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

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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
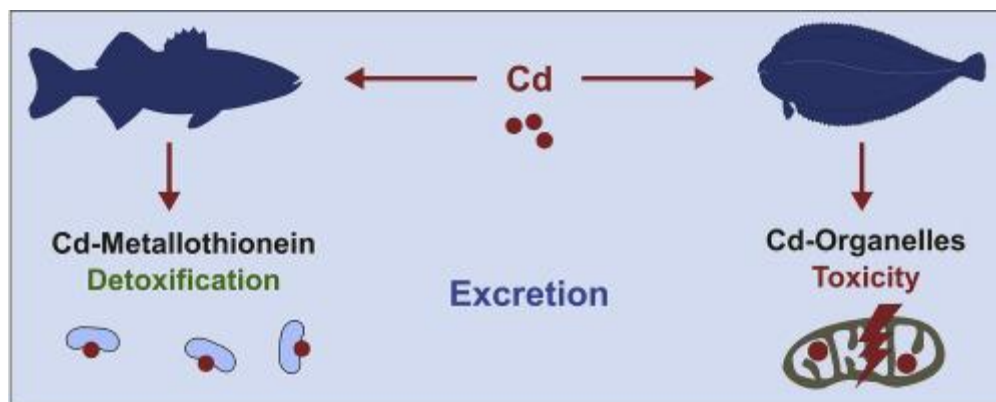
Highlights

► 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

46 depression of the immune system, which can finally affect growth and survival (McGeer et

47 al., 2011).

48 Recently, attention has been focused on fish species ability to cope with metal toxicity,

49 depending on subcellular partitioning of the element (Eyckmans et al., 2012; Leonard et al.,

50 2014). At the cellular level, Cd can take different toxic chemical forms including: free or

51 complexed ion forms (*e.g.* Cd^{2+}, $CdCl_2$), bound to enzymes (*e.g.* cytochromes), bound to

52 organic acids (*e.g.* citrates), or bound to cellular constituents causing damages (*e.g.* DNA)

53 (Vijver et al., 2004). Today, only a few forms of Cd speciation are considered as detoxified:

54 Cd complexed to peptides (*e.g.* glutathione) or functional, transport or sequestration

55 proteins (*e.g.* metallothioneins), and Cd trapped in vesicles of the lysosomal system or

56 precipitated in mineral granules (Wang and Rainbow, 2006). Very little is however known

57 about the intracellular mechanisms leading to Cd elimination (Moulis et al., 2014; Zalups and

58 Ahmad, 2003).

59 While one of the adverse effects of toxic metals is their interference with essential elements

60 (Martelli et al., 2006; Moulis, 2010a), some of them confer a protective role against metal

61 toxicity, directly through formation of detoxifying complexes (Sasakura and T. Suzuki, 1998)

62 or by indirectly preventing oxidative stress due to their association with antioxidant enzymes

63 (Martínez-Álvarez et al., 2005; Talas et al., 2008). The significance of essential element

64 subcellular partitioning in their protective role against toxic metals has never yet been

65 considered. Moreover, despite evidence of the influence of cellular components like MT in

66 metal accumulation kinetics in marine species (Wang and Rainbow, 2010), most previous

67 studies only emphasize on the influence of metal intracellular handling on sensitivity of

68 organisms to toxic elements (Campbell et al., 2008; Eyckmans et al., 2012; Giguère et al.,

69 2006; Leonard et al., 2014; Wang and Rainbow, 2006). Very few studies have therefore

70 investigated the link between metal subcellular partitioning and kinetics in fish (Glynn, 1991)

71 and information about the relationships between Cd intracellular handling and excretion

72 pathways is lacking for marine fish.

73 In order to fill these gaps, the present study aimed to investigate Cd intracellular distribution

74 in the liver of two different marine fish species which are naturally present in the CCLME, a

75 region particularly prone to Cd contamination. For this purpose, European sea bass

76 *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis*, were exposed for two months

77 to an environmentally realistic dietary cadmium (Cd) dose followed by a depuration period

78 of a further two months. Analyses were conducted at the end of the uptake period and at

79 the end of the depuration period and Cd partitioning was examined in six major subcellular

80 fractions: cellular debris, metal-rich granules, mitochondria, organelles, cytosolic enzymes,

81 cytosolic proteins and peptides, as well as in the storage lipid fraction of the liver when

82 present. Sensitivity of the two species was assessed according to: (1) Cd subcellular

83 partitioning during accumulation and depuration periods, (2) impairments to essential metal

84 subcellular distribution, (3) essential metal concentrations in the whole liver and their

85 partitioning within hepatocytes, (4) growth and hepatosomatic index calculation. Finally,

86 hypotheses were made regarding the influence of Cd cellular speciation in the liver and Cd

87 biliary excretion or transport to other tissues.

88

89 **MATERIALS AND METHODS**

90 All animal procedures were in accordance with the French and EU guidelines for animal

91 research (project approval number: 03266.03).

92 **Fish and experimental procedures**

93 All details about animal procedures and experimental design can be found in Le Croizier et

94 al., 2018. Briefly, immature sea bass *Dicentrarchus labrax* (length: 14.1 ± 0.7 cm; weight 29.8

95 ± 4.5 g) used in this experiment were obtained from a commercial hatchery (Aquastream,

96 Ploemeur, France), whereas immature Senegalese sole *Solea senegalensis* (length: 14.9 ± 1.1

97 cm; weight 36.4 ± 7.9 g) were provided from a marine farm (Ferme marine de l'Adour,

98 Anglet, France). The fish were transported to the Cedre (Centre of Documentation, Research

99 and Experimentation on Accidental Water Pollution, Brest, France). After receiving

100 anaesthesia by bathing in a 0.05 mL·L^{-1} solution of tricaine methanesulfonate (MS-222)

101 (Ackerman et al., 2005), each fish was randomly assigned to one of twelve high density

102 polyethylene tanks that had a 300 L volume (six tanks for each species, 40 sole and 50 sea

103 bass per tank to ensure equivalent biomass) at the Cedre's marine animal facility. This facility

104 is an independent greenhouse that is submitted to a natural photoperiod and supplied with

105 a continuous seawater flow from the bay of Brest. The temperatures in the experimental

106 tanks followed the outdoor temperature. Fish were first acclimated to the experimental

107 conditions for one month, during which they were fed daily with dried commercial pellets

108 (Turbot label rouge 1.4 mm, Le Gouessant Aquaculture).

