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1 **Molecular and phenotypic responses of Japanese medaka**
2 **(*Oryzias latipes*) early life stages to environmental**
3 **concentrations of cadmium in sediment**

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18

19 **Abstract**

20 Japanese medaka embryos were exposed to environmental concentrations of cadmium (Cd) to investigate
21 adverse and adaptive responses in fish early life stages. Embryos were exposed during their whole development
22 by static sediment-contact to environmental Cd concentrations (2 and 20 µg/g dry weight). Cd bioaccumulation,
23 developmental defects, biochemical and biomolecular (qRT-PCR) responses were analyzed in embryos and
24 hatchlings. A dose-dependent increase of Cd bioaccumulation and developmental defects was observed at
25 hatching. Cd had clear impacts on heart beat and cardiac morphogenesis and also induced to spinal deformities.
26 The profile and the level of gene transcription were differentially modulated according to the Cd concentration,
27 the duration of exposure and/or the developmental stage of fish. Pro-apoptotic *bax* and DNA repair *rad51*
28 transcripts were significantly repressed in embryos exposed to the highest Cd concentration. Repression of these
29 genes was correlated to the increase of heart rate in 6 day-old embryos. NADH-dehydrogenase *nd5* gene
30 transcription was inhibited in larvae at the lowest concentration suggesting mitochondrial respiratory chain
31 impairment, in association with Cd-induced teratogenicity. Finally, *wnt1* gene was overexpressed indicating
32 putative deregulation of Wnt signaling pathway, and suggested to be implied in the occurrence of some spinal
33 and cardiac deformities. Results of this study permitted to propose some promising markers at the transcriptional
34 and phenotypical level, responding to environmental concentrations of Cd. The present work also highlights the
35 usefulness of the modified version of the Medaka Embryo-Larval Assay with Sediment-contact exposure
36 (MELAc) to investigate the toxicity and the modes of action of sediment-bound pollutants.

37

38 *Keywords:* Cd-spiked sediment; fish embryos; Cd bioaccumulation; gene transcription; metallothionein content;
39 heart rate; developmental abnormalities.

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41

42 **1. Introduction**

43 Cadmium (Cd) is a non-essential metallic trace element whose dispersion in the environment increased over past
44 decades due to its widespread industrial use and as a by-product of zinc and lead production. Several field
45 studies showed that Cd contamination can persist many years in the environment because of its storage in
46 sediment and its further release into the water column under favorable hydrodynamic conditions (Audry et al.
47 2004a; Coynel et al. 2007). This phenomenon could result in a long-term Cd bioaccumulation in various aquatic
48 organisms, even in those living several hundreds of kilometers from the initial source of contamination
49 (Baudrimont et al. 2005).

50 In addition to its widely recognized carcinogenicity and mutagenicity, Cd was also reported as an important
51 teratogenic and embryotoxic chemical in several fish species (Hallare et al. 2005; Brinkman and Hansen, 2007;
52 Benaduce et al. 2008; Cao et al. 2009; Barjhoux et al. 2012) and several studies already investigated gene
53 expression modulation (Weil et al. 2009; Hsu et al. 2010) after exposure of fish embryos to Cd. However, little is
54 known about the effects of Cd store in the sediment at a molecular level in fish early life stages (ELS) and the
55 possible link between gene transcription disturbances and phenotypic impairments. *In vitro* studies demonstrated
56 the inhibitory potential of Cd on the mitochondrial electron transfer chain, resulting in reactive oxygen species
57 (ROS) overproduction (Wang et al. 2004). Moreover, Cd-induced oxidative stress was shown to lead to DNA
58 damage and apoptotic cell death (Risso-de Faverney et al. 2004). Cd is also known to disturb cell cycle
59 progression, cell proliferation and differentiation (Bertin and Averbeck 2006). Among Cd detoxification
60 mechanisms, the one most studied relies on low molecular weight cytosolic proteins named metallothioneins
61 (MT). Their cysteine-rich structure gives them a high potential to sequester metals such as Cd (Coyle et al. 2002;
62 Baudrimont et al. 2003). They are also known for their antioxidant properties (Yang et al. 2009).

63 In the present study, we propose a cross-analysis of Cd-spiked sediments impacts on Japanese medaka (*Oryzias*
64 *latipes*) embryos both at phenotypic and molecular levels. Indeed, to our knowledge very few studies, if any,
65 analyzed in parallel phenotypic and gene expression together with Cd accumulation. The aim is thus to
66 understand the molecular and biochemical mechanisms underlying –or behind– a specific phenotypic response
67 and to identify molecular or biochemical markers as early warning tools of subsequent negative effects at a
68 higher level in the biological organization (i.e. at the individual scale).

69 To meet this objective, we used a modified version of the Japanese medaka embryo-larval assay with sediment-
70 contact exposure (MELAc) (Vicquelin et al. 2011; Barjhoux et al. 2012). This test was improved adding several
71 biochemical analyses to the range of existing non-invasive markers of embryotoxicity and teratogenicity. One

72 day-old medaka embryos were exposed to Cd-spiked sediment at 2 and 20 µg/g dry weight (dw) for 7 days post-
73 fertilization (dpf; embryonic stage analyses) or during their whole embryonic development (i.e. up to hatching at
74 9 dpf, larval stage analyses). These concentrations of Cd can be measured in areas of low to moderate metallic
75 contamination and have already been characterized as sublethal but highly teratogenic to medaka ELS (Barjhoux
76 et al. 2012). Cd bioaccumulation was monitored in embryos and newly hatched larvae. Survival, hatching, heart
77 rate, biometric measurements and developmental abnormalities were recorded during the course of exposure. In
78 parallel, qRT-PCR analysis of target gene transcription level was carried out in embryos and newly hatched
79 larvae in order to investigate Cd mechanisms of toxicity. A panel of 12 genes was selected for this purpose due
80 to their involvement in metal sequestration, antioxidant defense, mitochondrial metabolism, DNA repair, cell
81 cycle regulation and apoptosis. Potential induction of metal-specific detoxification mechanism was examined
82 through MT protein level measurement in association with their gene (*mt*) transcription level. The response to
83 oxidative stress was studied through cytoplasmic (*sod(Cu/Zn)*) and mitochondrial superoxide dismutase
84 (*sod(Mn)*) genes transcription. The impact of Cd on the mitochondrial respiratory chain was investigated using
85 NADH-dehydrogenase subunit V (*nd5*) and cytochrome C oxidase subunit I (*cox1*) transcripts. Two other genes,
86 the *oggl* (8-oxoguanine glycosylase 1) and *rad51* genes, were selected for their involvement in DNA repair
87 mechanisms. Cd-induced apoptosis was studied through Bcl-2 associated X protein (*bax*) and *p53* gene
88 transcription levels. The last pair of genes was selected to examine the impact of Cd on the transcription of genes
89 involved in embryogenesis and morphogenesis: the empty spiracles homolog 2 (*emx2*) gene for its involvement
90 in central nervous system development, and the wingless integration site 1 (*wnt1*) gene for its key role in cell
91 differentiation and proliferation.

92 The novelty of this work relies in that: (i) it combines the analysis of Cd accumulation, developmental effects
93 and gene expression at two different developmental stages (e.g. embryo and pro-larva) of a model fish species;
94 (ii) it uses a realistic sediment-contact exposure scenario to mimic exposure of fish embryos developing in direct
95 contact to sediment. The most important issue is to underline the link between the early molecular responses and
96 those observed at the individual level in order to describe the mechanisms implied in the phenotypic endpoints
97 measured and to consider if these responses can be used as early markers of these impacts.

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102 **2. Material and Methods**

103 *2.1. Experimental design*

104 Medaka embryos at one dpf were exposed to three different Cd-spiked sediments: a C0 control condition at 0 µg
105 Cd/g dw of sediment; a C1 condition at 2 µg Cd/g dw; and a C2 condition at 20 µg Cd/g dw. Each treatment
106 consisted of six replicates of 100 embryos each. The first three replicates of each treatment were dedicated to
107 sampling at the embryonic stage (7 dpf, T7 i.e. corresponding to the last steps of embryonic organogenesis at
108 26 °C (Iwamastu 2004)) and the three remaining ones kept for larval stage samplings (9 dpf, T9 i.e.
109 corresponding to the hatching day at 26 °C, thus the end of the sediment-contact exposure and the very early
110 larval stage).

111 Medaka embryos remained in direct contact with the sediment up to sampling time, i.e. for 7 dpf (T7) or
112 hatching time (T9), depending on the replicate under consideration. Embryos and larvae were incubated in a
113 climate cabinet (Snijders Scientific; 26 ± 0.3 °C; 12L:12D; 5000 lx white light) under static non-renewal
114 exposure conditions, in egg rearing solution (ERS: 17.11 mM NaCl; 0.4 mM KCl; 0.36 mM CaCl₂; 1.36 mM
115 MgSO₄; pH 7.0). The experiment ended when all T9-samples were completed, i.e. at 9 dpf.

