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Copper and Cadmium administration induce toxicity and oxidative stress in the marine flatworm *Macrostomum lignano*

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Abstract

The contamination of coastal regions with different toxicants, including heavy metal ions such as copper and cadmium jeopardizes health and survival of organisms exposed to this habitat. In order to study the effects of high copper and cadmium concentrations in these marine environments, we used the flatworm *Macrostomum lignano* as a model. This platyhelminth lives in shallow coastal waters and is exposed to high concentrations of all toxicants that accumulate in these shallow sea floors. We show that both, cadmium and copper induce toxicity at high concentrations, with copper being more toxic than cadmium. At concentrations below those inducing acute toxicity, long-term survival rates were reduced for both metal ions. The effects of sublethal doses comprise reduced physical activities and an increase in ROS levels within the worms.

Key words: Reactive oxygen species (ROS), cadmium, copper, oxidative stress, flatworm

Introduction

Metal contamination of waters results in serious environmental problems around the world and are mostly due to human activities, including the use of fertilizers, industrial mining and smelting, as well as the release of urban waste [1]. These metal contaminations are persistent and they can also undergo bio-magnifications and accumulation in food chains [2].

In general, metals (metal ions) can be divided into essential and nonessential ones. The nonessential metals (e.g. aluminum (Al), cadmium (Cd), mercury (Hg), tin (Sn) and lead (Pb)) have no proven biological function and their toxicity rise with increasing concentration [3]. These types of metals (or metal ions) are also xenobiotics. On the other hand, essential metals (e.g. copper (Cu), zinc (Zn), chromium (Cr), nickel (Ni), cobalt (Co), molybdenum (Mo) and iron (Fe)) are vital for many biological processes, and at high concentrations their negative effects include the induction of metabolic deficiencies or toxicity [3]. These toxic effects can develop at slightly higher accumulations compared to optimal levels [4]. The most commonly found metals in waters are Cd, Hg, Cu, Zn, Ni, Co, Mo, Cr and Sn [5]. Under normal conditions, both essential and nonessential metals are present in water in trace amounts, and the typical (uncontaminated) values of Cd and Cu range 0.02-0.41 nM and 2.8-8.5 nM, respectively [6]. Environmental levels often overpass these levels. For example, Polak-Juszczak [7] recorded much higher concentrations of Cd (2500 μM) and Cu (150 μM) in coast sediments of the Baltic Sea. The Cu values were even more concentrated in the sediments of the Portuguese Atlantic coast (300 μM). From the data reported by the International Atomic Energy Agency, the maximum levels of Cd and Cu in 433 marine sediments were 6 μM and 375 μM , respectively, these values being much higher than the those observed in pristine regions [8]. Extremely high concentrations of cadmium and copper are observed in floating fuel patches on the sea surface [6].

The intertidal region is an area that is confronted with heavy coastal development, industry, and marine traffic, all of which contribute to chronic exposures to environmental chemicals. In order to survive in these habitats, organisms depend on regulatory mechanisms to cope with these toxic components. Considerable attention has been directed towards metal ions because of their properties, which enable them to alter the structure and function of important biological targets [9, 10]. Overload with metal ions will affect post-translational modifications of proteins and also their production and/or scavenging. These metabolic processes inevitably produce more redox-active species (RAS). RAS could catalyze the reduction of molecular oxygen to the superoxide anion ($\text{O}_2^{\cdot-}$) or hydrogen

peroxide (H_2O_2) to the hydroxyl radical ($\cdot\text{OH}$). The $\text{O}_2^{\cdot-}$ and H_2O_2 are not only dominating reactive oxygen species (ROS), but also facilitate other forms of ROS generation [11]. Fan et al. [12] showed that mitochondria can be damaged by triggering a burst of ROS. Nevertheless, the reactive nature of ROS renders them potentially lethal [13]. Several experimental studies showed that ROS-induced oxidative damage plays an important role in metal toxicity. Excess metal may lead to peroxidative damage to membrane lipids via the reaction of lipid radicals and oxygen to form peroxy radicals [14], and can cause peroxidation of lysosomal membranes [15, 16]. Metal overload also reduces the activity of cytochrome c oxidase and impairs liver mitochondrial respiration [17]. Moreover, metal overloaded rats exhibit oxidative injury including decreased levels of hepatic GSH and α -tocopherol, increased levels of mitochondrial lipid peroxidation products, decreases in state 3 respiration and the respiratory control ratio in hepatic mitochondria, and decreased complex IV (cytochrome C oxidase) activity [16, 18-20].

Macrostomum lignano, is a marine free-living flatworm that lives in intertidal and estuarine areas. Because of its small size, its transparent body, and the easiness of culturing, *M. lignano* has become a suitable model in many fields of biological research [21]. Rivera-Ingraham et al. [22] used live-imaging techniques to study the mechanisms of salinity and hypoxic stress by using these worms in *in-vivo* assays. As a transparent meiofauna organism, it should be ideally suited for toxicological studies, especially those aiming to understand the associated underlying mechanisms. A great number of studies have described the adverse effects of metals on various metabolic processes and the resulting developmental and fertility impairments [23, 24]. However, *in-vivo* studies of the toxicological significance of metals exert in aquatic organisms have not been a major focus to now [25-27]. Moreover, those studies using whole animals with aiming to understand the toxicological effects at the molecular and cellular level are especially lacking at present. Here, we used the flatworm *Macrostomum lignano* to study the effects of two different metals, Cu and Cd on major life history traits, with a focus on the effects on the generation of ROS.

Material and Methods

Animal culturing and experimental treatments

Macrostomum lignano were grown in artificial seawater (ASW) (Seasalts, Sigma S-9883) and fed with the diatom (*Nitzschia curvilineata*) for 3 weeks. Both animals and diatoms were routinely kept at 20°C

with 12/12 h dark/light cycles. To exclude developmental effects, all animals used in this study were adults with less than 1 month of age.

Our experiments were performed with pretested concentrations of CdSO₄ and CuSO₄, where no mortality was observed during 24 hours after exposure. Three concentrations of CdSO₄ (730 μM, 460 μM, 330 μM) and CuSO₄ (15 μM, 12 μM and 10 μM) were finally chosen for most experiments. All animals were exposed to the toxic conditions for 48 hours, except for two higher concentrations (730 μM CdSO₄ and 15 μM CuSO₄), where exposure time was shortened to 24 hours.

Lifespan and measurement

Experiments employed 20 adult animals per treatment with 5 replicates, meaning that in total 100 worms were used. Worms were kept in 24-well plates without any food supply. In order to avoid any other effects, such as water evaporation, degradation product from dead animals etc., animals were transferred to new metal confronted medium every 3 days.

Hatching rate measurement

Animals were transferred to new plates without food supply, where animals laid eggs. Twenty freshly laid eggs (< 1.5h) were selected in each group. They were kept in the selected concentrations of heavy metals until the end of the experiment (7 days).

Total RNA extracting

Worms were transferred from the original culture medium to sterile artificial seawater (ASW) for 48h, reducing any other interference from the gut content on gene expression analyses. *M. lignano* was treated with different experimental concentrations of CuSO₄ and CdSO₄ for 24-48h. Each group consisted of 150 animals and their total RNA were extracted using NucleoSpin®RNA (Macherey-Nagel, Düren, Germany) following the manufacturer's advices. 1μl of each sample was run in a 1% agarose gel for quality control, while the RNA concentration was measured spectro-photometrically.

Genomic DNA (gDNA) digestion

This step was employed only if gDNA contamination was detected in samples during the process of RNA quality control. In particular, no more than 1 μg of RNA was mixed with 1 μl 10× reaction buffer containing MgCl₂, 1 U of DNase I (RNase-free) and 0.25 μl of RNase inhibitor. HPLC water was added up to a total volume of 10 μl. The mixture was incubated 30 min at 37°C, and then it was incubated at 65°C for another 10 min with 1 μl of 50 mM EDTA to prevent the RNA hydrolyses.