109 To obtain an environmental relevant Cd concentration in the fish food, a Cd-enriched diet

110 was prepared in order to reach a Cd level around 25 μg·g^{-1}, which corresponds to the Cd

111 level reported in potential prey within the natural distribution of the two fish species (Bodin

112 et al., 2013; Maanan, 2008). For this purpose, the commercial pellets were spiked with a 100

113 mg·L^{-1} Cd (as CdCl$_2$ in milliQ water) solution for 30 min. The diet was then placed at -20°C,

114 freeze dried and broken into small pellets before usage. This preparation led to a Cd

115 concentration of 22.9 ± 0.3 μg·g^{-1} dw in the Cd-enriched food. The control diet was prepared

116 in the same way, but with the addition of milliQ water only, where the measured

117 background Cd level was 0.71 ± 0.00 µg·g^{-1} dw. For each of the four conditions (*i.e.*, Cd-

118 exposed and control fish, for both species) there were three replicate tanks. Dietary Cd

119 exposure was initiated by randomly assigning three of the six tanks per species to the Cd-

120 enriched food. The six remaining tanks were fed the control diet. Cd exposure was

121 conducted for 60 days, after which a depuration period was conducted that lasted for 60

122 days. During the depuration period, all of the tanks were fed the control diet. Daily food

123 distribution was performed slowly and continuously over 24 h with a clockwork feeder

124 (COFA, Paris, France) to prevent pellets from remaining in the tank and thus avoid Cd

125 desorption in water. Sea bass were fed at 1.7 % body weight while sole were fed at 1 % body

126 weight per day to meet the physiological requirements of each species (Danion et al., 2011;

127 Salas-Leiton et al., 2010). The bottom of each tank was siphoned every day to avoid Cd

128 leaching from faeces.

129 Physico-chemical parameters (O2, pH, temperature, salinity) and water quality (nitrates,

130 nitrites) were measured every ten days. During the experiment, the water temperature

131 decreased from 20.7 to 12.8 °C, oxygen saturation increased from 81.2% to 98.2%, pH

132 increased from 8.0 to 8.2 and salinity increased from 35.2 to 35.7. The water was free of

133 nitrate and nitrite (Colorimetric test JBL) in both experiments.

134 **Biological sampling**

135 After the two months of Cd exposure, three fish per tank (nine for each treatment) were

136 anesthetized by bathing in a 0.05   mL·L^{-1} solution of MS-222 before being euthanized by

137 bathing in a 0.2   mL·L^{-1} solution of MS-222 (Ackerman et al., 2005). They were weighed,

138 measured and dissected with ceramic tools to avoid metal contamination. The liver was

139 collected, weighed and put in acid-washed (10% HNO$_3$) individual plastic microcentrifuge

140 tubes, flash frozen in liquid nitrogen and stored at -80°C until analysis. Another sampling was

141 made in the same way after a further two months of depuration.

Subcellular partitioning procedure

143 This procedure generally followed the protocol of Rosabal et al. (2015), adapted from that of

144 Wallace et al. (2003), which has been validated and extensively used on various fish species

145 (Campbell et al., 2008; Eyckmans et al., 2012; Giguère et al., 2006; Lapointe et al., 2009a).

146 Briefly, liver samples were fractionated into six operationally-defined subcellular fractions:

147 cell membranes (cellular debris), metal-rich granules (MRG); mitochondria, microsomes +

148 lysosomes (organelles), heat-denatured proteins (HDP) including cytosolic enzymes, and

149 heat-stable proteins and peptides (HSP) such as metallothionein (MT) and glutathione (GSH).

150 Three liver samples of fish from the same tank were pooled to obtain approximately 200 mg

151 of wet sample and homogenized in 1.5 mL of a solution containing Tris-HCl 20 mmol·L^{-1} pH

152 8.6, 0.01% DTT (dithiothreitol) as a reducing agent and 1 % of an antiproteolytic agent

153 (Protease Inhibitor Mix, GE Healthcare). A 100 μL aliquot was removed from the liver

154 homogenate for determining total trace metal concentrations in the liver and assessing

155 metal recovery from the subcellular fractions. The remainder of the liver homogenate was

156 centrifuged at 1450×g for 15 min at 4°C. The supernatant (S1) was transferred to an acid-

157 washed 1.5-mL polypropylene microcentrifuge tube for further separations. The pellet from

158 this centrifugation was suspended in 0.5 mL of ultrapure water, heated to 100°C for 2 min,

159 digested with an additional 500 μL of 1 N NaOH at 65°C for 60 min. Centrifugation at

160 10,000×g for 10 min at ambient temperature (∼20°C) was performed to separate the NaOH-

161 resistant fraction (referred to as "MRG") from the cellular debris fraction, which includes cell

162 membranes, unbroken cells and nuclei. The S1 supernatant was centrifuged at 10,000×g for

163 30 min at 4°C to produce the mitochondrial fraction. The resulting supernatant was

164 ultracentrifuged at 100,000×g for 60 min at 4°C, giving a pellet containing other organelles

165 (microsomes and lysosomes) and the cytosolic fraction in the supernatant. To separate the

166 heat-stable peptides and proteins (HSP) from the heat-denatured proteins (HDP), the

167 cytosolic fraction was held at 80°C for 10 min, left on ice for 1 h and then centrifuged at

168 50,000×g for 10 min at 10°C. The HSP fraction, which includes MT, was collected from the

169 supernatant. Each fraction was finally kept at -20°C until the metal analyses were performed.

Metal analyses

171 Subcellular fractions were digested in a mixture of 2 mL 70% HNO_3 and 0.5 mL 30% H_2O_2

172 (both of ultrapure quality) in closed Teflon vessels. Mineralization was performed on a

173 hotplate for 4 h at 100°C. A 100-µL aliquot was removed from the digestate and diluted to 15

174 mL with Milli-Q quality water (Merck Millipore). Cadmium and essential metals (Ca, Cu, Fe,

175 Mg, Mn, Se and Zn) were analysed using an ICP Q-MS (X Series 2, Thermo Scientific) at the

176 Pôle de Spectrométrie Océan (PSO, Plouzané, France) with an internal standard solution of

177 Rhodium (2 µg·L^{-1}). Reference materials (fish protein DORM-4 and dogfish liver DOLT-5,

178 NRCC) were treated and analysed in the same way as the samples. The results for reference

179 materials displayed mean metal recoveries of 85 ± 6 % for DORM-4 and 91 ± 6 % for DOLT-5.

