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**A comprehensive study of the toxicity of natural multi-contaminated sediments: new insights brought by the use of a combined approach using the Medaka embryo-larval assay and physico-chemical analyses**

*Iris Barjhoux<sup>✉1</sup>, Christelle Clérandeau, Karyn Le Menach, Pierre Anschutz, Patrice Gonzalez, Hélène Budzinski, Bénédicte Morin, Magalie Baudrimont and Jérôme Cachot*

Univ. Bordeaux, EPOC UMR CNRS 5805, allée Geoffroy Saint-Hilaire, 33615 Pessac Cedex,  
France

E-mail contacts:

✉ Iris Barjhoux (PhD, corresponding author): [iris.barjhoux@univ-reims.fr](mailto:iris.barjhoux@univ-reims.fr) / [irisbarjhoux@hotmail.com](mailto:irisbarjhoux@hotmail.com) (permanent). Christelle Clérandeau (MEnvSc): [c.clerandeu@epoc.u-bordeaux1.fr](mailto:c.clerandeu@epoc.u-bordeaux1.fr). Karyn Le Menach (MEnvSc): [k.lemenach@epoc.u-bordeaux1.fr](mailto:k.lemenach@epoc.u-bordeaux1.fr). Pierre Anschutz (Prof): [p.anschutz@epoc.u-bordeaux1.fr](mailto:p.anschutz@epoc.u-bordeaux1.fr). Patrice Gonzalez (PhD): [p.gonzalez@epoc.u-bordeaux1.fr](mailto:p.gonzalez@epoc.u-bordeaux1.fr). Hélène Budzinski (RD PhD) : [h.budzinski@epoc.u-bordeaux1.fr](mailto:h.budzinski@epoc.u-bordeaux1.fr). Bénédicte Morin (PhD): [b.morin@epoc.u-bordeaux1.fr](mailto:b.morin@epoc.u-bordeaux1.fr). Magalie Baudrimont (Prof.): [m.baudrimont@epoc.u-bordeaux1.fr](mailto:m.baudrimont@epoc.u-bordeaux1.fr). Jérôme Cachot (Prof.): [jerome.cachot@u-bordeaux.fr](mailto:jerome.cachot@u-bordeaux.fr).

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<sup>1</sup> Present adress: Univ. Reims Champagne-Ardenne, UMR-I 02 INERIS-URCA-ULH SEBIO, Moulin de la Housse, BP 1039, 51687 Reims Cedex 2, France. Phone: +33 (0)326913719. Fax: +33 (0)326913342

## 1 **Abstract**

2 Sediment compartment is a long term sink for pollutants and a secondary source of  
3 contamination for aquatic species. The abiotic factors controlling the bioavailability and thus  
4 the toxicity of complex mixtures of pollutants accumulated in sediments are poorly  
5 documented. To highlight the different factors influencing sediment toxicity, we identified  
6 and analyzed the physico-chemical properties, micro-pollutant contents, and toxicity level of  
7 six contrasted sediments in the Lot-Garonne continuum. Sediment toxicity was evaluated  
8 using the recently described Japanese medaka (*Oryzias latipes*) embryo-larval assay with  
9 direct exposure to whole sediment (MELAc). Multiple toxicity endpoints including  
10 embryotoxicity, developmental defects and DNA damage were analyzed in exposed embryos.  
11 Chemical analyses revealed significant variations in the nature and contamination profile of  
12 sediments, mainly impacted by metallic trace elements and, unexpectedly, polycyclic  
13 aromatic hydrocarbons. Exposure to sediments induced different toxic impacts on medaka  
14 early life stages when compared with the reference site. Principal component analysis showed  
15 that the toxic responses following exposure to sediments from the Lot River and its tributary  
16 were associated with micro-pollutant contamination: biometric measurements, hatching  
17 success, genotoxicity, craniofacial deformities and yolk sac malabsorption were specifically  
18 correlated to metallic and organic contaminants. Conversely, the main biological responses  
19 following exposure to the Garonne River sediments were more likely related to their physico-

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*Abbreviations:* Bdx, Bordeaux station; Bo, Bouillac station; DBT, dibenzothiophene; dpf, days post-fertilization; dph, days post-hatching; dw, dry weight; ELS, early life stages; ERS, egg rearing solution; GC-ECD, gas chromatography coupled to electron capture detection; GC-MS, gas chromatography coupled to mass spectrometry; Jo, Joanis station; LR, La Réole station; LT, Le Temple station; MELAc, Medaka Embryo-Larval Assay in sediment-contact; MPhe, methylphenanthrenes; Mrc, Marcenac station; MTE, metallic trace elements; PAHs, polycyclic aromatic hydrocarbons; PBDEs, polybrominated diphenyl ethers; PCA, principal component analysis; PCBs, polychlorobiphenyls; PEC, probable effect concentration; POC, particulate organic carbon; POPs, persistent organic pollutants; qPEC, probable effect concentration quotients; SD, standard deviation; SI, supporting information; TEC, threshold effect concentration.

20 chemical properties than to their contamination level. Time to hatch, cardiovascular injuries  
21 and spinal deformities were correlated to organic matter content, fine particles and dissolved  
22 oxygen levels. These results emphasize the necessity of combining physico-chemical analysis  
23 of sediment with toxicity assessment to accurately evaluate the environmental risks associated  
24 with sediment contamination.

25

26 *Keywords:* sediment toxicity, pollutant mixture, physico-chemical properties, fish embryo-  
27 larval assay, multivariate statistics.

28

## 29 **1. Introduction**

30 As the final receptacle of pollutants from various origins (industrial, urban and agricultural)  
31 and their ability to accumulate substances such as persistent organic pollutants (POPs) and  
32 metallic trace elements (MTE), sediments are considered as both a sink and a long-term  
33 source of contamination for aquatic organisms (Burton Jr, 1991). Such contamination could  
34 threaten not only benthic organisms living on direct contact to sediment but also the entire  
35 aquatic ecosystem and finally human health *via* bioaccumulation, trophic transfer and/or  
36 drinking water (Fent, 2004). The evaluation of sediment toxicity is thus of major concern in  
37 environmental risk assessment strategies. While integrated procedures combining chemical  
38 analyses and biological responses are widely recommended (Ahlf et al., 2002), there is still no  
39 system of universal toxicity bioassay(s) capable of predicting the potential hazard of  
40 contaminated sediments. Because this particular matrix is very complex to analyze, several  
41 test phases have been used for sediment toxicity testing as, for instance, extractable pollutants  
42 and pore water (Burton Jr, 1991). However, whole sediment exposure is generally considered  
43 the most realistic and integrative method to mimic contamination of organisms *in situ* as the  
44 uptake route (including sediment-contact and aqueous phase exposure) is very similar to that  
45 found in environmental conditions, taking sediment characteristics and physico-chemical  
46 properties governing pollutant bioavailability into account (Hollert et al., 2003; Kosmehl et  
47 al., 2006).

