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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 methylsulfonate; NMP: normal melting point agarose; NOEC: no observed effect concentration; PBS: phosphate buffered-saline; PNEC: probable no effect concentration; POPs: persitent organic pollutants; ww: wet weight

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Chemicals

$CdCl_2$:$2H_2O$, phosphate-buffered saline (PBS), nitric acid 65% and normal melting point agarose (NMP) were purchased from Fluka Chemie (Buchs, CH). Cu standard 1000mg Titrisol® ($CuCl_2$ in H2O) was purchased from Merck (Darmstadt, DE). Tricaine methanesulfonate (MS222), low melting point agarose (LMP) and collagenase

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

2.2 Reference sediment characterisation

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

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

2.3 Sediment spiking

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

The 30X Cd-stock solution (2.5 mgCd/mL) was obtained by adding 48.79 mg of $CdCl_2$:$2H_2O$ to 10mL of ultrapure water (Milli-Q Maxima, Elga Labwater, Veolia water, Blagnac FR). The Cu-stock solution was made by diluting Cu standard 1000mg ($CuCl_2$) in 1L of ultrapure water. Dilutions of these Cd and Cu stock solutions were performed in ultrapure water to obtain the three contamination solutions used for sediment spiking.

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

2.4 Fish breeding and embryos collection

Mature Japanese medakas were reared and maintained in 40L-tanks with a male:female ratio of 1:2, at a temperature of 26°C with a photoperiod of 12h light:12h dark. Tanks were cleaned using a siphon and 30 % was renewed with stalling water (1/3 24h-dechlorinated tap water and 2/3 purified water by reverse osmosis; pH 7.5; 53.4 ppm $CaCO_3$; 0.025 ppm NO_2; 1.5 ppm PO_4; 5 ppm NO_3; <0.1ppm NH_4) twice a week. Fishes were fed once a day with dry flakes (Tetramin® Tropical, Tetra, Melle, Germany) and once a day with brine shrimp nauplii *Artemia sp*. nauplii (Ocean Nutrition, Assen, Belgium). It was visually checked daily and unhealthy fish individuals were removed and euthanized using MS222 solution at 1g/L. During the spawning stimulation period, medaka genitors were reared at 28°C under a 16h:8h light:dark photoperiod and fed four times a day. Females were examined for eggs 1h after onset of light and egg clusters were gently removed by hand and placed in Egg Rearing Solution (ERS; 17.11mM NaCl; 0.4mM KCl; 0.36mM $CaCl_2$; 1.36mM $MgSO_4$; pH 7.0). Harvested eggs were pooled and then individualised by rolling them softly to sever attaching filaments. Finally, they were staged according to Iwamatsu (2004) and Gonzalez-Doncel et al. (2005) under a stereomicroscope (Leica MZ75, Leica Microsystems, Nanterre, France) and cold light source (Intralux® 4100, Volpi AG, Schlieren, Switzerland) to discard unfertilised or dead individuals and only pre-morula stage embryos (2-4hours post fertilisation, hpf) were selected for the study. Animal breeding and experiments on embryos and larvae were conducted in accordance to the EU directive 210/63/EU on the protection of animals used for scientific purposes.

2.5 Embryos exposure by sediment-contact

Two distinct experiments were performed for Cd and Cu toxicity analysis. Both experiments followed the same general protocol, which consisted of three independent replicates for each condition. As mentioned above, 5 g dw sediment were placed in a 35 mm diameter plastic Petri dish for each replicate and each concentration. 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 the beginning of the experiment.

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

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

During the whole experiment, embryos and larvae were maintained in a climate cabinet (Economic Delux, Snijders Scientific, Tilburg, NL) at 26°C ± 0.3 with a 12h:12h photoperiod and 5000Lux white light.