180 Mean recoveries (± SD) of DORM-4 reference samples (n = 3) were 81 ± 2 % for Cd, 78 ± 3 %

181 for Ca, 95 ± 2 % for Mg, 89 ± 5 % for Mn, 86 ± 5 % for Fe, 81 ± 2 % for Cu, 79 ± 1 % for Zn, 92

182 ± 3 % for Se. Mean recoveries (± SD) of DOLT-5 reference samples (n = 3) were 99 ± 2 % for

183 Cd, 83 ± 1 % for Ca, 96 ± 4 % for Mg, 92 ± 4 % for Mn, 98 ± 6 % for Fe, 86 ± 3 % for Cu, 85 ± 2

184 % for Zn, 86 ± 2 % for Se. Blanks were included in each analytical batch. The limits of

185 detection (ng·g^{-1} dry wt), corresponding to the mean concentration of the blank solutions,

186 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

187 metal concentrations in the liver ($\mu g \cdot g^{-1}$) are given on a dry weight basis ($\mu g \cdot g^{-1}$ dw), based

188 on a previous study involving the same fish and where the liver samples were freeze-dried

189 before analysis (Le Croizier et al., 2018). The proportion of metal in each fraction is

190 expressed as a percentage of the sum of the concentrations of all fractions. As it was not

191 present in all of the liver samples, the storage lipid fraction was not taken into account in the

192 calculation of metal partitioning. Metal proportion in this fraction was expressed as a

193 percentage of the total metal concentration in the liver. "Sensitive fractions" gathered the

194 Cd contained in the sensitive fractions (*i.e.* mitochondria + HDP + organelles, while the

195 "metal-detoxified fractions" gathered the Cd contained in the detoxifying fractions (*i.e.* MRG

196 + HSP) (Eyckmans et al., 2012; Leonard et al., 2014). The total metal concentration recovery

197 in the liver from the sum of the metal concentrations measured in all fractions, including

198 lipids, was 95 ± 11% for sea bass and 96 ± 6% for sole.

Data analysis

200 All data tested statistically were first checked for normality (Shapiro–Wilks tests) (Shapiro

201 and Wilk, 1965) and homogeneity of variances (Bartlett tests) (Bartlett, 1937). When these

202 conditions were met, raw data were used and one-way ANOVAs, followed by Tukey's HSD

203 tests, were performed to test for differences between treatments and species. Otherwise,

204 non-parametric analogues were used, *i.e.* Kruskal-Wallis tests (KW), followed by Conover-

205 Iman multiple comparison tests with Bonferroni's adjustment (Conover and Iman, 1979). All

206 of the statistical analyses were performed using the open source software R (version 3.4.3, R

207 Core Team, 2017).

208

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 ± 5.9% to 51.5 ± 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 ± 1.4% in organelles while it significantly decreased from 16.9 ± 3.2% to 9.2 ± 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 ± 4.1% and 32.6 ± 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).

232   The simultaneous increase in proportion of Cd and essential metals in organelles and

233   decrease of essential metals in MRG may be caused by sequestration of MRG by ER, which is

234   part of the organelle fraction (Table 1; Figure 2). This hypothesis seems reasonable since

235   MRG in our species contained the highest proportion of Ca, and ER is deeply involved in Ca

236   storage (Görlach et al., 2006). Supplementing fish diet has furthermore been shown to

237   increase Cd in MRG while decreasing it in organelles (Ng et al., 2009). Although the authors

238   did not draw this conclusion, our hypothesis is that increasing cellular Ca concentration will

239   reduce the probability for Cd bound to calcium granules to be sequestered by ER during Ca

240   uptake. The changes in essential metal cellular repartition would thus be a side effect of Cd

241   transport from MRG to organelles (*i.e.* ER, GA and vesicles, in that order) before final

242   exocytosis (Table 1; Figure 2).

243   Conversely, no change in Cd partitioning was observed in sea bass liver despite effective Cd

244   elimination as well as Cd biliary excretion observed in this species during the depuration

245   period (Le Croizier et al., 2018). In mammals, Cd bound to glutathione (GSH) was shown to

246   be excreted from hepatocytes to bile while Cd bound to metallothionein (MT) was released

247   into the blood before reaching other organs like kidney (Ballatori, 1991; Chan et al., 1993;

248   Klaassen, 1978; Nordberg, 1978). In fish, MT were reported in bile (Hauser-Davis et al., 2012)

249   and were shown to transport metal during biliary excretion in Cu- and Se-exposed fish

250   (Hauser-Davis et al., 2016, 2014). As more than half of the Cd (e.g. 61.8 ± 5.6% after the

251   depuration period) was bound to HSP regardless of condition in the sea bass (Table 1), this

252   may suggest that Cd complexed to components of the HSP fraction (*i.e.* MTLP or GSH) was

253   excreted directly from cytoplasm to bile or blood without passing through another cellular

254   compound (Figure 2).

255 This elimination of Cd-saturated MT may lead to a new pool of free MT, which would be

256 available for binding other metals. In accordance with the well-identified large Zn-binding

257 capacities of MT and the competition between Cd and Zn (Maret, 2011; Moulis, 2010b), MT

258 turnover during exocytosis of Cd-MT complexes may have enhanced Zn binding to newly-

259 synthesized MT, as suggested by the significant increasing proportion of Zn (from 21.1 ±

260 1.9% to 27.7 ± 2.1%) in the HSP fraction at the end of the depuration period (Table 1).

261 Finally, it should be noted that more disturbances in metal homeostasis were observed in

262 the sole liver following Cd exposure (*i.e.* changes in Fe, Mg, Mn and Zn partitioning; Table 1),

263 indicating a greater sensitivity to Cd toxicity by interference with essential elements in this

264 species than in sea bass, which only showed a modification in the Zn distribution.

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

266 While more than half of the Cd (61.8 ± 5.6%) wasbound to HSP in the sea bass after the

267 depuration period, Cd partitioning in sole showed a significantly greater Cd pool in the

268 sensitive fractions (74.3 ± 6.2%) compared with the detoxifying fractions (13.3 ± 4.0%). The

269 different storage strategies imply that different supposed paths for Cd exocytosis, discussed

270 above (*i.e.* direct transport of MT-bound Cd to the membrane in sea bass *versus* passing of

271 MRG-bound Cd through ER and GA before excretion by vesicles in sole), may thus be

272 responsible for a greater sensitivity of sole to Cd toxicity (Figure 2; Figure 3).

