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

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Abstract

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

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

1. Introduction

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

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

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

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

day-old medaka embryos were exposed to Cd-spiked sediment at 2 and 20 µg/g dry weight (dw) for 7 days post-fertilization (dpf; embryonic stage analyses) or during their whole embryonic development (i.e. up to hatching at 9 dpf, larval stage analyses). These concentrations of Cd can be measured in areas of low to moderate metallic contamination and have already been characterized as sublethal but highly teratogenic to medaka ELS (Barjhoux et al. 2012). Cd bioaccumulation was monitored in embryos and newly hatched larvae. Survival, hatching, heart rate, biometric measurements and developmental abnormalities were recorded during the course of exposure. In parallel, qRT-PCR analysis of target gene transcription level was carried out in embryos and newly hatched larvae in order to investigate Cd mechanisms of toxicity. A panel of 12 genes was selected for this purpose due to their involvement in metal sequestration, antioxidant defense, mitochondrial metabolism, DNA repair, cell cycle regulation and apoptosis. Potential induction of metal-specific detoxification mechanism was examined through MT protein level measurement in association with their gene (*mt*) transcription level. The response to oxidative stress was studied through cytoplasmic (*sod(Cu/Zn*)) and mitochondrial superoxide dismutase (*sod(Mn)*) genes transcription. The impact of Cd on the mitochondrial respiratory chain was investigated using NADH-dehydrogenase subunit V (*nd5*) and cytochrome C oxidase subunit I (*coxI*) transcripts. Two other genes, the *ogg1* (8-oxoguanine glycosylase 1) and *rad51* genes, were selected for their involvement in DNA repair mechanisms. Cd-induced apoptosis was studied through Bcl-2 associated X protein (*bax*) and *p53* gene transcription levels. The last pair of genes was selected to examine the impact of Cd on the transcription of genes involved in embryogenesis and morphogenesis: the empty spiracles homolog 2 (*emx2*) gene for its involvement in central nervous system development, and the wingless integration site 1 (*wnt1*) gene for its key role in cell differentiation and proliferation.

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

2. Material and Methods

2.1. Experimental design

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

Medaka embryos remained in direct contact with the sediment up to sampling time, i.e. for 7 dpf (T7) or hatching time (T9), depending on the replicate under consideration. Embryos and larvae were incubated in a climate cabinet (Snijders Scientific; 26 ± 0.3 °C; 12L:12D; 5000 lx white light)under static non-renewal exposure conditions, in egg rearing solution (ERS: 17.11 mM NaCl; 0.4 mM KCl; 0.36 mM $CaCl_2$; 1.36 mM $MgSO_4$; pH 7.0). The experiment ended when all T9-samples were completed, i.e. at 9 dpf.

Sampled embryos/larvae were divided into pools of adequate size to perform several analyses including MT proteins content determination, gene transcription analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Cd bioaccumulation measurement in the whole body of embryos (T7) and larvae (T9). A wide range of non-invasive phenotypic endpoints were also investigated to complete this biochemical approach and as described in previous work from our laboratory (Barjhoux et al. 2012). They included embryonic survival, heart beat, biometric measurements, hatching success, time taken to hatch and the occurrence of developmental abnormalities.

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

2.2. Reference sediment characterization

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

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

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

2.3. Sediment spiking procedure

Cd spiking levels were selected according to Cd concentrations measured in the Lot-Garonne-Gironde continuum, well-known for its historical contamination with metals. The C2 concentration (20 µg Cd/g dw) was selected according to Cd levels in sediments from moderate to highly contaminated sites along the Lot river whereas the C1 concentration (2 µg Cd/g dw) represents the current level of contamination found, for instance, in sediments from the Garonne river (Blanc et al. 1999; Audry et al. 2004a, b). Moreover, these concentrations are representative of those found in several contaminated sediments from various locations worldwide (Table 2). The spiking procedure was similar to the protocol proposed by Barjhoux et al. (2012) with some adjustments related to the larger amount of sediment needed. The spiking solutions were obtained by dilution of a Cd-stock solution at 2.5 mg Cd/mL ($CdCl_2$:$2H_2O$, Fluka Chemie) in ultrapure water (Milli-Q Maxima, Elga Labwater, Veolia water). Crushed and freeze-dried sediment (65 g dw) was placed in a 1L-beaker. One mL of Cd spiking solution (or ultrapure water for the control treatment) was added, followed by 35 mL of ultrapure water. The sediment remained in contact with the Cd solution for 1 h under agitation. Then, most of the supernatant was removed and the sediment was kept all night at room temperature for partial drying. The sediment moisture content was gravimetrically determined to calculate the wet weight equivalent to 17 g dw for each spiking condition. Finally, sediment from the same treatment was divided into six aliquots of 17 g dw equivalent (for embryos exposure) and one aliquot of approximately 5 g dw equivalent dedicated to Cd analysis.

