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# Effects of copper and cadmium spiked-sediments on embryonic development of Japanese medaka (*Oryzias latipes*)

Iris Barjhoux<sup>a</sup>, Magalie Baudrimont<sup>b</sup>, Bénédicte Morin<sup>a</sup>, Laure Landi<sup>a</sup>, Patrice Gonzalez<sup>b</sup>, Jérôme Cachot<sup>a</sup>

1 a Univ. Bordeaux, EPOC/LPTC, UMR 5805, F-33400 Talence, France

2 b Univ. Bordeaux, EPOC/EA, UMR 5805, F-33400 Talence, France

Contact author: Jérôme Cachot

Postal address: UMR 5805 EPOC, CNRS, LPTC group, University of Bordeaux, 351 cours de la Libération, 33405 Talence Cedex, France

Email address: [jerome.cachot@u-bordeaux1.fr](mailto:jerome.cachot@u-bordeaux1.fr)

Phone: +33 (0)5 40 00 38 30

Fax: +33 (0)5 40 00 22 67

dpf: days post fertilization; dw: dry weight; ELS: early life stages; ERS: egg rearing solution; hpf: hours post fertilization; LMP: low melting point agarose; LOEC: lowest observed effect concentration; MELA: Medaka Embryo-Larval Assay; MEM: minimum essential medium; MS222: tricaine methanesulfonate; NMP: normal melting point agarose; NOEC: no observed effect concentration; PBS: phosphate buffered-saline; PNEC: probable no effect concentration; POPs: persistent organic pollutants; ww: wet weight

## Abstract

3 Because of their high capacity to accumulate contaminants such as persistent organic pollutants and heavy  
4 metals, aquatic sediments are considered as a long-term source of contamination for aquatic organisms. In  
5 compliance with the increasing interest both for sediment quality evaluation and the use of fish early life stage  
6 (ELS) toxicity assays, we proposed an embryo-larval test to evaluate embryotoxicity and genotoxicity of  
7 sediment-bound contaminants. Pre-blastula stage medaka (*Oryzias latipes*) embryos were exposed by static  
8 sediment contact to two model heavy metals (cadmium and copper) at environmental concentrations during the  
9 whole 10-day embryonic development. Lethal and sub-lethal effects were recorded in both embryos and larvae  
10 for 20dpf (days post fertilisation) using several global toxicity and phenotypic endpoints. The comet assay was  
11 also performed on medaka prolarvae to evaluate genotoxic effects of the tested chemicals. Environmental  
12 concentrations of cadmium (Cd) and copper (Cu) did not affect embryo and larval survival. However, both  
13 heavy metals significantly induced morphological abnormalities, particularly spinal and cardiovascular  
14 deformities. Cd but not Cu induced tachycardia. Both heavy metals induced a significant increase in DNA  
15 damage at all tested concentrations. Resulting LOEC values for Cd and Cu corresponded to 1.9 and 8.5 µg/g dw  
16 sediment respectively. Although metal bioavailability is probably lower for naturally-contaminated sediments,  
17 the relatively low toxicity thresholds for both Cd and Cu raise the question of possible risk for fish embryos  
18 developing in direct contact to sediments. This study demonstrates the applicability, sensitivity and relevance of  
19 the Japanese medaka embryo-larval assay (MELA) to evaluate sediment hazardous potency at environmental  
20 concentrations of heavy metals.

21 *Keywords: Medaka embryo-larval assay, sediment, teratogenicity, genotoxicity, heavy metals*

22

## 23 1. Introduction

24 The European Water Framework Directive (WFD) aims to reach a good ecological and chemical status in all  
25 European surface waters by 2015 (EC, 2000). However, chemical characterisation and toxicity evaluation of  
26 sediments are not clearly required by the WFD. Actually, sediments are well-known to be important secondary  
27 sources of contamination for aquatic environment because of their high capacity to sequester and then to release  
28 a great number of persistent chemicals such as POPs (persistent organic pollutants) and heavy metals (Burton,  
29 1991). For benthic organisms, exposure can occur via both aqueous phase (water column and porewater) and  
30 direct contact to or ingestion of contaminated particles (Kosmehl et al., 2006). For these reasons, sediments play  
31 a non-negligible role in the pollutants' bioavailability and route of exposure especially for benthic organisms.  
32 Consequently, they represent key compartments that have to be taken into account in risk assessment approaches  
33 recommended by the WFD.

34 Several test phases have been used for sediment toxicity testing (Burton, 1991) as for instance extractable phase,  
35 pore water and *in situ* assays (sediment test chamber, *in situ* mesocosm...). However, whole sediment exposure  
36 is generally considered as the most realistic integrative method to mimic *in situ* contamination of organisms  
37 because the uptake route (including sediment-contact and aqueous phase exposure) is very similar to that in  
38 environmental conditions, taking sediment characteristics and chemical properties into account (Hollert et al.,  
39 2003; Kosmehl et al., 2006; Mages et al., 2008).

40 In recent years, fish embryos have gained interest in risk assessment procedures because of their high sensitivity  
41 to pollutants and their ecological relevance (e.g. recruitment, population wellness, Burton, 1991; Cao et al.,  
42 2009) and also because of the new European regulation on the protection of animals used for scientific purpose  
43 (EC, 2010). Considering that sediments are putative spawning substrates for many pelagic and benthic organisms  
44 including fish, the use of fish early life stage (ELS) to evaluate sediment contamination would enable effects on  
45 hatchability, development and growth, which are considered as critical endpoints, to be monitored (Burton,  
46 1991).

47 In this context, an increasing number of studies proposed whole sediment-contact bioassays using fish embryos,  
48 (Hollert et al., 2003; Hallare et al., 2005a; Kosmehl et al., 2006). In these studies, the potentially lethal and  
49 teratogenic effects of the tested sediments were assessed using classical endpoints such as embryonic survival,  
50 hatching rate, growth retardation, heart rate and developmental abnormalities. An adaptation of the comet assay  
51 was also proposed to evaluate the genotoxicity of whole sediments (Kosmehl et al., 2006; Kosmehl et al., 2008).  
52 All these works clearly demonstrate the suitability of such embryo-larval assays for natural sediment testing as it  
53 enables complex environmental matrices containing several chemicals in mixture to be analysed and the sole  
54 bioavailable fraction of particle-bound pollutants to be evaluated. However, to our knowledge, very few studies  
55 used this approach to measure the toxicity of single compounds artificially coated onto sediments and only  
56 hydrophobic organic pollutants were concerned (Vicquelin et al., 2011).

57 Japanese medaka embryos offer the same advantages than zebrafish embryos for developmental toxicity testing  
58 *i.e.*: transparent chorion, lack of pigments in embryos which facilitates non-invasive observations during the  
59 development; sensitivity to toxicant exposure (e.g.: Gonzalez-Doncel et al., 2003a; Farwell et al., 2006;  
60 Gonzalez-Doncel et al., 2008); well-described stages of development (Iwamatsu, 2004; Gonzalez-Doncel et al.,

61 2005); small size; adaptability to various environmental conditions (Carlson et al., 2002; Wittbrodt et al., 2002;  
62 Yao et al., 2010); low maintenance cost; short maturation time (Wittbrodt et al., 2002) and spawning all over the  
63 year in good breeding conditions (Iwamatsu, 2004). However, whereas zebrafish embryos hatch after 2-3 days of  
64 development, medaka embryos development extends up to 9 days at 26°C. This longer embryo development in  
65 the egg provides the possibility to extend the duration of embryonic exposure up to 9-10 days instead of 48  
66 hours, which can mimic a more chronic exposure to contaminants. Furthermore, Japanese medaka is tolerant to a  
67 wide range of temperatures and salinities allowing experiments in very contrasted thermal and salinity  
68 conditions. Finally, one of the major advantages of the Japanese medaka is the low baseline embryo and larval  
69 mortality.

70 Heavy metals are among the most widespread pollutants in environment and have a high potency to accumulate  
71 in sediments (Burton, 1991; Chapman et al., 1998). It has been well documented that excessive aqueous  
72 cadmium (Cd) or copper (Cu) exposure could lead to dramatic effects on fish embryos such as high mortality,  
73 low hatchability, delay in time to hatch, reduced length, weight and head height, cardiac activity disturbances  
74 and numerous developmental impairments including craniofacial alterations, yolk sac abnormalities, spinal  
75 deformities, oedemata, cardiovascular disturbances and lack of pigmentation (reviewed by Jezierska et al.,  
76 2009). In addition, both heavy metals have been reported as potent genotoxicants in several fish cell lines,  
77 generating reactive oxygen species (ROS) that can induce DNA strand breaks and trigger apoptosis and/or cell  
78 death (Risso-de Faverney et al., 2001; Manzl et al., 2004; Sandrini et al., 2009).