116 Sampled embryos/larvae were divided into pools of adequate size to perform several analyses including MT
117 proteins content determination, gene transcription analysis by quantitative reverse transcription polymerase chain
118 reaction (qRT-PCR) and Cd bioaccumulation measurement in the whole body of embryos (T7) and larvae (T9).

119 A wide range of non-invasive phenotypic endpoints were also investigated to complete this biochemical
120 approach and as described in previous work from our laboratory (Barjhoux et al. 2012). They included
121 embryonic survival, heart beat, biometric measurements, hatching success, time taken to hatch and the
122 occurrence of developmental abnormalities.

123 Finally, the time-course of Cd contamination was followed in both the sediment and aqueous phases (i.e. in egg
124 rearing solution, ERS) at T0 (just before exposure began) and at each sampling time dedicated to the molecular
125 approach (T7 and T9).

126

127 *2.2. Reference sediment characterization*

128 The reference sediment was collected in the Lot river on the Marcenac site (December 2008, SW France), which
129 is considered as a pristine site for metal contamination in the Lot/Garonne/Gironde continuum (Audry et al.

130 2004a, 2010). The reference sediment was stored at -20 °C, then freeze-dried and crushed slightly using a mortar
131 and a pestle to eliminate larger particles and homogenize the grain size before use.

132 The physico-chemical characteristics of the sediment were analyzed using the process described by Vicquelin et
133 al. (2011). As shown in Table 1, Marcenac sediment can be defined as a very fine sand (according to the
134 modified Wentworth sediment classification) with low organic carbon content. Chemical analyses of this
135 sediment showed a weak presence of trace metallic elements and very marginal contamination with persistent
136 organic compounds (Table 1).

137

138 *2.3. Sediment spiking procedure*

139 Cd spiking levels were selected according to Cd concentrations measured in the Lot-Garonne-Gironde
140 continuum, well-known for its historical contamination with metals. The C2 concentration (20 µg Cd/g dw) was
141 selected according to Cd levels in sediments from moderate to highly contaminated sites along the Lot river

142 whereas the C1 concentration (2 µg Cd/g dw) represents the current level of contamination found, for instance,
143 in sediments from the Garonne river (Blanc et al. 1999; Audry et al. 2004a, b). Moreover, these concentrations
144 are representative of those found in several contaminated sediments from various locations worldwide (Table 2).

145 The spiking procedure was similar to the protocol proposed by Barjhoux et al. (2012) with some adjustments
146 related to the larger amount of sediment needed. The spiking solutions were obtained by dilution of a Cd-stock
147 solution at 2.5 mg Cd/mL (CdCl₂:2H₂O, Fluka Chemie) in ultrapure water (Milli-Q Maxima, Elga Labwater,

148 Veolia water). Crushed and freeze-dried sediment (65 g dw) was placed in a 1L-beaker. One mL of Cd spiking
149 solution (or ultrapure water for the control treatment) was added, followed by 35 mL of ultrapure water. The
150 sediment remained in contact with the Cd solution for 1 h under agitation. Then, most of the supernatant was

151 removed and the sediment was kept all night at room temperature for partial drying. The sediment moisture
152 content was gravimetrically determined to calculate the wet weight equivalent to 17 g dw for each spiking
153 condition. Finally, sediment from the same treatment was divided into six aliquots of 17 g dw equivalent (for

154 embryos exposure) and one aliquot of approximately 5 g dw equivalent dedicated to Cd analysis.

155

156 *2.4. Medaka embryo exposure*

157 Each aliquot of 17 g dw of spiked sediment was laid in a 65 mm-diameter plastic Petri dish and immersed by
158 adding 5 ml of ERS. The resulting system was then maintained at 26 °C for a 4–5 h equilibration period before

159 the beginning of the experiment. Medaka embryos (i.e. 24 h post-fertilization, hpf) were purchased from GIS
160 AMAGEN (Gif-sur-Yvette, FR). Healthiness and developmental stage synchronism of the embryos were
161 checked using a stereomicroscope (Leica MZ75, Leica Microsystems) and cold light source (Intralux® 4100,
162 Volpi AG). Immediately after sorting, embryos were randomly placed on a Nytex® mesh (mesh opening
163 1000 µm, Sefar Filtration Inc.). The Nytex® grid was then slightly sunk into the sediment. Afterwards, embryos
164 were kept exposed to the sediment until T7- or T9-sampling time. At the end of the experiment, the remaining
165 embryos or larvae were euthanized using MS222 (Sigma-Aldrich) solution at 1 g/L.
166 During the exposure period, the level of aqueous media was re-adjusted in case of evaporation, adding an
167 adequate volume of clean ERS. Dissolved oxygen was also checked daily at the water-sediment interface using
168 an oxygen optical microsensor (NeoFox® Foxy probe, Ocean Optics sensors, Idle Fibres optiques). This
169 measurement confirmed good oxygenation of the medium with values always superior to 80 % saturation (data
170 not shown).

171

172 2.5. *Phenotypic endpoints*

173 The different procedures performed for phenotypic endpoint assessments were previously described in Barjhoux
174 et al. (2012). Viability was checked daily for all individuals and all conditions. Heart rate was monitored in 6-
175 and 7-dpf embryos (5 randomly selected individuals per replicate, analyses were performed in the same
176 replicates for the two series of measurements) while biometric measurements and developmental anomalies
177 (spinal, craniofacial, ocular, cardiovascular, yolk-sac and edema) were observed in newly hatched larvae (15
178 randomly selected individuals per replicate). All the observations were done using a stereomicroscope (MZ75,
179 Leica Microsystem) and a cold light source (Intralux® 4100, Volpi AG), in an air-conditioned room at 23 ± 1 °C.
180 For heart beat measurements, developmental abnormalities observations and biometric measurements, the
181 stereomicroscope was also equipped with a color CDD camera (Leica DFC 420C) connected to an image
182 analysis software program (Leica Application Suite v2.8.1.).

183

184 2.6. *Metallothionein (MT) proteins content*

185 The total level of MT proteins was determined in medaka embryos (T7 sampling) and larvae (T9 sampling) after
186 exposure to Cd-spiked sediments. This analysis was conducted on two samples (15 pooled individuals each) per
187 replicate, and on three replicates per treatment for each sampling time.

188 Sampled organisms were briefly dried on absorbent paper and the total weight was recorded. Afterwards,
189 samples were immediately stored in liquid nitrogen to minimize MT oxidation until analysis.
190 MT concentrations were measured with the mercury-saturation assay using cold inorganic Hg as described by
191 Baudrimont et al. (2003) with few adaptations. In summary, tissue was homogenized in 300 μ L of Tris-HCl
192 10 mM (Sigma-Aldrich, pH 7.5 at 20 °C) using an Ultra-Turrax (T10 Basic, Ika®) on ice and under N₂
193 atmosphere. The cytosolic fraction was then extracted by centrifugation (20 000 g for 1 h at 4 °C) and divided
194 into two 100 μ L-aliquots for a duplicated analysis. Then, 100 μ L of HgCl₂ 50 mg Hg/L in trichloroacetic acid
195 (10 %) was added to each sample to saturate MTs with Hg and to denature non-MT proteins. Excess Hg not
196 bound to the MT proteins was removed by adding 200 μ L of lyophilized pig hemoglobin (Sigma-Aldrich)
197 prepared in 30 mM Tris-HCl (pH 8.2 at 20 °C), mixing and immediate centrifugation (20 000 g for 20 min at
198 room temperature).
199 The final supernatant was then quantitatively recovered and used for Hg determination by flameless atomic
200 absorption spectrometry (AMA 254, Altec). The detection limit was estimated at 0.01 ng Hg. Owing to the fact
201 that the exact quantity of Hg binding sites per MT molecule is unknown for the medaka species, MT
202 concentrations are expressed in nmol sites Hg/g wet weight (ww).
203 Each analytical run included three reference samples (blanks) prepared to monitor the Hg complexation
204 efficiency of the hemoglobin. The mean of the three blank values was deducted from the Hg burden measured in
205 the samples. In addition, a recovery percentage from purified rabbit liver MT (Alexis biochemical) was
206 systematically determined. For the analysis performed in the present study, the recovery percentages (between
207 94 % and 97 %) were consistently within the certified ranges (100 \pm 20 %) of the method.

208

209 2.7. *Gene transcription analysis*

210 Gene transcription analysis was conducted on three samples of 8 individuals per replicate, and on three replicates
211 per treatment at each sampling time (T7 and T9). Immediately after sampling, each pool of embryos or larvae
212 was entirely immersed in RNase-free microtubes containing 200 μ L RNA Later® (Qiagen) and quickly frozen
213 by dipping in liquid nitrogen. Samples were then stored at -80 °C until RNA extraction.
214 Total RNA extraction was performed using an Absolutely RNA® Miniprep kit (Stratagene, Agilent) according
215 to the manufacturer's instructions, with an additional phenol-chloroform-isoamyllic acid (25:24:1, v/v)
216 purification step. The quality and the quantity of the extracted RNA were determined by spectrophotometry at
217 260 and 280 nm.