48 In conventional ecotoxicity testing strategies, fish represent an indispensable component of  
49 integrated toxicity assessments because of their high sensitivity to contaminants, their critical  
50 role in the aquatic food chain and their socio-economic importance (Lammer et al., 2009). In  
51 the light of European regulations on the protection of animals used for scientific purpose (EC,  
52 2010), fish early life stages (ELS) bioassays have gained interest in environmental risk  
53 assessments and (eco)toxicological evaluations. Indeed, their higher sensitivity to a wide

54 range of chemicals when compared with adult or juvenile stages (Hutchinson et al., 1998),  
55 their ecological relevance (e.g. recruitment, population wellness) (Burton Jr, 1991; Cao et al.,  
56 2009) and the possibility they offer to perform small-scale, high-throughput analyses with an  
57 excellent correlation to conventional *in vivo* testing (Lammer et al., 2009) make fish ELS a  
58 promising tool to replace traditional acute fish tests (Braunbeck et al., 2005). As a result, fish  
59 ELS-based bioassays found their way into the laboratories not only to test chemical  
60 (eco)toxicity, but also to assess the hazard of environmental samples such as sediments  
61 (Hallare et al., 2005; Hollert et al., 2003; Kosmehl et al., 2008, 2006). These studies  
62 investigated embryotoxic, teratogenic and genotoxic potencies of sediments in zebrafish ELS  
63 demonstrating the suitability of such whole sediment-contact embryo-larval assays for natural  
64 sediment testing as it enables to evaluate the impacts of the sole bioavailable fraction of  
65 particle-bound pollutants from complex environmental matrices containing a mixture of a  
66 multitude of chemicals. Japanese medaka embryos offer similar practical advantages to  
67 zebrafish embryos for developmental toxicity testing, but its longer development time in the  
68 egg provides the possibility to extend the duration of embryonic exposure to 9–10 days at  
69 26 °C (as opposed to 48-72 h for zebrafish), which can mimic a more chronic exposure to  
70 contaminants (Barjhoux et al., 2012).

71 The Medaka Embryo-Larval Assay in sediment-contact (MELAc) was developed in our  
72 laboratory. It consisted in an incubation of Japanese medaka (*Oryzias latipes*) embryos at the  
73 surface of sediment for the whole duration of the embryonic phase. As recommended, several  
74 non-invasive lethal and sublethal endpoints of embryotoxicity and teratogenicity are analyzed  
75 during the bioassay. Additional genotoxicity assessment is also performed on 2-days post-  
76 hatching (dph) larvae using the Comet assay. This procedure proved its applicability and its  
77 relevance for hydrophobic substances, MTE and sediment organic extract toxicity testing  
78 (Barjhoux et al., 2014, 2012; Cachot et al., 2007; Vicquelin et al., 2011). In the present study,

79 we propose an application of the MELAc to the evaluation of whole raw sediment (i.e. with  
80 no prior extraction procedures) from a multi-contaminated environment: the Lot-Garonne  
81 continuum (South West of France).

82 This area is highly impacted by historical polymetallic pollution, first highlighted in the early  
83 1970s by the National Observation Network, and characterized by very high Cd  
84 bioaccumulation in bivalves collected downstream, in the Gironde estuary, revealing it was in  
85 fact the most contaminated along the European coastline. The main source of Cd was  
86 identified in the upper part of the Lot River (Latouche, 1992), in a small Lot tributary (the  
87 Riou-Mort River) draining the waste area of a now-abandoned factory previously specializing  
88 in zinc ore treatment, which had been active for over a century in the Decazeville industrial  
89 basin. Although mining activities stopped for several decades and remediation procedures are  
90 in progress, the Lot-Garonne-Gironde fluvial-estuarine system remains clearly impacted by  
91 MTE such as Cd, Pb, Cu and Zn (Audry et al., 2004). These MTE are mainly transported in  
92 the particulate phase along the Lot-Garonne-Gironde continuum and constitute the major  
93 metallic inputs in the estuary (Audry et al., 2004). Sediments within the Lot-Garonne system  
94 represent a real storage compartment for MTE that can be remobilized in the water column  
95 during natural (flood, storm) or anthropogenic (dredging, dam flush) events and salinity  
96 gradient rising in the Gironde estuary (Audry et al., 2010). While the effects of the  
97 polymetallic contamination of the water column have been investigated in several aquatic  
98 organisms such as diatoms, bivalves and fish (e.g. Arini et al., 2012, 2011; Orieux et al.,  
99 2011), to our knowledge there is no study directly investigating the toxicity of sediments from  
100 this area.

101 The present study aimed to assess the embryotoxicity, teratogenicity and genotoxicity of six  
102 multi-contaminated sediments from the Lot-Garonne system using the MELAc. In parallel, a  
103 comprehensive characterization of sediments was carried out including MTE and POP

104 contaminant levels and physico-chemical properties. Correlation and principal component  
105 analyses were conducted to investigate the possible interrelationships between physico-  
106 chemical and contamination parameters between themselves as well as with biological  
107 responses. Finally, a discussion on the toxic potential of the studied sediments and the  
108 relevance of the considered biomarkers is proposed.

109

## 110 **2. Material and Methods**

### 111 *2.1. Study sites and sample collection*

112 Six stations were selected along the polymetallic gradient of the Lot-Garonne continuum in  
113 South-West France (Fig. 1). The Marcenac (Mrc) station (44°35'53.5" N, 2°14'29.4" E) was  
114 located on the right bank of the Lot River, above its confluence with the Riou-Mort River.  
115 This sediment was used as the reference, as Marcenac is considered as a pristine site for MTE  
116 contamination in the Lot-Garonne-Gironde continuum (Audry et al., 2010). Moreover,  
117 previous work in our laboratory already demonstrated that Marcenac sediment was an  
118 adequate substrate for medaka embryonic development (Barjhoux et al., 2012).  
119 Joanis (Jo) station (44°33'56.9" N, 2°12'41.1" E) was located on the banks of the Riou-Mort  
120 River, about 2 km downstream from the ancient zinc factory. At this site, MTE contamination  
121 is expected to be the highest of all selected sampling sites (Audry et al., 2004). Bouillac (Bo)  
122 station (44°34'54.3" N, 2°12'02.8" E) is situated on the Lot River at about 20 m downstream  
123 from its confluence with the Riou-Mort, is representative of the MTE discharge in the Lot  
124 River system from the old mining activities in the industrial basin of Decazeville. Still on the  
125 Lot River, Le Temple (LT) station (44°23'43.3"N 0°32'33.1"E), located at around 15 km from  
126 the confluence with the Garonne River, thus integrating the outlet of the Lot River watershed  
127 before it reaches the Garonne River (Audry et al., 2010, 2004).