2.4. Medaka embryo exposure

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

the beginning of the experiment. Medaka embryos (i.e. 24 h post-fertilization, hpf) were purchased from GIS AMAGEN (Gif-sur-Yvette, FR). Healthiness and developmental stage synchronism of the embryos were checked using a stereomicroscope (Leica MZ75, Leica Microsystems) and cold light source (Intralux® 4100, Volpi AG). Immediately after sorting, embryos were randomly placed on a Nytex® mesh (mesh opening 1000 µm, Sefar Filtration Inc.). The Nytex® grid was then slightly sunk into the sediment. Afterwards, embryos were kept exposed to the sediment until T7- or T9-sampling time. At the end of the experiment, the remaining embryos or larvae were euthanized using MS222 (Sigma-Aldrich) solution at 1 g/L.

During the exposure period, the level of aqueous media was re-adjusted in case of evaporation, adding an adequate volume of clean ERS. Dissolved oxygen was also checked daily at the water-sediment interface using an oxygen optical microsensor (NeoFox® Foxy probe, Ocean Optics sensors, Idle Fibres optiques). This measurement confirmed good oxygenation of the medium with values always superior to 80 % saturation (data not shown).

2.5. Phenotypic endpoints

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

2.6. Metallothionein (MT) proteins content

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

Sampled organisms were briefly dried on absorbent paper and the total weight was recorded. Afterwards, samples were immediately stored in liquid nitrogen to minimize MT oxidation until analysis.

MT concentrations were measured with the mercury-saturation assay using cold inorganic Hg as described by Baudrimont et al. (2003) with few adaptations. In summary, tissue was homogenized in 300 µL of Tris-HCl 10 mM (Sigma-Aldrich, pH 7.5 at 20 °C) using an Ultra-Turrax (T10 Basic, Ika®) on ice and under N_2 atmosphere. The cytosolic fraction was then extracted by centrifugation (20 000 g for 1 h at 4 °C) and divided into two 100 µL-aliquots for a duplicated analysis. Then, 100 µL of $HgCl_2$ 50 mg Hg/L in trichloroacetic acid (10 %) was added to each sample to saturate MTs with Hg and to denature non-MT proteins. Excess Hg not bound to the MT proteins was removed by adding 200 µL of lyophilized pig hemoglobin (Sigma-Aldrich) prepared in 30 mM Tris-HCl (pH 8.2 at 20 °C), mixing and immediate centrifugation (20 000 g for 20 min at room temperature).

The final supernatant was then quantitatively recovered and used for Hg determination by flameless atomic absorption spectrometry (AMA 254, Altec). The detection limit was estimated at 0.01 ng Hg. Owing to the fact that the exact quantity of Hg binding sites per MT molecule is unknown for the medaka species, MT concentrations are expressed in nmol sites Hg/g wet weight (ww).

Each analytical run included three reference samples (blanks) prepared to monitor the Hg complexation efficiency of the hemoglobin. The mean of the three blank values was deducted from the Hg burden measured in the samples. In addition, a recovery percentage from purified rabbit liver MT (Alexis biochemical) was systematically determined. For the analysis performed in the present study, the recovery percentages (between 94 % and 97 %) were consistently within the certified ranges (100 ± 20 %) of the method.

2.7. Gene transcription analysis

Gene transcription analysis was conducted on three samples of 8 individuals per replicate, and on three replicates per treatment at each sampling time (T7 and T9). Immediately after sampling, each pool of embryos or larvae was entirely immerged in RNAse-free microtubes containing 200 µL RNA Later® (Qiagen) and quickly frozen by dipping in liquid nitrogen. Samples were then stored at -80 °C until RNA extraction.