79 However, to our knowledge, there is a lack of data on the bioavailability and effects of sediments specifically  
80 contaminated by heavy metals on fish embryos development. Furthermore, this kind of approach could be  
81 extremely useful to calculate toxicity thresholds (LOEC) of particle-bound pollutants in fish and to derive  
82 sediment quality guidelines (PNEC).

83 The objective of the present work is to characterise both the teratogenic and genotoxic effects of Cd and Cu  
84 using a modified version of the Japanese medaka embryo-larval assay (MELA) (Helmstetter and Alden, 1995)  
85 and spiked sediments as source of contaminants. Medaka embryos were exposed to spiked sediments during  
86 their whole embryonic development. In the course of the exposure several lethal and sublethal endpoints were  
87 followed at both embryonic and larval stages, including cardiac activity, hatching, developmental impairments  
88 and survival. In addition, a comet assay was performed on 2-day old larvae to evaluate DNA damage induced by  
89 heavy metals.

90

## 91 **2. Materials and Methods**

92

### 93 **2.1 Chemicals**

94 CdCl<sub>2</sub>·2H<sub>2</sub>O, phosphate-buffered saline (PBS), nitric acid 65% and normal melting point agarose (NMP) were  
95 purchased from Fluka Chemie (Buchs, CH). Cu standard 1000mg Titrisol® (CuCl<sub>2</sub> in H<sub>2</sub>O) was purchased from  
96 Merck (Darmstadt, DE). Tricaine methanesulfonate (MS222), low melting point agarose (LMP) and collagenase

97 (Collagenase from *Clostridium histolyticum* Type IV) were purchased from Sigma Aldrich (Lyon, FR). MEM  
98 (Minimum Essential Medium) was purchased from Gibco (Invitrogen, Cergy Pontoise, FR)  
99

## 100 **2.2 Reference sediment characterisation**

101 The reference sediment was collected in the Lot river, on Marcenac site (December 2008, SW France), which is  
102 considered as a pristine site for heavy metal contamination in the Lot/Garonne/Gironde continuum (Audry et al.,  
103 2004; Audry et al., 2010). The reference sediment was stored at -20°C, then freeze-dried and finally slightly  
104 crushed using a mortar and a pestle to eliminate larger particles and homogenise particle size before use.

105 The particle-size distribution and particulate organic carbon concentration of the freeze-dried crushed sediment  
106 were determined by diffractometry and infrared spectroscopy (Etcheber et al., 1999) respectively. Dissolved  
107 ammonium and sulphide were measured in pore waters extracted from the sediment using colorimetric  
108 procedures (Strickland and Parsons, 1972) and spectrophotometrical analyses with the methylene blue method  
109 (Cline, 1969) respectively. Detailed protocols used for the forementioned analyses are described by Vicquelin et  
110 al. (2011).

111

## 112 **2.3 Sediment spiking**

113 Four spiking concentrations were determined using Cd contents recorded in sediments along the Lot-Garonne-  
114 Gironde continuum (Table 1). A 0.3X-concentration (2 µg/g dw sediment) mimicked a low Cd-impacted area  
115 (e.g. La Reole on Garonne river), a 1X-concentration (6.5 µg/g dw) was selected as a mean Cd-level present in  
116 the fluvial-estuarine system and a 3X-concentration (20 µg/g dw) was not set to represent the highest  
117 concentration but a moderate Cd content as measured along the Lot river (e.g. Carjac site). A control treatment  
118 (0 µg/g dw) was also added to the conditions cited above. The same concentrations were tested for the Cu  
119 treatment to allow comparison of effects between the two metals.

120 The 30X Cd-stock solution (2.5 mgCd/mL) was obtained by adding 48.79 mg of CdCl<sub>2</sub>·2H<sub>2</sub>O to 10mL of  
121 ultrapure water (Milli-Q Maxima, Elga Labwater, Veolia water, Blagnac FR). The Cu-stock solution was made  
122 by diluting Cu standard 1000mg (CuCl<sub>2</sub>) in 1L of ultrapure water. Dilutions of these Cd and Cu stock solutions  
123 were performed in ultrapure water to obtain the three contamination solutions used for sediment spiking.

124 For each treatment, 25 g dw of sediment was spiked by adding 2 mL of contamination solution (or ultrapure  
125 water for control treatment) and 11mL of ultrapure water as homogenising solvent. The whole mixture was kept  
126 under agitation for 1h then, once supernatant removed at most, kept all night at room temperature for partial  
127 drying. The spiked-sediment moisture content was gravimetrically determined to calculate the wet weight (ww)  
128 equivalent to 5 g dw for each condition. After manual homogenisation, the sediment of a same spiking-  
129 concentration was divided into five aliquots of 5g dw equivalent. Three aliquots were kept for embryo-toxicity  
130 testing, another one was used for chemical analysis (T0 measurement) and the last one was saved as a  
131 supplement.

132

## 133 **2.4 Fish breeding and embryos collection**

134 Mature Japanese medakas were reared and maintained in 40L-tanks with a male:female ratio of 1:2, at a  
135 temperature of 26°C with a photoperiod of 12h light:12h dark. Tanks were cleaned using a siphon and 30 % was  
136 renewed with stalling water (1/3 24h-dechlorinated tap water and 2/3 purified water by reverse osmosis; pH 7.5;  
137 53.4 ppm CaCO<sub>3</sub>; 0.025 ppm NO<sub>2</sub>; 1.5 ppm PO<sub>4</sub>; 5 ppm NO<sub>3</sub>; <0.1ppm NH<sub>4</sub>) twice a week. Fishes were fed once  
138 a day with dry flakes (Tetramin® Tropical, Tetra, Melle, Germany) and once a day with brine shrimp nauplii  
139 *Artemia sp.* nauplii (Ocean Nutrition, Assen, Belgium). It was visually checked daily and unhealthy fish  
140 individuals were removed and euthanized using MS222 solution at 1g/L. During the spawning stimulation  
141 period, medaka genitors were reared at 28°C under a 16h:8h light:dark photoperiod and fed four times a day.  
142 Females were examined for eggs 1h after onset of light and egg clusters were gently removed by hand and  
143 placed in Egg Rearing Solution (ERS; 17.1mM NaCl; 0.4mM KCl; 0.36mM CaCl<sub>2</sub>; 1.36mM MgSO<sub>4</sub>; pH 7.0).  
144 Harvested eggs were pooled and then individualised by rolling them softly to sever attaching filaments. Finally,  
145 they were staged according to Iwamatsu (2004) and Gonzalez-Doncel et al. (2005) under a stereomicroscope  
146 (Leica MZ75, Leica Microsystems, Nanterre, France) and cold light source (Intralux® 4100, Volpi AG,  
147 Schlieren, Switzerland) to discard unfertilised or dead individuals and only pre-morula stage embryos (2-4hours  
148 post fertilisation, hpf) were selected for the study. Animal breeding and experiments on embryos and larvae were  
149 conducted in accordance to the EU directive 210/63/EU on the protection of animals used for scientific purposes.

150

## 151 **2.5 Embryos exposure by sediment-contact**

152 Two distinct experiments were performed for Cd and Cu toxicity analysis. Both experiments followed the same  
153 general protocol, which consisted of three independent replicates for each condition. As mentioned above, 5 g  
154 dw sediment were placed in a 35 mm diameter plastic Petri dish for each replicate and each concentration.  
155 Sediment was then covered with 1 ml of ERS buffer at pH 7 and then maintained at 26°C for 4 to 5 hours before  
156 the beginning of the experiment.

157 Immediately after sorting, 30 embryos per replicate were randomly placed on a Nitex® mesh (mesh opening  
158 1000µm, Sefar Filtration Inc., Depew, NY, US) which was then slightly sunk into the sediment to allow a good  
159 contact between the embryos and the sediment without any risk that embryos might get entirely buried into the  
160 sediment and die from hypoxia. ERS levels in exposure dishes were daily checked and topped in case of  
161 evaporation. Dissolved oxygen was measured daily throughout the 10-day exposure period in the reference  
162 sediment (Marcenac freeze-dried sediment) using a Clark-type sensor equipped with a guard cathode (Unisense,  
163 Aarhus, DK) and connected to a high sensitivity picoammeter (PA2000, Unisense). Good oxygenation of the  
164 medium was confirmed with values always superior to 80% saturation in the water column and at the water-  
165 sediment interface (Data not shown).