128 Finally, two sites were selected on the Garonne River banks: La Réole (LR) station  
129 (44°34'41.1" N, 0°02'01.6" W), at around 50 km downstream from the junction of the Lot and  
130 Garonne Rivers and located just upstream of the tidal limit; and Bordeaux (Bdx) station  
131 (44°51'02.0" N, 0°33'46.7" W), located in the fluvial part of the Gironde Estuary, which  
132 integrates both polymetallic contamination from the Lot River and anthropogenic pollution  
133 from the Bordeaux conurbation (~1,000,000 inhabitants) (Schäfer et al., 2009).  
134 All the sediments were sampled in April 2011 with the exception of Marcenac sediment,  
135 which was collected during a previous sampling campaign in March 2009. At each sampling  
136 station, only the superficial layer (0–2 cm) of sediment was collected. Samples were packed  
137 in aluminum boxes and cool-transported to the laboratory where they were immediately  
138 stored at -20 °C. Sediments were kept at 4 °C overnight for slow defrost. An aliquot was  
139 sieved using a 1-mm mesh to eliminate debris and homogenized prior to use in the bioassay  
140 and chemical analyses. Another subsample was used to extract pore water. The sediment was  
141 put in a 0.2 µm VIVASPIN20 centrifuge vial. Pore waters were extracted by centrifugation at  
142 4000 rpm for 20 min.

143

## 144 2.2. *Physico-chemical characterization and contamination levels of sediments*

145 Physical and chemical analyses were conducted on sediment from each sampling stations to  
146 determine their natural characteristics (Table 1).

147 The grain-size distribution was measured using a Malvern laser diffraction particle size  
148 analyzer. Particulate organic carbon (POC) content was determined on freeze-dried  
149 homogenized sediment by infrared spectroscopy (LECO C-S analyzer) after removal of  
150 carbonates with 2 M HCl from 50 mg powdered sample (Etcheber et al., 1999). Dissolved  
151 ammonium (NH<sub>4</sub>) was analyzed in pore water using the phenol reaction followed by

152 colorimetric measurement (Grasshoff and Johanssen, 1972; Koroleff, 1969). Detailed  
153 protocols used for the above-mentioned analyses are described by Vicquelin et al. (2011).  
154 MTE analysis was performed on each sediment (0.5 g dry wet of sediment, dw) after a  
155 digestion step using 70% nitric acid for 2 h at 100 °C. Then, the sample volumes were made  
156 up to 25 mL with Milli-Q water. Ten MTE including Ag, As, Cd, Co, Cr, Cu, Mn, Ni, Pb and  
157 Zn were measured by ICP-MS (4500 Series, Agilent Technologies, Wilmington, DE, USA),  
158 using external calibration. A 1 g/L multi-elemental solution was used to prepare the standard  
159 solutions. Rhodium was used as an internal standard to evaluate the instrumental drift. The  
160 accuracy of the determination procedure was assessed by analysis of the standard reference  
161 PACS2 (NRCC, CNRC, marine sediment). The concentrations measured for the standard  
162 sediment were consistent with the certified values. Hg was also analyzed in sediment using an  
163 Automated Mercury Analyzer (LECO, AMA-254) as previously described by Baudrimont et  
164 al. (2005). The analytical results were quality-checked by analyzing international certified  
165 TORT-2 reference materials (NRCC, CNRC, lobster hepatopancreas) after each set of  
166 samples. Measured concentrations were consistently within the certified ranges.

167 Organic contaminants were quantified in freeze-dried sediments from each station after  
168 homogenization and a micro-waved extraction step using dichloromethane. Detailed  
169 procedures concerning extraction, purification and analysis of organic compounds have been  
170 widely described in previous works from our laboratory (Devier et al., 2005; Tapie et al.,  
171 2008). Briefly, organic extracts were purified on alumina micro-columns containing activated  
172 copper. A second purification step took place on acidified silica micro-column during which  
173 polycyclic aromatic hydrocarbons (PAHs) and organohalogenated compounds were co-eluted  
174 using a mixture of pentane and dichloromethane (90/10, v/v). The final extract was re-  
175 concentrated and divided in two aliquots for PAH and organohalogen analysis. Quantification  
176 of PAHs (listed in Table 1), including 21 individual PAHs, 5 methylphenanthrenes (MPhe)

177 and dibenzothiophene (DBT), was performed by gas chromatography coupled to mass  
 178 spectrometry (GC-MS) whereas organohalogen content (listed in Table 1), including 8  
 179 polychlorobiphenyls (PCBs) congeners, 4 polybrominated diphenyl ethers (PBDEs) and  
 180 lindane, was analyzed by gas chromatography coupled to electron capture detection (GC-  
 181 ECD).

182 The quality of the analytical procedure was systematically controlled in each batch of  
 183 analysis. Specific PAH and organohalogen standards were gravimetrically added in one hand,  
 184 prior to extraction as internal standards and others prior to GC-MS or GC-ECD analysis as  
 185 syringe standards, on the other hand. An extraction blank was also added with each series of  
 186 extractions. To test the accuracy and validity of the quantification method, standard solutions  
 187 (for PAHs and organohalogens separately) of compounds to be quantified in mixture with the  
 188 related internal standards are regularly run on the GC-MS and GC-ECD systems.

189

190 **Table 1** Physico-chemical characteristics of the six sediments sampled along the Lot/Garonne continuum  
 191 and sediment ranking (in order of increasing toxicity) according to the calculated mean qPEC values and  
 192 the biological responses from the MELAc

	Marcenac	Bordeaux	La Réole	Le Temple	Bouillac	Joanis	TEC <sup>a</sup>	PEC <sup>a</sup>
D(0.50) (µm)	151	27.0	32.1	392	274	325		
Fine particles (<63 µm) (%)	9.6	81.0	71.9	4.6	19.3	11.3		
POC (%)	0.1	1.2	2.1	0.1	0.8	0.3		
NH <sub>4</sub> (µM)	24.9	290	743	114	424	164		
<i>Metallic trace elements (MTE) (µg/g dw)</i>								
Ag	0.0	0.1	0.1	0.0	1.0	2.4	-	-
As	17.5	8.9	10.7	3.0	42.6	28.5	9.8	33.0
Cd	0.1	1.3	1.5	0.4	17.9	14.6	1.0	5.0
Co	5.6	8.9	8.3	5.1	10.4	9.8	-	-

