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Gaël Le Croizier, Camille Lacroix, Sébastien Artigaud, Stéphane Le Floch, Jean-Marie Munaron, et al.. Metal subcellular partitioning determines excretion pathways and sensitivity to cadmium toxicity in two marine fish species. *Chemosphere*, 2019, 217, pp.754-762. 10.1016/j.chemosphere.2018.10.212 . hal-02494936

HAL Id: hal-02494936

<https://hal.science/hal-02494936>

Submitted on 16 Jun 2020

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Metal subcellular partitioning determines excretion pathways and sensitivity to cadmium toxicity in two marine fish species.

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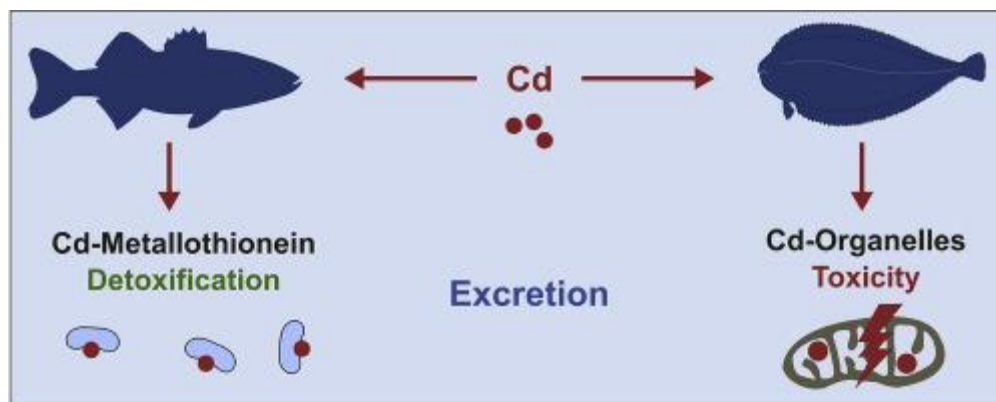
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Abstract :

Subcellular cadmium (Cd) partitioning was investigated in the liver of two marine fish species, the European sea bass *Dicentrarchus labrax* and the Senegalese sole *Solea senegalensis*, dietary exposed to an environmentally realistic Cd dose for two months followed by a two-month depuration. Cd exposure did not modify Cd cellular partitioning for either species, refuting the spillover hypothesis. Both species contained most of the Cd in the detoxifying fraction but displayed different handling strategies. Cd was largely bound to heat stable proteins (HSP) including metallothioneins (MT) in sea bass while Cd was more linked to metal rich granules (MRG) in sole. Whole liver concentrations and subcellular partitioning were also determined for essential elements. The greatest impairment of essential metal homeostasis due to Cd exposure was found in sole. These elements followed the Cd partitioning pattern, suggesting that they are involved in antioxidant responses against Cd toxicity. Cd consumption diminished sole growth in terms of body weight, probably due to lipid storage impairment. During the depuration period, the two species showed contrasting partitioning patterns, implying different pathways for Cd elimination from the liver. In sea bass, MT-bound Cd would be excreted through bile or released into blood, crossing the cell membrane via a protein transporter. In sole, MRG-bound Cd would be sequestered by organelles before being released into the blood via vesicular exocytosis. These distinct strategies in cellular Cd handling in the liver might account for differential sensitivity to Cd toxicity and differential Cd excretion pathways between the two marine fish species.

Graphical abstract



Highlights

► Sea bass and sole displayed different Cd subcellular partitioning in the liver. ► Cd was largely bound to metallothionein-like proteins in the sea bass liver. ► Cd was mainly linked to metal rich granules in the sole liver. ► Essential metals followed the Cd partitioning pattern in hepatic cells. ► Handling strategies might account for sensitivity and Cd excretion.

Keywords : Sub-cellular fractionation ; *Dicentrarchus labrax* ; *Solea senegalensis* ; Essential element distribution ; Elimination ; Depuration

28 INTRODUCTION

29 Aside from natural sources, marine ecosystems can be subjected to metal contamination
30 from urban effluents and industrial activities. Cadmium (Cd) is a common by-product of the
31 mining industry and can reach high levels in some regions (World Health Organization,
32 2010). Coastal regions in West Africa, which belong to the Canary Current Large Marine
33 Ecosystem (CCLME), are thereby particularly subjected to Cd residue due to the direct
34 release of phosphogypsum into water by the phosphate industry (Auger et al., 2015;
35 Cheggour et al., 1999; Gaudry et al., 2007). Marine consumers such as fish mainly
36 accumulate metals through trophic pathway, which can lead to significant Cd levels in the
37 organs of fish from the CCLME. Moreover, fish species from this region display a wide range
38 of Cd concentrations (Afandi et al., 2018; Diop et al., 2016), resulting partially from
39 differences in foraging habitats and dietary habits (Borrell et al., 2016; Goutte et al., 2015; Le
40 Croizier et al., 2016; Metian et al., 2013). In addition to ecological traits, the observed
41 interspecific variability in terms of Cd bioaccumulation results from different physiological
42 characteristics, including the presence of metal binding proteins like metallothioneins (MT)
43 (Le Croizier et al., 2018; Moulis et al., 2014; Zalups and Ahmad, 2003). Cd is a toxic element
44 responsible for numerous impairments in fish, such as oxidative damage, disruption of
45 essential metal homeostasis, endocrine and ionoregulatory disruption, histopathology and

depression of the immune system, which can finally affect growth and survival (McGeer et al., 2011).

Recently, attention has been focused on fish species ability to cope with metal toxicity, depending on subcellular partitioning of the element (Eyckmans et al., 2012; Leonard et al., 2014). At the cellular level, Cd can take different toxic chemical forms including: free or complexed ion forms (*e.g.* Cd^{2+} , CdCl_2), bound to enzymes (*e.g.* cytochromes), bound to organic acids (*e.g.* citrates), or bound to cellular constituents causing damages (*e.g.* DNA) (Vijver et al., 2004). Today, only a few forms of Cd speciation are considered as detoxified: Cd complexed to peptides (*e.g.* glutathione) or functional, transport or sequestration proteins (*e.g.* metallothioneins), and Cd trapped in vesicles of the lysosomal system or precipitated in mineral granules (Wang and Rainbow, 2006). Very little is however known about the intracellular mechanisms leading to Cd elimination (Moulis et al., 2014; Zalups and Ahmad, 2003).

While one of the adverse effects of toxic metals is their interference with essential elements (Martelli et al., 2006; Moulis, 2010a), some of them confer a protective role against metal toxicity, directly through formation of detoxifying complexes (Sasakura and T. Suzuki, 1998) or by indirectly preventing oxidative stress due to their association with antioxidant enzymes (Martínez-Álvarez et al., 2005; Talas et al., 2008). The significance of essential element subcellular partitioning in their protective role against toxic metals has never yet been considered. Moreover, despite evidence of the influence of cellular components like MT in metal accumulation kinetics in marine species (Wang and Rainbow, 2010), most previous studies only emphasize on the influence of metal intracellular handling on sensitivity of organisms to toxic elements (Campbell et al., 2008; Eyckmans et al., 2012; Giguère et al., 2006; Leonard et al., 2014; Wang and Rainbow, 2006). Very few studies have therefore

investigated the link between metal subcellular partitioning and kinetics in fish (Glynn, 1991) and information about the relationships between Cd intracellular handling and excretion pathways is lacking for marine fish.