Total RNA extraction was performed using an Absolutely RNA® Miniprep kit (Stratagene, Agilent) according to the manufacturer's instructions, with an additional phenol-chloroform-isoamylic acid (25:24:1, v/v) purification step. The quality and the quantity of the extracted RNA were determined by spectrophotometry at 260 and 280 nm.

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

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

The amplification of cDNA was monitored using the DNA intercaling dye SyberGreen I. Real-time PCR reactions were performed using a LightCycler® (Roche) and LC FastStart DNA Master SybrGreen kit (Roche) according to the manufacturer's instructions. PCR reactions were prepared in glass capillaries adding 1 µL of master mix containing SyberGreen fluorescent dye, 3.2 µL of $MgCl_2$ (25mM), 12.8 µL of ultra-pure water, 2 µL of primer-pair mix (3 µM) and 1 µL of cDNA. Afterwards, PCR reactions consisted of an activation cycle (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 (Ct), i.e. the number of cycles required for the fluorescent signal to cross the background level, was determined for each studied gene. The specificity of each amplification was determined from the dissociation curve of the PCR product. These dissociation curves were obtained by following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95 °C (0.05 °C/s). Relative quantification of each gene transcription level was normalized according to *β-actin* gene transcription level. Indeed, relative gene transcription level was determined using the $2^{-\Delta Ct}$ method described by Livak and Schmittgen (2001), where ΔCt represents the difference between the cycle threshold of a specific gene and the Ct of the *β-actin* gene. The so obtained relative transcription level of each studied gene is reported in the online supplementary data file (Online Resource 1) for each treatment and each sampling time. Then, the induction factor (IF) of each studied gene for Cd treatments in comparison with the control treatment was calculated using the following equation:

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

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

2.8. Cd analysis in sediments, water phase and tissues

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

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

Cd bioaccumulation in tissues was analyzed in two pools of 15 individuals per replicate (three replicates per treatment) at T7 (on embryos) and T9 (on larvae). Sampled organisms were dried slightly on absorbent paper and weighted before being stored at -20 °C until Cd analysis. Samples were then digested with 200 µL of 65 % nitric acid for 3 h at 100 °C. Then, each sample was diluted by adding 1 mL of ultrapure water. As described for Cd analysis in ERS medium and sediment, each series included method blanks and Tort-2 certified samples which showed the recovery percentage to be within certified ranges (values comprised between 98 % and 101 %). Cd concentrations were measured by atomic absorption spectrophotometry with Zeeman correction, using a graphite tube atomizer (M6 Solaar FS98, Thermo Elemental®). In order to avoid interference, analyses were carried out in a tube atomizer with a blend of Pd (2 g/L) and $Mg(NO_3)_2$ (1 g/L).

2.9. Statistical analysis

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

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

3. Results and discussion

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

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

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

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

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

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

Moreover, a 93 % reduction in Cd concentration was observed in the present study when comparing embryonic and larval bioaccumulation. This decrease of Cd load in organisms could be the result of the loss of Cd bound to the chorion occurring at hatching. Indeed, Michibata (1981) demonstrated that more than 94 % of the total accumulated Cd was actually adsorbed onto the chorion in *O. latipes* embryos exposed to 10 mg Cd/L. This phenomenon could be explained by the presence of negatively charged macromolecules such as mucopolysaccharides and glutamic acid in the chorion which could sequester Cd and thus limit Cd penetration into the embryo (Meteyer et al. 1988). Moreover, Meteyer et al. (1988) reported that Cd accumulation varies over time, probably due to physiological changes and chorion permeability modifications during embryo development. Indeed, the chorion could be more permeable and present less adsorption sites to Cd before hatching, which facilitates its passage to the perivitellin fluid (Meteyer et al. 1988; Gonzalez-Doncel et al. 2003).

Pearson's coefficient calculation demonstrated significant correlations between Cd content in embryos and ERS medium ($r = 0.81$, $p < 0.001$) or sediment ($r = 0.90$, $p < 0.0001$). The higher correlation coefficient for sediment might indicate that the sediment contact also acts as a non-negligible route of Cd exposure for medaka embryos.

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

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

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

3.3. Cd exposure induces teratogenic effects

No significant impact was observed on larva body size following Cd exposure. Indeed, the average total body 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-C2 treatments, respectively. Furthermore, average head size and head/body ratio were very similar for all 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 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-C2 treatments, respectively.