Cr	10.0	24.1	23.7	10.2	11.3	23.2	43.4	111
Cu	6.2	16.5	20.1	4.1	78.7	96.7	31.6	149
Mn	261	535	649	183	843	883	-	-
Ni	9.5	16.6	16.9	16.2	22.8	17.5	22.7	48.6
Pb	12.5	30.2	25.8	3.9	273	318	35.8	128
Zn	35.0	103	116	16.8	2,041	2,293	121	459
Hg	0.05	0.03	0.16	0.08	0.09	0.25	0.18	1.06
ΣMTE	357	745	872	243	3,342	3,687		
<i>Organic contaminants (OC) (ng/g dw)</i>								
ΣLPAHs <sup>b</sup>	3.2	85.4	127	2,603	214	15,649		
ΣHPAHs <sup>c</sup>	9.4	1,114	788	21,585	814	9,352		
ΣMPhe <sup>d</sup>	1.7	39.3	46.2	984	75.5	849		
ΣPAHs <sup>e</sup>	13.8	1,239	960	25,173	1,103	25,850	1,610	22,800
DBT	0.3	3.6	4.5	113	7.0	309		
Lindane	0.1	0.1	0.1	<dl	<dl	<dl	2.37	4.99
ΣPCBs <sup>f</sup>	1.0	8.9	14.4	0.8	3.3	3.7	59.8	676
ΣPBDEs <sup>g</sup>	<dl	0.1	<dl	<dl	0.1	0.4		
ΣOHCs <sup>h</sup>	1.1	9.0	14.4	0.8	3.4	4.1		
ΣOC <sup>i</sup>	15.2	1,252	979	25,286	1,114	26,163		
Mean qPEC1 <sup>j</sup>	0.10	0.16	0.18	0.18	1.27	1.38		
Mean qPEC2 <sup>j</sup>	0.06	0.11	0.13	0.68	0.74	1.88		
Mean qPEC-based ranking <sup>k</sup>	#1	#2	#3	#4	#5	#6		
MELAc-based ranking <sup>l</sup>	#1 (0; reference)	#3 (4)	#5 (7)	#3 (4)	#2 (3)	#6 (11)		

193 <sup>a</sup> values taken from MacDonald et al. (2000)  
194 <sup>b</sup> sum of low molecular weight PAHs (LPAHs; three or fewer aromatic rings) includes naphthalene,  
195 acenaphthene, acenaphthylene, fluorene, phenanthrene and anthracene  
196 <sup>c</sup> sum of high molecular weight PAHs (HPAHs; four or more aromatic rings) includes fluoranthene,  
197 pyrene, benzo[a]anthracene, chrysene, triphenylene, benzo[b]fluoranthene, benzo[k]fluoranthene,  
198 benzo[j]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-c,d]pyrene,  
199 dibenzo[a,h]anthracene, dibenzo[a,c]anthracene and benzo[g,h,i]perylene  
200 <sup>d</sup> sum of methylphenanthrenes (MPhe) includes 1-, 2-, 3-, and 9-methylphenanthrene and 1-  
201 methylantracene  
202 <sup>e</sup> sum of PAHs includes  $\Sigma$ LPAHs,  $\Sigma$ HPAHs and  $\Sigma$ MPhe  
203 <sup>f</sup> sum of PCBs includes congeners 50, 28, 52, 101, 118, 153, 138 and 180  
204 <sup>g</sup> sum of PBDEs includes congeners 47, 119, 99 and 153  
205 <sup>h</sup> sum of organohalogenated compounds (OHCs) includes  $\Sigma$ PCBs,  $\Sigma$ PBDE and lindane  
206 <sup>i</sup> sum of organic contaminants (OC) includes  $\Sigma$ PAHs, DBT and  $\Sigma$ OHCs  
207 <sup>j</sup> values calculated according to MacDonald et al. (2000) recommendations  
208 <sup>k</sup> sediment ranking according to their calculated mean qPEC values. Note that similar ranking of  
209 sediments was obtained using mean qPEC1 or mean qPEC2 values  
210 <sup>l</sup> sediment ranking based on the number of significant sublethal effects (indicated in brackets) recorded in  
211 each treatment using the MELAc  
212 D(0.50), median particle size; dw, dry weight of sediment; DBT, dibenzothiophene; MPhe,  
213 methylphenanthrenes; PAHs, polyaromatic hydrocarbons; PBDEs, polybrominated diphenyl ethers;  
214 PCBs, polychlorinated biphenyls; PEC, probable effect concentration; mean qPEC1, mean PEC quotient  
215 value calculated for each sediment using  $\Sigma$ PAH concentrations; mean qPEC2, mean PEC quotient value  
216 calculated for each sediment using individual PAH concentrations; TEC, threshold effect concentration.

217

### 218 2.3. *Medaka embryo-larval assay with sediment-contact exposure (MELAc)*

219 Toxicity assessment of six sediments from the Lot-Garonne system was performed using the  
220 Medaka embryo-larval assay with a direct sediment-contact exposure (Barjhoux et al., 2012;  
221 Vicquelin et al., 2011). For each studied station, 5 g of freshly defrost sediment were laid in a 35

222 mm-diameter plastic Petri dish and immersed by adding 2 ml of egg rearing solution (ERS;  
223 17.11 mM NaCl, 0.4 mM KCl, 0.36 mM CaCl<sub>2</sub>; 1.36 mM MgSO<sub>4</sub>, pH 7.0). The resulting system  
224 was then maintained at 26 °C for a 4-5 h equilibration period before the beginning of the  
225 experiment.

226 Japanese medaka (*O. latipes*) embryos of the CAB strain were purchased from GIS Amagen  
227 (Gif-sur-Yvette, France) and received 24 hours post-fertilization. Upon receipt, healthiness and  
228 developmental synchronism of 1 day post-fertilization (dpf) embryos were checked using a  
229 stereomicroscope (Leica MZ75, Leica Microsystems) and cold-light source (Intralux® 4100,  
230 Volpi AG). Immediately after sorting, embryos (27 per replicate and three replicates per  
231 treatment) were placed on a Nytex® mesh (mesh opening 1000 µm, Sefar Filtration Inc.) which  
232 was then slightly sunk into the sediment to ensure a good contact between eggs and particles.  
233 The level of ERS buffer was adjusted to ensure complete immersion of embryos in the medium.  
234 The buffer was then completely renewed every day. Dissolved oxygen was also measured daily  
235 throughout the 10-day exposure period at the water-sediment interface using a Clark-type sensor  
236 equipped with a guard cathode (Unisense, Aarhus, DK) and connected to a high sensitivity  
237 picoammeter (PA2000, Unisense) as detailed by Vicquelin et al. (2011).