In order to fill these gaps, the present study aimed to investigate Cd intracellular distribution in the liver of two different marine fish species which are naturally present in the CCLME, a region particularly prone to Cd contamination. For this purpose, European sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis*, were exposed for two months to an environmentally realistic dietary cadmium (Cd) dose followed by a depuration period of a further two months. Analyses were conducted at the end of the uptake period and at the end of the depuration period and Cd partitioning was examined in six major subcellular fractions: cellular debris, metal-rich granules, mitochondria, organelles, cytosolic enzymes, cytosolic proteins and peptides, as well as in the storage lipid fraction of the liver when present. Sensitivity of the two species was assessed according to: (1) Cd subcellular partitioning during accumulation and depuration periods, (2) impairments to essential metal subcellular distribution, (3) essential metal concentrations in the whole liver and their partitioning within hepatocytes, (4) growth and hepatosomatic index calculation. Finally, hypotheses were made regarding the influence of Cd cellular speciation in the liver and Cd biliary excretion or transport to other tissues.

MATERIALS AND METHODS

All animal procedures were in accordance with the French and EU guidelines for animal research (project approval number: 03266.03).

Fish and experimental procedures

All details about animal procedures and experimental design can be found in Le Croizier et al., 2018. Briefly, immature sea bass *Dicentrarchus labrax* (length: 14.1 ± 0.7 cm; weight 29.8 ± 4.5 g) used in this experiment were obtained from a commercial hatchery (Aquastream, Ploemeur, France), whereas immature Senegalese sole *Solea senegalensis* (length: 14.9 ± 1.1 cm; weight 36.4 ± 7.9 g) were provided from a marine farm (Ferme marine de l'Adour, Anglet, France). The fish were transported to the Cedre (Centre of Documentation, Research and Experimentation on Accidental Water Pollution, Brest, France). After receiving anaesthesia by bathing in a $0.05 \text{ mL}\cdot\text{L}^{-1}$ solution of tricaine methanesulfonate (MS-222) (Ackerman et al., 2005), each fish was randomly assigned to one of twelve high density polyethylene tanks that had a 300 L volume (six tanks for each species, 40 sole and 50 sea bass per tank to ensure equivalent biomass) at the Cedre's marine animal facility. This facility is an independent greenhouse that is submitted to a natural photoperiod and supplied with a continuous seawater flow from the bay of Brest. The temperatures in the experimental tanks followed the outdoor temperature. Fish were first acclimated to the experimental conditions for one month, during which they were fed daily with dried commercial pellets (Turbot label rouge 1.4 mm, Le Gouessant Aquaculture).

To obtain an environmental relevant Cd concentration in the fish food, a Cd-enriched diet was prepared in order to reach a Cd level around $25 \mu\text{g}\cdot\text{g}^{-1}$, which corresponds to the Cd level reported in potential prey within the natural distribution of the two fish species (Bodin et al., 2013; Maanan, 2008). For this purpose, the commercial pellets were spiked with a $100 \text{ mg}\cdot\text{L}^{-1}$ Cd (as CdCl_2 in milliQ water) solution for 30 min. The diet was then placed at -20°C , freeze dried and broken into small pellets before usage. This preparation led to a Cd concentration of $22.9 \pm 0.3 \mu\text{g}\cdot\text{g}^{-1}$ dw in the Cd-enriched food. The control diet was prepared in the same way, but with the addition of milliQ water only, where the measured

background Cd level was $0.71 \pm 0.00 \mu\text{g}\cdot\text{g}^{-1}$ dw. For each of the four conditions (*i.e.*, Cd-exposed and control fish, for both species) there were three replicate tanks. Dietary Cd exposure was initiated by randomly assigning three of the six tanks per species to the Cd-enriched food. The six remaining tanks were fed the control diet. Cd exposure was conducted for 60 days, after which a depuration period was conducted that lasted for 60 days. During the depuration period, all of the tanks were fed the control diet. Daily food distribution was performed slowly and continuously over 24 h with a clockwork feeder (COFA, Paris, France) to prevent pellets from remaining in the tank and thus avoid Cd desorption in water. Sea bass were fed at 1.7 % body weight while sole were fed at 1 % body weight per day to meet the physiological requirements of each species (Danion et al., 2011; Salas-Leiton et al., 2010). The bottom of each tank was siphoned every day to avoid Cd leaching from faeces.

Physico-chemical parameters (O_2 , pH, temperature, salinity) and water quality (nitrates, nitrites) were measured every ten days. During the experiment, the water temperature decreased from 20.7 to 12.8 °C, oxygen saturation increased from 81.2% to 98.2%, pH increased from 8.0 to 8.2 and salinity increased from 35.2 to 35.7. The water was free of nitrate and nitrite (Colorimetric test JBL) in both experiments.

Biological sampling

After the two months of Cd exposure, three fish per tank (nine for each treatment) were anesthetized by bathing in a $0.05 \text{ mL}\cdot\text{L}^{-1}$ solution of MS-222 before being euthanized by bathing in a $0.2 \text{ mL}\cdot\text{L}^{-1}$ solution of MS-222 (Ackerman et al., 2005). They were weighed, measured and dissected with ceramic tools to avoid metal contamination. The liver was collected, weighed and put in acid-washed (10% HNO_3) individual plastic microcentrifuge

tubes, flash frozen in liquid nitrogen and stored at -80°C until analysis. Another sampling was made in the same way after a further two months of depuration.

Subcellular partitioning procedure

This procedure generally followed the protocol of Rosabal et al. (2015), adapted from that of Wallace et al. (2003), which has been validated and extensively used on various fish species (Campbell et al., 2008; Eyckmans et al., 2012; Giguère et al., 2006; Lapointe et al., 2009a). Briefly, liver samples were fractionated into six operationally-defined subcellular fractions: cell membranes (cellular debris), metal-rich granules (MRG); mitochondria, microsomes + lysosomes (organelles), heat-denatured proteins (HDP) including cytosolic enzymes, and heat-stable proteins and peptides (HSP) such as metallothionein (MT) and glutathione (GSH). Three liver samples of fish from the same tank were pooled to obtain approximately 200 mg of wet sample and homogenized in 1.5 mL of a solution containing Tris-HCl 20 mmol·L⁻¹ pH 8.6, 0.01% DTT (dithiothreitol) as a reducing agent and 1 % of an antiproteolytic agent (Protease Inhibitor Mix, GE Healthcare). A 100 µL aliquot was removed from the liver homogenate for determining total trace metal concentrations in the liver and assessing metal recovery from the subcellular fractions. The remainder of the liver homogenate was centrifuged at 1450×g for 15 min at 4°C. The supernatant (S1) was transferred to an acid-washed 1.5-mL polypropylene microcentrifuge tube for further separations. The pellet from this centrifugation was suspended in 0.5 mL of ultrapure water, heated to 100°C for 2 min, digested with an additional 500 µL of 1 N NaOH at 65°C for 60 min. Centrifugation at 10,000×g for 10 min at ambient temperature (~20°C) was performed to separate the NaOH-resistant fraction (referred to as "MRG") from the cellular debris fraction, which includes cell membranes, unbroken cells and nuclei. The S1 supernatant was centrifuged at 10,000×g for

30 min at 4°C to produce the mitochondrial fraction. The resulting supernatant was ultracentrifuged at 100,000×g for 60 min at 4°C, giving a pellet containing other organelles (microsomes and lysosomes) and the cytosolic fraction in the supernatant. To separate the heat-stable peptides and proteins (HSP) from the heat-denatured proteins (HDP), the cytosolic fraction was held at 80°C for 10 min, left on ice for 1 h and then centrifuged at 50,000×g for 10 min at 10°C. The HSP fraction, which includes MT, was collected from the supernatant. Each fraction was finally kept at -20°C until the metal analyses were performed.