First-Strand cDNA synthesis

The following components were used: 250 to 500 ng of total RNA, 0.5 μ l Oligo-dT7 (10 pmol/ μ l), 0.5 μ l dNTPs mix (10 mM), add HPLC water up to 6.75 μ l. The mixture was incubated at 65°C for 5 min, then placed on ice for at least 1 min. The following components were added to the mixture in the indicated order: 2 μ l 5 \times first strand buffer, 0.25 μ l RNase Inhibitor (10U), 0.5 μ l DTT (0.1M) and 0.5 μ l Superscript III Reverse Transcriptase (100U). Samples were gently mixed and incubated at room temperature for 5 min, and then at 50°C for 1 h. Finally, the reaction was terminated at 70°C for 15 min to inactivate the transcriptase, before it was chilled on ice for immediate usage or stored at -20°C.

Relative quantification of qRT-PCR

For relative quantitative PCR, the reaction was performed using the StepOnePlus™ Real-Time PCR System for the primer efficiency check and relative expression level of gene of interest determination. For primer efficiency tests, the template cDNA was treated as a series of 2-fold dilution from a starting quantity of 50 ng/ μ l to the final concentrations of 3.125 ng/ μ l, while for the gene expression level determination template cDNA was diluted to 20 ng/ μ l. Then 5 μ l 2 \times qPCRBIOSyGreen Mix Hi-ROX, 0.5 μ l sense primer (5 μ M), 0.5 μ l anti-sense primer (5 μ M), 1 μ l template cDNA and 3 μ l HPLC H₂O were mixed for one reaction. The amplification was run as below: 95 °C, 10 min, (95 °C, 15 sec; 60 °C, 20 sec; 17 °C, 35 sec) 40 cycles, (95 °C, 5 min; 60 °C + 0.3 °C until 95 °C), 95°C, 15 sec.

The primer efficiencies were calculated according to published protocols [28]. *Rpl12* (ribosomal protein L12) served as a reference gene for all of assays [22]. Five biological replicates were performed and all primers used in the present study are listed in Table 1.

Animal activity

The animal activity procedure was performed as in Rivera-Ingraham et al [22]. Briefly, 5 animals that had previously been exposure to different conditions were stained with Mitotracker Deep Red 633 (Ex: 633nm; Em: 660 nm) at a concentration of 0.33 μ M for at least 1 h before the analysis and were kept in this medium until the end of the experiment. Animals were observed individually in a \approx 200 μ m-deep slide and 3 μ l medium, all covered by a coverslip. This ensured that animals could only move in the X-Y axis while staying under focus and permanently in the field of vision of the microscope objective. Animals were scanned for 2 min with at least 1800 frames at a frequency of 67 msec using an Olympus SZX microscope coupled with a CellSens camera. Pictures were stacked using ImageJ (NIH, Rasband WS) and then imported into Imaris, with which behavioral parameters were analyzed using

the “Imaris Track” module (Bitplane)

Fluorescence intensity

For monitoring the heavy metal response, treated animals were incubated for 1-0.5 hour with different types of fluorescence probes (Table 2) and then imaged using a Zeiss Axio Imager Z1 microscope equipped with an apotome (Zeiss Oberkochen, Germany). The pictures were analyzed using ImageJ software. Images were converted to 8-bits, and to standardize all measurements only the head region of each worm was considered for analysis, following Rivera-Ingraham et al. [29]. In the present study, all experiments were carried out using 10 animals per treatment with 3 replicates each, resulting in 27-30 animals in total. In order to avoid inevitable interferences with and among dyes, exposure times were kept as brief as possible and all dyes were used separately. The median and maximum value of the signal was analyzed using ImageJ software.

Phos-H3 labeling

Worms were collected from the culturing medium and were washed twice with f/2 and relaxed in 1:1 7.14% MgCl₂: artificial sea water for 5 minutes, followed by 15 minutes in pure 7.14% MgCl₂. Animals were fixed for 1 hour at RT in 4% PFA in 0.1 M PBS, followed by multiple washing with PBS-T (0.1%). Protease treatment (0.22 µg/ml Protease XIV (30 min, 37°C) was followed by 10minute incubation in 0.1 N HCl on ice and further denaturation in 2 N HCl (1 h, 37°C). A 3 × 5 minutes wash in PBS-T was carried out to completely remove the acid and samples were treated with BSA-T for 30 minutes. Samples were incubated at 4°C overnight with a primary antibody (rabbit anti- phosphorylated H3, Roche), diluted 1:150 in PBS-BSA-T. After washing with PBS-T (3 × 5 minutes), specimens were incubated at RT in darkness for 1 h with a secondary antibody (goat-anti-rabbit IGG - Cy3-conjugated (Jackson ImmunoResearch)), diluted 1:150 and washed again with PBS-T for 3 × 5 minutes. Specimens were mounted in Vectashield (Vector Laboratories) and animals observed with AxioVision SE64 fluorescent microscope.

Death cell number counts

In order to assess levels of cell death, worms were incubated with ethidium homodimer III. Every treatment group contained 7 animals with 3 replicates, in total 21 animals. To quantify the number of positive cells at different treatments, pictures were taken using an AxioVision 4.9.1. Resulting pictures

were combined in Z-stacks , and the ethidium- positive cells were counted in each layer.

Statistical analysis

In all experiments, Prism version 6.0 was used for statistical analysis. For comparison between two groups, the unpaired two-tailed Student's t-test was used, while if multiple comparisons was required, one-way ANOVA followed by a Tukey *post-hoc* test were performed. Two-way ANOVA was employed when two different categorical independent variables on one continuous dependent variable had to be analyzed. All histograms were represented as means \pm S.D. The survival curves of lifespan were analyzed with the log-rank (Mantel-Cox) test. Statistical analysis of significant difference were defined as n.s. (not significant), *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Results

1 CdSO₄ and CuSO₄ treatments affect worm endurance

Metals accumulate in aquatic animals and become toxic when concentrations reach certain thresholds. The toxicity may vary considerably between different metal cations. To identify the concentrations of Cd²⁺ and Cu²⁺ that are not directly toxic, 8 different concentrations were tested. Survival curves revealed that animals can survive more than 100 minutes only when CdSO₄ concentrations are lower than 1000 μ M (Fig. 1A). However, worms were even more susceptible to CuSO₄, where CuSO₄ concentrations above 25 μ M were lethal (Fig. 1B).

Furthermore, hatching rates of newly laid eggs also showed a disparate tolerance towards Cu and Cd. The hatching rate of *M. lignano* dramatically decreased when CdSO₄ concentrations were above 500 μ M (Fig. 1A2). CuSO₄, on the other hand showed a massive reduction in hatching rates at \sim 14 μ M (Fig. 1B2).

2 Lifespan

Macrostomum lignano can survive for about 50 days without food supply. When animals suffer from a toxic environment, lifespan is greatly reduced. As expected, lifespan showed a negative correlation with the presence of heavy metals. Worms confronted with 730 μ M CdSO₄ were able to survive only 4 days, which is significantly less than in the control group (Fig. 2A, p < 0.0001). Animals exposed to lower 460 μ M and 330 μ M CdSO₄ concentrations, showed slightly longer lifespans up to 8 days

(CdSO₄_460 μM vs. CdSO₄_730 μM, $p < 0.0001$; CdSO₄_330 μM vs. CdSO₄_730 μM, $p < 0.0001$). Worms treated with CuSO₄ also showed a same trend, lifespan dramatically decreased at slightly higher concentrations of 14 μM and 12 μM, respectively (Fig. 2B). In addition, most animals died very quickly at 10 μM CuSO₄, while a small fraction of worms can live almost as long as control animals (Fig. 2B). Are they starving to death? If exposed animals get food do they live longer? Detoxification needs a lot energy, which may be the reason for dying soon. All animals used in the lifespan experiments were starved animals, without any food supply. It is possible that detoxification needs a lot energy, which may be the reason for dying soon. Toxicity induced the mitochondrial reactive oxygen species (ROS) production, which in turn regulated the energy expenditure (Chouchani et al., 2016).