218 First-strand cDNA was synthesized using an AffinityScript™ Multiple temperature cDNA Synthesis kit
219 (Stratagene, Agilent). Briefly, 1 µL of oligo(dT) (1 µM), 1 µL of random primers (1µM), 0.8 µL of dNTPs
220 (10 mM) and 2 µL of AffinityScript™ RT buffer (10X) were mixed together with 14 µL of the previously
221 extracted RNA (approximately 5 µg). The mixture was then incubated in a thermocycler (MasterCycler pro™,
222 Eppendorf) for 5 min at 65 °C. cDNA synthesis was performed by adding 1 µL of reverse transcriptase (1 U/µL)
223 and 0.5 µL of RNase block (0.5 U) and then by incubating the mixture at 42 °C for 1 h. cDNA samples were
224 stored at -20 °C until real-time PCR was performed.

225 The coding sequences of the 12 selected genes were obtained from the GenBank (PubMed – NCBI) and HGNC
226 (Ensembl, EMBL – EBI) databases. The accession number of each coding sequence is reported in Table 3. For
227 each gene, specific primer pairs were determined using the LightCycler probe design software (v1.0, Roche) and
228 are mentioned in Table 3. Primers were purchased from Sigma-Aldrich (Easy Oligo™).

229 The amplification of cDNA was monitored using the DNA intercalating dye SyberGreen I. Real-time PCR
230 reactions were performed using a LightCycler® (Roche) and LC FastStart DNA Master SybrGreen kit (Roche)
231 according to the manufacturer's instructions. PCR reactions were prepared in glass capillaries adding 1 µL of
232 master mix containing SyberGreen fluorescent dye, 3.2 µL of MgCl₂ (25mM), 12.8 µL of ultra-pure water, 2 µL
233 of primer-pair mix (3 µM) and 1 µL of cDNA. Afterwards, PCR reactions consisted of an activation cycle
234 (10 min at 95 °C) and 45 amplification cycles (5 s at 95 °C, 5 s at 60 °C and 20 s at 72 °C). The cycle threshold
235 (Ct), i.e. the number of cycles required for the fluorescent signal to cross the background level, was determined
236 for each studied gene. The specificity of each amplification was determined from the dissociation curve of the
237 PCR product. These dissociation curves were obtained by following the SyberGreen fluorescence level during a
238 gradual heating of the PCR products from 60 to 95 °C (0.05 °C/s). Relative quantification of each gene
239 transcription level was normalized according to *β-actin* gene transcription level. Indeed, relative gene
240 transcription level was determined using the 2^{-ΔCt} method described by Livak and Schmittgen (2001), where ΔCt
241 represents the difference between the cycle threshold of a specific gene and the Ct of the *β-actin* gene. The so
242 obtained relative transcription level of each studied gene is reported in the online supplementary data file (Online
243 Resource 1) for each treatment and each sampling time. Then, the induction factor (IF) of each studied gene for
244 Cd treatments in comparison with the control treatment was calculated using the following equation:

$$245 \quad IF = \frac{2^{-\Delta Ct (Cd \text{ treatment})}}{2^{-\Delta Ct (Control \text{ treatment})}}$$

246 An IF < 1 indicates gene transcription repression whereas an IF > 1 illustrates gene transcription induction.

247

248 2.8. *Cd analysis in sediments, water phase and tissues*

249 Immediately after sampling, each aqueous sample (3-4 mL of ERS medium) was separated from the sediment
250 through a 15 min-centrifugation at 4000 rpm at room temperature and then acidified with 1 % final (v/v) of 65 %
251 nitric acid. Sediment and ERS samples were stored at -20 °C until Cd analysis.

252 Just prior to Cd measurement, approximately 1 g ww of each sediment sample was dried for 48 h at 60 °C and
253 then digested with 3 mL of 65 % nitric acid for 3 h at 100 °C. After mineralization, samples were diluted by
254 adding 15 mL of ultrapure water (Milli-Q). Cd measurements in the ERS medium and sediment were performed
255 using an atomic absorption spectrophotometer (Varian SpectrAA 220FS, Agilent Technologies, detection
256 limit = 0.01 µg/L). Each series of analyses included method blanks and certified reference materials (Tort-2,
257 lobster hepatopancreas, NRCC-CNRC). Values from standards were consistently within certified ranges
258 (recovery percentages ranging between 105 % and 110 %).

259 Cd bioaccumulation in tissues was analyzed in two pools of 15 individuals per replicate (three replicates per
260 treatment) at T7 (on embryos) and T9 (on larvae). Sampled organisms were dried slightly on absorbent paper
261 and weighted before being stored at -20 °C until Cd analysis. Samples were then digested with 200 µL of 65 %
262 nitric acid for 3 h at 100 °C. Then, each sample was diluted by adding 1 mL of ultrapure water. As described for
263 Cd analysis in ERS medium and sediment, each series included method blanks and Tort-2 certified samples
264 which showed the recovery percentage to be within certified ranges (values comprised between 98 % and
265 101 %). Cd concentrations were measured by atomic absorption spectrophotometry with Zeeman correction,
266 using a graphite tube atomizer (M6 Solaar FS98, Thermo Elemental®). In order to avoid interference, analyses
267 were carried out in a tube atomizer with a blend of Pd (2 g/L) and Mg(NO₃)₂ (1 g/L).

268

269 2.9. *Statistical analysis*

270 The data is expressed as mean ± standard deviation (SD). All statistical analyses were conducted using Statistica
271 7.1 software (Statsoft). Results were initially tested for normality (Shapiro-Wilk's test on residues with 1 % risk)
272 and homoscedasticity (Brown-Forsythe's test, 5 % risk). When necessary, data was transformed to fulfill
273 normality and homoscedasticity criteria. Afterwards, significant differences between treatments were tested with
274 a one-way or two-way ANOVA analysis followed by post-hoc Tukey HSD or Dunnett test ($p < 0.05$). If data
275 transformation was not sufficient to perform parametric analysis, non-parametric Kruskal-Wallis' test followed
276 by Bonferroni-Dunn's post-hoc test were used ($p < 0.05$). Relative gene transcription data (expressed as control-

277 relative induction factor) was statistically analyzed using *t* test for independent samples (only pairing
278 comparisons with the control treatment were performed). Finally, correlation analysis were performed to
279 investigate potential significant ($p < 0.05$) associations between (i) Cd bioaccumulation and concentrations in
280 ERS medium and sediment (Pearson correlation analysis on log-transformed data), and (ii) gene transcription
281 levels and phenotypical endpoints (Spearman rank order correlation analysis on raw data).

282

283 **3. Results and discussion**

284 The present study proposes a combined analysis of phenotypic and molecular responses induced by Cd in
285 medaka early life stages. A wide range of endpoints were investigated in embryos and larvae, including
286 phenotypic markers of embryotoxicity and teratogenicity, MT protein dosage, Cd bioaccumulation and gene
287 transcription analysis at the mRNA level, in order to link the different responses observed.

288

289 *3.1. Concentration-dependent increase of Cd in sediment, water column and fish*

290 Cd concentration measurement in spiked sediments and water column revealed a clear concentration-dependent
291 increase of Cd content resulting in significant differences between treatments (Table 4). Spiking efficiencies,
292 calculated by comparison between measured and nominal Cd contents in sediment at T0 (for these calculations,
293 Cd background level in Marcenac sediment was deduced) were satisfying with values ranging from 75% (C1) to
294 80 % (C2). Cd concentrations measured for the same condition at T0, T7 and T9 were not statistically different
295 for each treatment, indicating that Cd contamination in sediments remained stable over time.

296 Cd concentration in the aqueous phase of the control was low and remained stable throughout the exposure
297 period (Table 4). This marginal contamination of the ERS medium is probably due to partial desorption of the
298 natural background Cd present in Marcenac sediment. Cd content in the ERS medium significantly increased in
299 C1 and C2 treatments in comparison to control condition. The mean Cd-C1 concentration in the medium was
300 higher at T7 than at T9 whereas it declined over time for the C2 treatment (Table 4). However these time-
301 dependent variations were not statistically significant. Nonetheless, these results indicate a significant
302 contamination of the water column for C1 and C2 treatments in comparison to the controls at both sampling
303 times. Aqueous phase contamination could be the result of both Cd labile fraction desorption from the sediment
304 particles and Cd diffusion from the sediment pore-water.