2.6 Lethal and sub-lethal endpoints

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

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

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

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

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

2.7 Comet assay

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

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

2.8 Heavy metals analysis

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

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 for 3h at 100°C. Each batch of samples included method blanks and certified reference materials (Tort-2, lobster hepatopancreas, NRCC-CNRC, Ottawa, CA) and measured values were consistently within certified ranges (data not shown). After mineralisation, samples were diluted by addition of 15 ml ultrapure water (MilliQ, Bedford, MA, USA) and metal determinations were performed using an atomic absorption spectrophotometer (Varian SpectrAA 220 FS, Agilent Technologies, Santa Clara, US).

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

2.9 Statistical analysis

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

3. Results

3.1 Reference sediment characterisation

Particle sizes of Marcenac sediment ranged from very fine sand to silt with a median value of 111.5 µm. The particulate organic carbon content was low (0.11 %) and dissolved ammonium and sulphide in pore water reached 62 µM and 17 µM respectively (Table 2).

3.2 Heavy metal content in water and sediments

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

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

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

3.2 Embryonic and larval survivals

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

3.3 Hatching rate and time to hatch

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

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

3.4 Cardiac activity

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

3.5 Biometric measurements

Neither Cd nor Cu exposure induced modifications of larval biometric parameters, as compared to the respective control groups. Total body length of Cd-exposed larvae ranged from 4.95 to 5.00 mm on average (Fig. 4a), head 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).

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

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

3.6 Morphological abnormalities

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

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

3.7 Genotoxic effects

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

Although no acute effect was observed in medaka embryos, a significant increase in morphological deformities 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 µg/L Cd and 29 µg/L Cu in water). More precisely, Cu significantly enhanced spinal deformities (kyphosis and lordosis mainly), cardiac anomalies (in particular abnormal positioning and heart looping) and yolk-sac malabsorption while Cd only affected the vertebral column and cardio-vascular system.

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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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Figure captions

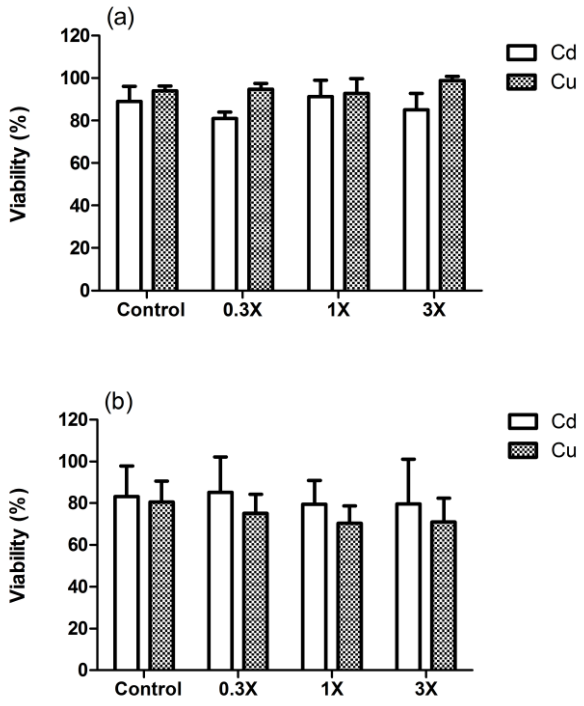
Fig. 1. Embryonic (a) and larval (b) viabilities following medaka embryos exposure to Cd or Cu-spiked sediments. Values represent the mean response (±SD) from three replicates. No significant difference was observed between treatments for both heavy metals.

