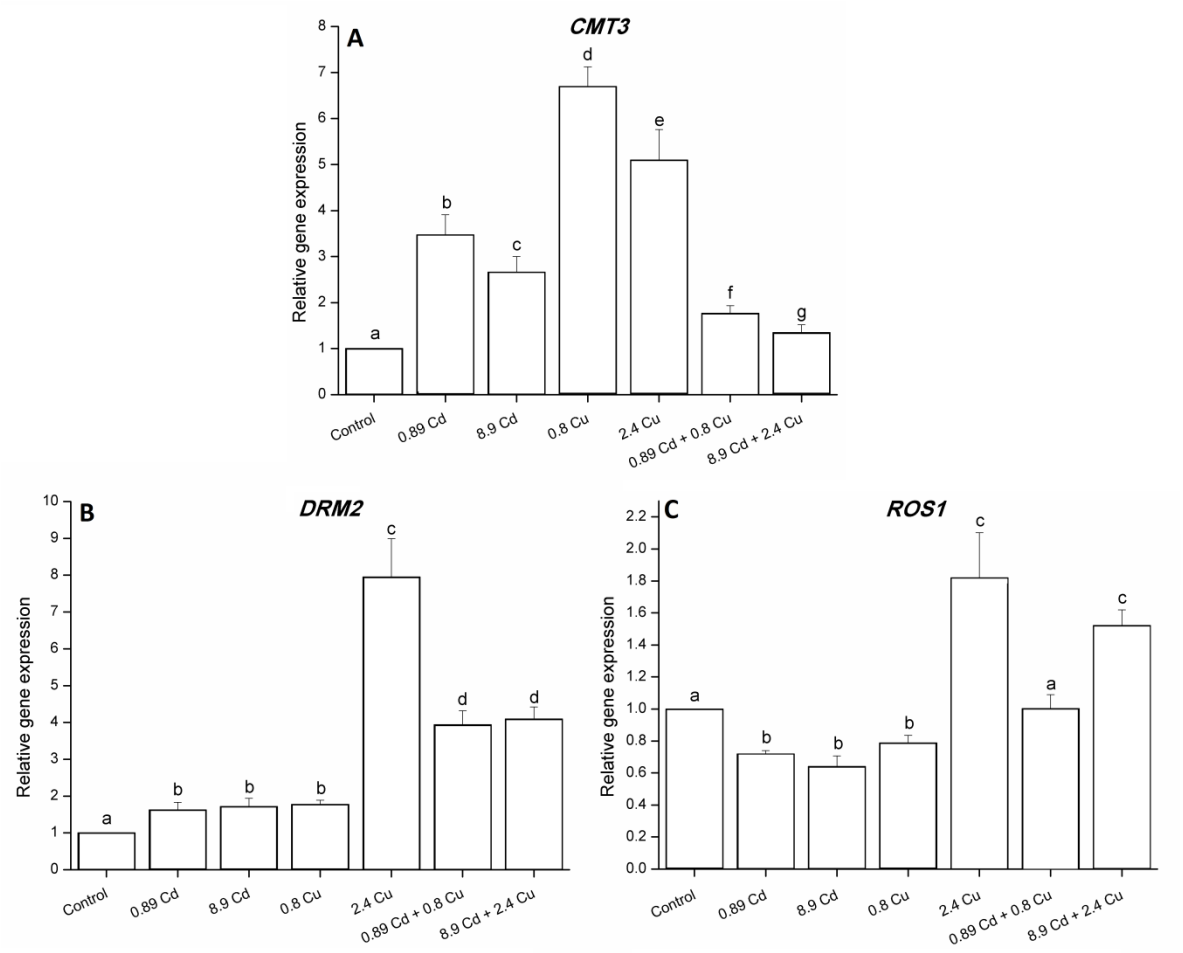

Figure 6

Cu accumulation is always higher than Cd, under single and combined treatments

GSH and PCs levels increased upon the accumulation of Cd and/or Cu

Zostera marina displayed up to PC5 under Cd and/or Cu exposure

Expression of *GR, APX* and *CAT* was the greatest under Cu, alone or combined with Cd

Expression of CMT, DRM2 and ROS1 showed epigenetic-mediated tolerance mechanisms

Table 1: list of primers used for qRT-PCR analyses on *Zostera marina*.

Primer sequences (5'-3') **Primer Pair**	**Putative Genes ID**	**Accession Number**
CCAGCAATGGCAGTTTCGT CAGATGGAACCGATGAGATTGA	*ELONGATION FACTOR (ELO_F)*	AM268885
GATTTGCCTGGTCTTCGTGT ACAGTTTCGTCCCACCAGAG	*CHROMOMETHYLASE3* (*CMT3*)	Q94F88
CCGATTAAGTCCAACCCAAA GAACGAATACGCCAACTGGT	*DOMAIN REARRANGED METHYLASE2* (*DRM2*)	Q9M548
TACCCAGCCCAGTCTAACGCA GCCCCACCTGACAAAGTAAAGG	*REPRESSOR OF SILENCING 1* (*ROS1*)	Q9SJQ6
GTGGAGGAAAGTGTGGGTGT TCACAGGGGAAACTCCAGTC	*METALLOTHIONEIN-LIKE PROTEIN 2A (MT)*	P25860
TTCAACCTGTTGGACGTCTG CGTTGAGTGTCGGCATAAGA	*CATALASE (CAT)*	O24339
ACAATCTTGCCACGACCTTC ATTGGGAGGTTCTCATGGC	*GLUTATHIONE REDUCTASE (GR)*	P80461
ATCGGTCTGGTTTTGAAGGA TATCAACAAGAGGGCGGAAC	*L-ASCORBATE PEROXIDASE 1 (APX1)*	Q05431