273 When present, the storage lipid fraction accounted for a non-negligible part of the

274 internalized Cd in liver (44.1 ± 9.8% in control sole and 24.9 ± 0.5% in control sea bass after

275 the depuration period) (see Supporting Information), highlighting the need to consider this

276 novel fraction in studies on metal subcellular partitioning. Cd is known to stimulate the lipid

277 peroxidation process, defined as oxidative deterioration of polyunsaturated fatty acids and

278 resulting in alteration of cell membranes (Roméo et al., 2000; Viarengo et al., 1989). Cd

279 binding to this fraction may thus cause oxidation of neutral lipids, preventing their use in

280 membrane structure. Cd was also shown to reduce lipid storage efficiency, increasing

281 utilisation of triglycerides, which finally led to a lower growth in terms of body weight in

282 exposed fish (Pierron et al., 2007). Although no growth impairment was observed according

283 to length measurement or HSI calculation, a significant weight decrease was found in Cd-

284 exposed sole compared with controls (38.8 ± 5.6 g *vs.* 46.0 ± 7.2 g, respectively) (Figure 5;

285 Table S2). Moreover, no storage lipid fraction was found in the livers of Cd exposed soles

286 (see Supporting Information). In addition to the disturbances in essential metal homeostasis,

287 the greater sensitivity of the sole to Cd toxicity was thus revealed by an alteration of the

288 whole-body condition due to fat consumption.

289 **Essential metal protection against Cd toxicity in the two studied species**

290 One of the main mechanisms for Cd cellular toxicity is the induction of oxidative stress by

291 production of oxygen free radicals (Almeida et al., 2002; Roméo et al., 2000). On the other

292 hand, some elements are essential for the activity of antioxidant enzymes like glutathione

293 peroxidases (GPx), catalases (CAT) and superoxide dismutases (SOD), which contain Se, Fe

294 and Mn-Cu-Zn as cofactors, respectively (Vural et al., 2010). These enzymes are highly

295 involved in preventing oxidative stress in fish and a relative higher level of essential metals

296 may thus provide a better protection against Cd toxicity through higher activity of

297 antioxidant enzymes (Basha and Rani, 2003; Janz, 2011; Martínez-Álvarez et al., 2005). For

298 instance, oxidative stress caused by Cd was reduced by Se treatment in the liver of the

299 rainbow trout (Talas et al., 2008), while a Zn-deficiency was responsible for oxidative stress

300 in the same species (Hidalgo et al., 2002). Besides the well documented role of Se in the

301   detoxification of mercury (Hg) through formation of Hg-Se complexes (Khan and Wang,

302   2009; Pelletier, 1986), it has been suggested that Se could also complex with Cd and

303   subsequently bind to selenoprotein P, thus reducing Cd availability and toxicity (Sasakura

304   and T. Suzuki, 1998; Siscar et al., 2014). The resistance of a species to Cd toxicity may thus

305   depend partially on the level of elements enhancing antioxidant response or able to complex

306   Cd. The two fish species investigated in our study presented some differences in hepatic

307   concentrations of such elements (Figure 3). While sea bass liver contained significantly more

308   Fe (27.8 ± 6.6 µg·g^{-1} in sea bass *vs.* 8.3 ± 2.5 µg·g^{-1} in sole), sole showed higher

309   concentrations of Cu, Mn and Se (*e.g.* 66 ± 35.6 µg·g^{-1} of Cu in sole *vs.* 9.9 ± 2.0 µg·g^{-1} of Cu in

310   sea bass) (see Supporting Information and Figure 3). Regarding essential metal

311   concentrations, the two species thus seem to possess contrasting defense capabilities,

312   involving different antioxidant enzymes.

313   Despite significantly higher levels of Cd in two sensitive fractions in sea bass (12 ± 4.2% in

314   mitochondria and 6.3 ± 1.8% in cytosolic HDP) compared with sole (3.8 ± 1.2% in

315   mitochondria and 0.5 ± 0.0% in HDP) after Cd exposure, sea bass also contained more

316   antioxidant metals (Cu, Se and Zn) in these fractions (*e.g.* 12.3 ± 3.7% *vs.* 3.2 ± 0.2% of Cu in

317   mitochondria for sea bass and sole, respectively) (Figure 1). Similarly, after depuration, large

318   proportions of Cu, Mg, Mn, Se and Zn were observed alongside the significantly higher Cd

319   proportions in two sensitive fractions (51.5 ± 4.8% in organelles and 18.8 ± 1.1% in cytosolic

320   HDP) in sole compared with sea bass (15.4 ± 4.7% in organelles and 9.0 ± 1.4% in HDP). As

321   the three types of antioxidant enzyme (GPx, CAT and SOD) are all found in cytosol,

322   mitochondria and organelles (Bai et al., 1999; Martínez-Álvarez et al., 2005; Orbea et al.,

323   2000), the fact that essential metals followed Cd distribution in sensitive fractions may

324 reflect their mobilization to activate antioxidant defences and a role in preventing Cd

325 damage to cellular components.

326 **Link between Cd partitioning and hepatic excretion**

327 In a previous study, higher MT concentration combined with higher Cd biliary excretion and

328 relocation to muscle were found in sea bass than in sole, suggesting that MT level would

329 enhance Cd excretion from the liver (Le Croizier et al., 2018). These differences between the

330 two species were attributed to metabolism and/or phylogenetic divergences. Indeed,

331 seabass is characterized by a higher metabolism, which can increase the need for essential

332 metals and thus the need for binding sites such as MT. Moreover, the two species exhibited

333 significant variation in the MT sequence, potentially leading to difference in terms of

334 function. Finally, these evolutionary divergences have probably been accentuated by

335 contrasting ecological niches (*i.e.*, demersal for sea bass *versus* benthic for sole), leading sea

336 bass to be more adapted to manage Cd sequestration in MT compared to sole. Even if Cd

337 was also excreted from sole hepatocytes, Cd elimination seemed higher in sea bass (not

338 significant) since around 60% of the Cd in liver was eliminated in this species after two

339 months of depuration *versus* around 40% in sole (Le Croizier et al., 2018) (Figure S1,

340 Supporting Information). Regarding subcellular partitioning, two main mechanisms were

341 likely to give sea bass a greater Cd elimination from hepatocytes.

342 First, the large proportion of Cd bound to HSP may facilitate biliary excretion via transport of

343 Cd/GSH complexes through a specific transporter of GSH present in the canalicular

344 membrane (Zalups and Ahmad, 2003), or via transport of Cd/MT complexes as suggested by

345 recent observations of MT in bile of metal-exposed fish (Hauser-Davis et al., 2016, 2014,

346 2012) (Figure 2).