Metal analyses

Subcellular fractions were digested in a mixture of 2 mL 70% HNO₃ and 0.5 mL 30% H₂O₂ (both of ultrapure quality) in closed Teflon vessels. Mineralization was performed on a hotplate for 4 h at 100°C. A 100-μL aliquot was removed from the digestate and diluted to 15 mL with Milli-Q quality water (Merck Millipore). Cadmium and essential metals (Ca, Cu, Fe, Mg, Mn, Se and Zn) were analysed using an ICP Q-MS (X Series 2, Thermo Scientific) at the Pôle de Spectrométrie Océan (PSO, Plouzané, France) with an internal standard solution of Rhodium (2 μg·L⁻¹). Reference materials (fish protein DORM-4 and dogfish liver DOLT-5, NRCC) were treated and analysed in the same way as the samples. The results for reference materials displayed mean metal recoveries of 85 ± 6 % for DORM-4 and 91 ± 6 % for DOLT-5. Mean recoveries (± SD) of DORM-4 reference samples (n = 3) were 81 ± 2 % for Cd, 78 ± 3 % for Ca, 95 ± 2 % for Mg, 89 ± 5 % for Mn, 86 ± 5 % for Fe, 81 ± 2 % for Cu, 79 ± 1 % for Zn, 92 ± 3 % for Se. Mean recoveries (± SD) of DOLT-5 reference samples (n = 3) were 99 ± 2 % for Cd, 83 ± 1 % for Ca, 96 ± 4 % for Mg, 92 ± 4 % for Mn, 98 ± 6 % for Fe, 86 ± 3 % for Cu, 85 ± 2 % for Zn, 86 ± 2 % for Se. Blanks were included in each analytical batch. The limits of detection (ng·g⁻¹ dry wt), corresponding to the mean concentration of the blank solutions,

were 0.2 (Cd), 1.3 (Ca), 0.4 (Cu), 0.1 (Fe), 0.2 (Mg), 0.01 (Mn), 0.1 (Se) and 0.4 (Zn). Total metal concentrations in the liver ($\mu\text{g}\cdot\text{g}^{-1}$) are given on a dry weight basis ($\mu\text{g}\cdot\text{g}^{-1}$ dw), based on a previous study involving the same fish and where the liver samples were freeze-dried before analysis (Le Croizier et al., 2018). The proportion of metal in each fraction is expressed as a percentage of the sum of the concentrations of all fractions. As it was not present in all of the liver samples, the storage lipid fraction was not taken into account in the calculation of metal partitioning. Metal proportion in this fraction was expressed as a percentage of the total metal concentration in the liver. “Sensitive fractions” gathered the Cd contained in the sensitive fractions (*i.e.* mitochondria + HDP + organelles, while the “metal-detoxified fractions” gathered the Cd contained in the detoxifying fractions (*i.e.* MRG + HSP) (Eyckmans et al., 2012; Leonard et al., 2014). The total metal concentration recovery in the liver from the sum of the metal concentrations measured in all fractions, including lipids, was $95 \pm 11\%$ for sea bass and $96 \pm 6\%$ for sole.

Data analysis

All data tested statistically were first checked for normality (Shapiro–Wilks tests) (Shapiro and Wilk, 1965) and homogeneity of variances (Bartlett tests) (Bartlett, 1937). When these conditions were met, raw data were used and one-way ANOVAs, followed by Tukey’s HSD tests, were performed to test for differences between treatments and species. Otherwise, non-parametric analogues were used, *i.e.* Kruskal-Wallis tests (KW), followed by Conover-Iman multiple comparison tests with Bonferroni’s adjustment (Conover and Iman, 1979). All of the statistical analyses were performed using the open source software R (version 3.4.3, R Core Team, 2017).

RESULTS AND DISCUSSION

Impact of Cd elimination on Cd and essential metal partitioning

After being exposed to cadmium, the two-month depuration period led to many changes in metal partitioning (Table 1).

First, Cd proportion significantly increased in the organelle fraction (from $37.3 \pm 5.9\%$ to $51.5 \pm 4.8\%$) in sole liver at the end of the depuration period. The organelle fraction includes cellular components involved in the vesicular transport, which is a major pathway for extracellular excretion: Golgi apparatus (GA), endoplasmic reticulum (ER) and lysosomes. ER manages the sequestration of molecules and their binding to excretory proteins, which are transported through the GA and cytosol by vesicles (including lysosomes) and finally excreted across the plasma membrane. Increasing Cd in the organelle fraction during the depuration period may reflect Cd exocytosis via vesicles, which could lead to total Cd elimination from the liver tissue, as described in sole in a previous study (Le Croizier et al., 2018).

Second, Cd exposure followed by a depuration period also led to a displacement of essential elements (Fe, Mg, Mn and Zn) from metal rich granules (MRG) to organelles in sole liver (*e.g.* Fe proportion significantly increased from 30.1 ± 2.4 to $41.2 \pm 1.4\%$ in organelles while it significantly decreased from $16.9 \pm 3.2\%$ to $9.2 \pm 2.6\%$ in MRG). In marine organisms, MRG are inorganic insoluble concretions containing mainly Ca phosphate (George et al., 1980), as reflected by the high proportion of Ca in the MRG fraction in both species ($69.3 \pm 4.1\%$ and $32.6 \pm 13.7\%$ for sea bass and sole, respectively, during the depuration period). These structures can play an important role in accumulation and detoxification of metals in fish (Lapointe et al., 2009b; Leonard et al., 2014).

The simultaneous increase in proportion of Cd and essential metals in organelles and decrease of essential metals in MRG may be caused by sequestration of MRG by ER, which is part of the organelle fraction (Table 1; Figure 2). This hypothesis seems reasonable since MRG in our species contained the highest proportion of Ca, and ER is deeply involved in Ca storage (Görlach et al., 2006). Supplementing fish diet has furthermore been shown to increase Cd in MRG while decreasing it in organelles (Ng et al., 2009). Although the authors did not draw this conclusion, our hypothesis is that increasing cellular Ca concentration will reduce the probability for Cd bound to calcium granules to be sequestered by ER during Ca uptake. The changes in essential metal cellular repartition would thus be a side effect of Cd transport from MRG to organelles (*i.e.* ER, GA and vesicles, in that order) before final exocytosis (Table 1; Figure 2).

Conversely, no change in Cd partitioning was observed in sea bass liver despite effective Cd elimination as well as Cd biliary excretion observed in this species during the depuration period (Le Croizier et al., 2018). In mammals, Cd bound to glutathione (GSH) was shown to be excreted from hepatocytes to bile while Cd bound to metallothionein (MT) was released into the blood before reaching other organs like kidney (Ballatori, 1991; Chan et al., 1993; Klaassen, 1978; Nordberg, 1978). In fish, MT were reported in bile (Hauser-Davis et al., 2012) and were shown to transport metal during biliary excretion in Cu- and Se-exposed fish (Hauser-Davis et al., 2016, 2014). As more than half of the Cd (e.g. $61.8 \pm 5.6\%$ after the depuration period) was bound to HSP regardless of condition in the sea bass (Table 1), this may suggest that Cd complexed to components of the HSP fraction (*i.e.* MTLP or GSH) was excreted directly from cytoplasm to bile or blood without passing through another cellular compound (Figure 2).