238 As described by Barjhoux et al. (2012), embryos were kept exposed to sediments until hatching  
239 peak (10 dpf) in the reference treatment (Marcenac). Afterwards, unhatched embryos were  
240 transferred to new Petri dishes with 3 mL of clean ERS. Similarly, newly hatched larvae were  
241 transferred in 50 mL of clean stalling water (1/3 v/v of dechlorinated tap water and osmosed  
242 water, 26 °C, pH 7.5, 53.4 mg/L CaCO<sub>3</sub>, 0.025 mg/L NO<sub>2</sub>; 1.5 ppm PO<sub>4</sub>; 5 mg/L NO<sub>3</sub>;  
243 <0.1 mg/L NH<sub>4</sub>). Embryos and larvae mediums were 100%-renewed every two days. Larvae  
244 were fed twice a day with TetraMin® Baby (Tetra, Melle, DE) flakefood until the end of the  
245 experiment (20 dpf). During the whole experiment, organisms were maintained in a climate

246 cabinet (Economic Delux, Snijders Scientific, Tilburg, NL) at 26 °C ± 0.3 with a 12h: 12 h  
247 photoperiod and 5000 lx white light.

248 The different procedures performed for phenotypic endpoint assessments have been previously  
249 detailed by Barjhoux et al. (2012). Viability was checked daily in all individuals from each  
250 treatment over the experimental period and dead organisms were systematically counted and  
251 removed. Heart rate was monitored in 6- and 7-dpf embryos (five randomly selected individuals  
252 per replicate). Biometric measurements (total body length, head size and head/body length ratio)  
253 and developmental anomalies (spinal, craniofacial, ocular, cardiovascular, yolk-sac and edema)  
254 were observed in 15 randomly selected newly hatched larvae per replicate. All these observations  
255 were carried out in an air-conditioned room at 23 °C ± 1 using a stereomicroscope (MZ75, Leica  
256 Microsystem) equipped with a color CCD camera (Leica DFC 420C) and cold-light source  
257 (Intralux® 4100, Volpi AG), connected to an image analysis software program (Leica  
258 Application Suite v2.8.1.).

259

#### 260 *2.4. Comet assay*

261 Genotoxicity of sediments was evaluated on 2 dph-larvae (five per pool sampled in each  
262 replicate) using the comet assay. Cell dissociation and comet assay procedures were carried out  
263 following the protocol described by Morin et al. (2011). Briefly, pools of larvae were digested in  
264 a MEM-Collagenase IV 0.125% (w/v) medium, and cell viability was checked using a trypan  
265 blue exclusion test (only cell suspensions with viability superior to 80% were used). Once  
266 embedded in a 1%-low melting point agarose gel, cells were lysed and immersed in an  
267 electrophoresis buffer (0.3 M NaOH, 1 mM EDTA; pH >13) for 15 min to allow DNA  
268 unwinding. Then, electrophoresis was carried out at 25 V, 300 mA for 15 min. Ethidium bromide  
269 (20 mg/L) was used as DNA fluorescent tag and coded-slides were blind-analyzed for 75 nuclei  
270 per gel (two gels per experimental replicate) using an Olympus epi-fluorescent microscope (400x

271 magnification) equipped with a grayscale CCD camera (Zeiss, DE) and the Komet 5.5 software  
272 program (Kinetic Imaging, Liverpool, UK). As recommended by Hartmann et al. (2003), the Tail  
273 DNA (percentage of DNA which migrates from the nucleus i.e. the head of the comet) was  
274 selected to measure the rate of DNA damage. Heavily DNA-damaged nuclei displaying a small  
275 or inexistent head and a large diffuse tail, also known as ‘hedgehog’ cells, were not taken into  
276 account in the comet measurement, according to the recommendations of Kumaravel et al.  
277 (2009). However, the percentage of ‘hedgehog’ cells, which have been reported as apoptotic or  
278 necrotic cells (Olive and Banath, 1995), was visually scored on a total of 100 cells per gel.  
279

## 280 2.5. *Statistical analysis*

281 The data is expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were conducted  
282 using Statistica 7.1 software (Statsoft, Maisons-Alfort, FR). Results were initially tested for  
283 normality (Shapiro-Wilk’s test on residues with 1% risk) and homoscedasticity (Brown-  
284 Forsythe’s test, 5% risk). Afterwards, significant differences between treatments were tested with  
285 a one-way or two-way ANOVA analysis followed by a post-hoc Tukey’s test ( $p < 0.05$ ).

286 The existence and significance of relationships between parameters were determined using  
287 parametric Pearson’s product-moment correlation (Pearson  $r$ ) analysis. Principal component  
288 analysis (PCA) on standardized data was used to obtain an overview of the spatial distribution  
289 pattern of the different studied parameters (physico-chemical and toxicological endpoints) and to  
290 assess the relative implication of these factors in sediment discrimination. Only principal  
291 component axes with an eigenvalue superior to the mean of the eigenvalues were considered.  
292 Variables and cases contributions were considered as significant when superior to the mean of  
293 contributions (i.e.  $> 1/\text{nb. of variables or cases}$ ). Factor-variable correlations (equals to factor  
294 coordinate of the variable for the considered component) were considered as significant when  
295 above 0.6. We considered that a principal component was significantly loaded by variables



296 showing both significant contribution and factor-variable correlation coefficient. Key values  
297 obtained from the different PCA conducted in this study are given in Supporting Information (SI)  
298 (Tables i to v).

299

### 300 **3. Results**

#### 301 *3.1. Sediment physico-chemistry*

302 The physico-chemical characteristics of the sediments from the Lot-Garonne continuum are  
303 summarized in Table 1. Principal component analysis and Pearson's coefficient calculations  
304 were performed on POC content, NH<sub>4</sub> concentration and the percentage of fine particles  
305 (<63 μm) (SI Fig. i and Table i). In agreement with the inter-correlation of sediment physico-  
306 chemical endpoints, the first component of the PCA explained almost 90% of the total  
307 variability. As a result, the factor scores of cases on this first component were used to define a  
308 new synthetic variable called 'GPN' equal to the minus factor score value obtained for each  
309 station (SI Table i). This GPN variable was used in subsequent PCA analysis as an integrative  
310 representation of the three physico-chemical characteristics of each sediment: stations showing a  
311 positive GPN value are associated to high fine particles <63 μm, NH<sub>4</sub> and POC contents (Bdx  
312 and LR) and, on the contrary, sites with negative GPN value are associated to low fine particles  
313 <63 μm, NH<sub>4</sub> and POC contents (Mrc, Jo and LT). GPN value for Bo sediment was close to 0 as  
314 this sediment showed intermediate physico-chemical characteristics (SI Fig. i and Table i).