166 Embryos were maintained in contact to the sediment during their whole embryonic development until 'hatching  
167 peak' usually around day 9-10 post-fertilisation (dpf) in control condition. Afterwards, unhatched embryos were  
168 transferred to new Petri dishes with 3mL of clean ERS and sediments were collected for T10 heavy metal  
169 analysis. Once hatched, larvae were placed in beakers containing 50mL of clean stalling water which was  
170 renewed (100%) every 2 days. 48hours post-hatching (hph) larvae were fed twice a day with TetraMin® Baby  
171 (Tetra, Melle, DE) flake food until 20dpf.

172 During the whole experiment, embryos and larvae were maintained in a climate cabinet (Economic Delux,  
173 Snijders Scientific, Tilburg, NL) at  $26^{\circ}\text{C} \pm 0.3$  with a 12h:12h photoperiod and 5000Lux white light.

174

## 175 **2.6 Lethal and sub-lethal endpoints**

176 Viability was checked daily at embryonic and larval stages, during the whole experiment (day 1 to 20pf). Dead  
177 embryos (whitish opaque appearance or absence of cardiac contraction after 3dpf) were counted and removed  
178 from each replicate.

179 Cardiac activity was assessed at day 6 and 7pf . Five randomly selected embryos per replicate were taken into  
180 account for this parameter. Heartbeats were counted in three 20s-intervals per individual using Leica MZ75  
181 stereomicroscope (Leica Microsystems, Nanterre, France) and a cold light source (Intralux® 4100, Volpi AG,  
182 Schlieren, Switzerland). Room temperature was maintained at  $23 \pm 1^{\circ}\text{C}$ . These three measurements were then  
183 added to obtain cardiac activity in beats per minute. The five individual measurements were finally averaged to  
184 determine the mean cardiac activity for a replicate.

185 Time to hatch and hatching success were checked daily from the day of first hatching to the end of the  
186 experiment at day 20pf. Only embryos able to fully exit chorions were considered as ‘hatched’, others were  
187 counted as ‘not hatched’. Successfully hatched larvae were taken into account for time to hatch determination,  
188 noting the date of hatching and considering that the day of fertilisation was day 0.

189 Biometric measurements at hatching were performed every day on all newly hatched larva. Larvae were  
190 observed under a stereomicroscope (MZ75) equipped with a colour CDD camera (Leica DFC 420C), connected  
191 to a computer. A picture of whole body larvae was taken, and total body length (from the end of lower jaw to the  
192 end of caudal fin) as well as head size (from the end of lower jaw to pectoral fin attachment level) were assessed  
193 thanks to an image analysis software program (Leica Application Suite v2.8.1). The ratio between head size and  
194 total body length (called further ‘head/length ratio’, expressed in percentage) was also calculated to follow  
195 possible changes in larva proportions.

196 Development abnormalities were observed every day on each newly hatched larva. Six different categories of  
197 abnormalities were recorded including: pericardial, perivitelline and cranial oedemata; spinal deformities  
198 (scoliosis, lordosis, kyphosis, C-shaped larvae and tail malformations); craniofacial deformities (skull and jaw  
199 deformities including underdevelopment); eye anomalies (hyper- hypo- or dystrophia, pigmentation alteration,  
200 hypopigmentation, absence of eye); cardio-vascular anomalies (hyper- hypo- or dystrophia, positioning  
201 abnormality, incomplete or abnormal heart looping, tubular heart characterised by an absence of chamber  
202 differentiation, anemia resulting in an absence of blood circulating cells, haemorrhage, local accumulation of  
203 motionless blood cell) and yolk-sac anomalies (mainly yolk malabsorption).

204

## 205 **2.7 Comet assay**

206 The comet assay was performed at day 12pf on a pool of 5 larvae per replicate. Pools of larvae were digested in a  
207 MEM-Collagenase IV 0.125% (w/v) medium, and after cell viability (superior to 80%) evaluation using a trypan  
208 blue exclusion test, the comet assay was carried out following Morin et al. (2011) protocol. Ethidium bromide  
209 ( $20\mu\text{g/L}$ ) was used as DNA fluorescent tag and all coded-slides were randomly analysed for 75 nuclei per gel

210 (two gels per experimental replicate) using an Olympus epi-fluorescent microscope (400x magnification)  
211 equipped with a grayscale CCD camera (Zeiss, DE) and Komet 5.5 software program (Kinetic Imaging,  
212 Liverpool, UK). As recommended by Hartmann et al. (2003), %tail DNA (percentage of DNA which migrates  
213 from the nucleus *i.e.* the head of the comet) was the selected parameter to be used for DNA damage  
214 measurement. Heavily DNA damaged nuclei displaying small or inexistent head and large diffuse tail and known  
215 as hedgehogs, were not taken into account in the comet measurement as recommended by Kumaravel et al.  
216 (2009). However, the percentage of hedgehogs which have been reported as apoptotic or necrotic cells (Olive  
217 and Banath, 1995) was visually scored on a total of 100 cells per gel.

218

## 219 **2.8 Heavy metals analysis**

220 For each sample, ERS buffer was separated from sediment by a 15 min centrifugation at 4000 rpm at room  
221 temperature.

222 About 1 g ww of each sediment sample was dried for 48 h at 60°C and then digested with 3 ml 65% nitric acid  
223 for 3h at 100°C. Each batch of samples included method blanks and certified reference materials (Tort-2, lobster  
224 hepatopancreas, NRCC-CNRC, Ottawa, CA) and measured values were consistently within certified ranges (data  
225 not shown). After mineralisation, samples were diluted by addition of 15 ml ultrapure water (MilliQ, Bedford,  
226 MA, USA) and metal determinations were performed using an atomic absorption spectrophotometer (Varian  
227 SpectrAA 220 FS, Agilent Technologies, Santa Clara, US).

228 For ERS buffer analysis, about 3 mL of each sample was acidified with 1% final v/v 65% nitric acid. Samples  
229 were then analysed for Cd and Cu contents by ICP-AES (Varian Vista ProAxial, Agilent Technologies, Santa  
230 Clara, US) user standard conditions. Method blanks were added to each set of samples. The quality of the  
231 analytical method was checked by analysing the certified international reference water.

232

## 233 **2.9 Statistical analysis**

234 The data is expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were conducted using Statistica 7.1  
235 software program (Statsoft, Maisons-Alfort, FR). Results were initially tested for normality (Shapiro-Wilk test  
236 on residues with 1% risk) and equality of variance (Levene test, 5% risk). If necessary, data were log-  
237 transformed to fulfil normality and equality of variance criteria. Afterwards, significant differences between  
238 treatments were tested with one way ANOVA – Variance analysis followed by post-hoc Tukey test ( $p < 0.05$ ). If  
239 data transformation was not sufficient to perform parametric analysis, non-parametric Kruskal-Wallis ANOVA  
240 and its post-hoc test (analogous to Bonferroni-Dunn's test) were used ( $p < 0.05$ ).

241

# 242 **3. Results**

243

## 244 **3.1 Reference sediment characterisation**

245 Particle sizes of Marcenac sediment ranged from very fine sand to silt with a median value of 111.5  $\mu\text{m}$ . The  
246 particulate organic carbon content was low (0.11 %) and dissolved ammonium and sulphide in pore water  
247 reached 62  $\mu\text{M}$  and 17  $\mu\text{M}$  respectively (Table 2).

248

### 249 **3.2 Heavy metal content in water and sediments**

250 Table 3 shows Cd and Cu concentrations determined in the sediments at the beginning (T0) and at the end (T10)  
251 of the embryos' exposure. Heavy metal natural background (mean of control measurement) in sediment was  
252 evaluated at  $0.17 \pm 0.04 \mu\text{g/g dw}$  for Cd and  $7.18 \pm 0.49 \mu\text{g/g dw}$  for Cu. In addition, several other trace metals  
253 were present in the reference sediment (Table 3) including Co, Mn, Ni, Zn, Cr, As, Ag and Pb. In contrast,  
254 PAHs, PCBs and organochlorinated pesticides were detected at extremely low concentrations (Data not shown).