305 Cd bioaccumulation in embryos (T7) and larvae (T9) increased in a concentration-dependent manner with Cd
306 concentration in the sediment (Fig. 1a). Cd accumulation was significantly higher ($p < 0.001$) in embryos
307 exposed to C1 and C2 treatments (0.27 ± 0.05 and 12.9 ± 8.16 ng Cd/mg ww, respectively) when compared to
308 the corresponding control group (0.01 ± 0.00 ng Cd/mg ww). Cd concentrations in newly hatched larvae were
309 significantly lower than in embryos (0.02 ± 0.01 and 0.94 ± 1.28 ng Cd/mg ww for C1 and C2 groups
310 respectively). However, larvae exposed to the two Cd treatments showed a significant ($p < 0.02$) Cd
311 bioaccumulation when compared to control larvae.

312 Several studies have previously reported such concentration-dependent increases of Cd accumulation in fish ELS
313 (Meteyer et al. 1988; Burnison et al. 2006). The levels of Cd bioaccumulation observed in the present study were
314 very similar to those reported in zebrafish *Danio rerio* larvae exposed for 3 h to Cd concentrations comprised
315 between $0.2 \mu\text{M}$ ($22 \mu\text{g/L}$) and $125 \mu\text{M}$ (14 mg/L) (Matz et al. 2007). In fact, these authors reported a
316 concentration-dependent increase of Cd accumulation in exposed organisms with values ranging from 0.01 to
317 11 ng/mg ww.

318 Moreover, a 93 % reduction in Cd concentration was observed in the present study when comparing embryonic
319 and larval bioaccumulation. This decrease of Cd load in organisms could be the result of the loss of Cd bound to
320 the chorion occurring at hatching. Indeed, Michibata (1981) demonstrated that more than 94 % of the total
321 accumulated Cd was actually adsorbed onto the chorion in *O. latipes* embryos exposed to 10 mg Cd/L. This
322 phenomenon could be explained by the presence of negatively charged macromolecules such as
323 mucopolysaccharides and glutamic acid in the chorion which could sequester Cd and thus limit Cd penetration
324 into the embryo (Meteyer et al. 1988). Moreover, Meteyer et al. (1988) reported that Cd accumulation varies
325 over time, probably due to physiological changes and chorion permeability modifications during embryo
326 development. Indeed, the chorion could be more permeable and present less adsorption sites to Cd before
327 hatching, which facilitates its passage to the perivitellin fluid (Meteyer et al. 1988; Gonzalez-Doncel et al. 2003).
328 Pearson's coefficient calculation demonstrated significant correlations between Cd content in embryos and ERS
329 medium ($r = 0.81$, $p < 0.001$) or sediment ($r = 0.90$, $p < 0.0001$). The higher correlation coefficient for sediment
330 might indicate that the sediment contact also acts as a non-negligible route of Cd exposure for medaka embryos.

331

332 3.2. *Absence of acute effects in Cd-exposed medaka embryos*

333 In spite of the significant Cd bioaccumulation in embryos exposed to Cd-spiked sediments, the tested
334 concentrations did not induced any lethal effect with average embryonic survival rates ranging from 94.1 % to

335 98.8 % (Table 5). These results are in agreement with those reported previously (Barjhoux et al. 2012). Hallare
336 et al. (2005) also reported no acute toxicity in *D. rerio* embryos exposed to Cd concentrations up to 10 mg/L.
337 Mean hatching success was above 90 % for all the tested concentrations and no significant differences were
338 noted between treatments (Table 5). The average time to hatch was also very similar for the different treatments
339 with mean values comprised between 8.6 and 8.8 dpf (Table 5). As previously observed, Cd did not affect the
340 kinetic of medaka *in ovo* development at the tested concentrations and under our exposure conditions (Barjhoux
341 et al. 2012). The absence of acute toxicity as well as hatching success and time to hatch impairments have
342 already been reported by Gonzalez-Doncel et al. (2003) after waterborne exposure of medaka late morula-staged
343 embryos (~5 hpf) to Cd concentrations up to 80 mg/L. However, in rainbow trout *Oncorhynchus mykiss*
344 embryos, precocious or delayed hatching was observed following exposure to 0.05-2.5 mg Cd/L (Lizardo-Daudt
345 and Kennedy 2008). All these observations seem to highlight differences in sensitivity between species. These
346 inter-specific variations in Cd-induced effects could also be magnified by the exposure conditions, in particular
347 physico-chemical parameters such as pH, water hardness and temperature which can widely influence metal
348 bioavailability (Hallare et al. 2005; Benaduce et al. 2008).

349

350 3.3. *Cd exposure induces teratogenic effects*

351 No significant impact was observed on larva body size following Cd exposure. Indeed, the average total body
352 length values were 4.66 ± 0.05 mm, 4.64 ± 0.05 mm and 4.81 ± 0.05 mm for the control, the Cd-C1 and the Cd-
353 C2 treatments, respectively. Furthermore, average head size and head/body ratio were very similar for all
354 treatments, with mean values of 1.04 ± 0.01 mm, 1.01 ± 0.03 mm and 1.05 ± 0.01 mm for the head size, and of
355 22.3 ± 0.24 %, 21.8 ± 0.55 % and 21.9 ± 0.10 % for the head/body ratio, for the control, the Cd-C1 and the Cd-
356 C2 treatments, respectively.

357 Although no acute toxicity was observed, Cd concentrations of 1.7 and 16.3 $\mu\text{g/g}$ dw sediment (up to 25.8 and
358 138 $\mu\text{g Cd/L}$ in water, respectively) induced a significant increase of the percentage of larval abnormalities (Fig.
359 1b). Indeed, 50 % (Cd-C1) to 60 % (Cd-C2) of the observed larvae at hatching presented at least one
360 developmental abnormality against 13 % in the control group. Such high percentages of abnormal larvae have
361 already been reported in several fish ELS following waterborne Cd exposure (Benaduce et al. 2008; Cao et al.
362 2009).

363 Cd exposure resulted in a significant increase in the percentage of larvae developing spinal deformities ($44.4 \pm$
364 7.7 %) and cardiovascular injuries (36.7 ± 14.1 %) in the C1 and C2 treatments, respectively (Fig. 1b). The

365 observed spinal deformities included mainly kyphosis, lordosis and C-shaped larvae. Similar developmental
366 abnormalities have been reported in fish ELS exposed to Cd (Blechinger et al. 2002; Cao et al. 2009). Cd-
367 induced spinal curvature is thought to be linked to a reduction of myosin heavy chains and a concomitant
368 disorganization of the myotomes in the somites (Chow and Cheng 2003).

369

370 3.4. Cd exposure affects both heart morphogenesis and functioning

371 Over a third of the newly hatched larvae exposed to the highest Cd concentration showed cardiovascular
372 impairments (Fig. 1b), mainly abnormal heart positioning and heart looping. However, the first sign of cardiac
373 disturbance was recorded in embryos when examining their heart rate. The highest tested Cd concentration
374 induced significant tachycardia ($p < 0.01$) in 6 dpf-embryos when compared to control embryos (Fig. 1c). A
375 similar impact on heart beat has already been reported in Medaka embryos and could be interpreted as a stress
376 response of the organism to metal exposure (Johnson et al. 2007; Barjhoux et al. 2012). Conversely, a significant
377 decrease ($p < 0.05$) in heart rate was observed in 7 dpf-embryos exposed to the same concentration of Cd in
378 comparison to the control (Fig. 1c). Bradycardia was also reported in red sea bream and zebrafish embryos
379 exposed up to 2.4 mg Cd/L (Hallare et al. 2005; Cao et al. 2009). These authors interpreted the inhibition of
380 heart beat as a result of impaired ionic channels, such as Ca^{2+} -ATPases causing reduced Ca^{2+} intake. Moreover,
381 blood flow disturbance was shown to affect cardiogenesis and resulted in cardiac morphological alterations
382 (Hove et al. 2003). Interestingly, the impairments described by Hove et al. (2003) are very similar to those
383 reported in the present study, and included abnormal positions of heart chambers in relation to each other and to
384 the cephalo-caudal axis, due to incomplete heart looping. These observations may indicate that Cd-induced
385 impact on heart rate could be, at least in part, involved in the developmental defects observed on the cardio-
386 vascular system of exposed larvae. Heart rate in 6 dpf-embryos proved to be positively correlated to the
387 occurrence of cardiovascular injuries in newly hatched larvae (Spearman r value = 0.747, $p < 0.05$), supporting
388 the above-mentioned causal relationship between cardiac function and morphogenesis. This also suggests that
389 heart beat measurement at the embryonic stage could serve as an early marker of cardiovascular injuries. Indeed,
390 as many aspects of cardiac remodeling and maturation are dependent on function (Glickman and Yelon 2002), it
391 is quite possible that alterations of cardiac function, even transiently, may impact later stages of heart
392 development, and perhaps in an irreversible way (Incardona et al. 2004).
393 Additionally, 6 dpf-heart rate was also negatively correlated ($p < 0.05$) to the transcription levels of *mt*
394 (Spearman r value = -0.810), *sod(Cu)* ($r = -0.738$), *rad51* ($r = -0.762$) and *bax* ($r = -0.738$) genes in embryos

395 (T7). These two later gene transcripts were significantly modulated following Cd exposure (see part 3.7 for
396 details). As a result, heart beat measurement in 6 dpf-embryos may also be considered as a phenotypical and
397 non-invasive marker of more general molecular and cellular injuries induced by Cd.