315

#### 316 *3.2. Organic pollutant distribution*

317 A comprehensive analysis of organic pollutants was conducted in sediment from each station and  
318 is summarized in Table 1. Organic contamination of sediments was nearly exclusively  
319 attributable to PAHs as the presence of organohalogenated compounds was very marginal at all

320 stations. The highest concentrations of PAHs were measured for Jo and LT sites, with a total  
321 PAH concentration over 25  $\mu\text{g/g dw}$ . The others sites were clearly less impacted by organic  
322 contaminants with a total load around 1  $\mu\text{g/g dw}$  for Bdx, LR and Bo and close to 15  $\text{ng/g dw}$  for  
323 Mrc.

324 The PCA based on organic contaminant levels gave two principal components accounting  
325 respectively for 74.5% and 25% of the variability among the data set (SI Fig. ii and Table ii ).  
326 The first principal component was significantly loaded by heavy PAHs (high molecular weight  
327 PAHs i.e. composed by four or more aromatic rings) and MPhe. In turn, the second component  
328 was significantly loaded by light PAHs (low molecular weight PAHs i.e. composed by three or  
329 fewer aromatic rings) and DBT (SI Fig. iiA). Consequently, sums of light and heavy PAHs, DBT  
330 and total MPhe concentrations (see Table 1 for the complete list of analyzed compounds) were  
331 used in further PCA as integrative variables efficiently representing organic compound  
332 distribution among the six sediments of the study.

333 The plot of the different sediments in the projection plan derived from the two principal  
334 components separated three groups of sites (SI Fig. iiB). The first group included Mrc, Bo, Bdx  
335 and LR stations on the right part of the plan, representing sites weakly impacted by organic  
336 contamination. Contrarily, Jo and LT projections were both situated on the left side of the plan,  
337 traducing the high organic contaminant content in these sediments. These two sites were also  
338 clearly separated from each other by the second component axis, highlighting a contamination  
339 dominated by light PAHs and heavy PAHs, respectively.

340

### 341 3.3. *Metallic trace element distribution*

342 Chemical analysis of 10 MTE carried out on the sediments from the Lot-Garonne system  
343 revealed significant metallic contamination at Bo and Jo stations, with total MTE concentrations  
344 above 3,000  $\mu\text{g/g dw}$  at each site (Table 1). The other sites were less subject to metallic

345 contamination with values below 900  $\mu\text{g/g dw}$  for Bdx and LR, and lower than 400  $\mu\text{g/g dw}$  for  
346 Mrc and LT (Table 1).

347 Consistently with the strong positive inter-correlation observed between each MTE  
348 concentrations, the PCA results showed that the first principal component accounted alone for  
349 more than 80% of the variability amongst the data (SI Fig. iii and Table iii). This axis was  
350 significantly negatively loaded by each individual MTE concentrations. Hence, the sum of MTE  
351 was used in further analysis as an integrative endpoint representing metallic contamination  
352 distribution among studied sediments. The plot of case factor coordinates for different sites on  
353 the first principal component separated three groups of sites: Jo and Bo sites (first group)  
354 characterized by high concentrations of MTE, Bdx and LR stations (second group) with an  
355 intermediate position, and Mrc and LT (third group) associated to low MTE contamination level.

356

### 357 *3.4. Global toxicity prediction based on contaminant analysis*

358 Concentrations of contaminants were compared to the threshold effect concentration (TEC) and  
359 probable effect concentration (PEC) established as consensus-based freshwater sediment quality  
360 guidelines (MacDonald et al., 2000). These thresholds were respectively intended to identify  
361 chemical concentrations below which harmful effects on organisms are unexpected, and above  
362 which these effects are expected to occur frequently.

363 Concerning organic contaminants, both TEC and PEC values established for total PAH  
364 concentration were substantially exceeded for Jo and LT. Individual PAH concentrations (for  
365 which quality criteria were available) were much higher than TEC values in Jo and LT (except  
366 for naphthalene) sediments. Similarly, PEC values were also exceeded for heavy PAHs and  
367 phenanthrene in LT, and for light PAHs, except naphthalene, in Jo sediments.

368 Among the studied MTE for which PEC and TEC values were available, Cd, Pb, Zn and As  
369 concentrations were above PEC respective values at both Bo and Jo (except As) stations. TEC

370 thresholds were exceeded for all available MTE in Bo (except for Cr and Hg) and Jo (except for  
371 Cr and Ni) stations. As concentrations were also above TEC value in Mrc and LR sediments.  
372 Similar observations were done for Cd in Bdx and LR sites.  
373 As recommended by MacDonald et al. (2000), the overall potential toxicity of sediments was  
374 evaluated calculating PEC quotients (qPEC, equals to measured concentration divided by the  
375 PEC value of the corresponding compound). The mean of qPECs was then calculated for each  
376 station (Table 1). Sediments were predicted to be toxic when the mean qPEC value exceeded 0.5  
377 as established by the authors. The mean qPEC value can be calculated using PEC value  
378 established for the sum of PAH concentrations (mean qPEC1) or PEC values for individual  
379 PAHs (mean qPEC2). Neither Mrc, LR nor Bdx sediments were predicted as toxic according to  
380 the calculated mean qPEC values. Whereas the mean qPEC1 was below the 0.5-limit in LT, the  
381 mean qPEC2 for this site exceeded this threshold mainly due to some heavy PAHs showing  
382 qPEC values around 2.0 as fluoranthene, pyrene and benzo[a]anthracene. Whatever the  
383 calculation method used, mean qPEC values were both over 0.5 at Bo and Jo stations, mostly  
384 owing to qPEC values between 2.0 to 5.0 for MTE as Cd, Pd and Zn. Moreover, very high qPEC  
385 values were observed for phenanthrene and anthracene (above 5.0 and 9.0 respectively) for Jo  
386 sediment. As a result, several chemicals including several PAHs and MTE may potentially  
387 represent a threat for aquatic organisms. The ecotoxicity of the sediments from the Lot-Garonne  
388 continuum was thus evaluated using the MELAc.

389

### 390 3.5. *Acute toxicity and impact on embryonic development*

391 Dissolved oxygen measurements were performed over the 10 day-exposure of the MELAc (SI  
392 Fig. iv). The 10 day-averaged dissolved oxygen levels in Bdx and LR treatments were lower in  
393 comparison to the reference (Mrc) with values around 88%. However, it must be emphasized that  
394 all the mean values and each dissolved oxygen daily measurement (data not shown) were over

395 80%, thus within the concentration range recommended by OECD for fish embryo-larval stage  
396 toxicity testing (OECD, 2013).