2 Physical activity

Treatment with metal ions, especially exposure to higher concentrations, had a significant impact on animal behavior (Fig. 3). Animals exposed to all tested cadmium and two higher concentrations of copper sulfate showed significantly lower movement activities than untreated controls. The average speed of control animals was about 80 μm/s, while it was greatly reduced to below ~ 25 μm/s under cadmium treatments (Fig. 3, $P < 0.05$). This obvious decrease was also found in response to copper treatment. Only the lowest copper concentration had no statistically significant effect (Fig. 3, control vs. CuSO₄_15μM, $P = 0.0128$; control vs. CuSO₄_10μM, $P = 0.0222$). Energy saving? It might be a gateway to lower the movement and energy expenditure on the detoxification. In addition to the detoxification, metals might direct interaction with essential macromolecules and minerals. Cu and Cd, as a transition metal ion, are capable to undergo redox-cycling reactions and accelerates the formation of reactive oxygen species (ROS) that modify the structure and/or function of crucial biomolecules (Halliwell and Gutteridge, 1984; Lippard, 1999). ROS itself might increases the energy expenditure.

4 Reactive oxygen species formation

The O₂⁻ formation increases with increasing metal ion concentrations, as indicated by the 2-OH-E⁺:DHE ratio results ($P < 0.001$) (Fig. 4A1 and A2). Furthermore, in accordance with the results observed when plotting the 2-OH-E⁺:DHE ratios, mitochondrial O₂⁻ formation (MitoSOX staining) also revealed a significant increase with heavy metal treatment ($P < 0.001$) (Fig. 4B1 and B2). These results are further supported by staining with C-H₂DFFDA, which detects ROS formation aside from O₂[•] (-) such

as H_2O_2), $\text{HOO}\cdot$ and ONOO^- (Fig. 4C1 and C2). This increased ROS levels after metal-treatment are consistent with hyperoxic exposure [29].

5 Mitochondrial density and energetic states

A significant increase was observed in the mitochondrial (mt) density of animals that were exposed to higher concentrations of heavy metal ions over a period of 24-48 hours (Fig. 5A1 and A2, $P < 0.001$). The mt density of copper treated animals increased by ~ 1.4 -fold regarding the maximum values and by ~ 1.1 -fold when mean values were considered (Fig. 5A1, $P < 0.001$). In cadmium treated animals these differences are higher, reaching up to ~ 1.8 -fold (Fig. 5A1, $P < 0.001$). The fluorescence intensity results indicate that higher concentrations of metal ions led to increased mitochondrial signals.

6 Cell mitosis

In order to test the effect of heavy metal ions on cell proliferation, we used the anti-phosphorylated histone 3 antibody to evaluate the number of mitotically active cells. Values were significantly reduced in the 730 μM and 460 μM CdSO_4 treatment groups, while an apparent increase was observed at 330 μM CdSO_4 (Fig. 6). No significant increases or decreases of mitotically active cell numbers were found for either of the Cu^{2+} -induced treatment groups.

7 Cell death quantification

Hartwig et al. [30] proposed that Cd acts as a mitogen, stimulating cell proliferation, inhibiting apoptosis and DNA repair. While other studies reported in Cd-treated mammalian cells, Cd also generates damage and affects differentiation and causes apoptosis and cell death [31]. It does this by inducing damage to DNA, cell membranes, and proteins, by inhibiting different types of DNA repair [32]. Compared with unexposed animals, in our study, the number of dead cells significantly increased in the 330 μM Cd^{2+} group, whereas no changes were observed for the other concentrations. Copper ion cytotoxicity was observed at higher concentrations. The dead cell numbers highly increased in 15 μM and 12 μM Cu-induced groups (Fig. 7).

8 Gene expression quantification of antioxidant enzymes and Multidrug resistance transporter genes

Transcription of major antioxidant enzymes was significantly down-regulated in response to heavy metal confrontation. These tested enzymes include microsomal glutathione-s-transferase (*mgst1*), *peroxiredoxin 1* (*prdx1*), *peroxiredoxin* (*prdx6*), mitochondrial *superoxide dismutase* (*sod2*) and glutathione-s-transferase pi-1 (*gstp1*). The later was the only anti-redox gene observed to significantly increase in expression (Fig. 8A). Gene expression was also down regulated for *caspase 3* (*cas3*), coding for an enzyme directly involved in programmed cell death. The mitochondrial cytochrome c oxidase subunit *cox5b* showed decreased expression in all tested metal groups. Mito fluorescence increased- mor Mitos. Why lower cox?. Cox, the last enzyme of the mitochondrial respiratory chain, is the major oxygen consumer enzyme in the cell. Regulation of cox assembly involves several reactive oxygen species and redox-regulated steps. Mito increases indicates higher ROS. Cox expression might later than the ROS expression and effect redox-regulated cox biogenesis steps.

The multi-drug-resistant transporter genes (*mdr*, adenosine triphosphate-binding cassette transporter) play a crucial role in protecting cells from xenobiotics, chemicals, and drugs [33, 34]. We therefore quantified transcript levels of three typical ABC transporter cassette family members, namely *mdr1*, *mdr5* and *mdr7*. As *mdr 5* showed no or reduced transcript levels in response to metal confrontation, *mdr1* and *mdr7* contrarily presented either no change or increased transcript levels (Fig. 8B).

Discussion

1 General toxicity of copper and cadmium

Copper is mostly present in biological systems as cupric (Cu^{2+}). It is an important mineral ion necessary for numerous biological processes [35, 36]. For example Cu^{2+} is required for cytochrome c oxidase activity, which catalyzes the reduction of oxygen to water, the vital step in cellular respiration, and is also a component of copper, zinc-superoxide dismutases (Cu, Zn-SOD), which scavenge free radical superoxide [37]. Non-specific Cu^{2+} binding to thiol enzymes may modify the catalytic activities of cytochrome P450 monooxygenases, and Cu^{2+} could both oxidize and bind to some amino acid residues of P450 monooxygenases [38]. Additionally, copper chaperones help to minimize the presence of unbound (free) copper from participating in redox reactions [39, 40] and guarantee delivery of copper

ions to specific target proteins [41-43]. The amount of copper absorption is relatively low and animals are able to control the amounts of copper in the body by either reducing absorption or inducing excretion.

However, Cu toxicity may result from exposure to excess copper caused by e.g. environmental contaminations. Gray et al. [44] investigated hepatoma cells (HepG2 line) and found that concentrations of 16 μM Cu^{2+} led to 125-fold increase in the cellular copper content. In addition to the direct interaction with essential macromolecules and minerals, several mechanisms, notably free radical-induced oxidative damage, have been proposed to explain Cu-induced cellular toxicity. Cu, as a transition metal ion, is able to undergo redox-cycling reactions and accelerates the formation of reactive oxygen species (ROS) that modify the structure and/or function of crucial biomolecules [45, 46]. As only single electrons are needed to reduce Cu^{2+} , Cu^{+} mediates the production of hydroxyl radicals [47]. The hydroxyl radical is the most powerful oxidizing radical likely to arise in biological systems. It is capable of initiating oxidative damage through abstracting the hydrogen from an amino-bearing carbon to form a carbon-centered protein radical and from an unsaturated fatty acid to form lipid radicals [14, 39, 48, 49] and thus should be a by-product of excessive Cu^{2+} intoxications. Moreover, it increases the risks of DNA strand breaks and oxidation of bases [50-53].