398

399 3.5. *Metallothionein content and mt gene transcription level remain unchanged following Cd* 400 *exposure*

401 MT concentrations in control organisms varied between 8.52 ± 1.19 and 12.2 ± 5.16 nmol sites Hg/g ww at T7
402 and T9, respectively (Data not shown). Levels observed in Cd-treated groups were slightly lower, with means
403 ranging from 4.99 ± 1.59 and 7.66 ± 2.66 nmol sites Hg/g ww. However, no significant difference was identified
404 in comparison to the controls ($p > 0.05$ according to the results of two-way ANOVA). In agreement with those
405 observations, *mt* gene transcription level remained unchanged following Cd exposure in both embryos and larvae
406 when compared to the corresponding control (Fig. 2).

407 MTs are low molecular weight and cysteine-rich proteins, giving them a high potential to sequester certain
408 metals. As a result, MTs are considered to play a key role in the homeostasis of several essential or toxic metallic
409 elements (Coyle et al. 2002). Riggio et al. (2003) demonstrated that waterborne exposure of *D. rerio* embryos to
410 $20 \mu\text{M}$ of Cd (2.2 mg/L) led to a significant increase of MT content at blastula stage but not at mid-gastrula stage
411 in comparison to the control. These observations suggest that the regulation of MT synthesis could depend on the
412 developmental stage of the embryo. We can also suppose that the maternal stock of MT in the embryos was
413 sufficient to prevent, at least in part, Cd accumulation. However, it must be underlined that the lack of
414 expression of MT proteins, under basal or Cd-stimulated conditions, has been regarded as one of the major
415 underlying causes of tissue susceptibility to Cd toxicity and carcinogenicity (Valko et al. 2005).

416

417 3.6. *Cd-exposure deregulates expression of genes involved in mitochondrial metabolism*

418 Gene transcription analysis performed on newly hatched larvae (T9) exposed to the C1 treatment revealed
419 significant repression ($p < 0.05$) of the *nd5* (NADH-dehydrogenase subunit V) gene transcription in comparison
420 to the control (Fig. 2). *Nd5* gene product is a mitochondria encoded subunit of complex I of the mitochondrial
421 respiratory chain. Repression of this gene, if also reflected at the protein level, could therefore impair electron
422 transport and ATP synthesis in Cd-exposed cells. Inhibition of electron transfer chain activity is considered one
423 of the main toxic effects of Cd on mitochondria, leading to ROS over-production (Wang et al. 2004; Bertin and

424 Averbach 2006). The present decrease of the *nd5* transcript level could thus result in the induction of oxidative
425 stress in medaka larvae exposed to Cd. The metal-induced overproduction of ROS is known to be involved in a
426 wide range of cellular injuries including lipid peroxidation, enzyme inactivation, essential element homeostasis
427 disturbance (e.g., Ca^{2+}), membrane injuries, DNA damage (strand breaks, base oxidation, mutations, etc.),
428 aberrant gene expression and induction of apoptosis (Bertin and Averbach 2006; Joseph 2009). Some of these
429 effects are known to actively participate in the development of cancer induced by Cd (Joseph 2009).
430 Spearman rank order correlation analysis showed that *nd5* transcription level in larvae (T9) was inversely
431 correlated with global teratogenicity (percentage of abnormal larvae; $r = -0.886$, $p < 0.01$) and the main observed
432 developmental defects including spinal ($r = -0.759$, $p < 0.05$), craniofacial ($r = -0.764$, $p < 0.05$) and
433 cardiovascular ($r = -0.831$, $p < 0.05$). These results suggest that impairment of mitochondrial electron transfer
434 chain and subsequent ROS overproduction could be involved in Cd-induced teratogenicity. Indeed, if not
435 efficiently eliminated, ROS could damage cellular macromolecules (e.g. DNA, RNA, proteins, lipids etc.),
436 which may in turn interfere with embryonic development (Wells et al. 2005). Moreover, embryonic development
437 also may be adversely affected by ROS reaction with transduction proteins, thereby altering embryonic signal
438 transduction pathways (Wells et al. 2005).
439 In the present study, no stimulation of the transcription level of the antioxidant genes (*sod(Mn)* and *sod(Cu/Zn)*)
440 was observed to corroborate the ROS overproduction hypothesis. However a strong induction of DNA damage
441 and of 'hedgehog cells' has been measured by the Comet assay in Medaka larvae after exposure to 2 and 20 μg
442 Cd/g dw (Barjhoux et al. 2012). Since Cd is not a Fenton metal, DNA damage induced by Cd exposure is likely
443 due to indirect ROS overproduction leading to oxidative stress generation (Joseph 2009; Riso-de Faverney et al.
444 2001).
445 Overall results suggest that the developmental defects observed in medaka ELS resulted more or less directly
446 from Cd-induced oxidative stress and that *nd5* transcription deregulation could be regarded as a marker of Cd
447 teratogenicity.

448

449 3.7. Cd-exposure deregulates expression of genes involved in DNA repair and apoptosis

450 At the embryonic stage (T7), exposure to the highest Cd concentration induced significant repression ($p < 0.05$)
451 of the transcription level of the pro-apoptotic gene *bax* and of the gene *rad51* involved in homologous
452 recombination DNA repair (Fig. 2). Conversely, these genes transcripts were not anymore down-regulated at the
453 larval stage (T9); rather they showed a non-significant tendency to increase (Fig. 2).

454 *In vitro* studies reported the induction of apoptosis by Cd through the activation of *p53* and *bax* pathways (Lag et
455 al. 2002; Risso-de Faverney et al. 2004). Actually, things are somewhat more complicated *in vivo*. Indeed it has
456 been shown in our laboratory that the *bax* gene transcription could be stimulated in the gills and in the skeletal
457 muscles of zebrafish exposed to high concentrations of Cd (9.6 µg Cd/L for 7 days) and repressed at lower
458 concentrations (1.9 µg Cd/L for 7 days) in the gills (Gonzalez et al. 2006). These observations indicate that *bax*
459 transcriptional response following Cd exposure could vary depending on the organ analyzed, the concentration
460 and the duration of exposure.

461 A concomitant reduction of *bax* and *rad51* gene transcripts levels has been reported in *Xenopus laevis* larvae
462 exposed to 30 µg Cd/L, whereas the same genes were overexpressed at the transcriptional level at the lower
463 concentration of 10 µg Cd/L (Mouchet et al. 2006). According to the authors, cells with damaged DNA are
464 expected to stop at the G1/S and G2/M checkpoints in order to repair DNA, and if the damage cannot be
465 repaired, to initiate apoptosis. Thus, the down-regulation of apoptotic genes transcription could be linked to the
466 repression of DNA repair by Cd (Mouchet et al. 2006). The inhibitory effect of Cd on NER- (nucleotide excision
467 repair), BER- (base excision repair) and MMR-type (mismatch repair) DNA repair mechanisms have been
468 widely described in the literature (see Giaginis et al. 2006 for review). However to our knowledge, the study by
469 Mouchet et al. (2006) and the present work are the only ones that highlight the transcriptional down-regulation
470 by Cd of genes involved in double-strand DNA repair mechanisms. The inhibition of DNA repair and apoptosis
471 mechanisms could lead to the fixation of DNA damage as mutations and is considered to play a critical role in
472 Cd-carcinogenesis (Joseph 2009).

473

474 3.8. *Cd-exposure deregulates Wnt-1 gene expression*

475 In the present study, a significant rise in the *wnt1* gene transcription level was noticed in newly hatched larvae
476 (T9) exposed to the C2 treatment (Fig. 2). If this transcriptional change has repercussions at the protein level, it
477 could lead to adverse effects on embryos development. Indeed, Wnt signaling is highly implicated in
478 embryogenesis and morphogenesis through cell differentiation and proliferation control (L'Allemain 2006).
479 Induction of the Wnt pathway activates cell survival pathways through the inhibition of *p53* activation and
480 forcing the overexpression of the anti-apoptotic *bcl-2* and *bcl-xL* genes (Venkatesan et al. 2010; Zeilstra et al.
481 2011). Conversely, inhibition of the Wnt-1 pathway promotes cell death by apoptosis (You et al. 2004). It has
482 also been shown that aberrant Wnt/ β -catenin pathway signaling is involved in the promotion of tumors (Polakis
483 2000).