255 A clear dose-dependent increase of Cu and Cd contents was observed for Cu- and Cd-spiked sediments  
256 respectively. Measured Cd concentrations were very close to nominal values while Cu concentrations were  
257 slightly above, likely because of the natural background level of Cu in Marcenac sediment. The comparison  
258 between T0 and T10 values showed that more than 80% of the initial Cu or Cd concentrations were still present  
259 in the sediments at the end of the exposure (Table 3). These results indicate that sediment contamination  
260 remained stable over time and that treatments are significantly different from one another.

261 Cd and Cu concentrations were also measured in the overlapping ERS buffer from the reference and spiked  
262 sediments (Table 4). The Cd content of the ERS buffer was below the quantification limit in both ERS and  
263 sediment controls but increased in a dose-dependent manner from 8 to 266  $\mu\text{g/L}$  for the three Cd-spiked  
264 sediments. Cu content was below the quantification limit for the control ERS but it reached 14.2  $\mu\text{g/L}$  for the  
265 control sediment. This unexpected contamination was likely issued from the desorption of particle-bound Cu in  
266 the control sediment. A slight increase of Cu content from 29 to 60  $\mu\text{g/L}$  was noticeable for the three Cu-spiked  
267 sediments. Data comparison between T1 and T9 showed that Cd concentrations remained globally constant  
268 while Cu concentrations decreased with time from 30 to 45%. The pH of the ERS buffer only slightly varied  
269 from 7.0 to 7.7 in the course of the experiment.

270

### 271 **3.2 Embryonic and larval survivals**

272 Cd and Cu tested concentrations did not induce lethal effects on both embryos and larvae. Viability for Cd-  
273 exposed fish ranged from 81 to 91 % at the embryonic stage (Fig. 1a) and from 80 to 85 % at the larval stage  
274 (Fig. 1b) according to exposure conditions. Similarly, Cu-exposed embryos' viability ranged from 93 to 99 %  
275 (Fig. 1a) and larval survival ranged from 71 to 81 % (Fig. 1b) for the various treatments.

276

### 277 **3.3 Hatching rate and time to hatch**

278 Almost all viable embryos hatched before the end of the experiment for both heavy metal exposures. As a result,  
279 hatching rates were very close to embryonic viability and no significant difference was observed in the  
280 treatments. Indeed, mean hatching success ranged from 80 to 91 % for Cd-exposed embryos and from 93 to 99  
281 % for Cu-exposed embryos (Fig. 2a).

282 Embryonic developmental time, corresponding to the time to hatch, was not affected by heavy metal exposures  
283 either. Actually, for both experiments, mean time to hatch was around  $10.2 \pm 0.17$  dpf (Fig. 2b) for all treatment  
284 groups.

285

### 286 **3.4 Cardiac activity**

287 Cu exposure did not affect the embryos' cardiac activity in comparison to that of the control group, neither at  
288 6dpf nor at 7 dpf. Conversely, heartbeats of 6dpf-embryos were significantly increased at Cd-3X concentration  
289 compared to those of the control group (156 and 131 beats/min on average respectively, Fig. 3a). This result was  
290 confirmed at 7 dpf, with a significant increase of cardiac activity in embryos exposed to Cd-1X and Cd-3X in  
291 comparison to the control group (156, 160 and 137 beats/min on average respectively, Fig. 3b).

292

### 293 **3.5 Biometric measurements**

294 Neither Cd nor Cu exposure induced modifications of larval biometric parameters, as compared to the respective  
295 control groups. Total body length of Cd-exposed larvae ranged from 4.95 to 5.00 mm on average (Fig. 4a), head  
296 size from 1.05 to 1.07 mm on average (Fig. 4b) and head/length ratio from 21.5 to 22.1% on average (Fig. 4c).

297 Interestingly, a slight significant increase of the three biometric parameters was observed on larvae from the Cu-  
298 3X group in comparison to Cu-0.3X group (average total length, 5.03 mm and 4.90 mm respectively; average  
299 head size, 1.11 mm and 1.05 mm respectively; average head/length ratio, 22.0 % and 21.5 % respectively; Fig.  
300 4abc).

301 Average values for these measurements were very similar in control groups of Cd and Cu exposures.

302

### 303 **3.6 Morphological abnormalities**

304 Morphological abnormalities on medaka larvae were recorded on hatching day for each individual and  
305 summarised in table 5. Percentages of abnormal larvae in control groups were around 20 %. Larval abnormalities  
306 in Cd-exposed groups were superior to 60 % and significantly different ( $p=0.01$ ) from those of the control  
307 treatment. More precisely, Cd exposure significantly increased the percentage of larvae developing spinal  
308 deformities (mainly kyphosis, lordosis and C-shaped larvae) and cardio-vascular anomalies (mainly abnormal  
309 positioning and heart looping) at 0.3X and 3X concentrations (for 1X group,  $p=0.074$ ).

310 Cu exposure had overall the same effect as Cd. The percentage of deformed larvae significantly increased  
311 ( $p=0.04$ ) up to 50 % for each contaminated groups in comparison to control treatment (17 % of deformed  
312 larvae). As observed during the Cd experiment, developmental abnormalities mainly concerned spinal column  
313 (kyphosis and lordosis) and cardiovascular system (abnormal positioning and heart looping). Percentage of  
314 individuals affected by this kind of deformities was significantly higher in Cu-3X ( $p=0.049$ ) and Cu-1X  
315 ( $p=0.020$ ) groups respectively in comparison to control treatment. Average percentage of individuals with spinal  
316 deformities in Cu-0.3X treatment was close to significance threshold with  $p=0.077$ . Moreover, yolk  
317 malabsorptions were significantly increased by Cu-1X treatment compared to control (23 % and 9 %  
318 respectively).  $p$  value between 3X- and control groups was not far from significance threshold ( $p=0.061$ ).

319

### 320 **3.7 Genotoxic effects**

321 The mean percentage of tail DNA measured in control groups was inferior to 10 % which confirmed that cells,  
322 even after dissociation treatment, were in satisfactory conditions for comet assay analysis (Collins, 2004).

323 Cd exposure led to a significant DNA damage induction ( $p < 0.001$ ) from the lowest concentration as shown by  
324 the significant increase in the percentage of tail DNA from 7 % for the control group up to 48 % - 60 % in  
325 average for contaminated groups (Fig. 5a). The percentage of 'hedgehog cells' showed the same response pattern  
326 as it raised to 25 - 28 % for Cd-exposed group against only 12 % in the control group (Fig. 5b).

327 Interestingly, only Cu-0.3X and Cu-3X concentrations induced significant DNA damage increase in comparison  
328 to control group (5 % of tail DNA in control *versus* 16 % in Cu exposed groups in average, Fig. 5a). As noticed  
329 in the Cd experiment, similar results were observed with the percentage of 'hedgehog cells', which was  
330 significantly increased at the lowest, and at the highest Cu doses (30 % and 25 % respectively, Fig. 5b)  
331 compared to control (11% in average).

332

## 333 **4. Discussion**

334 Medaka embryos that were allowed to develop in direct contact to control sediment in our test conditions showed  
335 survival rates around 90 % and 80 % at embryonic and larval stages respectively and hatching success superior  
336 to 85%. These results are in compliance with the guidelines for ELS assays which set minimum hatching success  
337 and survival rate after hatching at 80 % each (OECD, 1992; OECD, 1998).

338 Average times to hatch observed in control treatment were very similar in both experiments (10.2 and 10.2 dpf  
339 for Cd and Cu exposures respectively) with more than 70 % of embryos hatching at 10 dpf. These results are in  
340 compliance with observations reported in the literature which describe hatching as usually occurring between 9  
341 and 12 dpf at 25 - 26°C for the Japanese medaka (Gonzalez-Doncel et al., 2003a; Farwell et al., 2006; Gonzalez-  
342 Doncel et al., 2008).

343 Similarly, biometric measurements average values were very close to those of the control groups of both  
344 experiments. Several authors reported total body length of Japanese medaka larvae at hatching between 3.8- 4.7  
345 mm in average (Iwamatsu, 2004; Farwell et al., 2006; Oxendine et al., 2006). With an average total body length  
346 nearby 5 mm for both experiments, unexposed larvae from our stockbreeding are a bit longer in comparison with  
347 the data reported above. These biometric variations in unexposed individuals between experiments and  
348 laboratories might be closely linked to breeding conditions of genitors and embryos development environment.