Unlike Cu, Cd is nonessential, with no proven evidence for an association with biological processes. High concentrations of Cd impact cell proliferation, differentiation, and apoptosis [54]. These activities interact with the generation of ROS and DNA repair mechanism. Cadmium potentially gives rise to chromosomal deletions and mutations, which in turn leads to chromosomal aberrations [55]. Filipic [56] reported that Cd^{2+} does not only enhance the production of $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ but also inhibits the activity of antioxidant enzymes, such as catalase, MnSOD and Cu,Zn-SOD. Moreover, Cd disturbs the cellular calcium homeostasis [57] and the activities of caspases and nitrogen-activated protein kinases (MRPKs), these indirectly triggering apoptosis in cells [58].

Thus, both Cu and Cd can induce ROS production and result in severe oxidative stress. This dependency between high metal ion concentrations and reduced lifespan and increased ROS production was also seen in the present study. Thus, a combined activity of direct and indirect effects caused by Cu^{2+} and Cd^{2+} intoxications can lead to the observed reduced lifespan and fitness of the animals. These combined effects have a series of physiological outcomes comprising behavioral and cellular reactions. In the present study, metal ion treated animals show a reduced physical activity that

comes along with a shorter life span. The lower movement might be a result of energy being allocated to metal and ROS detoxification. Starving animals reduce lifespan during high energy on demand. ROS generation affects the function of the electron chain on the mitochondrial membrane, which inevitably influences ATP generation. Cadenas [59] reported that proton leaks will generate heat instead of ATP and cause therewith substantial energy dissipation that accounts for up to 25% of the basal metabolic rate??. Mitochondrial oxidative phosphorylation is incompletely coupled since protons translocated to the intermembrane space by specific respiratory complexes of the electron transport chain can return to the mitochondrial matrix independently of the ATP synthase- a process known as proton leak generating heat instead of ATP. Our DHE staining results imply an increased production of O_2^- after metal treatment. This correlation might explain the attenuated activity due to the energy loss. Meanwhile, increased intracellular accumulation of Cd^{2+} may also directly affect neuronal function. Kwakye et al. [60] demonstrated that Cd^{2+} exposure could cause neurotoxicity and neurodegeneration [57]. This may explain abnormal behaviors that can be observed in long time metal-induced worms, which lost light sensitivity and kept rotating constantly (results not shown). The results obtained for the programmed cell death showed differences between Cu^{2+} - and Cd^{2+} exposure, while we have no obvious explanation for this observation. We speculate that Cd^{2+} may be inducing neurotoxicity by disturbance of the cellular Ca^{2+} homeostasis, leading to abnormal behavior (rotating) and loss of sensory function (light sensitivity). Qi et al. [61] suggested that excessive ROS lead to mitochondrial membrane potential dissipation and promote the release of apoptotic factors from mitochondria.

Under normal physiological conditions, ROS formation is considered to account for 1-5% of the total oxygen consumed by mitochondria [62-64]. Another measurement of ROS production in mitochondria with disabled antioxidant systems revealed different ROS release values (0.25-11%), depending on the animal species and respiration rate [65]. Cellular ROS production is stimulated in response to metabolic imbalances imposed by abiotic and biotic stresses and proceeds through similar mechanism [66]. From the ROS staining experiments, we found increases in ROS production in both Cu^{2+} and Cd^{2+} -induced groups. Cd^{2+} can induce ROS through stimulating peroxisome biogenesis and also via altered ROS metabolism of both mitochondria and peroxisomes [67, 68]. Cu^{2+} is a core component or cofactor of a variety of antioxidant enzymes in animals [69, 70]. Under normal conditions, it can timely cleanup free radicals and reduces the peroxidative damage to the body [71]. In contrast, high

quantities of copper agents will act as exogenous pro-oxidants that generate ROS, which might result in upregulation of inflammatory markers that further contribute to pathophysiology [72].

The deleterious effects of heavy metals resulting from the formation of ROS in the mitochondrion are, to large extents, prevented by various antioxidant systems. Superoxide anion is enzymatically converted to H₂O₂ by a family of metalloenzymes called SODs [73]. There is compelling evidence that SODs are essential components of the biological defense against oxygen toxicity and maintaining the redox balance of immune system [74, 75]. Among all tested redox enzymes *gstp1* is the only significantly upregulated gene, which suggests that the corresponding enzyme might be relevant for the physiological reaction towards ROS and metal detoxification. Compared with other redox enzymes, *gstpi* is also the only antioxidant genes showing an increased expression in response to other stressors such as hypo/hyper salinity. Rivera-Ingraham et al. [22] speculated that this may relate to bioenergetics costs and it might be less costly to produce GSTs compared with other enzymes such as CAT or SOD. Interestingly, another *glutathione-s-transferase* (*mgst1*) is significantly decreased in all groups, which indicates a complex regulation of the different *glutathione-s-transferases* in *M. lignano* and that different functions can be attributed to these different genes.

The membrane transporters, which play important roles in pumping out toxicants, are also differentially regulated under stress conditions. In humans, the multidrug resistance protein is an ATP-binding cassette (ABC) transporter that confers resistance to many drugs and regulates redox homeostasis, inflammation and hormone secretion [76-78]. It uses ATP to transport drugs through a channel formed by the trans-membrane segments [79, 80]. Tietje et al. [81] confirmed the presence of functional multidrug resistance transporters in *M. lignano* that have a similar pharmacological profile as those known in mammals. Although most groups showed no transcriptional differences, we still detected both up and down regulated expression of *mdr* genes in some metal-induced groups. Up-regulated expression was found for *mdr1* and *mdr7* whereas *mdr5* showed a downregulation. The MDRP1 transports neutral as well as basic drugs that are both, hydrophobic and amphipathic compounds [82]. Multidrug resistance protein 7 (MDRP7, ABCC10) is able to transport amphipathic anions [83].

The presence of metal ions causes intra- and inter-mitochondrial redox-environment changes leading to ROS formation. Besides O₂⁻ present in mitochondria, other forms of ROS, such as hydrogen peroxide (H₂O₂) and peroxides (HOO⁻) also increased, suggesting that metals may also increase ROS

metabolism through changes in peroxisome function. Metals present in the water are crucial for aquatic organisms to maintain normal cellular physiology. Their accumulation leads to pro-oxidant conditions in cells and adverse effect such as organelle malfunctioning, energy loss, tissue damage and eventually cell death or apoptosis, which might be responsible for the reduced movement and increased mortality observed. The steady-state concentration of these oxidants is usually kept at non-toxic levels by a variety of antioxidant defenses and repair enzymes. The *gstp1* might be one of the important superoxide dismutases in precisely mediating the balance between antioxidant defenses and ROS production in *M. lignano*. Meanwhile, different types of multidrug resistance (*mdr*) genes are present in *M. lignano* and might be associated with metal ion transports. *M. lignano*, with bunches of advantage *in vivo* studies, might be an ideal model to unravel toxic mechanisms in marine waters making it a good bio-indicator for pollution detection.

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Figure legends:

Fig. 1 CdSO₄ and CuSO₄ treatments affect endurance of worms (A) and impact the hatching rate (B). Adult worms were treated with different concentrations of CdSO₄ and CuSO₄. The tested concentration of CdSO₄ ranged from 7340 μM to 333.6 μM in Fig. A1 and 734 μM to 83.5 μM in Fig. A2. Concentrations of CuSO₄ ranged from 173.3 μM to 10.4 μM in Fig. B1 and 14 μM to 2.5 μM in Fig. B2. Animal survival at higher metal concentrations were measured every 5 min and at lower concentrations they were measured every 20 min. Hatching rate was measured as total offspring of 20 worms after 7 days (B). When CdSO₄ concentrations were 734 μM (or above), eggs can hardly hatching. Eggs were much more sensitive to CuSO₄, which 14 μM was lethal to the eggs. Population size was determined under the different treatments. Animals were starved during the whole procedure.