397 In agreement with the good oxygenation levels, mean embryonic survival rates and hatching  
398 success were high (> 90%) in all treatments, with no significant difference between sites (SI  
399 Table iv). Similarly, mean larval and cumulative survival rates in the Mrc reference group  
400 remained high ( $\geq 95\%$ , SI Table iv). Although statistically similar, mean values for these  
401 endpoints were quite lower in the other treatments, respectively decreasing down to 70% and  
402 67% ( $p = 0.053$ ) in LR treatment (SI Table iv).

403 Additionally, exposure to the Garonne sediments significantly delayed embryonic development.  
404 Indeed, the mean time to hatch was around 11 dpf in Bdx and LR when the embryonic  
405 developmental time in the reference group was below 10 dpf in average (Fig. 2A). In the other  
406 treatments, time to hatch fluctuated around 10.3 dpf (Fig. 2A). Medaka *in ovo* growth was  
407 evaluated using biometric measurements at hatching (Fig. 2B). The mean total body length and  
408 the head size of hatchlings exposed to Jo sediment were significantly reduced by 6% and 5%  
409 respectively in comparison to the reference (Fig. 2B). Average head size of larvae in LR  
410 treatment was also statistically shorter than in Mrc group while it only represents a 1%-decrease.

411

### 412 3.6. Teratogenicity

413 The potential teratogenicity of sediments was evaluated examining the presence of  
414 morphological abnormalities in each newly hatched larvae. The total percentage of abnormal  
415 larvae and the type of deformities were recorded and are summarized on Table 2.

416 In the reference treatment (Mrc), the percentage of malformed larvae was around 13% in average  
417 with spinal and cardiovascular deformities as main types of anomalies recorded (Table 2). The  
418 percentage of malformed individuals was significantly increased in all the other treatments in  
419 comparison to reference (Table 2). The maximum mean values close to 70% or above were

420 observed for LR, Bo and Jo sites and were statistically higher than for Mrc and LT sediments  
 421 (Table 2). The same discrimination between sites was observed examining the occurrence of  
 422 cardiovascular anomalies which were the most frequent type of deformity, impacting up to more  
 423 than 60% of the larvae in Jo treatment (Table 2). These anomalies included abnormal positioning  
 424 of the heart chambers (in relation to each other and to the cephalo-caudal axis) as well as heart  
 425 hypo-, hyper-development, or dystrophies.

426 The appearance of edemas (mainly pericardial) was also significant in larvae exposed to Jo and  
 427 LR sediments, affecting 15-16% of the larvae, when compared to the reference (Table 2). Spinal  
 428 deformities, predominantly lordosis, kyphosis, C-shaped larvae and few scoliosis, were observed  
 429 in a fifth to one quarter of the organisms exposed to Bdx, LR and Jo sediments which is  
 430 significantly higher than for Mrc treatment (Table 2). Finally, yolk sac resorption defects  
 431 significantly damaged nearly 16% of the larvae in Jo group whereas this pathology was absent  
 432 from the reference population (Table 2).

433

434 **Table 2** Percentage of abnormal larvae following exposure to sediments from the Lot/Garonne  
 435 continuum, in total (Tot. D) and per type of deformities including edemas (Ed), spinal (Sp) and craniofacial  
 436 (Cf) deformities, ocular (Oc) abnormalities, cardiovascular anomalies (Cv) and yolk sac malabsorptions  
 437 (Ys)

Condition	Tot. D (%)	Ed (%)	Sp (%)	Cf (%)	Oc (%)	Cv (%)	Ys (%)
Mrc	12.7 ± 5.45 <sup>a</sup>	1.28 ± 2.22 <sup>a</sup>	5.13 ± 2.22 <sup>a</sup>	0.00 ± 0.00	0.00 ± 0.00	8.89 ± 5.54 <sup>a</sup>	0.00 ± 0.00 <sup>a</sup>
Bdx	<b>61.0 ± 3.83<sup>bc</sup></b>	0.00 ± 0.00 <sup>a</sup>	<b>22.4 ± 7.20<sup>bc</sup></b>	1.23 ± 2.14	0.00 ± 0.00	<b>49.0 ± 10.4<sup>bc</sup></b>	1.23 ± 2.14 <sup>ab</sup>
LR	<b>70.0 ± 7.96<sup>c</sup></b>	<b>15.7 ± 6.40<sup>b</sup></b>	<b>23.8 ± 5.01<sup>bc</sup></b>	1.28 ± 2.22	1.28 ± 2.22	<b>59.5 ± 10.5<sup>c</sup></b>	13.1 ± 5.80 <sup>ab</sup>
LT	<b>43.6 ± 5.57<sup>b</sup></b>	3.96 ± 0.23 <sup>a</sup>	13.3 ± 2.98 <sup>ab</sup>	6.89 ± 8.70	0.00 ± 0.00	<b>32.9 ± 0.77<sup>b</sup></b>	4.11 ± 4.17 <sup>ab</sup>
Bo	<b>65.2 ± 8.58<sup>c</sup></b>	2.57 ± 2.23 <sup>a</sup>	11.7 ± 4.03 <sup>ab</sup>	6.57 ± 8.38	1.23 ± 2.14	<b>56.1 ± 10.3<sup>c</sup></b>	13.0 ± 6.23 <sup>ab</sup>

Jo            **72.3 ± 7.88<sup>c</sup>**    **15.0 ± 1.84<sup>b</sup>**    **24.6 ± 3.30<sup>c</sup>**    7.97 ± 6.43    0.00 ± 0.00    **62.8 ± 5.70<sup>c</sup>**    **16.2 ± 10.7<sup>b</sup>**

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438 Values represent the mean response ( $\pm$  SD) for three replicates. Statistical analysis was performed on  
439 each endpoint independently. Different letters indicate significant differences between treatments using  
440 one-way ANOVA followed by Tukey's post-hoc test ( $p < 0.05$ ). In bold, statistical differences with control  
441 group (Mrc).

442

### 443        3.7.    *Cardiac activity*

444 Cardiac activity measurements were performed in 6 dpf- and 7 dpf-embryos from each treatment.  
445 Results showed a significant increase of heartbeat rate in 6 dpf-embryos exposed to LR and Jo  
446 sediments in comparison to Mrc treatment (Fig. 2C). Cardiac activity acceleration in 6 dpf-  
447 embryos was also close to significant threshold ( $p = 0.053$ ) in Bdx when compared to the  
448 reference. Conversely, a significant decrease in cardiac activity was observed in 7 dpf-embryos  
449 following exposure to LT, Bo and Jo sediments when compared to Mrc (Fig. 2C). As a result, the  
450 mean heart rate was significantly ( $p < 0.01$ ) lower at 7 dpf than at 6 dpf in embryos from the same  
451 treatment for LR, LT, Bo and Jo stations (Fig. 2C).