349 During both experiments, baseline cardiac activity in control embryos ranged between 130 and 140 beats per  
350 minute. This data is quite inferior to that reported by González-Doncel et al. (2005) who measured a cardiac  
351 rhythm stabilised at 180 beats per minutes in 7 dpf-embryos but very similar to the results obtained by Nassef et  
352 al. (2010) in 6 dpf-control embryos.

353 Developmental abnormalities are currently used as sensitive endpoints in embryo-larval bioassays. In this study,  
354 many kinds of defects were recorded from the optimal development (as described by Iwamatsu, 2004; Gonzalez-

355 Doncel et al., 2005) and the baseline level of abnormal larvae ranged from 16 to 20% depending on the  
356 considered experiment. Such a percentage of larval abnormalities have already been reported in unexposed  
357 larvae for various fish species including medaka (Farwell et al., 2006; Benaduce et al., 2008) but was almost  
358 twice higher than the previously reported baseline data (Vicquelin et al., 2011). It may be partly explained by the  
359 metal content differences between the two reference sediments. Indeed, the reference sediment from Marcenac  
360 which was used in the present study contained detectable levels of certain metal species including Cu (Table 3)  
361 and it was shown that at least a fraction of the particle-bound Cu was released in the water column within 24h  
362 after the beginning of the experiment (Table 4). Moreover, deformity categories (types of deformity and number  
363 of categories) recorded and recognition criteria (severity, minimum score for healthy/deformed classification) are  
364 variable between studies and strongly influence the number of individuals considered as 'deformed' or not. In  
365 this study, we selected a wide range of malformations categories and even weak abnormalities were taken into  
366 account.

367 Both lethal and sub-lethal endpoints observed in control groups enable this sediment-contact assay to be  
368 validated as it provides with acceptable conditions for medaka embryo development. As a result, natural Cu  
369 background level of 7 µg/g dw in sediment corresponding in our test conditions to about 14 µg/L of Cu released  
370 in the water column seems to have limited effects on medaka embryo survival and development.

371 Spiked Cu- or Cd-concentrations of 2, 6.5 and 20 µg/g of dw sediment (up to 266 µg/L Cd and 60 µg/L Cu in  
372 water) neither affected medaka survival nor hatching success or time to hatch. Absence of acute effects using the  
373 same endpoints was also reported after waterborne exposure of medaka late morula staged embryos (~5 hpf) to  
374 Cd at concentrations from 2.5 to 80 mg/L. In a second experiment, Cd exposure was initiated during fertilisation  
375 and led to a significant reduction of hatching success from 20 mg/L of Cd (Gonzalez-Doncel et al., 2003b). In  
376 rainbow trout embryos, premature or delayed hatching was observed following exposure to 0.05 - 2.5 µg/L Cd  
377 depending on the concentration level (Lizardo-Daudt and Kennedy, 2008).

378 Although no acute effect was observed in medaka embryos, a significant increase in morphological deformities  
379 was induced in newly hatched larvae at concentrations as low as 1.9 µg/g Cd or 8.5 µg/g Cu in sediment (1.9  
380 µg/L Cd and 29 µg/L Cu in water). More precisely, Cu significantly enhanced spinal deformities (kyphosis and  
381 lordosis mainly), cardiac anomalies (in particular abnormal positioning and heart looping) and yolk-sac  
382 malabsorption while Cd only affected the vertebral column and cardio-vascular system.

383 Numerous studies documented developmental effects of heavy metals and more specifically Cd and Cu in fish  
384 species. For instance, *Pagrus major* embryos exposed to 0.8 up to 3.2 mg/L of Cd led to 42 - 100 % of  
385 morphological abnormalities (Cao et al., 2009). Lugowska and Witeska (2004) reported a high percentage of  
386 deformed larvae, sometimes superior to 60% among common carp individuals exposed to 0.2 mg/L of Cu.  
387 Several categories of abnormalities have been reported including blastodermal lesions, yolk-sac and heart  
388 oedemata, haemorrhages, damaged blood vessels, hypopigmentation, craniofacial deformities including head and  
389 eye hypoplasia, cardiac abnormalities, deformed yolk sac and vertebral deformities including C-shaped larvae,  
390 shortened body and altered axial curvature (Cheng et al., 2000; Cheng et al., 2001; Lugowska and Witeska,  
391 2004).

392 Skeletal deformities are commonly observed after Cu and Cd exposures of fish embryos and are generally one of  
393 the predominant observed malformations (Chow and Cheng, 2003; Lugowska and Witeska, 2004), sometimes

394 representing more than 80 % of all recorded defects (Jeziarska et al., 2000). It had been reported that Cd  
395 exposure induces a reduction of myosin heavy chain production in the trunk which is correlated to a  
396 disorganisation of myotomes in the somites and results in altered spinal curvature (Cheng et al., 2000; Chow and  
397 Cheng, 2003).

398 As previously mentioned, fish cardiovascular system is also a target of heavy metal toxicity. Indeed various  
399 cardiovascular pathologies were described following exposures to heavy metals including haemorrhages,  
400 hypertension, oedemata (a result of an alteration of vascular permeability), megalocardias, circulatory collapse,  
401 tubular heart, heart rate alteration, red blood cells accumulation, atrium/ventricle morphology alteration,  
402 abnormal heart looping and aberrant vascular patterning (Cheng et al., 2001; Gonzalez-Doncel et al., 2003b; Cao  
403 et al., 2009; Li et al., 2009). Although these pathologies are frequently reported in the literature, little is known  
404 about the exact mechanisms involved in such cardiovascular dysfunctions. Several works previously showed an  
405 alteration of the cardiac functions following Cu or Cd exposure. For instance, zebrafish embryo heart rate was  
406 significantly increased by Cu exposure (Johnson et al., 2007) whereas Cd reduced the cardiac activity of the red  
407 sea bream (Cao et al., 2009) and zebrafish embryo (Hallare et al., 2005b). An explanation proposed by the  
408 authors for the alteration of cardiac functions is a perturbation of ionic channels such as  $\text{Ca}^{2+}$ -ATPases following  
409 Cd treatment or  $\text{Na}^+/\text{K}^+$ -ATPases following Cu exposure (Wong and Wong, 2000; Eyckmans et al., 2010). The  
410 respective results of such ionoderegulation would be a reduced  $\text{Ca}^{2+}$  uptake leading to the observed bradychardia  
411 and stress response increasing heart rate (Johnson et al., 2007; Cao et al., 2009). It also had been shown that  
412 cardiac function and hemodynamic conditions strongly influence cardiac morphogenesis and vascular  
413 endothelium modelling (Glickman and Yelon, 2002; Sidi and Rosa, 2004). Moreover, cardiac morphologic  
414 alterations described by Hove et al. (2003) following artificial perturbations of blood flow are very similar to  
415 those observed in the present study (abnormal positioning and heart looping) after Cu and Cd exposures. These  
416 observations seemed to confirm the strong relationship existing between cardiac functions and cardiac  
417 morphogenesis as mentioned by Incardona et al. (2004).

418 In the present study, yolk-sac malabsorptions were significantly increased following embryonic Cu exposure to  
419 23  $\mu\text{g/g}$  dw sediment (60  $\mu\text{g/L}$  in water). Lugowska and Witeska (2004) recorded yolk-sac resorption defects in  
420 common carp larvae after exposure to 0.2 mg/L of Cu. Yolk sac area in zebrafish larvae exposed to 190-464  
421  $\mu\text{g/L}$  of Cu was significantly larger in comparison to control larvae (Johnson et al., 2007). A concomitant  
422 decrease of zebrafish length was also observed and the authors concluded that Cu induced a reduction of yolk  
423 utilisation and embryonic rate of development impairment. However, in our study, no diminution of larvae size  
424 was observed after Cu exposure.