This elimination of Cd-saturated MT may lead to a new pool of free MT, which would be available for binding other metals. In accordance with the well-identified large Zn-binding capacities of MT and the competition between Cd and Zn (Maret, 2011; Moulis, 2010b), MT turnover during exocytosis of Cd-MT complexes may have enhanced Zn binding to newly-synthesized MT, as suggested by the significant increasing proportion of Zn (from $21.1 \pm 1.9\%$ to $27.7 \pm 2.1\%$) in the HSP fraction at the end of the depuration period (Table 1). Finally, it should be noted that more disturbances in metal homeostasis were observed in the sole liver following Cd exposure (*i.e.* changes in Fe, Mg, Mn and Zn partitioning; Table 1), indicating a greater sensitivity to Cd toxicity by interference with essential elements in this species than in sea bass, which only showed a modification in the Zn distribution.

Links between subcellular Cd handling and Cd toxicity in the two studied species

While more than half of the Cd ($61.8 \pm 5.6\%$) was bound to HSP in the sea bass after the depuration period, Cd partitioning in sole showed a significantly greater Cd pool in the sensitive fractions ($74.3 \pm 6.2\%$) compared with the detoxifying fractions ($13.3 \pm 4.0\%$). The different storage strategies imply that different supposed paths for Cd exocytosis, discussed above (*i.e.* direct transport of MT-bound Cd to the membrane in sea bass *versus* passing of MRG-bound Cd through ER and GA before excretion by vesicles in sole), may thus be responsible for a greater sensitivity of sole to Cd toxicity (Figure 2; Figure 3).

When present, the storage lipid fraction accounted for a non-negligible part of the internalized Cd in liver ($44.1 \pm 9.8\%$ in control sole and $24.9 \pm 0.5\%$ in control sea bass after the depuration period) (see Supporting Information), highlighting the need to consider this novel fraction in studies on metal subcellular partitioning. Cd is known to stimulate the lipid peroxidation process, defined as oxidative deterioration of polyunsaturated fatty acids and

resulting in alteration of cell membranes (Roméo et al., 2000; Viarengo et al., 1989). Cd binding to this fraction may thus cause oxidation of neutral lipids, preventing their use in membrane structure. Cd was also shown to reduce lipid storage efficiency, increasing utilisation of triglycerides, which finally led to a lower growth in terms of body weight in exposed fish (Pierron et al., 2007). Although no growth impairment was observed according to length measurement or HSI calculation, a significant weight decrease was found in Cd-exposed sole compared with controls (38.8 ± 5.6 g vs. 46.0 ± 7.2 g, respectively) (Figure 5; Table S2). Moreover, no storage lipid fraction was found in the livers of Cd exposed soles (see Supporting Information). In addition to the disturbances in essential metal homeostasis, the greater sensitivity of the sole to Cd toxicity was thus revealed by an alteration of the whole-body condition due to fat consumption.

Essential metal protection against Cd toxicity in the two studied species

One of the main mechanisms for Cd cellular toxicity is the induction of oxidative stress by production of oxygen free radicals (Almeida et al., 2002; Roméo et al., 2000). On the other hand, some elements are essential for the activity of antioxidant enzymes like glutathione peroxidases (GPx), catalases (CAT) and superoxide dismutases (SOD), which contain Se, Fe and Mn-Cu-Zn as cofactors, respectively (Vural et al., 2010). These enzymes are highly involved in preventing oxidative stress in fish and a relative higher level of essential metals may thus provide a better protection against Cd toxicity through higher activity of antioxidant enzymes (Basha and Rani, 2003; Janz, 2011; Martínez-Álvarez et al., 2005). For instance, oxidative stress caused by Cd was reduced by Se treatment in the liver of the rainbow trout (Talas et al., 2008), while a Zn-deficiency was responsible for oxidative stress in the same species (Hidalgo et al., 2002). Besides the well documented role of Se in the

detoxification of mercury (Hg) through formation of Hg-Se complexes (Khan and Wang, 2009; Pelletier, 1986), it has been suggested that Se could also complex with Cd and subsequently bind to selenoprotein P, thus reducing Cd availability and toxicity (Sasakura and T. Suzuki, 1998; Siscar et al., 2014). The resistance of a species to Cd toxicity may thus depend partially on the level of elements enhancing antioxidant response or able to complex Cd. The two fish species investigated in our study presented some differences in hepatic concentrations of such elements (Figure 3). While sea bass liver contained significantly more Fe ($27.8 \pm 6.6 \mu\text{g}\cdot\text{g}^{-1}$ in sea bass vs. $8.3 \pm 2.5 \mu\text{g}\cdot\text{g}^{-1}$ in sole), sole showed higher concentrations of Cu, Mn and Se (*e.g.* $66 \pm 35.6 \mu\text{g}\cdot\text{g}^{-1}$ of Cu in sole vs. $9.9 \pm 2.0 \mu\text{g}\cdot\text{g}^{-1}$ of Cu in sea bass) (see Supporting Information and Figure 3). Regarding essential metal concentrations, the two species thus seem to possess contrasting defense capabilities, involving different antioxidant enzymes.

Despite significantly higher levels of Cd in two sensitive fractions in sea bass ($12 \pm 4.2\%$ in mitochondria and $6.3 \pm 1.8\%$ in cytosolic HDP) compared with sole ($3.8 \pm 1.2\%$ in mitochondria and $0.5 \pm 0.0\%$ in HDP) after Cd exposure, sea bass also contained more antioxidant metals (Cu, Se and Zn) in these fractions (*e.g.* $12.3 \pm 3.7\%$ vs. $3.2 \pm 0.2\%$ of Cu in mitochondria for sea bass and sole, respectively) (Figure 1). Similarly, after depuration, large proportions of Cu, Mg, Mn, Se and Zn were observed alongside the significantly higher Cd proportions in two sensitive fractions ($51.5 \pm 4.8\%$ in organelles and $18.8 \pm 1.1\%$ in cytosolic HDP) in sole compared with sea bass ($15.4 \pm 4.7\%$ in organelles and $9.0 \pm 1.4\%$ in HDP). As the three types of antioxidant enzyme (GPx, CAT and SOD) are all found in cytosol, mitochondria and organelles (Bai et al., 1999; Martínez-Álvarez et al., 2005; Orbea et al., 2000), the fact that essential metals followed Cd distribution in sensitive fractions may

reflect their mobilization to activate antioxidant defences and a role in preventing Cd damage to cellular components.

Link between Cd partitioning and hepatic excretion

In a previous study, higher MT concentration combined with higher Cd biliary excretion and relocation to muscle were found in sea bass than in sole, suggesting that MT level would enhance Cd excretion from the liver (Le Croizier et al., 2018). These differences between the two species were attributed to metabolism and/or phylogenetic divergences. Indeed, seabass is characterized by a higher metabolism, which can increase the need for essential metals and thus the need for binding sites such as MT. Moreover, the two species exhibited significant variation in the MT sequence, potentially leading to difference in terms of function. Finally, these evolutionary divergences have probably been accentuated by contrasting ecological niches (*i.e.*, demersal for sea bass *versus* benthic for sole), leading sea bass to be more adapted to manage Cd sequestration in MT compared to sole. Even if Cd was also excreted from sole hepatocytes, Cd elimination seemed higher in sea bass (not significant) since around 60% of the Cd in liver was eliminated in this species after two months of depuration *versus* around 40% in sole (Le Croizier et al., 2018) (Figure S1, Supporting Information). Regarding subcellular partitioning, two main mechanisms were likely to give sea bass a greater Cd elimination from hepatocytes.

First, the large proportion of Cd bound to HSP may facilitate biliary excretion via transport of Cd/GSH complexes through a specific transporter of GSH present in the canalicular membrane (Zalups and Ahmad, 2003), or via transport of Cd/MT complexes as suggested by recent observations of MT in bile of metal-exposed fish (Hauser-Davis et al., 2016, 2014, 2012) (Figure 2).