347 Second, transport of Cd/MT through plasma membrane in the sea bass may be faster than

348 vesicular exocytosis occurring in sole. Although MT release from various organs to the blood

349 has been proven, the mechanisms for epithelial transport of MT (Chan et al., 1993; Moltedo

350 et al., 2000) and Cd/MT complex transport (Moulis et al., 2014; Sabolić et al., 2010; Zalups

351 and Ahmad, 2003) are unknown. FIHUREby the classical vesicular secretory pathway while

352 transport across membranes must occur directly from the cytoplasm to the exterior of the

353 cell through ATP-binding cassette transporters (ABC transporters) (De Lisle et al., 1996)

354 (Figure 2). Conversely, vesicular exocytosis of MRG-derived Cd in sole must involve

355 successive steps, including fusion of vesicle and plasma membranes, which induces a slower

356 removal of Cd compared to direct excretion in sea bass.

357

358 **CONCLUSIONS**

359 The present study identified two different Cd elimination pathways in European sea bass

360 *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis*, exposed for two months to an

361 environmentally realistic dietary Cd dose. The species-specific strategies imply differences in

362 metal transport and sensitivity to Cd toxicity. In sea bass, Cd was mainly bound to

363 metallothionein-like proteins and would be excreted through bile or released into the blood.

364 In sole, granules-bound Cd would be sequestered by organelles and may thus be responsible

365 for a greater sensitivity of sole to Cd toxicity, revealed by an alteration of the whole-body

366 condition due to fat consumption. These results moreover bring significant novelties

367 demonstrating that MT and MRG are involved in metal elimination from liver, through either

368 biliary excretion or transport to other tissues, as these cell components were so far

369 considered as long term metal storage fractions (Vijver et al., 2004; Wallace et al., 2003;

370 Wang and Rainbow, 2010). Future studies should therefore focus on the membrane

371 transport mechanisms of MT and MRG, to fully understand the implications of elimination

372 pathways on metal retention and sensitivity of marine fish species to toxic elements such as

373 Cd.

374

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380

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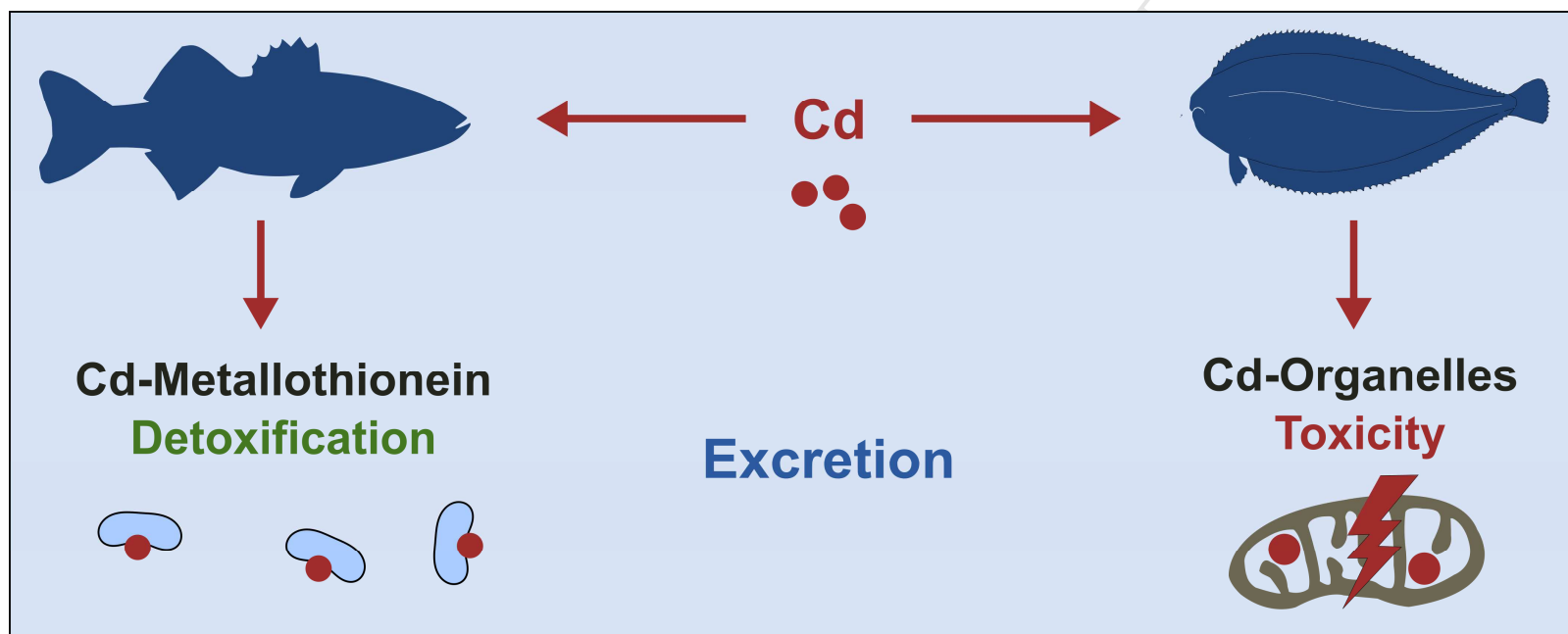
		Subcellular partitioning (%)					
		Organelles		**MRG**		**MTLP**	
Species	Element	Control	Exposed	Control	Exposed	Control	Exposed
D. labrax	Cd	20.1 ± 0.9	15.4 ± 4.7	1.3 ± 0.3	1.6 ± 0.7	53.7 ± 1.4	61.8 ± 5.6
	Ca	18.4 ± 16.3	12.9 ± 3.1	53.8 ± 42.8	69.3 ± 4.1	13.2 ± 12.6	9.6 ± 3.7
	Cu	20.2 ± 1.2	16.5 ± 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**
S. senegalensis	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 *Dicentrachus 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).
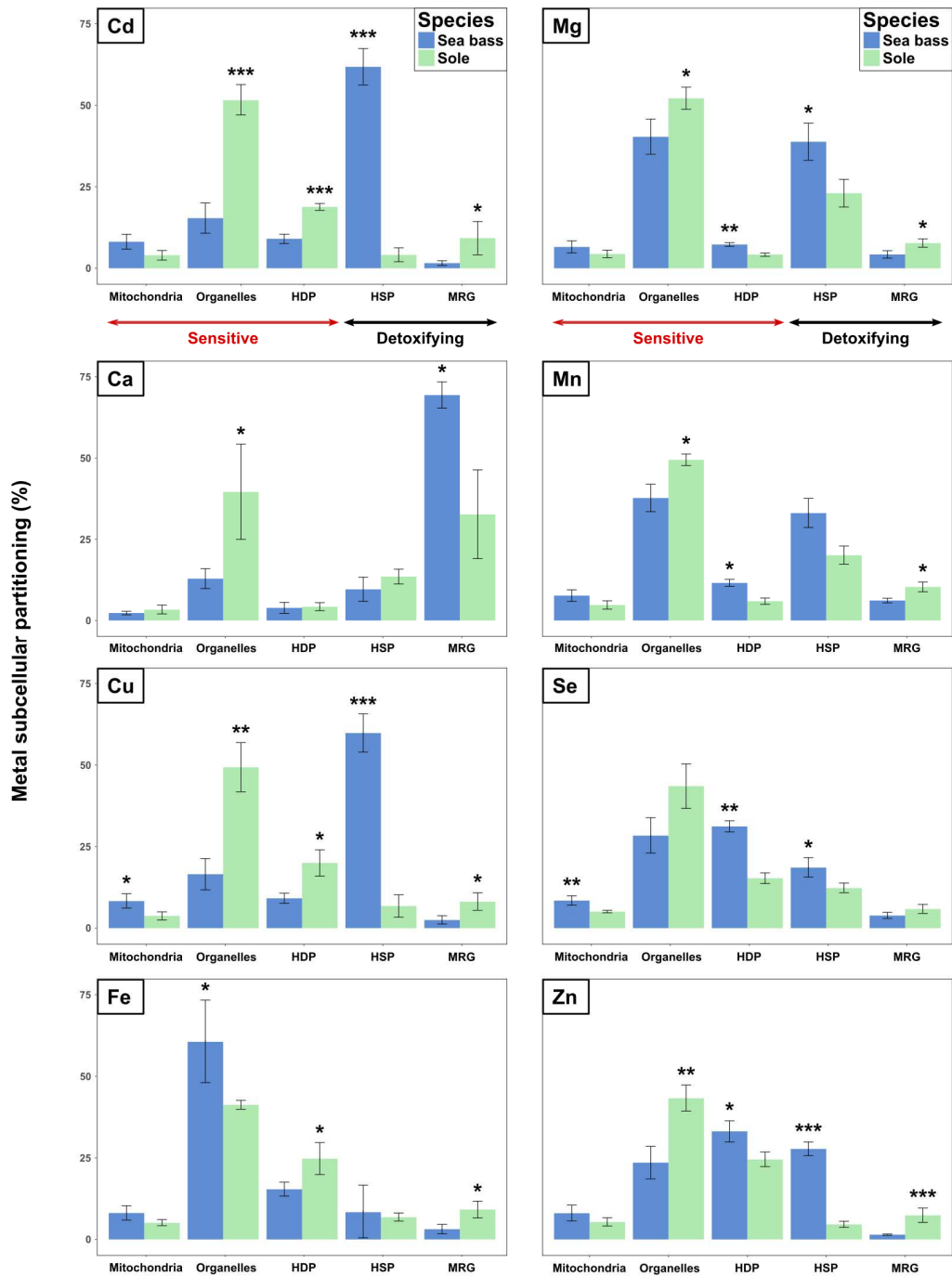
Graphical abstract