484 Recently, the impact of chronic exposure to Cd (100 mg/L in drinking water for 12 weeks) on the transcription
485 of Wnt family genes was studied in mouse kidneys (Chakraborty et al. 2010). The authors reported the
486 transcriptional activation of numerous *Wnt* genes, including *wnt1*, as well as the up-regulation of Wnt receptor
487 *frizzled (fz)*. Moreover, the activation of Wnt/ β -catenin dependent target genes such as *c-Myc*, *cyclin D1* and
488 *abcb1b*, was also observed. These genes promote cell proliferation and cell survival activation, but they could
489 also act as proto-oncogenes when aberrantly up-regulated. As a result, the Cd-induced activation of the Wnt/ β -
490 catenin pathway could lead to anarchic cellular proliferation, erratic activation of the cell survival pathway, and
491 consequently promote carcinogenesis (Joseph 2009). It is also suggested that disturbance of the Wnt/ β -catenin
492 pathway during embryonic development may contribute to teratogenic effects of Cd (Thévenod 2009). Chow
493 and Cheng (2003) hypothesized that some of the spinal deformities induced by Cd in *D. rerio* embryos exposed
494 to 1 mg/L could result from Wnt signaling disruption during somitogenesis. These results are in agreement with
495 our results showing a significant increase in the percentage of larvae developing spinal deformities ($44.4 \pm$
496 7.7%), including mainly kyphosis, lordosis and C-shaped larvae, as well as a significant rise in the *wnt1* gene
497 transcription level at the larval stage. However, Spearman rank order analysis showed that *wnt1* transcription
498 level at the larval stage (T9) was not significantly correlated to spinal deformities ($r = 0.554$, $p = 0.154$) but with
499 cardiovascular injuries ($r = 0.807$, $p < 0.05$).

500 Therefore, we could postulate that the disturbance of *wnt1* gene transcription during embryo-larval development
501 is only involved in a part of the spinal deformities observed in Cd-exposed larvae, and perhaps with a time-gap
502 between *wnt1* transcription level modulation and phenotypical impacts. We can also suggest that *wnt1*
503 transcription disturbance directly involved in the development of spinal deformities occurred very locally and
504 thus was not accurately revealed in our transcriptomic analysis performed on whole pooled individuals.

505 The Wnt/ β -catenin pathway is also highly involved in cardiogenesis acting biphasically promoting cardiac
506 differentiation when activated before gastrulation stage or inhibiting heart formation when signaling is activated
507 later in the development (Ueno et al. 2007). Moreover, Lin and Xu (2009) reported that the Wnt/ β -catenin
508 signaling regulates heart laterality in zebrafish ELS and that an over-activation of this pathway at the
509 transcriptional level resulted in both cardiac jogging and looping disruption affecting the left-right asymmetry of
510 the heart. In the light of these results, we could assume that some of the cardiovascular deformities, especially
511 abnormal positions of heart chambers in relation to each other and to the cephalo-caudal axis, observed in Cd-
512 exposed larvae in the present work are the result of Wnt/ β -catenin signaling pathway disruption. Analysis of Wnt
513 family genes transcription levels could thus be potentially used as early markers of cardiac development defects.

514

515 **4. Conclusion**

516 In the present study, we showed that exposure to environmental Cd concentrations resulted in significant
517 bioaccumulation in both medaka embryos and larvae and induced significant developmental defects and gene
518 transcription deregulation. Cd exposure resulted in abnormal cardiac morphogenesis and dysfunction and spinal
519 deformity increase. In addition, several genes involved in mitochondrial metabolism, DNA repair and apoptosis
520 were shown to be significantly deregulated. Deregulation of these cellular pathways could be involved in Cd-
521 induced teratogenicity, especially the modulation of the expression of the *wnt1* and *nd5* genes. These two genes
522 could probably be used in the future as an early warning tool to predict the rise of spinal or cardiovascular
523 developmental defects in fish, but causal relationship remains to be clearly demonstrated. Besides, heart beat
524 measurement proved to be a sensitive and non-invasive phenotypical marker correlated to more general injuries
525 at the molecular and cellular levels. These observations clearly highlight the complementarity of phenotypical
526 and molecular approaches in toxicity studies: on one hand some phenotypical markers such as cardiac rate and
527 developmental defects could integrate and reflect the modulation of several molecular responses, and on the
528 other hand, transcriptional gene levels such *nd5* and *wnt1* can illustrate alterations of specific cellular pathways
529 while being associated to phenotypical defects. The present work also reports successful adaptation of the
530 MELAc to the study of both the phenotypic and molecular impacts of Cd-contaminated sediments. With regards
531 to the efficiency of the MELA and the relevance of the route of exposure, this approach could be applied to the
532 investigation of the toxico-kinetics and toxico-dynamics of various particle-bound pollutants.

533

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540

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670

671 **Figure captions**

672 **Fig. 1 (a)** Cd bioaccumulation in medaka embryos (T7) and larvae (T9), (b) developmental abnormalities in
673 newly hatched larvae and (c) heart rate in 6 dpf and 7 dpf-embryos following Cd exposure. The data represents
674 the mean response (\pm SD) for three replicates. For Cd bioaccumulation and heart rate data, different letters
675 indicate significant differences between treatments ($p < 0.05$) according to the results of two-way ANOVA
676 followed by Tukey HSD post-hoc test. For developmental impairment data, significant differences in
677 comparison to control treatment are symbolized by an asterisk (*) according to the results of one-way ANOVA
678 followed by Dunnett post-hoc test.

679

680 **Fig. 2** Variation in gene transcription levels as compared to control observed in medaka embryos (T7; a) and
681 larvae (T9; b) after exposure to Cd-spiked sediments. Results are mentioned as control-relative gene induction ($>$
682 1) or repression (< 1) factors. The data represents the mean response (\pm standard error) for three replicates. The *
683 sign indicates a significant difference in comparison to the corresponding control, according to t test for
684 independent samples ($p < 0.05$).

685

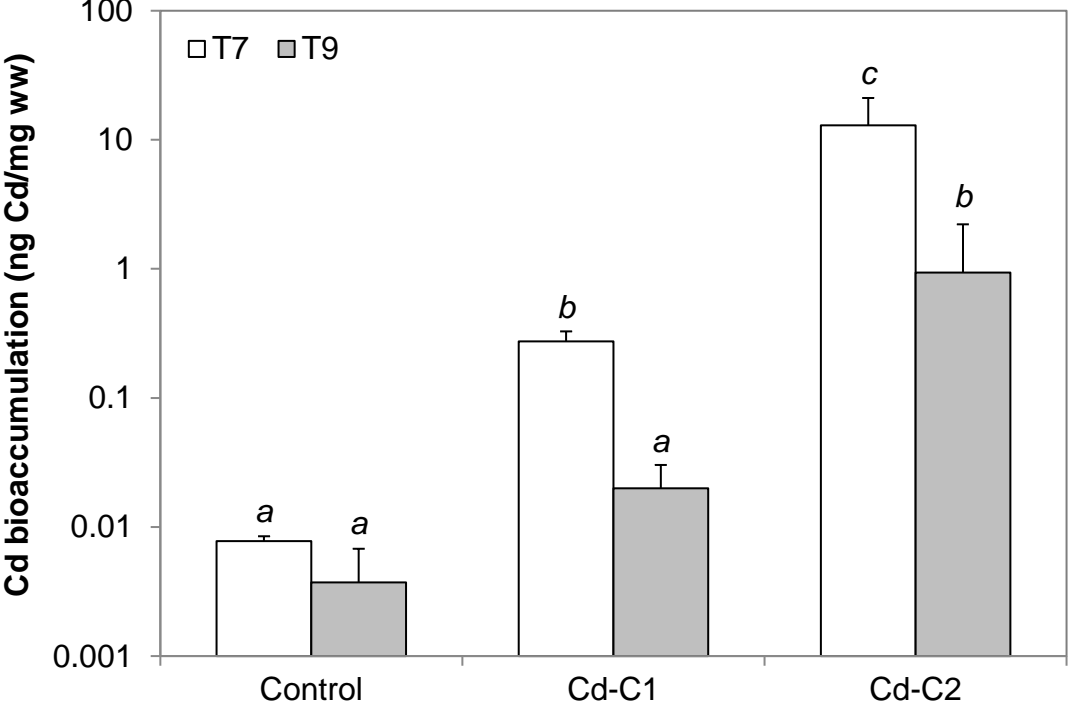
686 **Supplementary Material caption**

687 **Online Resource 1.** β -actin relative transcription levels observed in medaka embryos (T7) and larvae (T9) after
688 exposure to Cd-spiked sediments. The data represents the mean response (\pm SD) for three replicates.