Second, transport of Cd/MT through plasma membrane in the sea bass may be faster than vesicular exocytosis occurring in sole. Although MT release from various organs to the blood has been proven, the mechanisms for epithelial transport of MT (Chan et al., 1993; Moltedo et al., 2000) and Cd/MT complex transport (Moulis et al., 2014; Sabolić et al., 2010; Zalups and Ahmad, 2003) are unknown. By the classical vesicular secretory pathway while transport across membranes must occur directly from the cytoplasm to the exterior of the cell through ATP-binding cassette transporters (ABC transporters) (De Lisle et al., 1996) (Figure 2). Conversely, vesicular exocytosis of MRG-derived Cd in sole must involve successive steps, including fusion of vesicle and plasma membranes, which induces a slower removal of Cd compared to direct excretion in sea bass.

CONCLUSIONS

The present study identified two different Cd elimination pathways in European sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis*, exposed for two months to an environmentally realistic dietary Cd dose. The species-specific strategies imply differences in metal transport and sensitivity to Cd toxicity. In sea bass, Cd was mainly bound to metallothionein-like proteins and would be excreted through bile or released into the blood. In sole, granules-bound Cd would be sequestered by organelles and may thus be responsible for a greater sensitivity of sole to Cd toxicity, revealed by an alteration of the whole-body condition due to fat consumption. These results moreover bring significant novelties demonstrating that MT and MRG are involved in metal elimination from liver, through either biliary excretion or transport to other tissues, as these cell components were so far considered as long term metal storage fractions (Vijver et al., 2004; Wallace et al., 2003; Wang and Rainbow, 2010). Future studies should therefore focus on the membrane

transport mechanisms of MT and MRG, to fully understand the implications of elimination pathways on metal retention and sensitivity of marine fish species to toxic elements such as Cd.

Acknowledgements

The authors thank greatly Jean Raffray for the dissection of the fish. This study benefited of the technical support of the Centre of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE). This work was financially supported by the French National Research Agency project ANR-11-CEPL-0005 EPURE.

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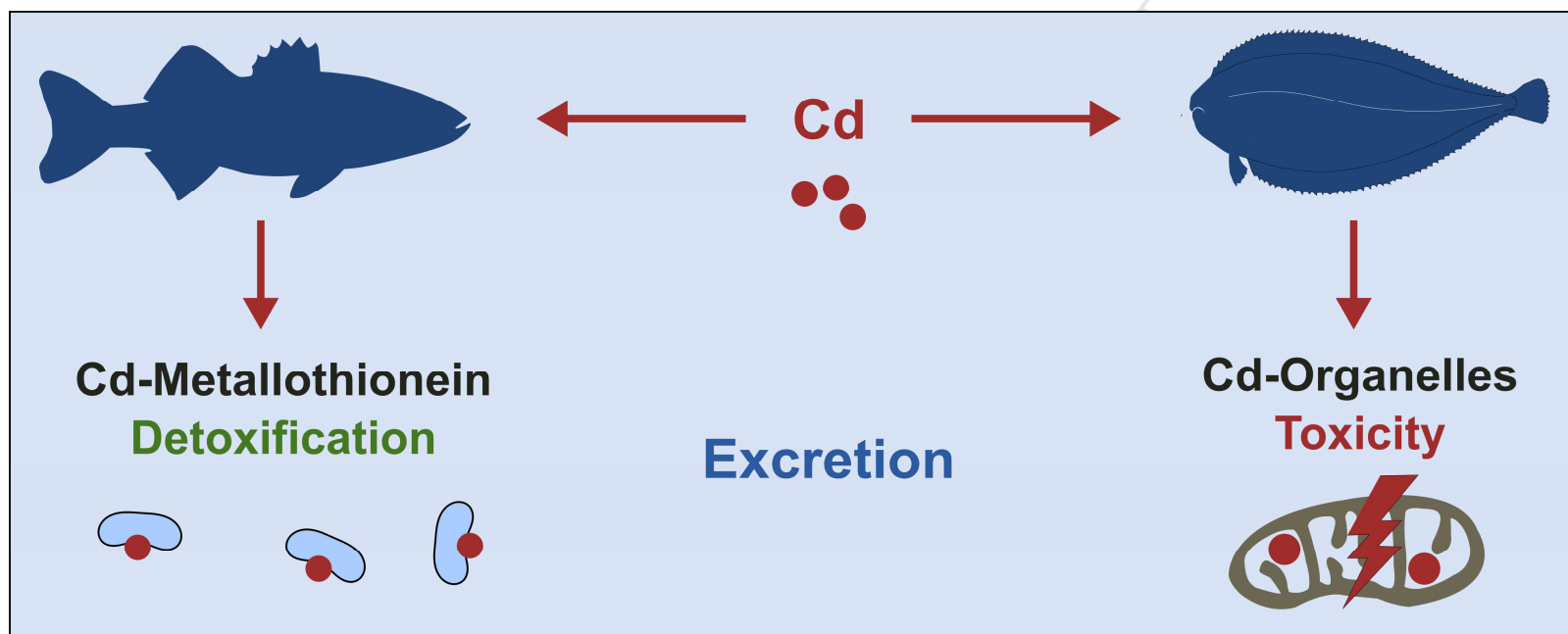
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Species	Element	Subcellular partitioning (%)					
		Organelles		MRG		MTLP	
		Control	Exposed	Control	Exposed	Control	Exposed
<i>D. labrax</i>	Cd	20.1 ± 0.9	15.4 ± 4.7	1.3 ± 0.3	1.6 ± 0.7	53.7 ± 1.4	61.8 ± 5.6
	Ca	18.4 ± 16.3	12.9 ± 3.1	53.8 ± 42.8	69.3 ± 4.1	13.2 ± 12.6	9.6 ± 3.7
	Cu	20.2 ± 1.2	16.5 ± 4.8	2.4 ± 0.7	2.5 ± 1.3	53.2 ± 1.2	59.8 ± 5.9
	Fe	56.6 ± 1.2	60.6 ± 12.8	2.7 ± 0.5	3.1 ± 1.5	6.7 ± 2	8.3 ± 8.3
	Mg	42.1 ± 1	40.3 ± 5.4	3.8 ± 1.3	4.2 ± 1.1	33.7 ± 1.8	38.8 ± 5.7
	Mn	38.2 ± 2.3	37.7 ± 4.2	5.8 ± 2.6	6.1 ± 0.7	29.3 ± 1.9	33 ± 4.5
	Se	31.7 ± 1.2	28.4 ± 5.5	3.1 ± 0.3	3.9 ± 1	16.6 ± 0.1	18.6 ± 3.1
	Zn	28.1 ± 0.8	23.5 ± 5	1.5 ± 0.1	1.5 ± 0.2	21.1 ± 1.9	27.7 ± 2.1
<i>S. senegalensis</i>	Cd	37.3 ± 5.9	51.5 ± 4.8	16.4 ± 9	9.2 ± 5.1	3.9 ± 2.4	4.1 ± 2.2
	Ca	28.3 ± 10.2	39.5 ± 14.7	37.7 ± 16.9	32.6 ± 13.7	17.5 ± 2	13.5 ± 2.3
	Cu	36.6 ± 6.3	49.3 ± 7.6	16.9 ± 9.6	8.1 ± 2.8	3.9 ± 3.3	6.8 ± 3.4
	Fe	30.1 ± 2.4	41.2 ± 1.4	16.9 ± 3.2	9.2 ± 2.6	8.9 ± 1.3	6.8 ± 1.3
	Mg	36.5 ± 6.4	52.1 ± 3.4	13.6 ± 2.7	7.7 ± 1.3	28.6 ± 2.9	23 ± 4.3
	Mn	36.8 ± 3.7	49.4 ± 1.8	16.8 ± 3	10.3 ± 1.5	23 ± 4.2	20.1 ± 2.8
	Se	32.8 ± 3.9	43.6 ± 6.8	10.6 ± 6.8	5.8 ± 1.4	11.6 ± 2.9	12.3 ± 1.5
	Zn	31.4 ± 3.2	43.3 ± 4.1	10.6 ± 7	7.4 ± 2.2	5.8 ± 0.5	4.6 ± 0.9