Figure 1: Subcellular partitioning of cadmium (Cd) and essential metals (% of the total metal contained in each fraction) in the liver of sea bass *Dicentrachus 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 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
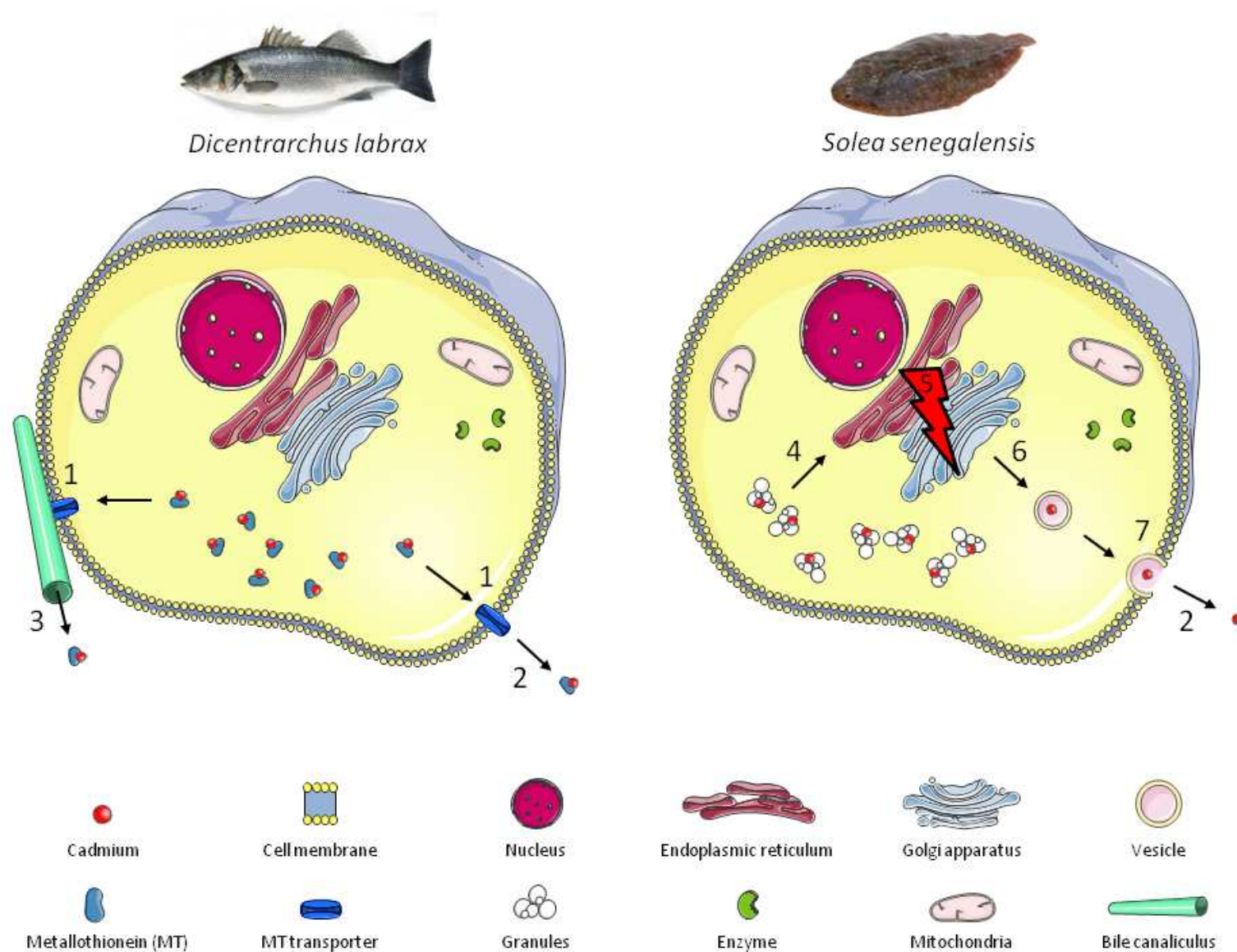
Figure 2: Subcellular Cd dynamics in hepatocytes of sea bass *Dicentrachus labrax* and Senegalese sole *Solea senegalensis*. Only the two main mechanisms of Cd handling are represented: binding to metallothionein in sea bass and binding to granules in sole. 1: Excretion of Cd/MT complexes through MT 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.