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

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

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

3.4. Cd exposure affects both heart morphogenesis and functioning

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

Additionally, 6 dpf-heart rate was also negatively correlated ($p < 0.05$) to the transcription levels of *mt* (Spearman r value = -0.810), *sod(Cu)* (r = -0.738), *rad51* (r = -0.762) and *bax* (r = -0.738) genes in embryos

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

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

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

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

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

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

Averbech 2006). The present decrease of the *nd5* transcript level could thus result in the induction of oxidative stress in medaka larvae exposed to Cd. The metal-induced overproduction of ROS is known to be involved in a wide range of cellular injuries including lipid peroxidation, enzyme inactivation, essential element homeostasis disturbance (e.g., Ca^{2+}), membrane injuries, DNA damage (stand breaks, basis oxidation, mutations, etc.), aberrant gene expression and induction of apoptosis (Bertin and Averbech 2006; Joseph 2009). Some of these effects are known to actively participate in the development of cancer induced by Cd (Joseph 2009).

Spearman rank order correlation analysis showed that *nd5* transcription level in larvae (T9) was inversely correlated with global teratogenicity (percentage of abnormal larvae; $r = -0.886$, $p < 0.01$) and the main observed developmental defects including spinal ($r = -0.759$, $p < 0.05$), craniofacial ($r = -0.764$, $p < 0.05$) and cardiovascular ($r = -0.831$, $p < 0.05$). These results suggest that impairment of mitochondrial electron transfer chain and subsequent ROS overproduction could be involved in Cd-induced teratogenicity. Indeed, if not efficiently eliminated, ROS could damage cellular macromolecules (e.g. DNA, RNA, proteins, lipids etc.), which may in turn interfere with embryonic development (Wells et al. 2005). Moreover, embryonic development also may be adversely affected by ROS reaction with transduction proteins, thereby altering embryonic signal transduction pathways (Wells et al. 2005).

In the present study, no stimulation of the transcription level of the antioxidant genes (*sod(Mn)* and *sod(Cu/Zn)*) was observed to corroborate the ROS overproduction hypothesis. However a strong induction of DNA damage and of 'hedgehog cells' has been measured by the Comet assay in Medaka larvae after exposure to 2 and 20 µg Cd/g dw (Barjhoux et al. 2012). Since Cd is not a Fenton metal, DNA damage induced by Cd exposure is likely due to indirect ROS overproduction leading to oxidative stress generation (Joseph 2009; Risso-de Faverney et al. 2001).

Overall results suggest that the developmental defects observed in medaka ELS resulted more or less directly from Cd-induced oxidative stress and that *nd5* transcription deregulation could be regarded as a marker of Cd teratogenicity.

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

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

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

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

3.8. Cd-exposure deregulates Wnt-1 gene expression

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

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

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

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

4. Conclusion

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

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Figure captions

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

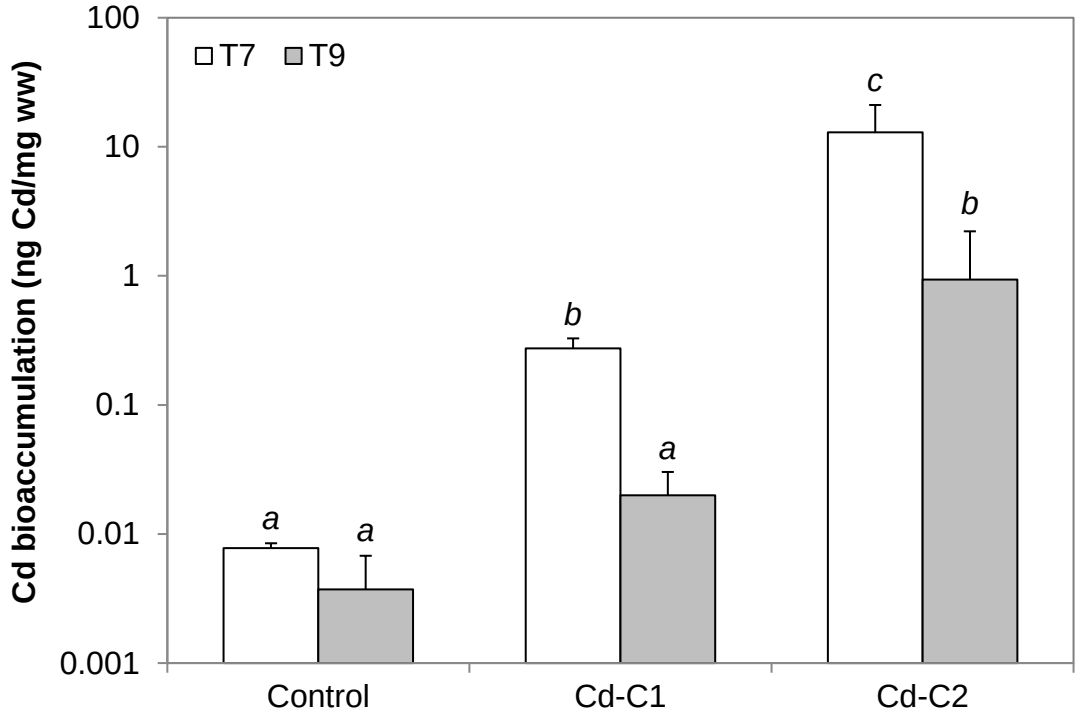
Fig. 2 Variation in gene transcription levels as compared to control observed in medaka embryos (T7; a) and larvae (T9; b) after exposure to Cd-spiked sediments. Results are mentioned as control-relative gene induction (> 1) or repression (< 1) factors. The data represents the mean response (± standard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