Table 1

Total metal concentration (ppm dry weight, mean ± sd) and subcellular partitioning (% of the total metal contained in each fraction, mean ± sd) of metal elements, after 60 days of cadmium depuration, in the liver of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* for both treatments (exposed and control fish, n = 3). Values are shown only for organelles, metal rich granules (MRG) and heat stable proteins (HSP) fractions where metal partitioning significantly differed between control and exposed fish (values in bold; ANOVA, p < 0.05).

Graphical abstract



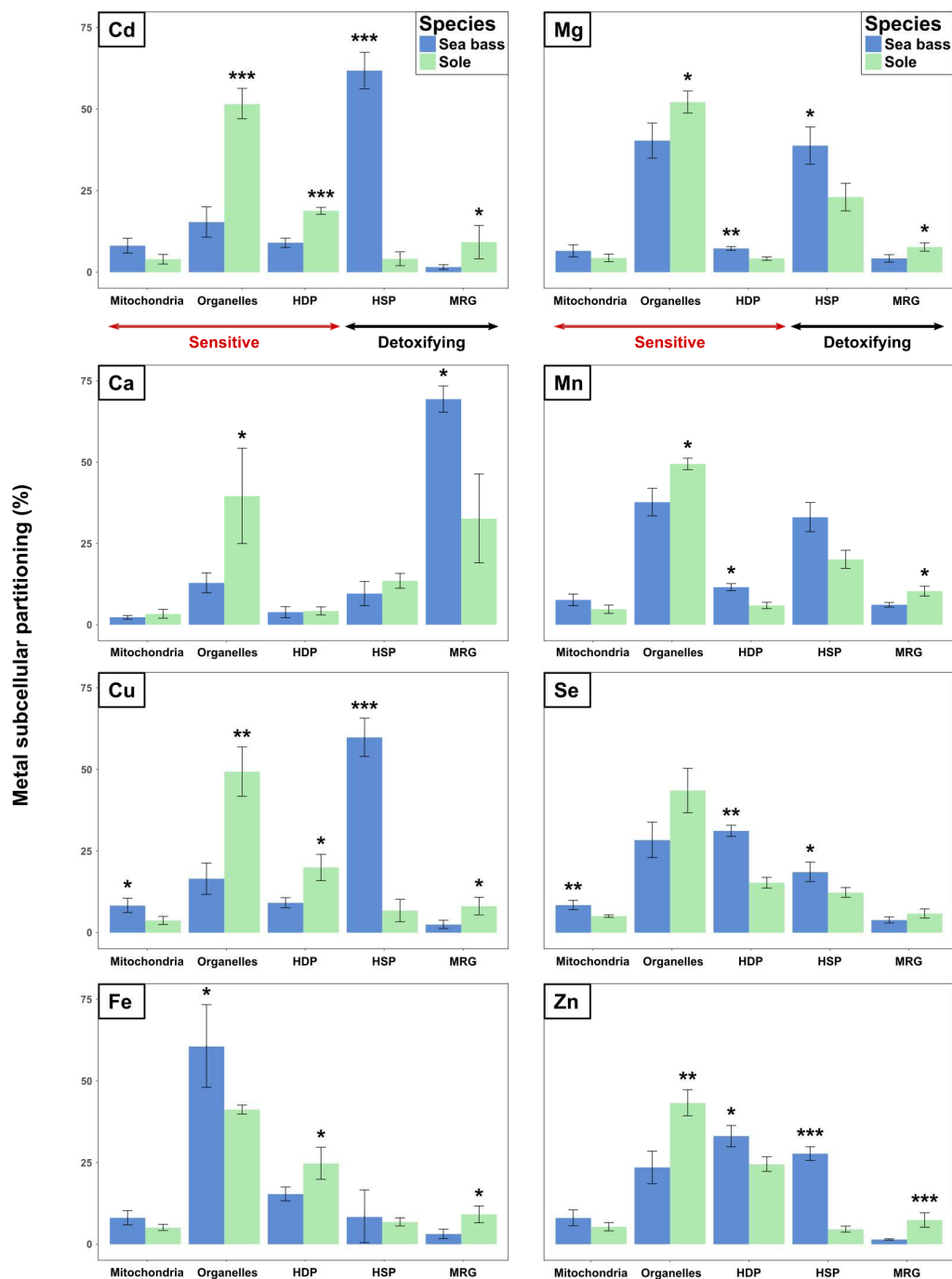


Figure 1: Subcellular partitioning of cadmium (Cd) and essential metals (% of the total metal contained in each fraction) in the liver of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* (n = 3) after 60 days of depuration. The cellular debris fraction is not shown as it is neither a sensitive nor a detoxifying fraction, but was taken into account in the calculation of the partitioning (for Cd proportions in cellular debris, see Supporting Information). Significant difference between species (ANOVA) are indicated by * p < 0.05, ** p < 0.01, *** p < 0.001).