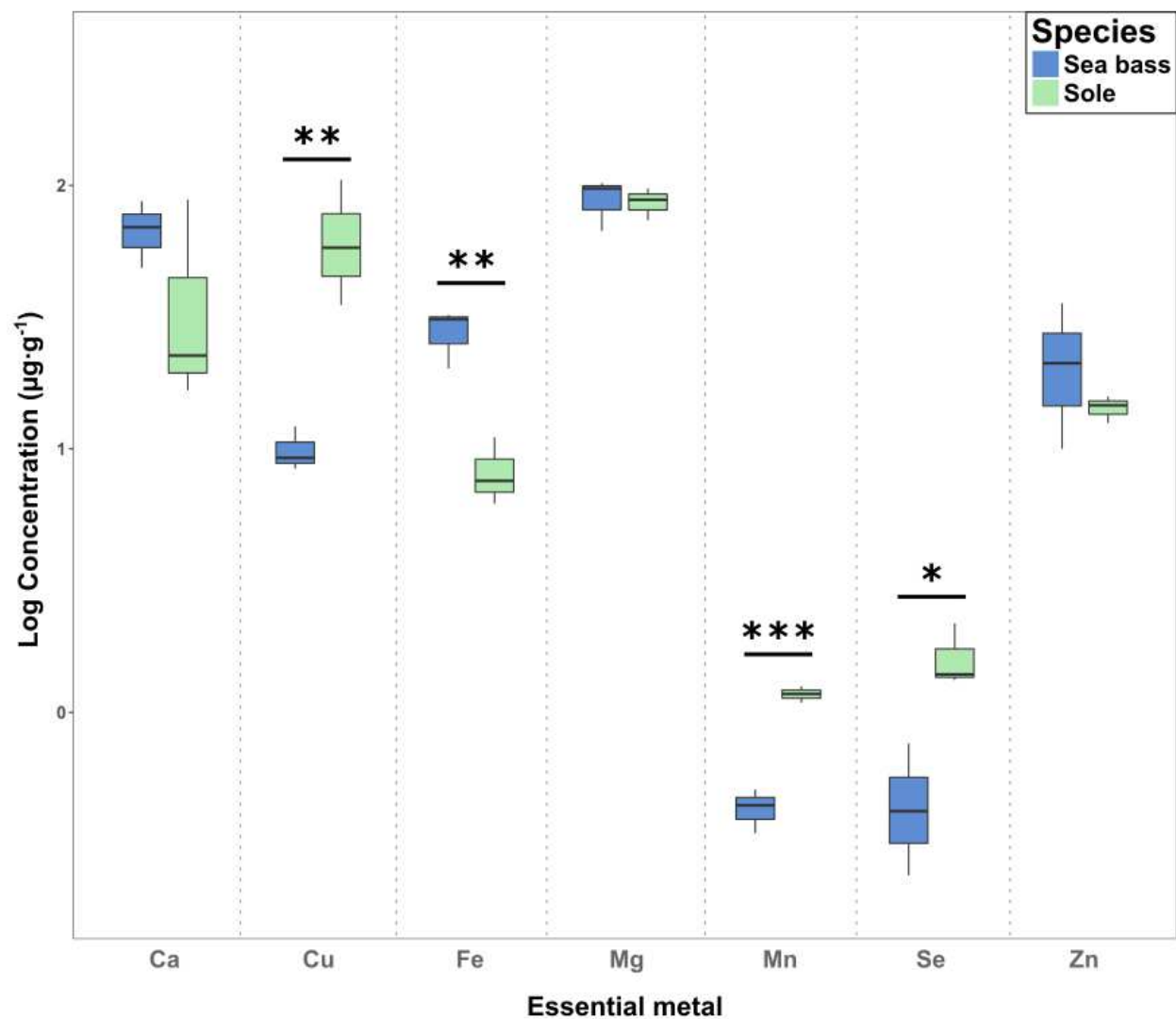
Figure 3: Total essential metal concentrations (log transformed values, μg·g^{-1} dw) in the liver of sea bass *Dicentrachus labrax* and Senegalese sole *Solea senegalensis* after 60 days of dietary Cd exposure (n = 3). Significant differences between species (ANOVA) are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. No differences were found in total essential metal concentrations between control and exposed fish of the same species (see Supporting Information).
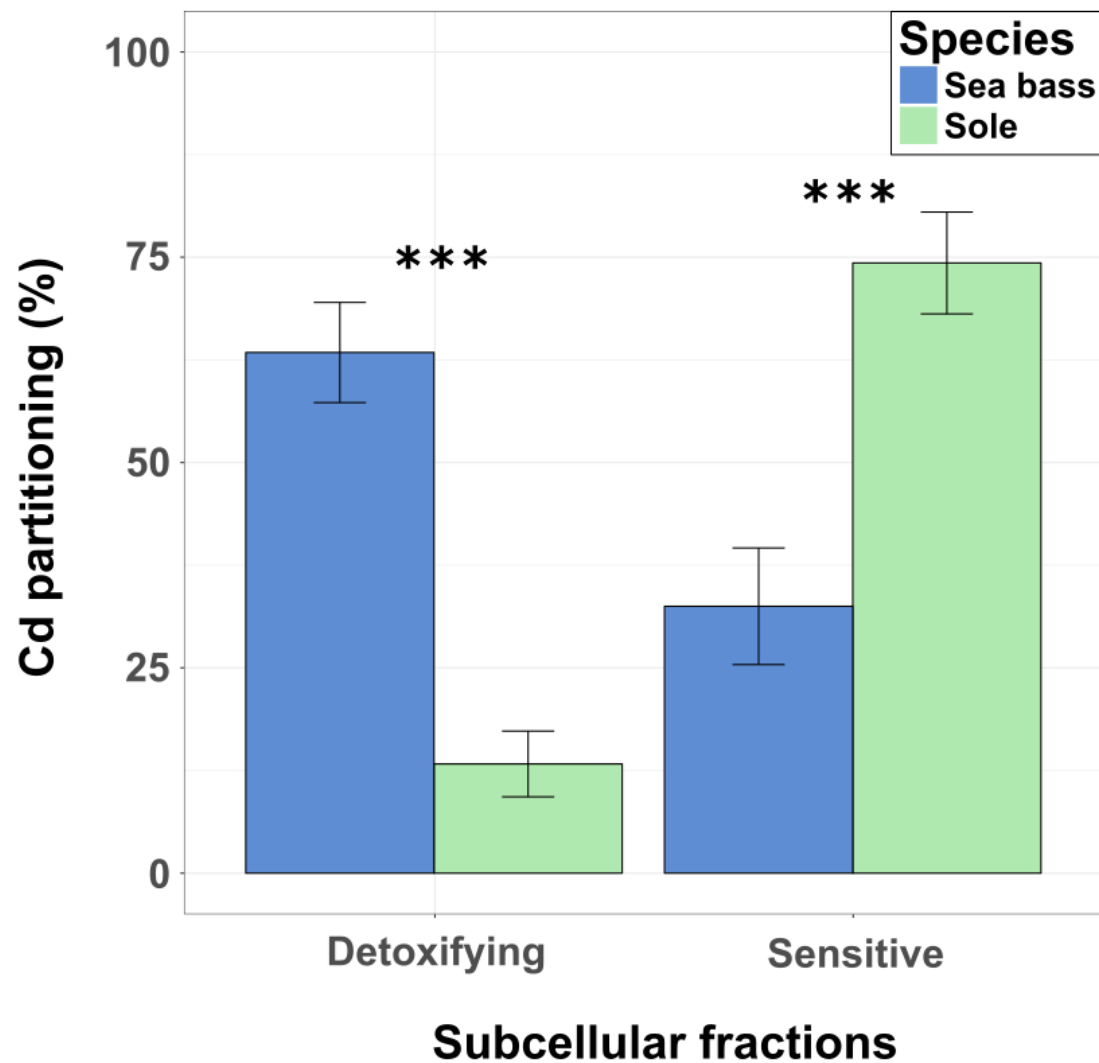