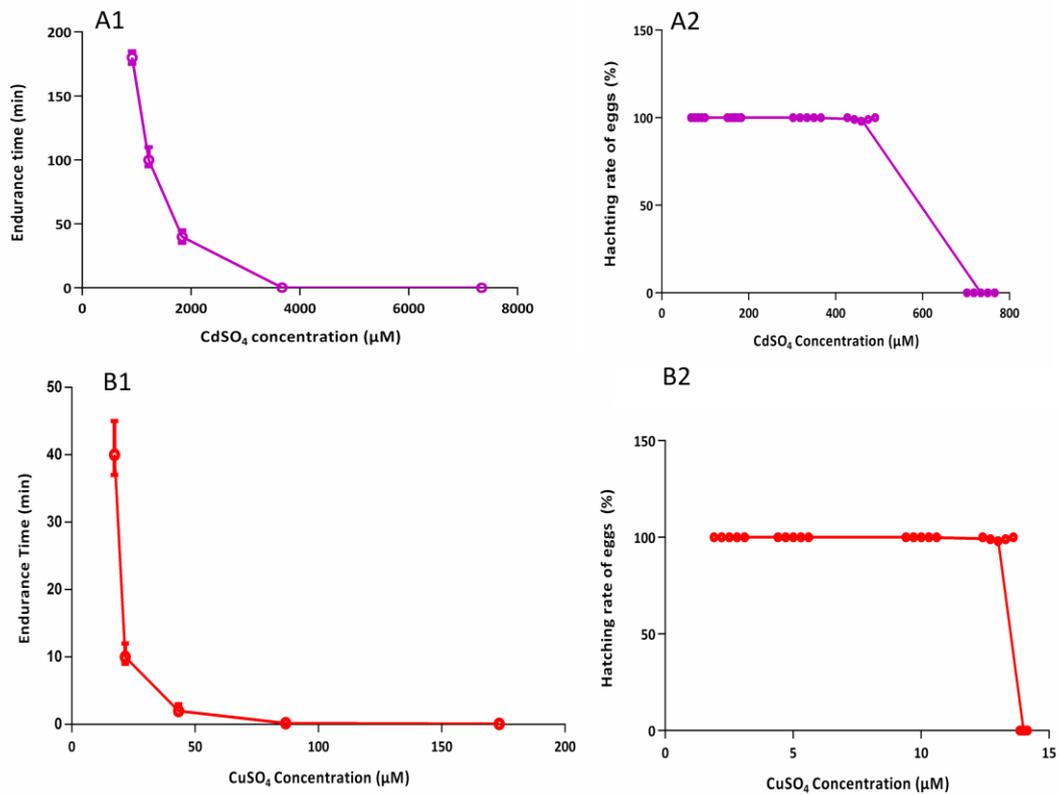


Fig. 2 Survival curves of Cu^{2+} and Cd^{2+} -treated animals. Animals were treated with three concentrations of CdSO_4 (730 μM , 460 μM , 330 μM , A) and CuSO_4 (15 μM , 12 μM , 10 μM , B), respectively. Experiments employed 20 adult animals per treatment with 5 replicates and livability was measured every 24h hours. The median survival rate of control animals was 41 days. Median survival rates of CdSO_4 treated animals from higher to lower concentrations are reduced to, 4 days and 6 days, respectively. Asterisks denote significant differences from control (Log-rank (Mantel-Cox) Test, ****, $p < 0.00001$). Colors highlight the different concentrations of metal stress.

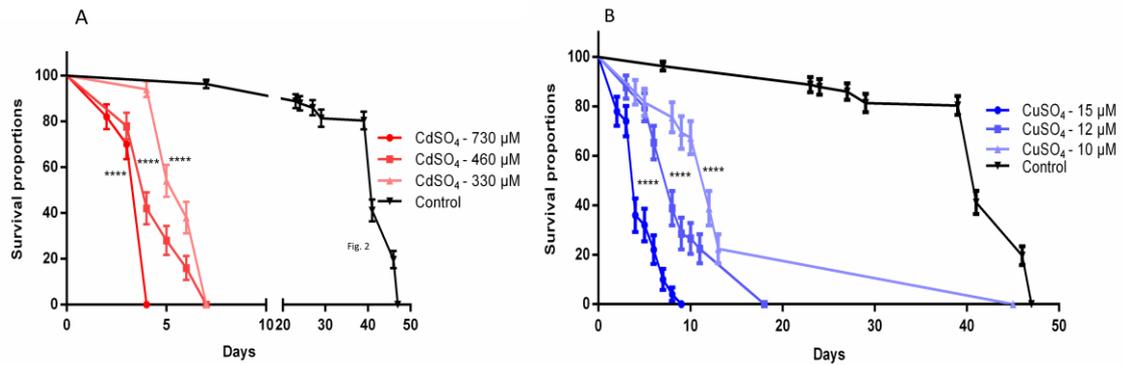


Fig. 3 Physical activities of Cu^{2+} and Cd^{2+} -treated animals. Animals were treated with three concentrations of CdSO_4 (730 μM _24h, 460 μM _48h, 330 μM _48h) and CuSO_4 (15 μM _24h, 12 μM _48h, 10 μM _48h), respectively. Five animals that had previously been exposed to different conditions were stained with Mitotracker Deep Red 633 at a concentration of 0.33 μM for at least 1 h before the analysis and were kept in this medium until the end of the experiment. Animals were observed individually and scanned for 2 min with at least 1800 frames at a frequency of 67 msec using an Olympus SZX microscope coupled with a CellSens camera. The 50 sec stacked pictures were shown above. Asterisks denote significant differences from non-drug control (Unpaired t-test, *, $p < 0.005$). Colors highlight the different concentrations of metal stress.