Supplementary Material caption

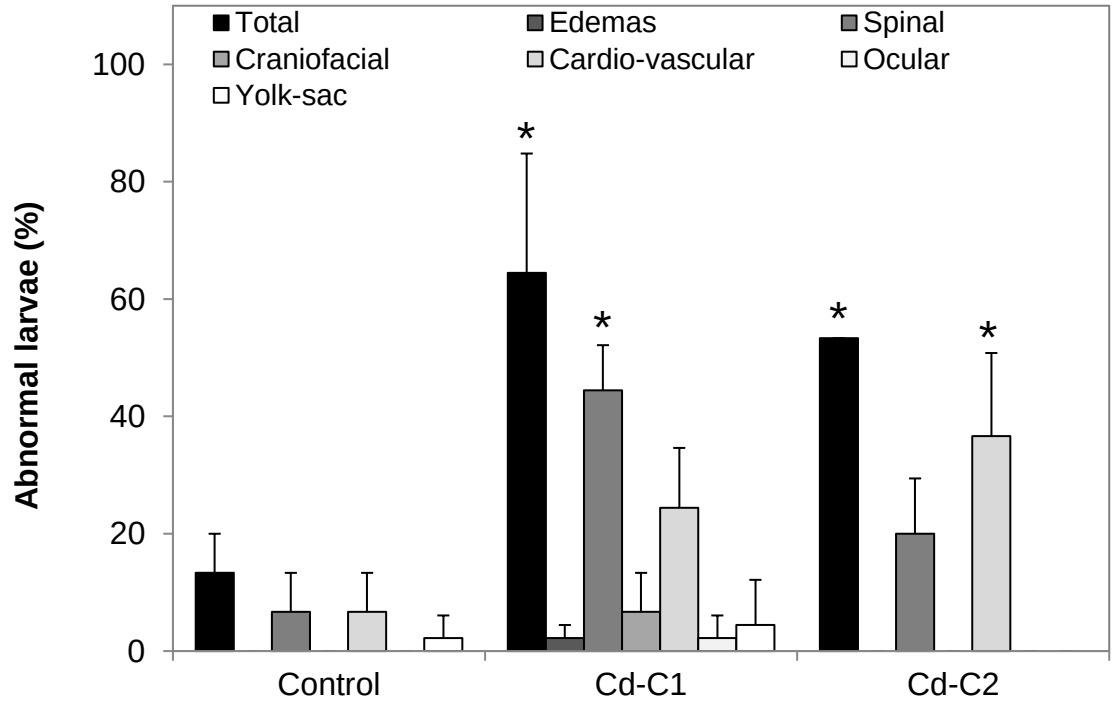
Online Resource 1. *β-actin* relative transcription levels observed in medaka embryos (T7) and larvae (T9) after exposure to Cd-spiked sediments. The data represents the mean response (± SD) for three replicates.