Figure 4: Subcellular partitioning (% of the Cd contained in each fraction type, mean ± sd) of Cd after 60 days of depuration in the liver of sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* (n = 3). Sensitive fractions = mitochondria + HDP + organelles; detoxifying fractions = MRG + HSP. Significant differences between species (ANOVA) are indicated by *** $p < 0.001$.
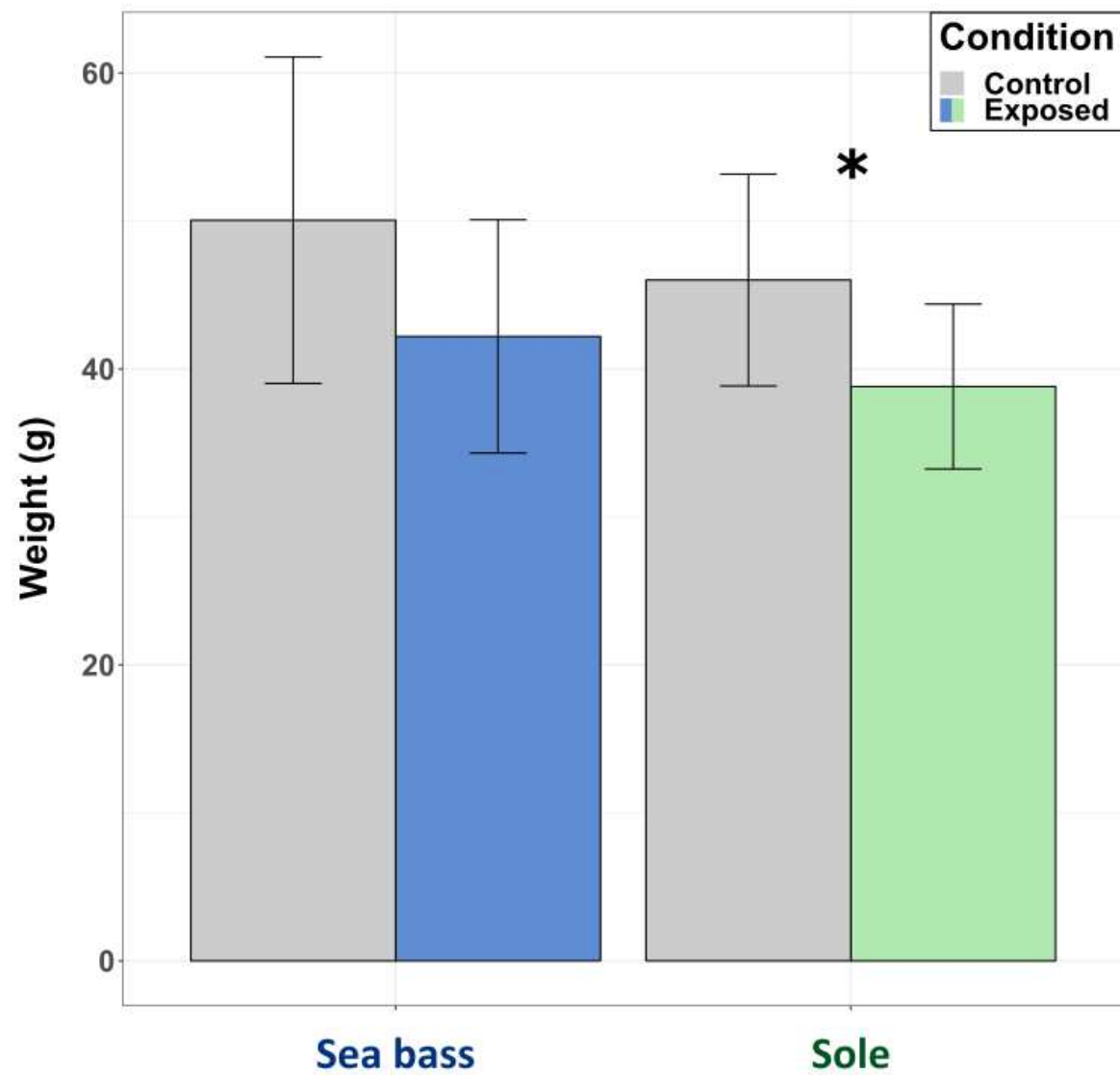
Figure 5 : Weight (g) (mean ± sd) of sea *bass Dicentrarchus labrax* and Senegalese sole *Solea senegalensis* after 60 days of Cd dietary exposure for both treatments (exposed and control fish, n = 3). Significant differences between conditions (ANOVA) are indicated by * p < 0.05.

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