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

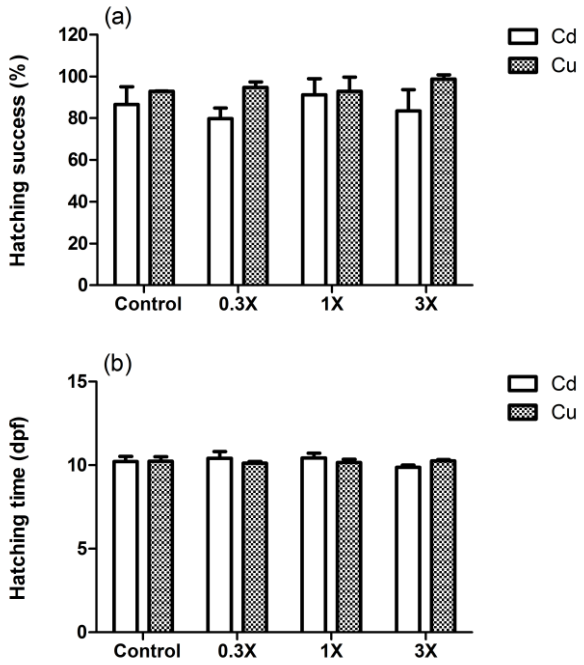
Fig. 3. Cardiac activity measured in 6 dpf- (a) and 7 dpf-embryos (b) following Cd- or Cu-spiked sediment exposures. Values represent the mean response (±SD) from three replicates. For each condition, means with different letters are significantly different ($p<0.05$) according to the results of one-way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

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

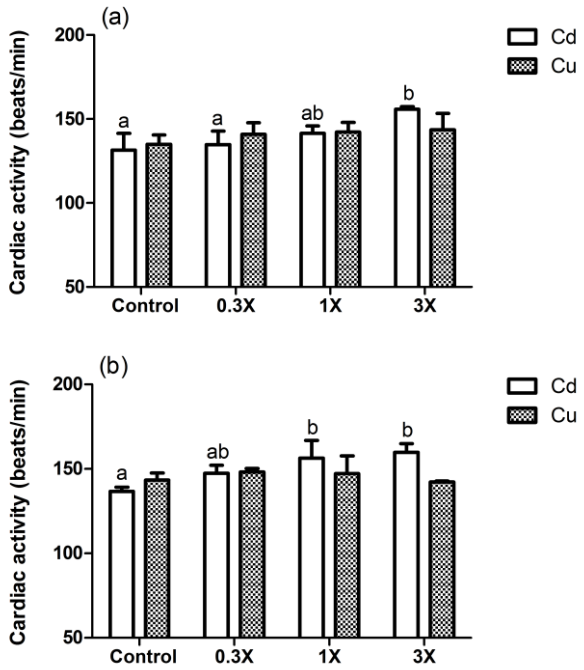
Fig. 5. Impact of Cd and Cu treatments on DNA integrity of medaka larvae assessed with the comet assay. Percentage of tail DNA (a) was recorded as an indicator of global genotoxic potency of chemicals and the percentage of 'hedgehog' cells (b) was determined to illustrate the presence of necrotic or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



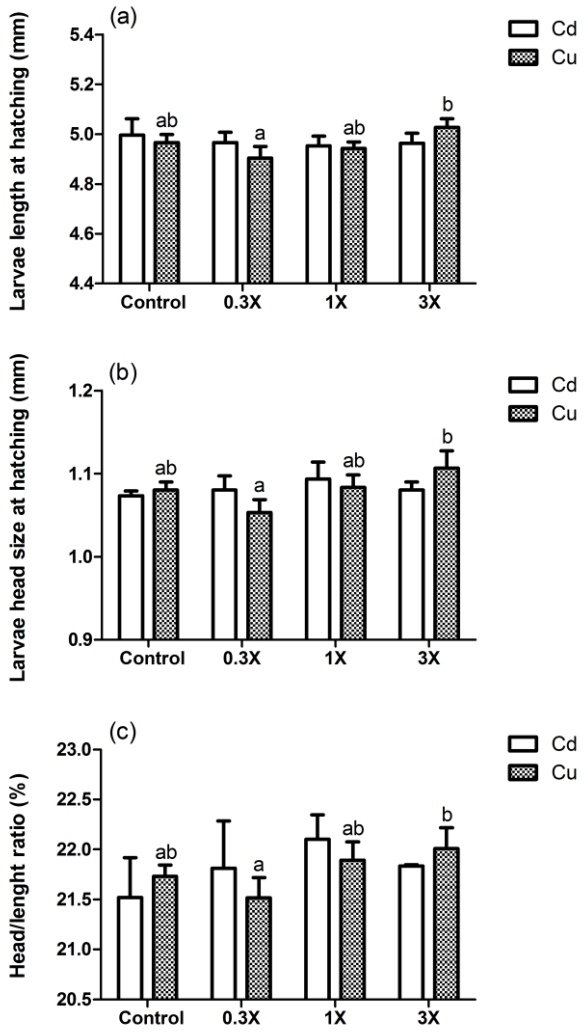
Figures 1a (embryonic viability) and 1b (larval viability)