425 Another aspect of heavy metal toxicity that was highlighted during the medaka embryo exposure to Cd and Cu  
426 was the genotoxic potency of these compounds. Actually, DNA damage was significantly increased in 2 dph-  
427 larvae following Cu and Cd exposure from the lowest concentration (1.9  $\mu\text{g/g}$  Cd and 8.5  $\mu\text{g/g}$  Cu). DNA  
428 damage assessed with the comet assay has already been reported in fish cells following Cu and Cd during *in vivo*  
429 or *in vitro* exposures (Risso-de Faverney et al., 2001; Bopp et al., 2008; Morin et al., 2011). It has been  
430 hypothesised and partially demonstrated that both heavy metals indirectly generate DNA strand breaks *via*  
431 reactive oxygen species production which could led to cell death and/or apoptosis (Risso-de Faverney et al.,  
432 2001; Manzl et al., 2004; Bopp et al., 2008). The significant increase of 'hedgehog cells' observed in the present

433 study seems to confirm this conclusion. However, the origin of such heavily damaged cells is still unclear.  
434 Previous works have demonstrated that apoptotic cells did not systematically result in 'hedgehog cells' after  
435 comet assay performing (Meintieres et al., 2003). Moreover, it has been reported that these comets, characterised  
436 with a small or inexistent head with a highly diffused tail physically separate from the head, could be the result  
437 of apoptosis or necrosis but also of high radiation doses or potent mutagen exposures (Burlinson et al., 2007;  
438 Kumaravel et al., 2009).

439 Overall observed effects of Cd and Cu were very comparable which seems to indicate similar mode of actions,  
440 probably based on oxidative stress generation and/or ionoregulation alteration as mentioned above. It had already  
441 been reported that equivalent concentrations of Cu and Cd caused similar teratogenic impairments in common  
442 carp larvae (Lugowska and Witeska, 2004; Lugowska, 2007). The results of the present study are used to  
443 establish the LOEC (Lowest Observed Effect Concentration) values for sedimentary compartment as regards the  
444 two tested heavy metals using the most sensitive endpoints. Indeed, both percentages of abnormal larvae and tail  
445 DNA were significantly increased following the medaka embryo exposure to even the weakest tested  
446 concentration of Cd and Cu, resulting in LOEC values of 1.9 µg/g (8 µg/L) and 8.5 µg/g (29 µg/L) respectively.  
447 The values take into account the overall metal content of sediment and indicate a higher toxicity of Cd compared  
448 to that of Cu. Present LOEC values are generally much lower than those reported in the literature, which varied  
449 from 0.5 µg/g to 861 µg/g for Cd (Table 6) and from 17.2 µg/g to more than 820 µg/g for Cu (Table 7). In fact,  
450 most of the LOEC were obtained in invertebrates. It can be hypothesised that fish ELS are much more sensitive  
451 to metals such as Cu and Cd. This implies that Cd and Cu accumulated in natural sediments may represent a  
452 threat for wild fish embryos developing in direct contact to sediments. Indeed, most of the sediments along the  
453 Lot-Garonne-Gironde fluvial system present Cd and/or Cu contents superior to the LOEC determined in the  
454 present study (Table 1). However it should be kept in mind that metal bioavailability likely differs between  
455 'naturally' and artificially contaminated sediments in particular for freshly spiked sediments. In the present  
456 study, embryo exposure was performed within 4 to 5 hours following sediment spiking. This probably results in  
457 a maximisation of Cd and Cu availability and thus to an overestimation of Cd and Cu toxic potencies. However,  
458 since sediment spiking was performed by simply adding metal-contaminated water to sediment under agitation,  
459 this may mimic an accidental and recent release of Cu or Cd in aquatic environment (Burton, 1991). Sediment  
460 ageing could be performed to better mimic

461 Several sublethal endpoints were significantly induced following Cd or Cu exposures and the most sensitive  
462 were the percentages of deformed larvae and tail DNA measurement. This kind of sensitive, integrative, low-cost  
463 and easy handling endpoints could be really useful in future normalised bioassays to assess the toxicity of  
464 sediments.

465 Finally, the present study demonstrated a successful application of MELA to the evaluation of the toxicity of  
466 metals accumulated in sediments. As proposed by these authors, a standardisation of the spiking matrix, using an  
467 artificial sediment and ageing process, could improve MELA's sensitivity and applicability. Moreover, the  
468 MELA could also be enriched by a mechanistic approach using molecular tools for instance, gene expression  
469 monitoring, enzymatic activity measurements and/or proteomics analysis performed on embryos and larvae.

470

## 471 **5. Conclusion**

472 Observations on control embryos demonstrate that the sediment-contact exposure protocol performed in this  
473 study provides acceptable conditions for medaka embryo development. Results obtained in contaminated groups  
474 showed obvious sublethal effects of both Cu and Cd on medaka development. Developmental defects mainly  
475 included spinal deformities, yolk-sac malabsorption and cardio-vascular injuries. Moreover, genotoxic effects  
476 were induced by both metals in 2-day old medakas. Consequently, the percentage of deformed larvae and tail  
477 DNA measurements appeared as relevant markers in sediment toxicity assessment. This study demonstrates the  
478 applicability of MELA to the evaluation of sediment hazardous potency at environmental concentrations of  
479 metals. Moreover, it confirms the importance of performing both chemical analyses and pertinent toxicity tests to  
480 evaluate hazards of pollutants accumulated in sediments since low concentrations of heavy metals could result in  
481 non-negligible deleterious effects on early developmental stages of various fish species.

482

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485

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## 661 **Figure captions**

662 **Fig. 1.** Embryonic (a) and larval (b) viabilities following medaka embryos exposure to Cd or Cu-spiked  
663 sediments. Values represent the mean response ( $\pm$ SD) from three replicates. No significant difference was  
664 observed between treatments for both heavy metals.

665 **Fig. 2.** Hatching success (a) and time to hatch (b) following medaka embryos exposure to Cd or Cu-spiked  
666 sediments. Values represent the mean response ( $\pm$ SD) from three replicates. No significant difference was  
667 observed between treatments for both heavy metals.

668 **Fig. 3.** Cardiac activity measured in 6 dpf- (a) and 7 dpf-embryos (b) following Cd- or Cu-spiked sediment  
669 exposures. Values represent the mean response ( $\pm$ SD) from three replicates. For each condition, means with  
670 different letters are significantly different ( $p < 0.05$ ) according to the results of one-way ANOVA followed by  
671 Tukey's test.

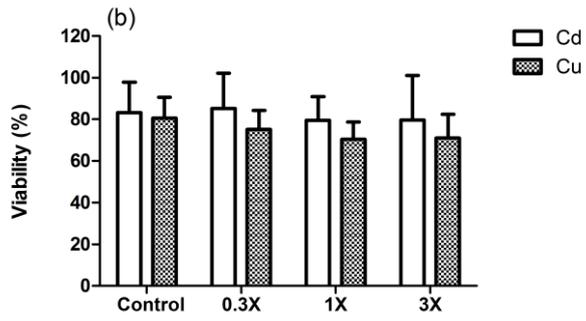
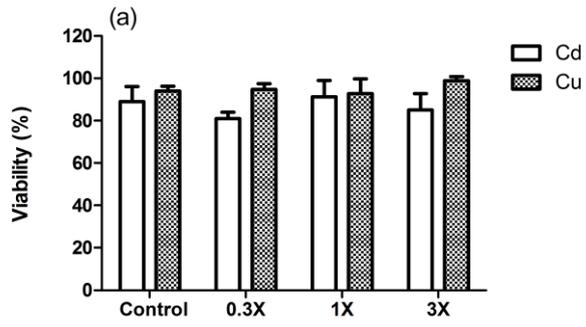
672 **Fig. 4.** Total body length (a), head length (b) and head/length ratio (c) of medaka larvae at hatching following  
673 Cd- or Cu-spiked sediment exposures. Values represent the mean response ( $\pm$ SD) from three replicates. For each  
674 experiment, means with different letters are significantly different ( $p < 0.05$ ) according to the results of one-way  
675 ANOVA followed by Tukey's test.

676 **Fig. 5.** Impact of Cd and Cu treatments on DNA integrity of medaka larvae assessed with the comet assay.  
677 Percentage of tail DNA (a) was recorded as an indicator of global genotoxic potency of chemicals and the  
678 percentage of 'hedgehog' cells (b) was determined to illustrate the presence of necrotic or apoptotic cells. Values  
679 represent the mean response ( $\pm$ SD) from three replicates. For each experiment, means with different letters are  
680 significantly different ( $p < 0.05$ ) according to the results of one-way ANOVA followed by Tukey's test.