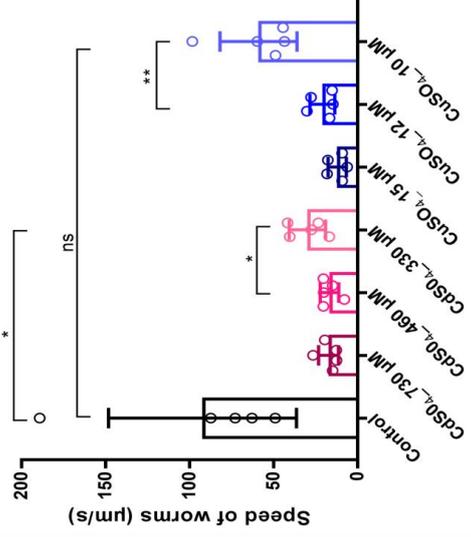
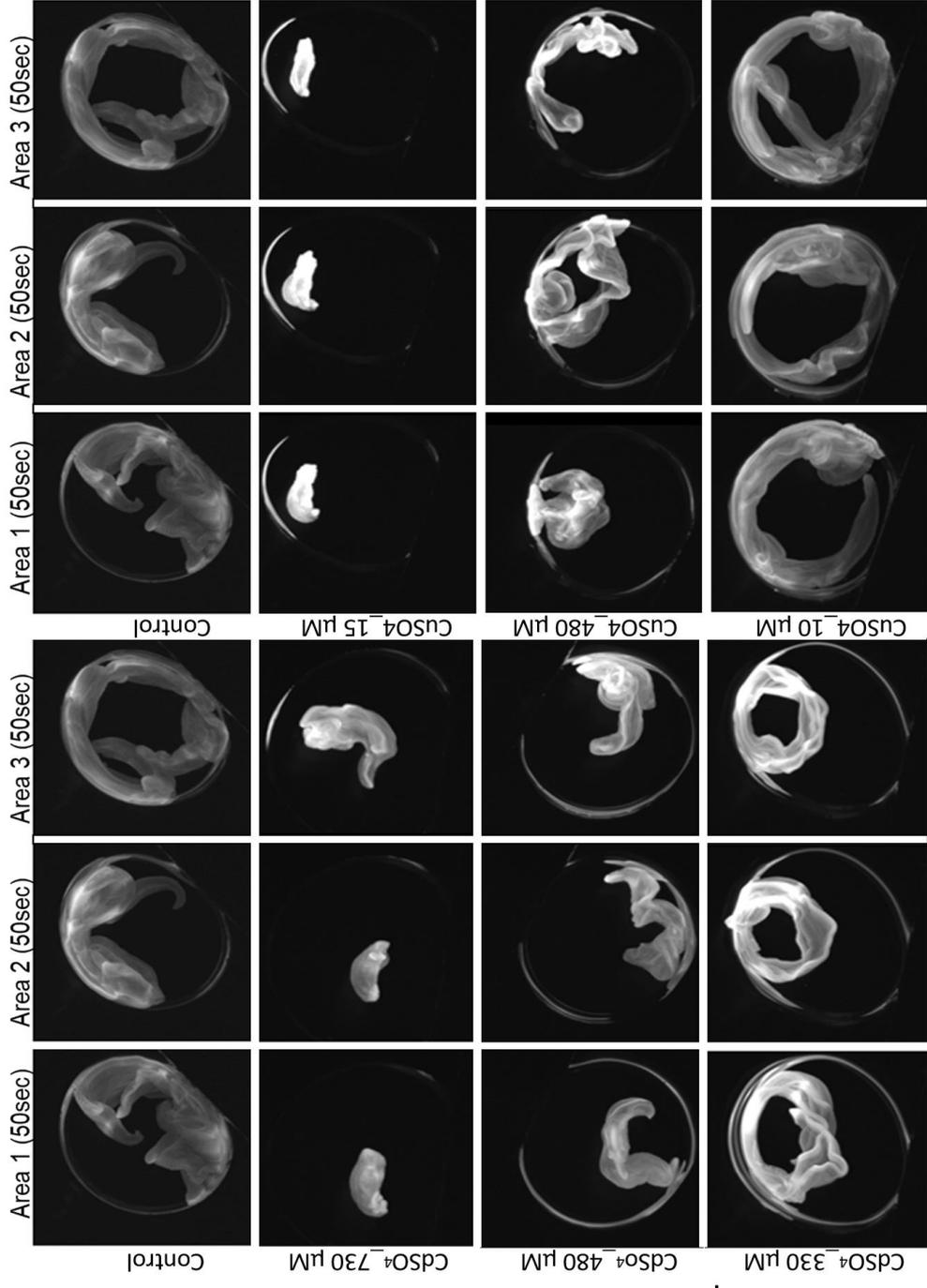
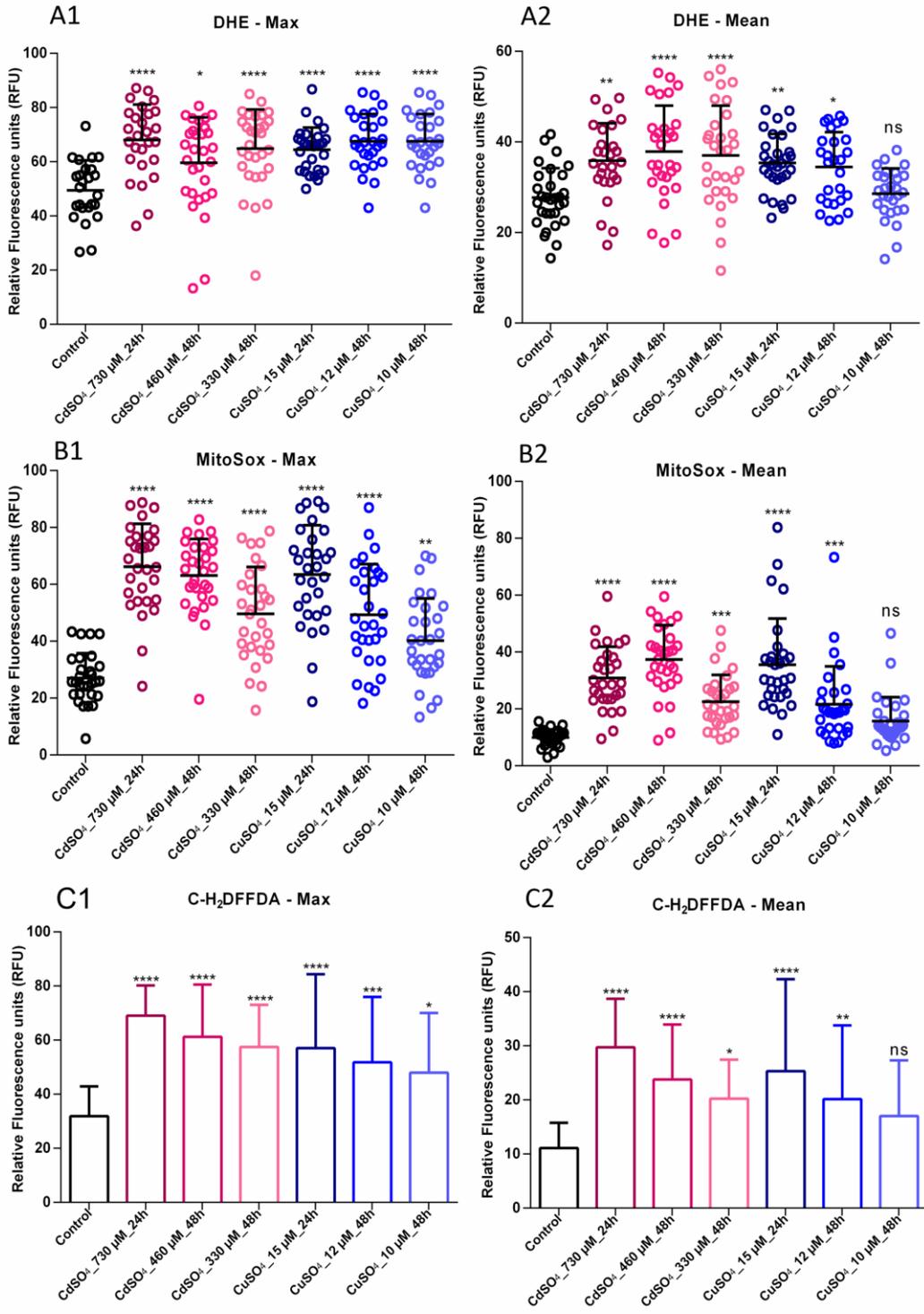


Fig. 4 Fluorescence reporters of reactive oxygen species in Cu²⁺ and Cd²⁺-treated animals. For monitoring the heavy metal response, treated animals were incubated for 30 min with 4 types of fluorescence probes and then imaged. In order to avoid any signal from the gut, quantification of the fluorescence intensity was done only in the head regions (before eyes), according to Rivera-Ingraham et al [29]. A1, A2, B1 and B2 show superoxide (O₂⁻) formation, as indicated by the 2-OH-E+: DHE ratio results, CellROS, MitoSOX staining. C1 and C2 shows other ROS types asides from O₂⁻ such as hydrogen peroxide (H₂O₂), HOO⁻ and ONOO⁻, vizualised by C-H₂DFDA. Asterisks denote significant differences from non-drug control (one-way ANOVA with Turkey Post-hoc test, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).