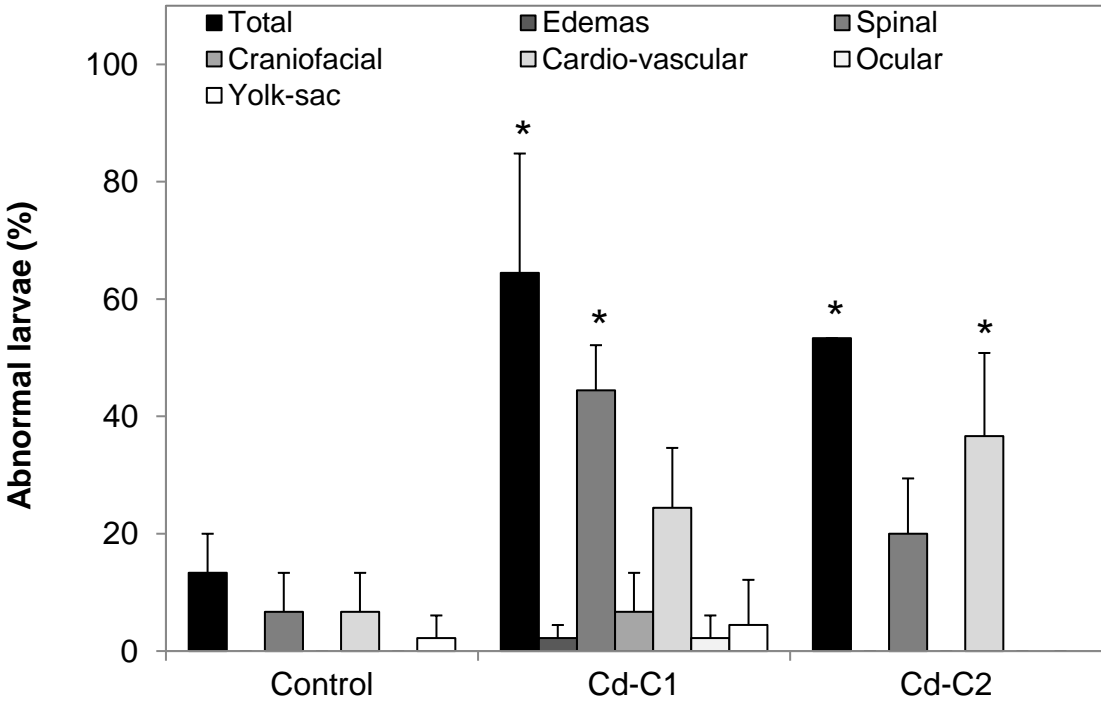
689

Figure 1

(a)



(b)



(c)

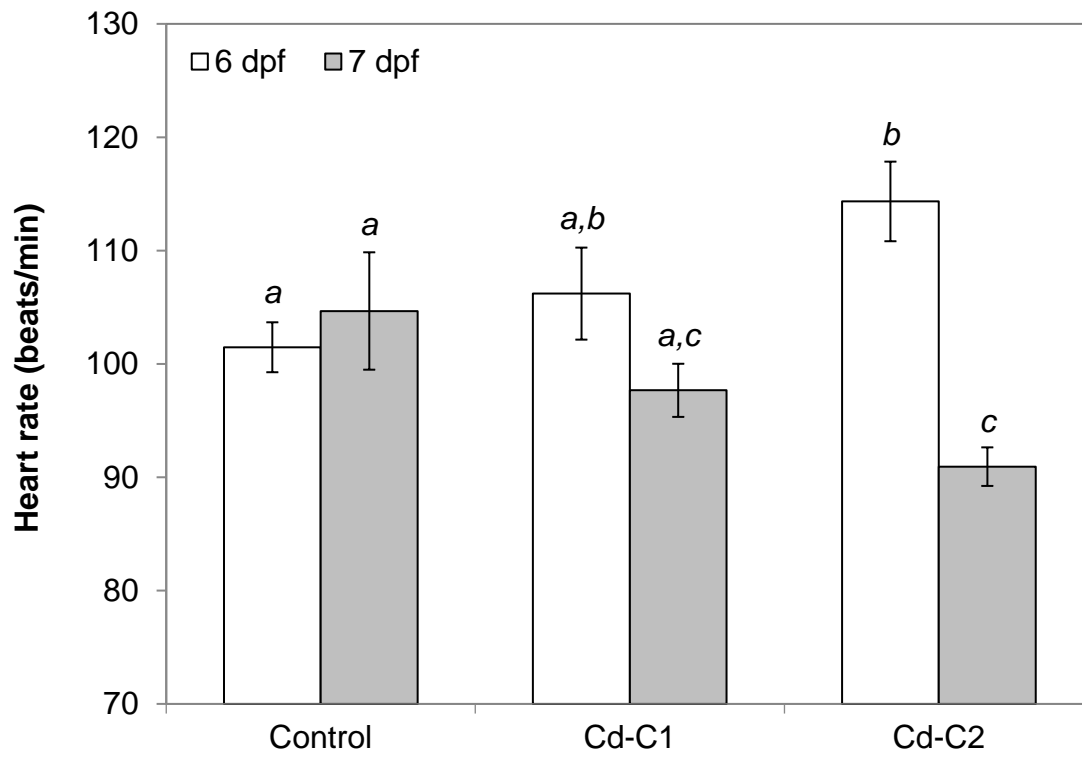
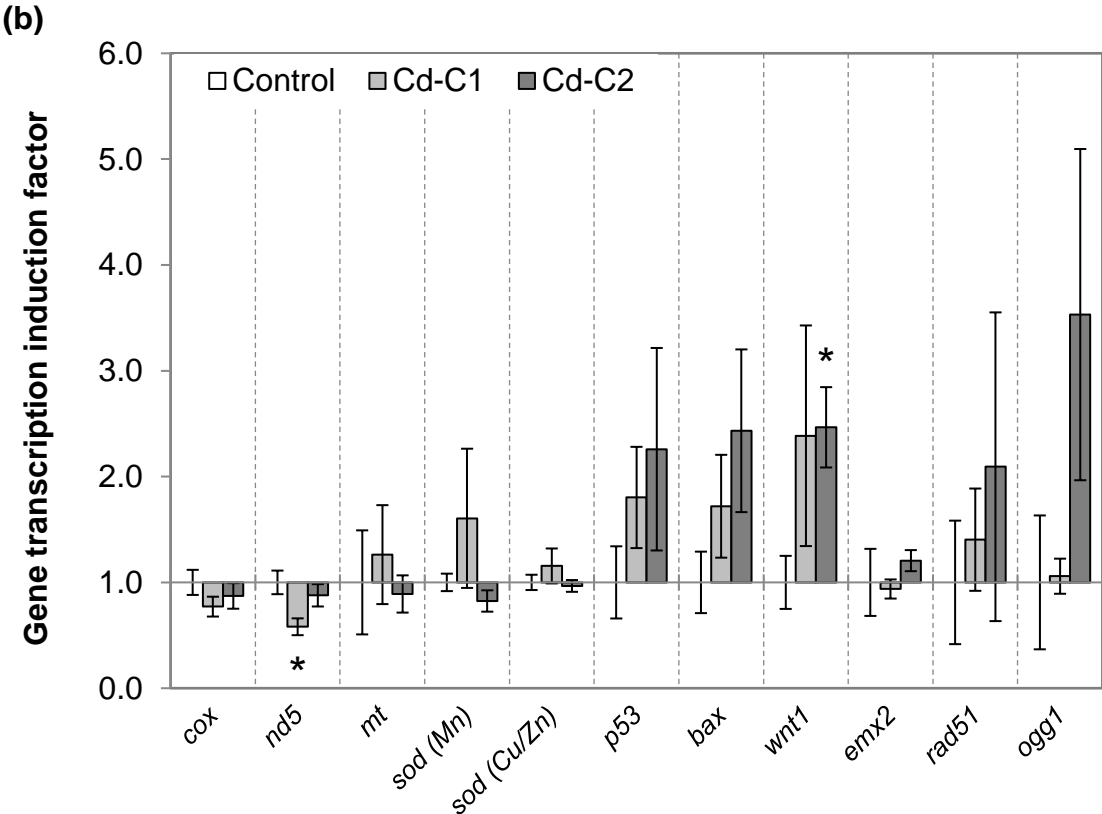
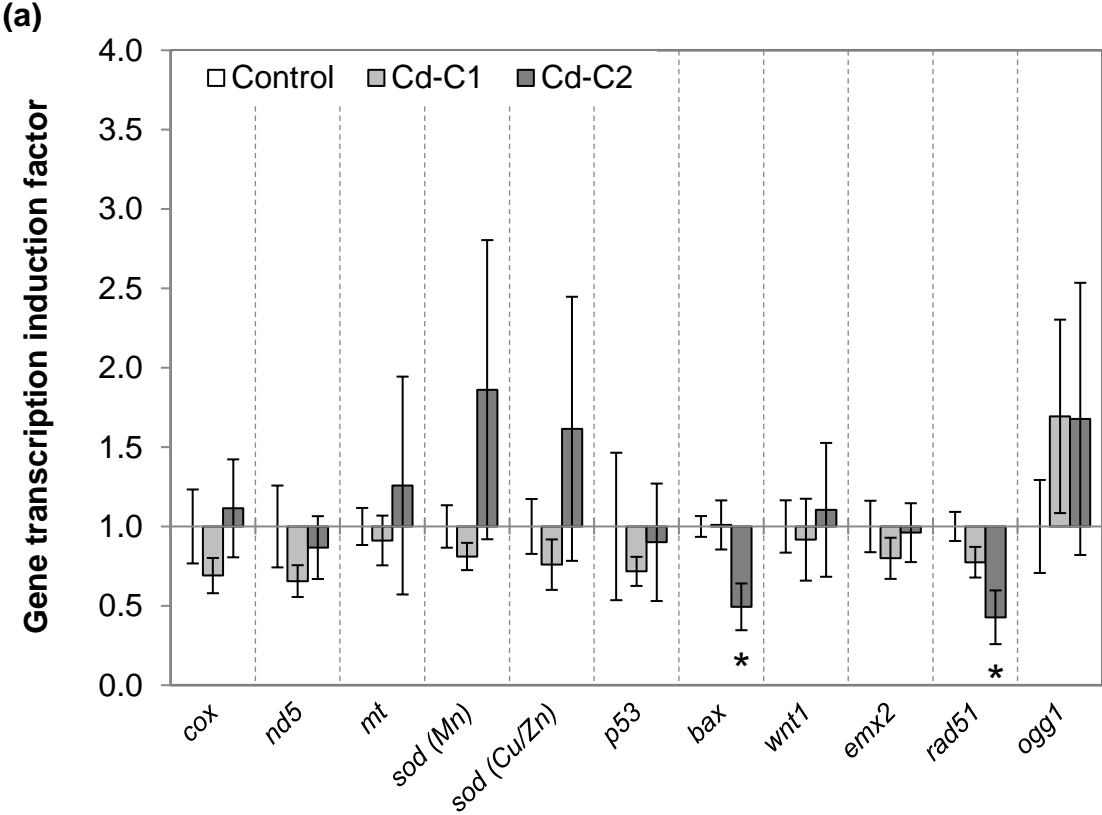