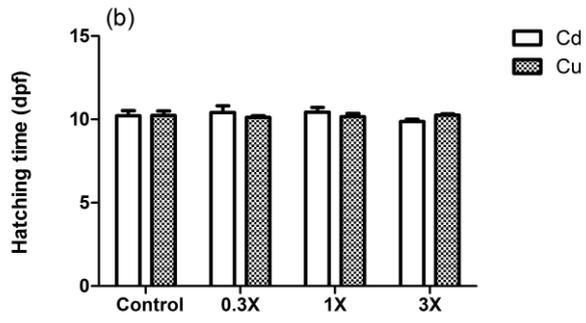
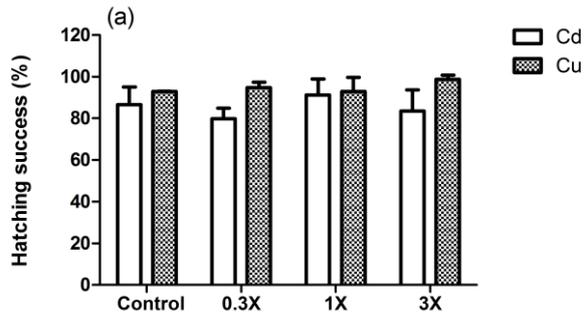
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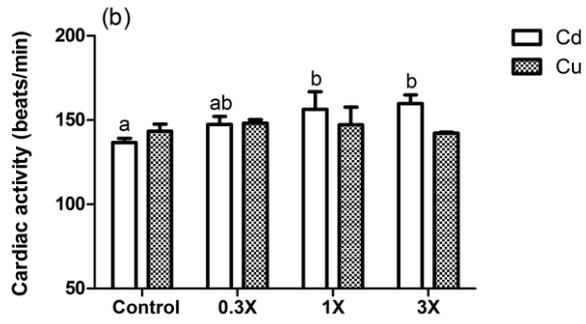
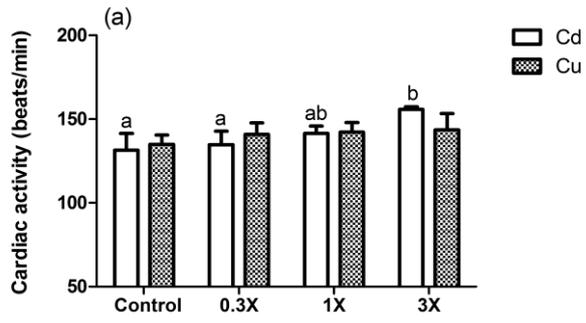
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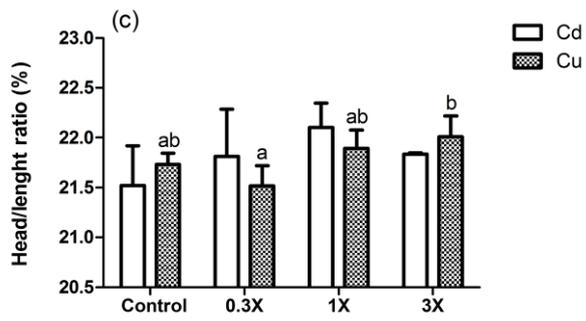
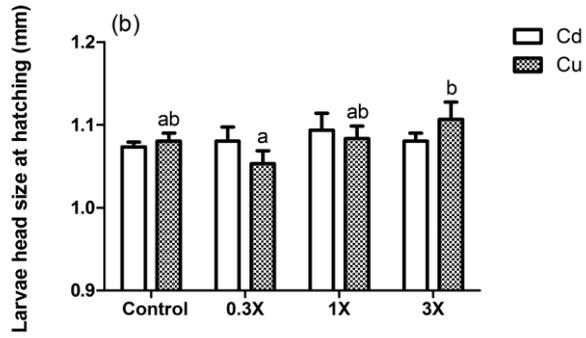
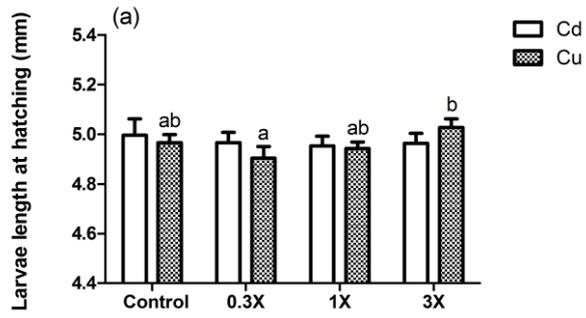
Figures 1a (embryonic viability) and 1b (larval viability)



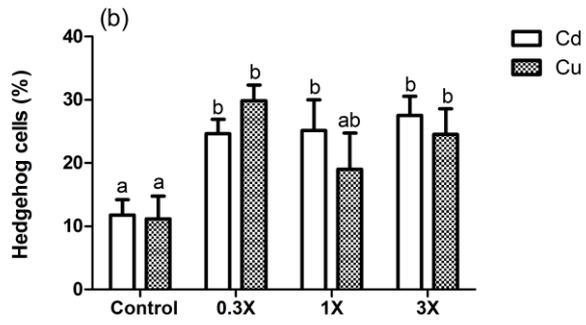
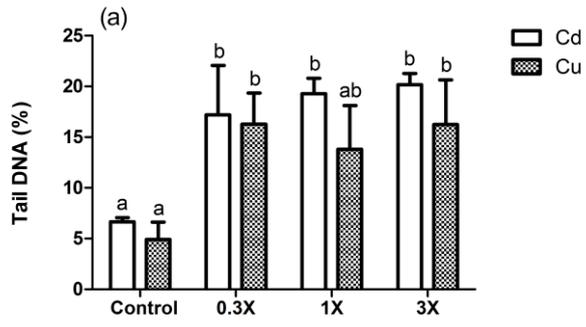
Figures 2a (Hatching success) and 2b (hatching time)



Figures 3a (cardiac activity on 6dpf-embryos) and 3b (cardiac activity on 7dpf-embryos)



Figures 4a (total body length), 4b (head size) and 4c (ratio head/length)



Figures 5a (Tai DNA) and 5b (Hedgehog cells)

**Table 1.** Reported Cd and Cu concentrations in sediments along the Lot-Garonne-Gironde continuum

<i>Studied area</i>	<i>Sampling site</i>	<i>Cadmium</i> ( $\mu\text{g/g d.w.}$ )	<i>Copper</i> ( $\mu\text{g/g d.w.}$ )	<i>Reference</i>
Gironde	Le Verdon	0.43	-	(Blanc et al., 1999)
	Cheyzin	0.67	33	(Geffard, 2001)
Garonne river	Bordeaux	0.7	29.5	(Grousset et al., 1999)
	La Réole	2.6	-	(Blanc et al., 1999)
	Tonneins	0.93	9.6	(Grousset et al., 1999)
	Saint Léger	0.44	17.1	(Grousset et al., 1999)
	Port Pascau	1.44	-	(Blanc et al., 1999)
	Thouars	0.41	10.3	(Grousset et al., 1999)
Lot river	Annual mean Temple/Luzech/Carjac 2006	14.8	24.4	(Shinn et al., 2009)
	Annual mean Temple/Luzech/Carjac 1987	41.7	31.7	(Shinn et al., 2009)
	Temple	20.4	30.7	(Audry et al., 2004)
	Saint Radegonde	17	22.8	(Grousset et al., 1999)
	Carjac	125	97.7	(Audry et al., 2004)
	Bouillac	26.4	-	(Blanc et al., 1999)
	Riou	16.1	25.4	(Grousset et al., 1999)
	Decazeville/Riou-Mort	460	-	(Blanc et al., 1999)
	Marcenac	0.17	7.18	This study

**Table 2.** Physico-chemical characteristics of the reference sediment (Marcenac, Lot river, France)

POC (%) <sup>*</sup>	NH <sub>4</sub> <sup>+</sup> (μM)	H <sub>2</sub> S <sup>*</sup> (μM)	D(v,0.10) <sup>*</sup> μm	D(v,0.50) <sup>*</sup> μm	D(v,0.90) <sup>*</sup> μm	<65 μm (%)
0.11	62.3	16.8	14.75	111.49	224.74	25.82

<sup>\*</sup>POC: particulate organic carbon; NH<sub>4</sub><sup>+</sup>: dissolved ammonia; H<sub>2</sub>S: dissolved sulfur; D(v,x): 10, 50 and 90 respective quantiles

**Table 3.** Heavy metal concentrations in spiked- and reference sediment at T0 and T10. T10 data are mentioned as mean±SD (n=3)