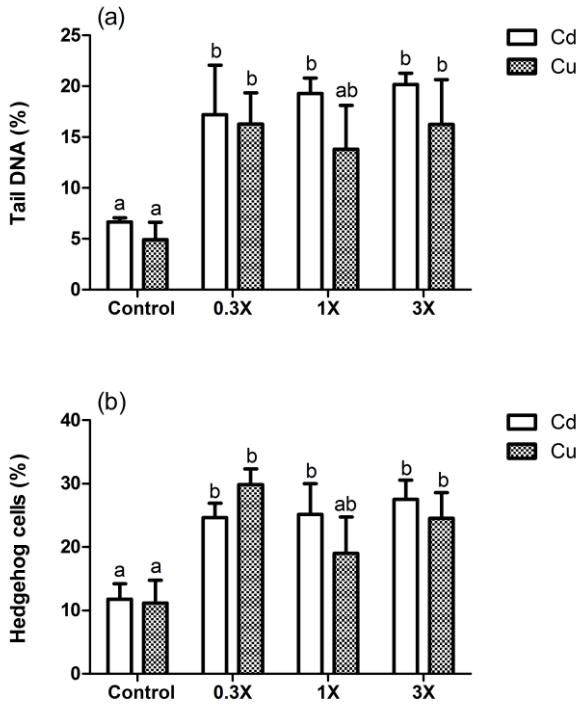
Figures 2a (Hatching success) and 2b (hatching time)



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 (Hedghog cells)

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

Studied area	*Sampling site*	*Cadmium (µg/g d.w.)*	*Copper (µg/g d.w.)*	*Reference*
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 (%)*	NH_4^{+*}(µM)	H_2S^*(µM)	D(v,0.10)*µm	D(v,0.50)*µm	D(v,0.90)*µm	≤65µm (%)
0.11	62.3	16.8	14.75	111.49	224.74	25.82

*POC: particulate organic carbon; NH_4^+: dissolved ammonia; H_2S: 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
Cd-spiked sediment				
Control	0.18	0.15 ± 0.00[a]	8.05	7.35 ± 0.14
Cd-0.3X	1.91	1.83 ± 0.02[b]	7.62	7.45 ± 0.11
Cd-1X	6.67	5.82 ± 1.69[c]	7.36	7.43 ± 0.19
Cd-3X	19.80	17.13 ± 1.35[d]	7.10	8.09 ± 0.52
Cu-spiked sediment				
Control	0.25	-	6.95	7.13 ± 0.08[a]
Cu-0.3X	0.19	-	8.49	8.94 ± 0.51[b]
Cu-1X	0.14	-	12.70	13.03 ± 0.13[c]
Cu-3X	0.21	-	23.11	28.43 ± 0.64[d]
Other heavy metals contents in reference sediment (µg/g d.w.)				
	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 (µg/L)		Cu concentration (µg/L)	
	T1	T9	T1	T9
Cadmium exposure				
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		
Copper exposure				
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±SD (N=3)[*]

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

[*]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

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

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

*NOEC value; **TEC = Threshold effect concentration