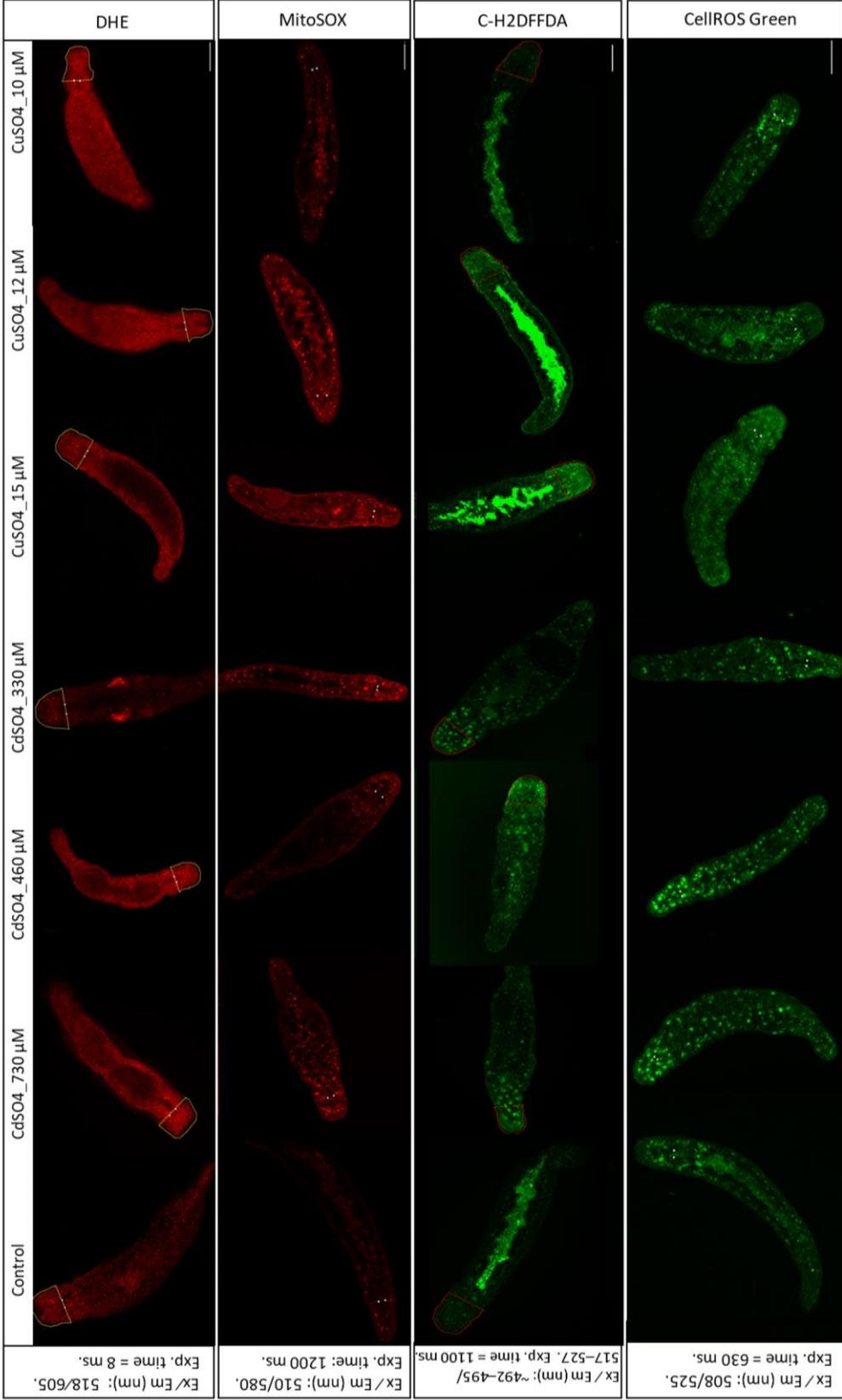


Fig. 5 Mitochondrial intensities of Cu^{2+} and Cd^{2+} -treated animals. Mitochondrial intensities were indicated by mitotracker deep red 633 (mitotracker), treated animals were soaked in $0.33 \mu\text{M}$ mitotracker for 1 hour. A1 and A2 shows mitochondrial (mt) densities of animals that were exposed to heavy metal ions over a period of 24-48 hours ($P < 0.001$). Asterisks denote significant differences from non-drug control (one-way ANOVA with Turkey Post-hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$).

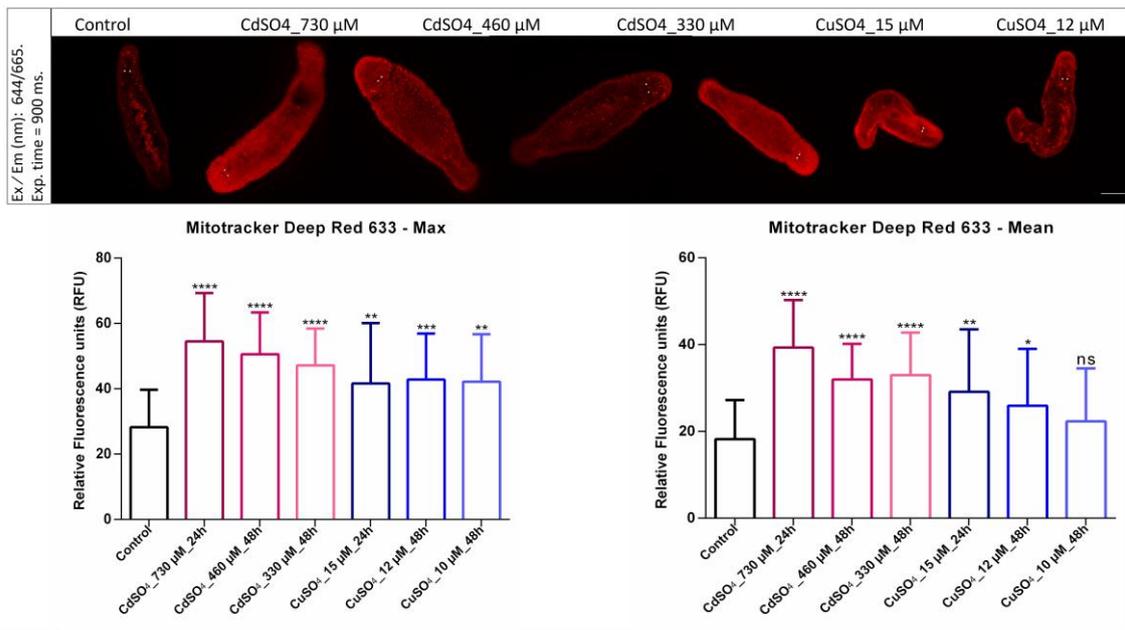


Fig. 6 Mitosis cell numbers of Cu^{2+} and Cd^{2+} -induced animals. The treated worms were immunostained for histone H3 phosphorylation to visualize mitotic cells. The white arrows from the above pictures are showing mitotic cells. Pictures were taken by combining 7 Z-stacks and each group employ 6 animals and three biological replicates were performed, 8-17 animals in total. Asterisks denote significant differences from non-drug control (Unpaired t-test, ** $P < 0.01$, **** $P < 0.0001$). The scale bar indicates 100 μm .

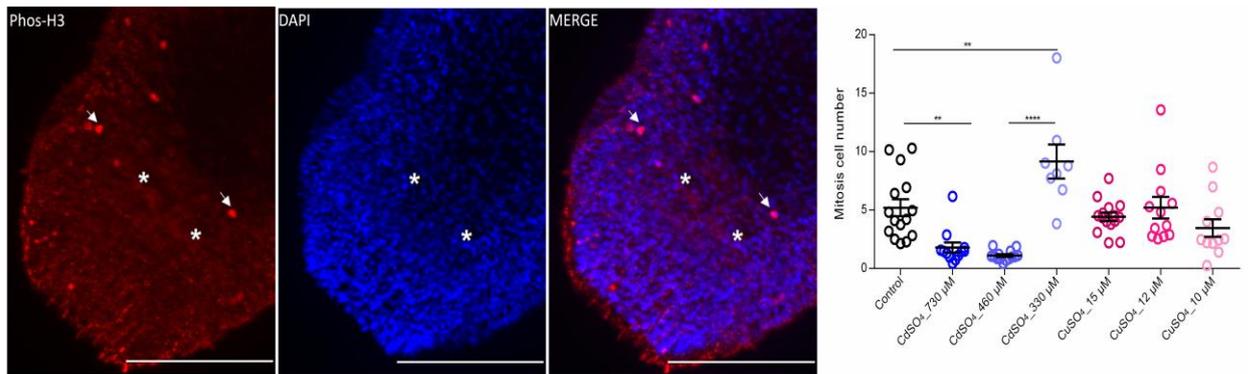


Fig. 7 Death cell numbers of Cu^{2+} and Cd^{2+} -treated animals. Cu^{2+} and Cd^{2+} treated animals were incubated for 30 min at 5 μM ethidium homodimer III and afterwards the dead cells were stained with red fluorescence. Pictures were taken by combining 7 Z-stacks. Asterisks denote significant differences from non-drug control (Unpaired t-test, * $P < 0.05$, ** $P < 0.01$). The scale bar indicates 100 μm .