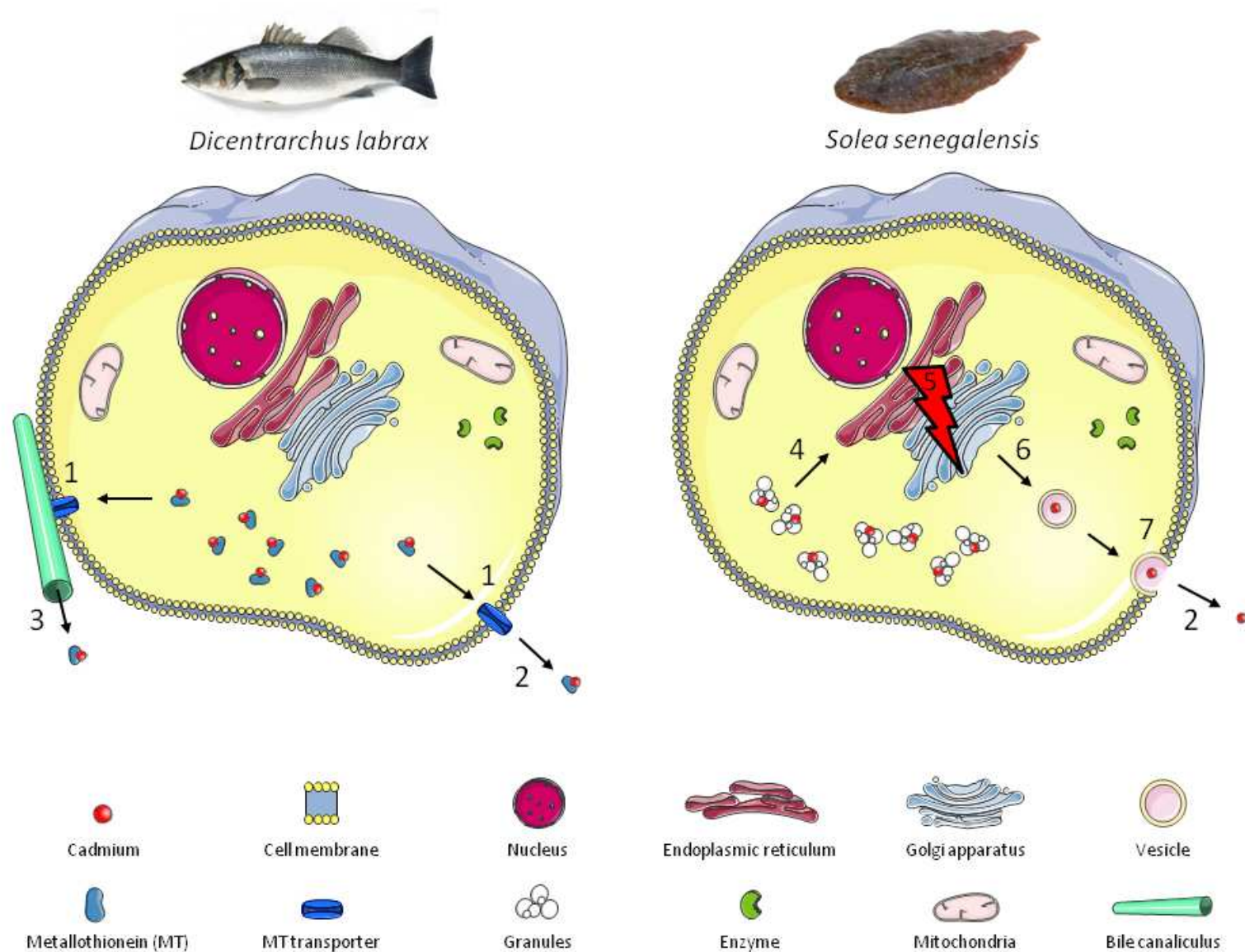


Figure 2: Subcellular Cd dynamics in hepatocytes of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis*. Only the two main mechanisms of Cd handling are represented: binding to metallothionein in sea bass and binding to granules in sole. 1: Excretion of Cd/MT complexes through MT transporters; 2: Release into the blood; 3: Release into bile; 4: sequestration of metal rich granules by the endoplasmic reticulum; 5: damage due to Cd toxicity; 6: vesicular transport; 7: vesicular exocytosis.

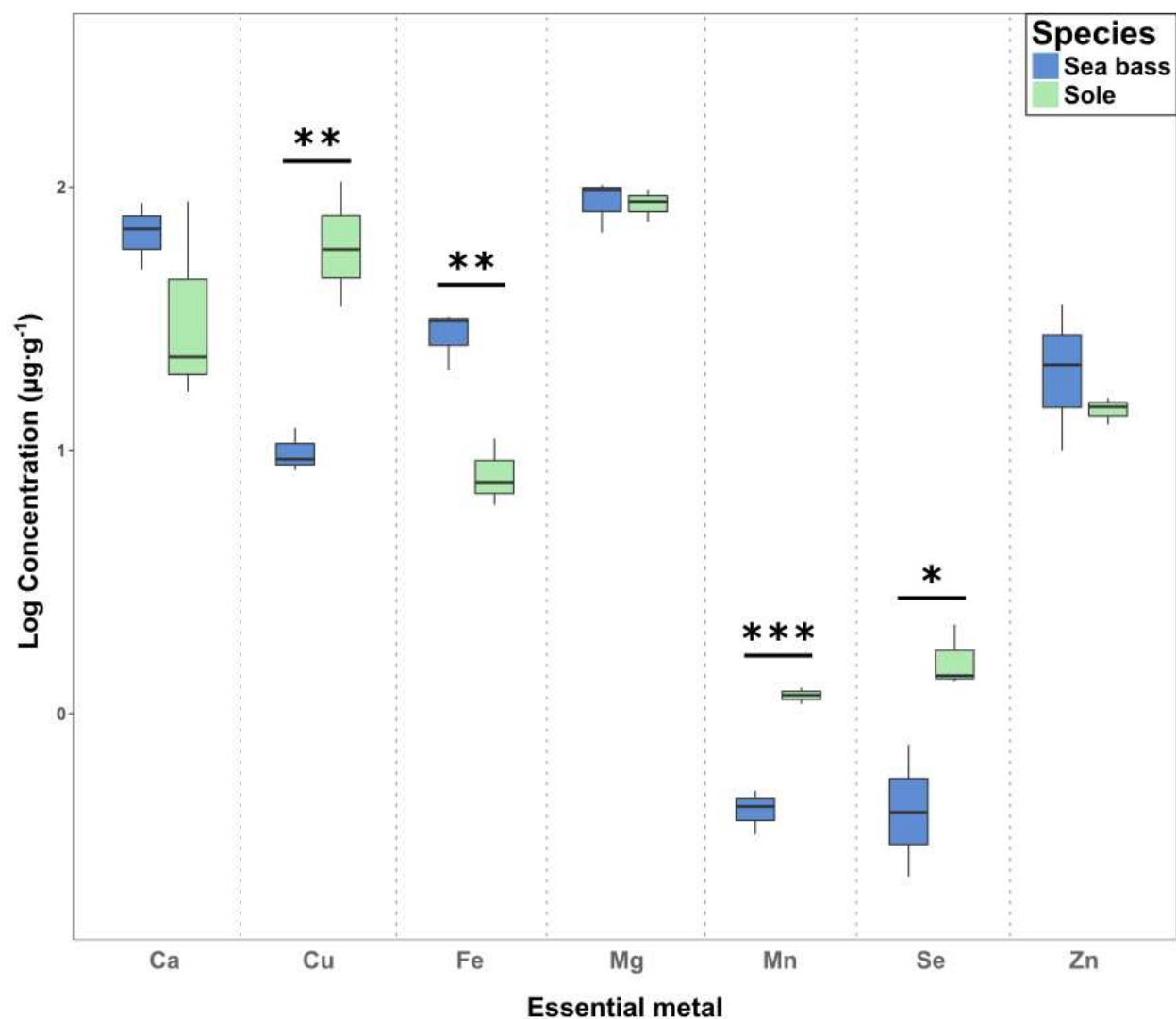


Figure 3: Total essential metal concentrations (log transformed values, $\mu\text{g}\cdot\text{g}^{-1}$ dw) in the liver of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* after 60 days of dietary Cd exposure (n = 3). Significant differences between species (ANOVA) are indicated by * p < 0.05, ** p < 0.01, *** p < 0.001. No differences were found in total essential metal concentrations between control and exposed fish of the same species (see Supporting Information).