Figure 1

(a)


100
10
1
0.1
0.01
0.001
Cd bioaccumulation (ng Cd/mg ww)
T7
T9
a
a
b
a
c
b
Control
Cd-C1
Cd-C2

(b)


Total
Edemas
Spinal
Craniofacial
Cardio-vascular
Ocular
Yolk-sac
100
80
60
40
20
0
Abnormal larvae (%)
*
*
*
*
Control
Cd-C1
Cd-C2

(c)

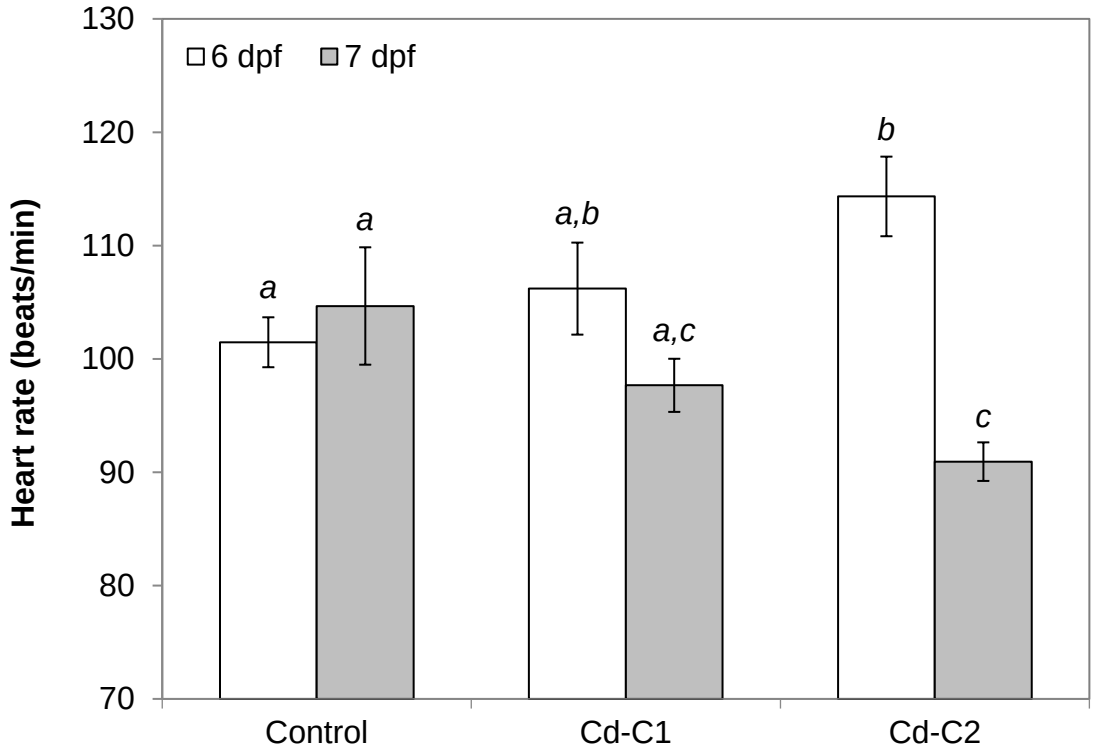
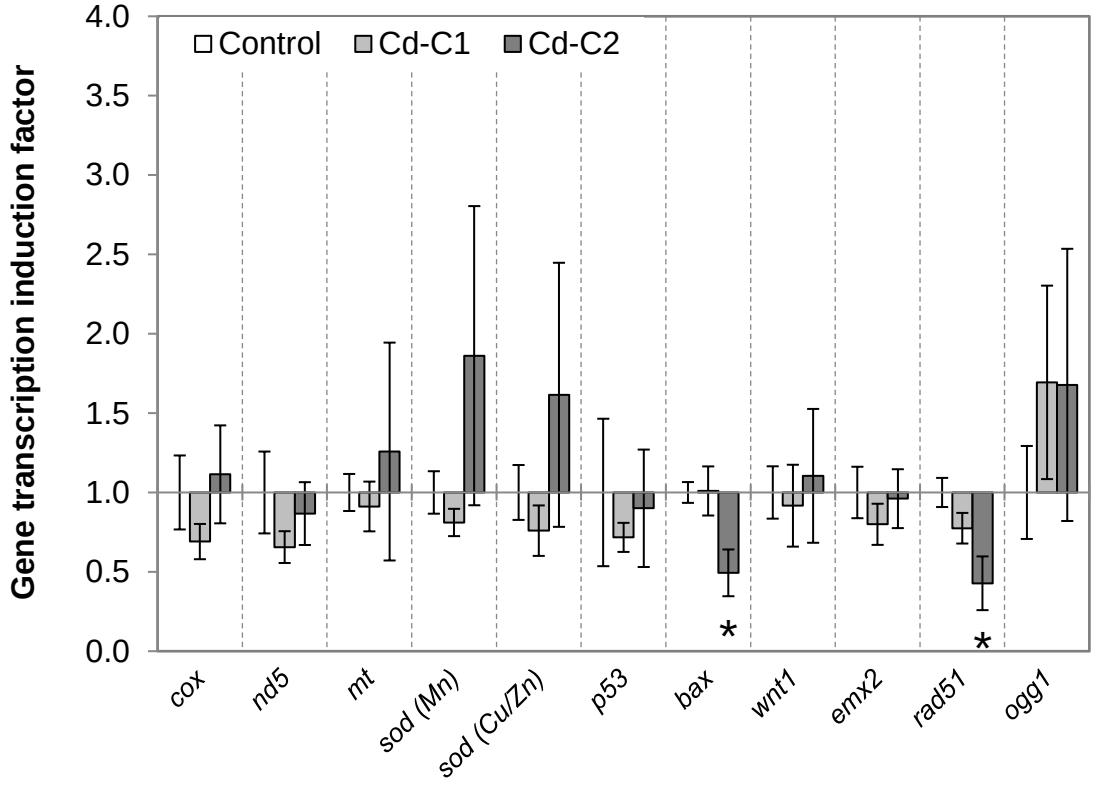
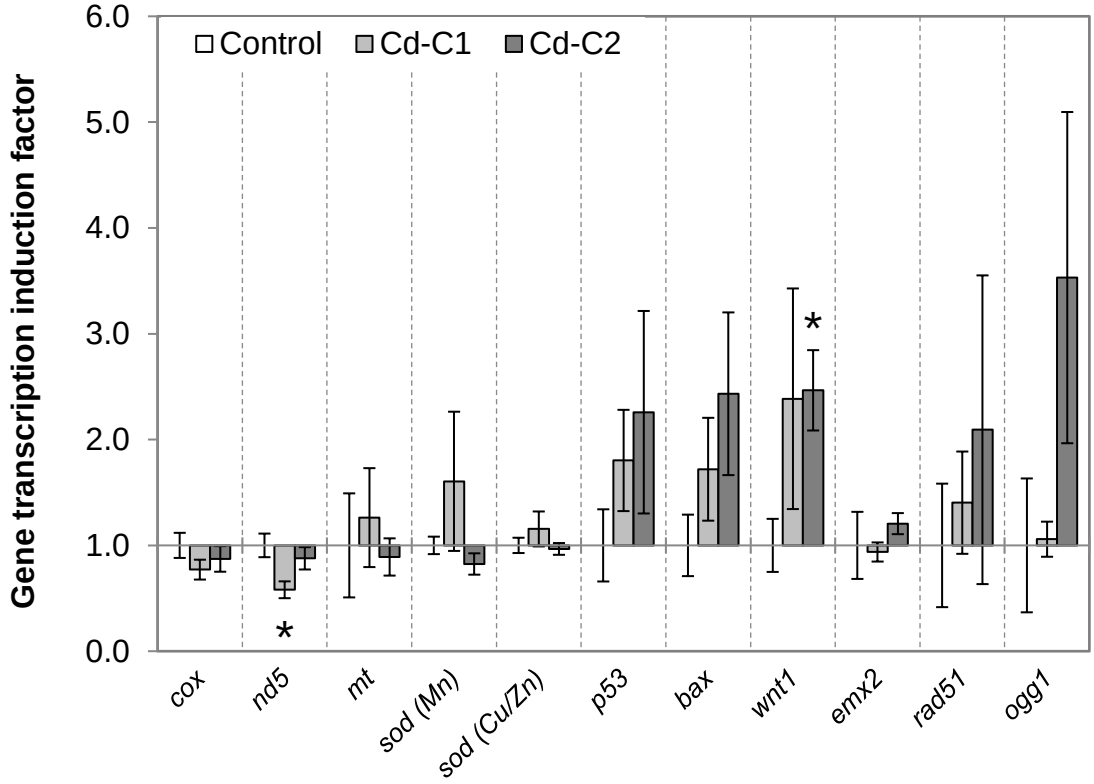