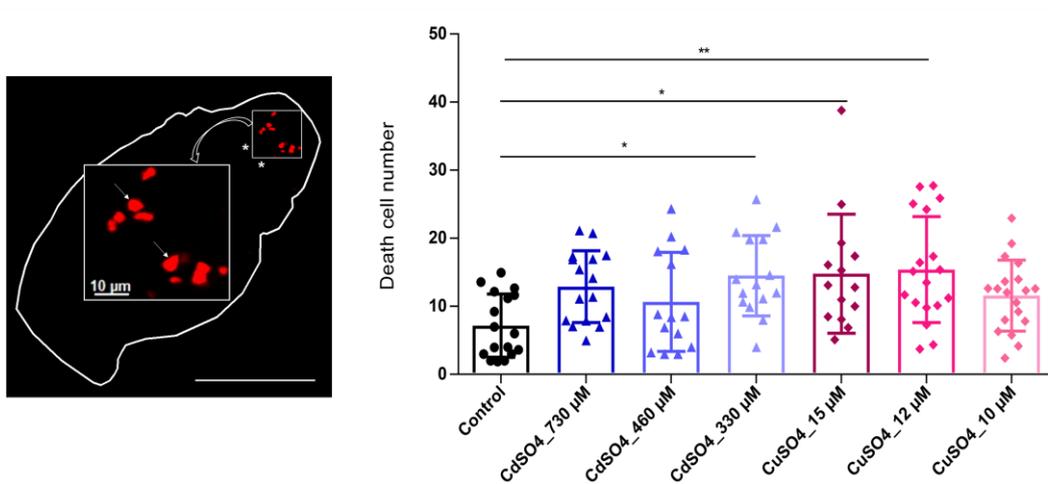


Fig. 8 Quantitative gene expression results for *M. lignano* under different metal stress. A Expression of antioxidant enzymes; B multi-drug-resistance transporter genes. Annotated groups have significant statistical differences with non-drug treatment control (value=1, dash line), and the groups shared with similar differences values are labeled with a line and shown together. A total of five biological replicates were performed.

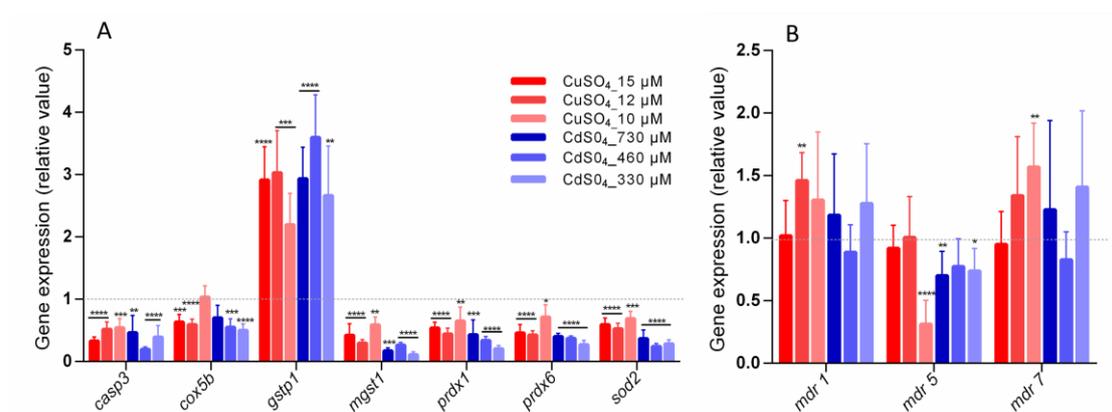


Table1 The qPCR primer information

Gene		SequenceID	length	Amplicon	length	Annealing	Reference
acronym	Gene name	database	Eff%	Primer sequence5' to 3' Forward/Reverse	(bp)	temp.(°C)	Reference
cox5b	Cytochrome c oxidase subunit 5B	RNA1310_41945	98.16	CGCGTCTAGACCGGTTC/CTGACGGTACCAGCACGGG	112	60	A.Rivera-Ingraham et al., 2016
cas3p3	Caspase 3	RNA1310_1479.1	78.78	GCCAGAAGACTCCAGCC/CTGCGTCTCTCCCTGGCAG	208	62	A.Rivera-Ingraham et al., 2016
gstp1	Glutathione S-transferase pi 1	RNA1310_29579.1	90.97	TTCGGTGCCAGGAAGAATCT/CCCATTAAAGATGCGGCGA	89	60	A.Rivera-Ingraham et al., 2016
mgst1	Microsomal glutathione S-transferase1	RNA1310_49001.2	87.09	ATGTGCTGCCGTTTGTACTG/CTACTGCGCGCAAGATTC	98	60	A.Rivera-Ingraham et al., 2016
prdx6	Peroxisdoxidin 6	RNA1310_35269	106.3	ACCAGGCCTTGTATCTCA/TGTCAATCTGAAGCCGGAGT	229	60	A.Rivera-Ingraham et al., 2016
prdx1	Peroxisdoxidin 1	RNA1310_28865.2	91.64	CGCGGCCTGTTCAATCAAA/GCTTGTGCGGTGAAGTGAAG	124	60	A.Rivera-Ingraham et al., 2016
sod2	Superoxide dismutase 2, mitochondrial	RNA1310_30405	105.6	AAGAACGTTGCCAGACAGTA/AGCTTAAGTCCGCCACTCTT	101	60	A.Rivera-Ingraham et al., 2016
rp12	Ribosomal protein L12	RNA1310_36703	91.21	GACAAGGTTAACGACGGCTC/TATAGCAGCCGGTGTGCAA	81	60	A.Rivera-Ingraham et al., 2016
mg2a	GM2 ganglioside activator	RNA1310_42438	64.26	CATCACCTGCCGAGATTC/TCTCAATGCGACCCCTCAAGC	132	62	A.Rivera-Ingraham et al., 2016
mdr5	multiple drug resistance-associated protein5	LFJF01002971.1	71.9	GGTAGATGTCGCTGTCTGGT/ATGCGACTGAGATCGGAGAG	99	60	present study
mdr1	multiple drug resistance-associated protein1	LFJF01003519.1	73.6	CCCTGCTGAGGAAGTCAAG/AACTCCCTGCGAATGGTCTG	100	60	present study
mdr7	multidrug resistance-associated protein 7	LFJF01001259.1	95.9	AGCCATCTCGTAGAACACGG/TCGGCTGAATACGGTGATGG	121	60	present study

Table 2 Fluorescence dyes used in our study.

Dye	Final conc.	Incubation	Ind/batch	N repetition (total N /treatment)	Excitation/Emission λ (nm)	Calculation
	Used (μ M)	time(min)				
Amplex [®] Red Hydrogen Peroxide/ Peroxidase Assay kit	4	30	10	3 (27-30)	570/585	Maximum and mean intensity
Cell ROS green	5	30	10	3 (27-30)	508/525	None
C-H2DFFDA (in Ethanol)	11	30	10	3 (27-30)	492/495	Maximum and mean intensity
Dihydroethidium (DHE) in DMSO	4	30	10	3 (27-30)	518/605	Maximum and mean intensity
Ethidium Homodimer iii in DMSO	5	30	10	3 (12-19)	530/620	Dead cell number
MitoTracker Deep Red 633 in DMSO	0.33	60	10	3 (27-30)	644/665	Maximum and mean intensity
MitoSOX in DMSO	5	30	10	3 (27-30)	510/580	Maximum and mean intensity