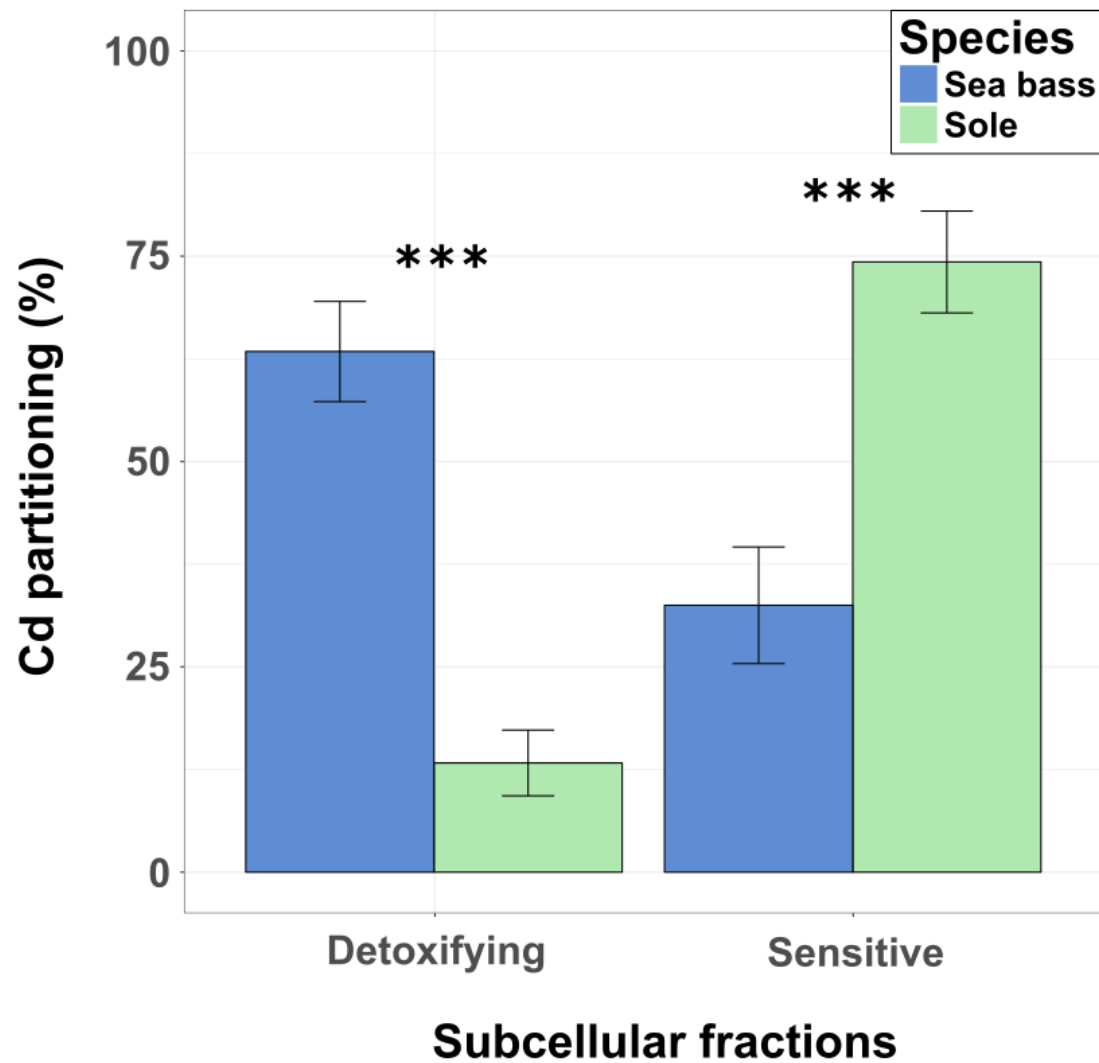


Figure 4: Subcellular partitioning (% of the Cd contained in each fraction type, mean \pm sd) of Cd after 60 days of depuration in the liver of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* (n = 3). Sensitive fractions = mitochondria + HDP + organelles; detoxifying fractions = MRG + HSP. Significant differences between species (ANOVA) are indicated by *** p < 0.001.

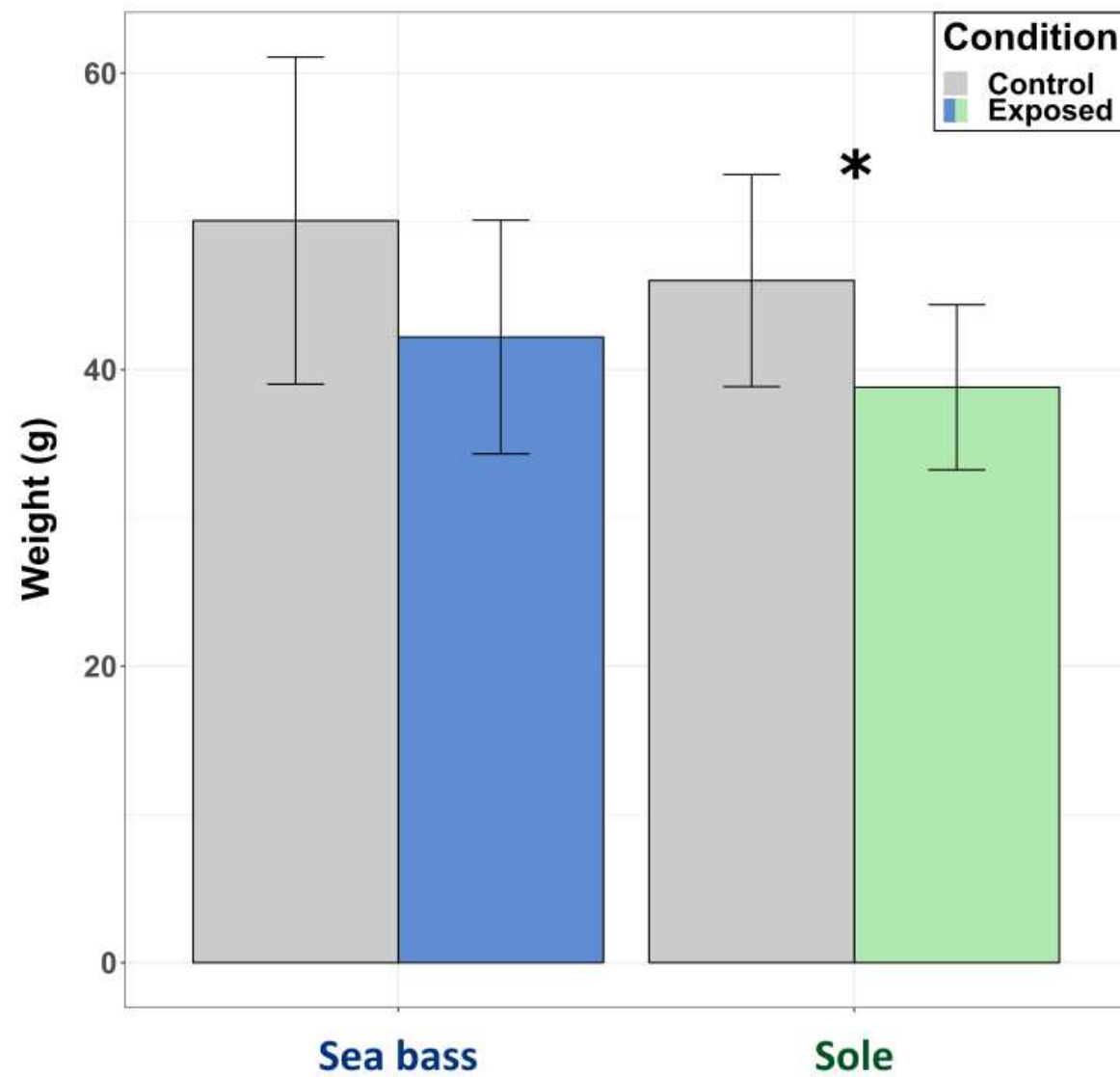


Figure 5 : Weight (g) (mean \pm sd) of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* after 60 days of Cd dietary exposure for both treatments (exposed and control fish, n = 3). Significant differences between conditions (ANOVA) are indicated by * $p < 0.05$.

HIGHLIGHTS

- Sea bass and sole displayed different Cd subcellular partitioning in the liver
- Cd was largely bound to metallothionein-like proteins in the sea bass liver
- Cd was mainly linked to metal rich granules in the sole liver
- Essential metals followed the Cd partitioning pattern in hepatic cells
- Handling strategies might account for sensitivity and Cd excretion