130
120
110
100
90
80
70
Heart rate (beats/min)
6 dpf
7 dpf
a
a
a,b
a,c
b
c
Control
Cd-C1
Cd-C2

Figure 2

(a)


Control
Cd-C1
Cd-C2
Gene transcription induction factor
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
cox
nd5
mt
sod (Mn)
sod (Cu/Zn)
p53
bax
wnt1
emx2
rad51
ogg1

(b)


Control
Cd-C1
Cd-C2
Gene transcription induction factor
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
cox
nd5
mt
sod (Mn)
sod (Cu/Zn)
p53
bax
wnt1
emx2
rad51
ogg1

Tables

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

Marcenac sediment (Lot river, Aveyron, SW France)									
Particulate organic carbon							0.11 %		
Dissolved ammonia (NH_4^+)							62.3 µM		
Dissolved sulfur (H_2S)							16.8 µM		
Granulometric distribution									
10th percentile diameter							14.8 µm		
50th percentile diameter							111.5 µm		
90th percentile diameter							224.7 µm		
≤ 65 µm fraction							25.8%		
Trace metals levels (µg/g dw)									
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
Organic compounds levels (ng/g dw)									
ΣPAH[a]							12 ng/g dw		
ΣPCB[b]							1 ng/g dw		
ΣPBDE[c]							Not detected		

a, cumulative concentration of 21 analyzed polycyclic aromatic hydrocarbon compounds

b, cumulative concentration of 8 analyzed polychlorobiphenyl congeners

c, cumulative concentration of 4 analyzed polybrominated diphenylethers

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

Concentrations in µg/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

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
β-actin	Cytoskeletal gene (housekeeping gene)	**S74868**	GTGACCCACACAGTGCC[a] GCGACGTAGCACAGCTTC[b]
coxI	Cytochrome C oxidase subunit I (complex IV of the mitochondrial respiratory channel)	**NC_004387** (gene ID 805432)	TTCCCCCAACACTTCTTAGGC[a] TGTGGCTGTTAGTTCGACTGA[b]
nd5	NADH-dehydrogenase subunit V (complex I of the mitochondrial respiratory channel)	**NC_004387** (gene ID 805441)	ACCCTCCCCTTACTCGG[a] AGCAAAGGCAGGTGGAC[b]
p53	Tumor suppressor gene P53	**AF212997**	TCTGGCACTGCAAAGTCTGT[a] CCTCGTTTTGGTGGTGGG[b]
mt	Metallothionein gene	**AY466516**	ACAAACTGCTCCTGCACC[a] ACTGACAACAAGTAGTGTCGC[b]
rad51	Homologous recombination gene (homologous RecA)	**ENSORLG00000017821**	CGCATGCTGCTGCGACTG[a] TTCCCCTCGACCTTTCCTC[b]
wnt1	Wingless integration site 1 (cell proliferation and somitogenesis)	**AJ243208**	CCGCTTTGACGGAGCAT[a] TTGAACCCACGCCCACAGC[b]
sod(Mn)	Mitochondrial Fe/Mn superoxide dismutase	**ENSORLG00000013261**	ATGGCTGGGCTATGACAAAG[a] TGGCTATCTGAAGACGCTCAC[b]
sod(Cu/Zn)	Cytosolic Cu/Zn superoxide dismutase	**ENSORLG00000008041**	GGGAAATGTGACCGCAGG[a] GCCAAACGCGCTCCAG[b]
bax	Bcl-2 associated X protein gene	**ENSORLG0000000456**	TCTTCGCTCAGTCCCTCC[a] GCCAACGTCTGCCAGCCA[b]
ogg1	8-oxoguanine glycosylase 1 gene (BER family)	**ENSORLG00000010758**	CTCGTATTCAGGGCATGGT[a] ACCCGTGGCTGTCTAAG[b]
emx2	Empty spiracles homolog 2 (nervous system development)	**ENSORLG00000014157**	CCATTACGTGGTGGGAGCA[a] GGGCTCGCCTGTTTAGT[b]

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

14

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

	Measured concentration			Nominal concentration	Spiking efficiency (%)
	T0	**T7***	**T9***		
Cd concentrations in sediment (µg/g dw)					
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
Cd concentration in ERS buffer (µg/L)					
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]	-	-

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

Table 5. Embryonic survival, hatching success and time to hatch following medaka embryo exposure to Cd-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

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