	Cd concentration (µg/g d.w.)		Cu concentration (µg/g d.w.)	
	T0	T10	T0	T10
<i>Cd-spiked sediment</i>				
Control	0.18	0.15 ± 0.00 <sup>a</sup>	8.05	7.35 ± 0.14
Cd-0.3X	1.91	1.83 ± 0.02 <sup>b</sup>	7.62	7.45 ± 0.11
Cd-1X	6.67	5.82 ± 1.69 <sup>c</sup>	7.36	7.43 ± 0.19
Cd-3X	19.80	17.13 ± 1.35 <sup>d</sup>	7.10	8.09 ± 0.52
<i>Cu-spiked sediment</i>				
Control	0.25	-	6.95	7.13 ± 0.08 <sup>a</sup>
Cu-0.3X	0.19	-	8.49	8.94 ± 0.51 <sup>b</sup>
Cu-1X	0.14	-	12.70	13.03 ± 0.13 <sup>c</sup>
Cu-3X	0.21	-	23.11	28.43 ± 0.64 <sup>d</sup>
<i>Other heavy metals contents in reference sediment (µg/g d.w.)</i>				
	Co	Mn	Ni	Zn
	5.6	261	10	35
	Cr	As	Ag	Pb
	10	17.5	0.04	12.5

**Table 4.** Concentrations of heavy metal in overlapping ERS buffer from Cu- and Cd-spiked and control sediment 1 day (T1) and 9 days (T9) after contamination

	Cd concentration ( $\mu\text{g/L}$ )		Cu concentration ( $\mu\text{g/L}$ )	
	T1	T9	T1	T9
<i>Cadmium exposure</i>				
Control buffer	< 1	-		
Control sediment	< 1	< 1		
Cd-0.3X	8.0	11.0		
Cd-1X	43.0	50.0		
Cd-3X	266.0	212.0		
<i>Copper exposure</i>				
Control buffer			< 1	-
Control sediment			14.2	8.7
Cu-0.3X			29.0	16.0
Cu-1X			55.0	28.0
Cu-3X			60.0	44.0

**Table 5.** Developmental deformities observed on larvae at hatching following Cd or Cu exposures. Results are expressed in percentage of impaired larvae among overall hatched individuals. Data are mentioned as mean $\pm$ SD (N=3)\*

	Deformed larvae	Oedematas	Spinal deformities	Craniofacial malformations	Eye abnormalities	Cardio-vascular anomalies	Yolk-sac deformities
<i>Cadmium exposure</i>							
Control	20.33 $\pm$ 4.51 <sup>a</sup>	4.19 $\pm$ 0.73	6.24 $\pm$ 5.57 <sup>a</sup>	2.52 $\pm$ 2.20	0.00 $\pm$ 0.00	10.90 $\pm$ 1.01 <sup>a</sup>	14.10 $\pm$ 6.00
Cd-0.3X	72.12 $\pm$ 19.42 <sup>b</sup>	11.36 $\pm$ 3.18	56.21 $\pm$ 23.40 <sup>b</sup>	1.67 $\pm$ 2.89	1.67 $\pm$ 2.89	25.61 $\pm$ 5.93 <sup>b</sup>	19.39 $\pm$ 1.05
Cd-1X	62.23 $\pm$ 6.34 <sup>b</sup>	7.07 $\pm$ 3.54	40.74 $\pm$ 8.49 <sup>ab</sup>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	22.11 $\pm$ 3.87 <sup>ab</sup>	18.41 $\pm$ 7.41
Cd-3X	67.97 $\pm$ 18.38 <sup>b</sup>	7.89 $\pm$ 3.16	58.60 $\pm$ 22.96 <sup>b</sup>	1.28 $\pm$ 2.22	0.00 $\pm$ 0.00	24.35 $\pm$ 6.05 <sup>b</sup>	8.69 $\pm$ 3.42
<i>Copper exposure</i>							
Control	16.54 $\pm$ 5.21 <sup>a</sup>	2.47 $\pm$ 4.28	7.55 $\pm$ 6.54 <sup>a</sup>	3.70 $\pm$ 6.42	2.47 $\pm$ 4.28	9.03 $\pm$ 2.57 <sup>a</sup>	8.84 $\pm$ 5.50 <sup>a</sup>
Cu-0.3X	51.24 $\pm$ 16.18 <sup>b</sup>	2.85 $\pm$ 2.48	32.42 $\pm$ 11.30 <sup>ab</sup>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	11.47 $\pm$ 6.67 <sup>a</sup>	13.63 $\pm$ 3.99 <sup>ab</sup>
Cu-1X	47.61 $\pm$ 2.61 <sup>b</sup>	5.44 $\pm$ 4.72	18.56 $\pm$ 12.82 <sup>ab</sup>	1.33 $\pm$ 2.31	0.00 $\pm$ 0.00	29.16 $\pm$ 4.85 <sup>b</sup>	22.99 $\pm$ 1.87 <sup>b</sup>
Cu-3X	51.92 $\pm$ 6.64 <sup>b</sup>	2.38 $\pm$ 4.12	35.23 $\pm$ 10.38 <sup>b</sup>	1.19 $\pm$ 2.06	0.00 $\pm$ 0.00	17.95 $\pm$ 9.30 <sup>ab</sup>	21.38 $\pm$ 7.17 <sup>ab</sup>

\*Means with different letters on each column are significantly different (p<0.05) according to the results of one-way ANOVA followed by Tukey's test

**Table 6.** Toxicity of cadmium to different aquatic organisms using Cd-spiked sediments

<i>Organism</i>	<i>Endpoint</i>	<i>Test duration (days)</i>	<i>LOEC<sub>Cd</sub> (µg/g d.w.)</i>	<i>References</i>
'Consensus-based TEC**'	-	-	0.99	(MacDonald et al., 2000)
Amphipods				
<i>Melita plumulosa</i> (adult)	Survival	10	>260	(King et al., 2006)
<i>Melita plumulosa</i> (juvenile)	Survival	10	820	(King et al., 2006)
<i>Chaetocorophium</i> cf. <i>lucasi</i>	Survival	10	748 ( <i>in situ</i> )	(Dewitt et al., 1999)
<i>Hyalella azteca</i>	Survival	4	128*	(Nebeker et al., 1986)
Gastropods				
<i>Helisoma</i> sp	Survival	10	340*	(Ditoro et al., 1992)
Insect larvae				
<i>Chironomus riparius</i>	Emergence	28	0.5	(Marinkovic et al., 2011)

\*NOEC value; \*\*TEC = Threshold effect concentration

**Table 7.** Toxicity of copper to different aquatic organisms using Cu-spiked sediments

<i>Organism</i>	<i>Endpoint</i>	<i>Test duration</i>	<i>LOEC<sub>Cu</sub></i> ( $\mu\text{g/g d.w.}$ )	<i>Reference</i>
'Consensus-based TEC**'	-	-	31.6	(MacDonald et al., 2000)
Amphipods				
<i>Hyalella azteca</i>	Survival	14days	180	(Roman et al., 2007)
<i>Hyalella azteca</i>	Growth	28days	95.4	(Roman et al., 2007)
<i>Gammarus pulex</i>	Survival/growth	35days	176	(Roman et al., 2007)
<i>Melita plumulosa (adult)</i>	Survival	10days	550	(King et al., 2006)
<i>Melita plumulosa (juvenile)</i>	Survival	10days	820	(King et al., 2006)
Insect larvae				
<i>Chironomus riparius</i>	Survival	14days	180	(Roman et al., 2007)
<i>Chironomus riparius</i>	Growth	28days	188	(Roman et al., 2007)
<i>Chironomus riparius</i>	Emergence	28days	89.2	(Roman et al., 2007)
<i>Chironomus riparius</i>	Emergence	28days	17.2	(Marinkovic et al., 2011)
Oligochaetes				
<i>Lumbriculus variegatus</i>	Survival	28days	140	(Roman et al., 2007)
<i>Lumbriculus variegatus</i>	Biomass	28days	103	(Roman et al., 2007)
<i>Lumbriculus variegatus</i>	Reproduction	28days	103	(Roman et al., 2007)
<i>Tubifex tubifex</i>	Survival	28days	158	(Roman et al., 2007)
<i>Tubifex tubifex</i>	Growth	28days	102	(Roman et al., 2007)
<i>Tubifex tubifex</i>	Reproduction	28days	102	(Roman et al., 2007)
<i>Tubifex tubifex</i>	Reproduction	28days	92-166	(Pasteris et al., 2003)

\*NOEC value; \*\*TEC = Threshold effect concentration