Figure 2



1 Tables

2 **Table 1.** Physico-chemical characteristics of the reference sediment (Marcenac, Lot River) used as
3 spiking matrix in the present study.

Marcenac sediment (Lot river, Aveyron, SW France)									
<hr/>									
Particulate organic carbon		0.11 %							
Dissolved ammonia (NH ₄ ⁺)		62.3 μM							
Dissolved sulfur (H ₂ S)		16.8 μM							
<i>Granulometric distribution</i>									
10 th percentile diameter		14.8 μm							
50 th percentile diameter		111.5 μm							
90 th percentile diameter		224.7 μm							
≤ 65 μm fraction		25.8%							
<i>Trace metals levels (μg/g dw)</i>									
Co	Mn	Ni	Zn	Cr	As	Ag	Pb	Cd	Cu
5.6	261	10	35	10	17.5	0.04	12.5	0.17	7.18
<i>Organic compounds levels (ng/g dw)</i>									
ΣPAH ^a		12 ng/g dw							
ΣPCB ^b		1 ng/g dw							
ΣPBDE ^c		Not detected							

4 a, cumulative concentration of 21 analyzed polycyclic aromatic hydrocarbon compounds

5 b, cumulative concentration of 8 analyzed polychlorobiphenyl congeners

6 c, cumulative concentration of 4 analyzed polybrominated diphenylethers

7

8 **Table 2.** Cadmium content in sediments from different places worldwide.

Concentrations in $\mu\text{g/g dw}$ (max and min values)	Place	References
0.05 – 1.4	Anzali Wetland, Iran	Janshidi-Zanjani and Saeedi, 2013
0.4 – 2.6	Garonne river, France	Barjhoux et al. 2012
0.17 – 460	Lot River, France	Barjhoux et al. 2012
17 – 37	Estuarine and coastal sediments in India	Chakraborty et al. 2012
0.5 – 4.2	Pearl River, Southern China	Wang et al. 2011
x – 5.8	River Nile, Egypt	El-Kammar et al. 2009

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11

12 **Table 3.** Accession number and specific primer pairs for the twelve *O. latipes* genes studied in the present study

Gene	Function	Accession number (EMBL or GenBank)	Primers sequences
<i>β-actin</i>	Cytoskeletal gene (housekeeping gene)	<u>S74868</u>	GTGACCCACACAGTGCC ^a GCGACGTAGCACAGCTTC ^b
<i>coxI</i>	Cytochrome C oxidase subunit I (complex IV of the mitochondrial respiratory channel)	<u>NC_004387</u> (gene ID 805432)	TTCCCCAACACTTCTTAGGC ^a TGTGGCTGTTAGTTCGACTGA ^b
<i>nd5</i>	NADH-dehydrogenase subunit V (complex I of the mitochondrial respiratory channel)	<u>NC_004387</u> (gene ID 805441)	ACCCTCCCCTTACTCGG ^a AGCAAAGGCAGGTGGAC ^b
<i>p53</i>	Tumor suppressor gene P53	<u>AF212997</u>	TCTGGCACTGCAAAGTCTGT ^a CCTCGTTTTGGTGGTGGG ^b
<i>mt</i>	Metallothionein gene	<u>AY466516</u>	ACAAACTGCTCCTGCACC ^a ACTGACAACAAGTAGTGTCCG ^b
<i>rad51</i>	Homologous recombination gene (homologous RecA)	<u>ENSORLG00000017821</u>	CGCATGCTGCTGCGACTG ^a TTCCCCTCGACCTTTCCTC ^b
<i>wnt1</i>	Wingless integration site 1 (cell proliferation and somitogenesis)	<u>AJ243208</u>	CCGCTTTGACGGAGCAT ^a TTGAACCCACGCCACAGC ^b
<i>sod(Mn)</i>	Mitochondrial Fe/Mn superoxide dismutase	<u>ENSORLG00000013261</u>	ATGGCTGGGCTATGACAAAG ^a TGGCTATCTGAAGACGCTCAC ^b
<i>sod(Cu/Zn)</i>	Cytosolic Cu/Zn superoxide dismutase	<u>ENSORLG00000008041</u>	GGGAAATGTGACCGCAGG ^a GCCAAACGCGCTCCAG ^b
<i>bax</i>	Bcl-2 associated X protein gene	<u>ENSORLG0000000456</u>	TCTTCGCTCAGTCCCTCC ^a GCCAACGTCTGCCAGCCA ^b
<i>oggl</i>	8-oxoguanine glycosylase 1 gene (BER family)	<u>ENSORLG00000010758</u>	CTCGTATTCAGGGCATGGT ^a ACCCGTGGCTGTCTAAG ^b
<i>emx2</i>	Empty spiracles homolog 2 (nervous system development)	<u>ENSORLG00000014157</u>	CCATTACGTGGTGGGAGCA ^a GGGCTCGCCTGTTTAGT ^b

13 a, upstream primer (5' → 3'); b, forward primer (3' → 5')

14

15 **Table 4.** Cd concentrations in spiked sediments and ERS medium.

	Measured concentration			Nominal concentration	Spiking efficiency (%)
	T0	T7*	T9*		
<i>Cd concentrations in sediment (µg/g dw)</i>					
Control	0.14	0.27 ± 0.09 ^a	0.23 ± 0.01 ^a	0	-
Cd-C1	1.67	1.71 ± 0.04 ^b	1.64 ± 0.15 ^b	2	75.0
Cd-C2	16.27	14.9 ± 1.09 ^c	15.0 ± 0.62 ^c	20	80.4
<i>Cd concentration in ERS buffer (µg/L)</i>					
Control	0.031	0.13 ± 0.14 ^a	0.31 ± 0.14 ^a	-	-
Cd-C1	-	8.14 ± 0.25 ^b	25.8 ± 5.46 ^{b,c}	-	-
Cd-C2	-	138 ± 2.73 ^c	96.4 ± 1.95 ^c	-	-

16 * T7 and T9 concentrations are mentioned as mean ± SD (N = 3). Means with different letters are significantly
 17 different (p < 0.05) according to the results of two-way ANOVA followed by Tukey HSD post-hoc test. Note
 18 that spiking efficiencies were calculated after subtraction of the natural Cd background in Marcenac sediment.

19
 20

21 **Table 5.** Embryonic survival, hatching success and time to hatch following medaka embryo exposure to Cd-
22 spiked sediments.

Condition	Embryonic survival (%)	Hatching success (%)	Time to hatch (dpf)
Control	94.8 ± 3.17	91.6 ± 5.90	8.76 ± 0.15
Cd-C1	94.1 ± 4.87	93.7 ± 3.79	8.64 ± 0.24
Cd-C2	98.8 ± 0.75	94.7 ± 3.06	8.63 ± 0.33

23 Values represent the mean responses (\pm SD) from six replicates for embryonic survival and from three replicates
24 for hatching success and time to hatch. No significant difference was observed between treatments for the three
25 endpoints according to the results of one-way ANOVA followed by Tukey HSD post-hoc test ($p > 0.05$).

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