452

### 453        3.8.    *Genotoxicity*

454 The potential induction of DNA damage following exposure to sediments from the Lot-Garonne  
455 system was evaluated in 2 dph-larvae using the comet assay. Basal DNA strand breaks (Tail  
456 DNA) and the percentage of 'hedgehog' cells in reference larvae were low with average values  
457 below 10% (Fig. 2D). The percentage of tail DNA slightly raised in the other treatments to reach  
458 a maximum value of 18% in average in Jo station, which was significantly different from Mrc  
459 (Fig. 2D). Likewise, the percentage of 'hedgehog' cells was quite similar (17-20%) for Bdx, LR  
460 and Bo stations and reached maximum values of 25% and 28% in Jo and LT treatments  
461 respectively, representing a significant raise of heavily DNA damaged cells (Fig. 2D).

462

463       3.9. *Crosslink analysis of physico-chemical characteristics of sediments and toxic*  
464               *responses in medaka ELS*

465 Principal component analysis was conducted on physico-chemical (GPN variable, dissolved  
466 oxygen concentrations), contamination (Sum of MTE, light PAHs and MPhe, DBT) and  
467 biological endpoints which included embryonic and larval survival rates, hatching success, time  
468 to hatch, cardiac activities, biometric measurements (total body length and head size), the  
469 percentage of abnormal larvae and genotoxicity endpoints (Tail DNA and ‘hedgehog’ cells).

470 Results of this analysis are summarized on Fig. 3 and SI Table v.

471 The two principal components (PC1 and PC2) accounted respectively for 53.1% and 26.9% of  
472 variability among the data set. Thus, the resulting projection plan PC1xPC2 explained 79.9% of  
473 variability in total. Total heavy PAHs did not significantly contributed to the two principal  
474 component determination and was thus excluded from the analysis.

475 The first component (PC1) was significantly negatively loaded by teratogenicity and  
476 genotoxicity endpoints as well as by some organic contamination variables including total light  
477 PAHs and DBT. In its positive direction, this axis was also significantly loaded by embryonic  
478 survival, hatching success and biometric measurements (Fig. 3A and SI Table v). Moreover,  
479 significant correlations with this principal component were observed with 7 dpf -cardiac activity  
480 (positive), total metallic and MPhe contamination levels (negative). In turn, the second  
481 component axis (PC2) was significantly loaded by larval survival and dissolved oxygen level in  
482 its negative sense and positively by GPN variable, time to hatch, and 6 dpf -cardiac activity (Fig.  
483 3A and SI Table v).

484 The plot of the different sites on the resulting projection plan separated four groups of sites (Fig.  
485 3B). A first group, including Mrc station only, was located on the bottom right of the plan. This  
486 area is associated with marginal contamination level, ‘good’ physico-chemical properties and



487 non-toxic responses. Bdx and LR stations (second group) were gathered in the upper part of the  
488 plan, principally characterized by ‘bad’ physico-chemical properties and specific toxic responses  
489 (according to the second component). Jo station (third group) was isolated at the bottom left of  
490 the plan. This area is associated with elevated contamination levels, ‘good’ physico-chemical  
491 properties and toxic responses (according to the first component only). Finally, the fourth group  
492 consisting of Bo and LT stations, occupied an intermediate position, close to the origin of the  
493 plan.

494 Pearson’s product-moment correlation analysis was conducted on the full set of selected  
495 variables and is summarized in Table 3 and Table 4. The extent of inter-correlations among  
496 biological endpoints was first examined. Embryonic survival, hatching success and biometric  
497 measurements (total body length and head size of larvae) proved to be significantly and  
498 positively correlated with one another (Table 3). Time to hatch and larval survival showed a  
499 significant negative correlation. The percentage of abnormal larvae was positively correlated to  
500 Tail DNA and 6 dpf -cardiac activity (Table 3) as well as with the main kind of developmental  
501 deformities observed in the present study which included spinal deformities, cardiovascular  
502 injuries and yolk sac resorption defects (Table 4). Additionally, Tail DNA presented a negative  
503 correlation with biometric measurements and 7 dpf -cardiac activity ( $p = 0.095$ ) (Table 3).

504 Lastly, the percentage of hedgehog cells was inversely correlated to 7 dpf -cardiac activity  
505 (Table 3). 6 dpf-cardiac activity was positively correlated to the percentage of deformed larvae  
506 (Table 3) and especially to the percentage of cardiovascular injuries ( $r = 0.926$   $p = 0.008$ ) and of  
507 spinal deformities ( $r = 0.965$   $p = 0.002$ ). 7 dpf-cardiac activity showed a negative correlation  
508 with the percentage of craniofacial deformities ( $r = -0.876$   $p = 0.022$ ).

509 Examination of the relationship between biological, contamination and physico-chemical  
510 variables gave the following results.

511 Hatching success displayed a negative correlation to DBT levels and more slightly with total  
512 light PAH concentrations ( $p = 0.061$ , Table 3) with a significant correlation with phenanthrene  
513 ( $r = -0.813$ ,  $p = 0.049$ ). Similar observations were done for embryonic survival with correlation  
514 coefficient around -0.7 although insignificant. Time to hatch, 6 dpf -cardiac activity and the  
515 percentage of abnormal larvae were all three negatively and significantly correlated with  
516 dissolved oxygen levels (Table 3). The two most frequent types of developmental anomalies  
517 (spinal deformities and cardiovascular anomalies) were also significantly negatively correlated  
518 with dissolved oxygen levels (Table 4). Cardiovascular injuries were also significantly correlated  
519 to Mn and Co levels (respective  $r$ -values equal to 0.85 and 0.84,  $p$ -values equal to 0.032 and  
520 0.037; data not shown). Time to hatch was also inversely correlated to GPN variable. Total body  
521 length of larvae showed a negative significant correlation with total MTE content, and more  
522 precisely with Ag, Cd, Cu, Pb and Zn ( $r$ -values between -0.85 to -0.94;  $p < 0.05$ ; data not shown).  
523 This endpoint was also inversely correlated with light PAH concentration (Table 3) including  
524 naphthalene, acenaphthene, fluorene and anthracene ( $r$ -values between -0.81 to -0.89;  $p < 0.05$ ;  
525 data not shown). Tail DNA was not significantly correlated to any physico-chemical or  
526 contamination variables despite correlation coefficients varying between 0.75 and 0.78 with  
527 several of them. The percentage of hedgehog cells proved to be positively correlated to the total  
528 concentrations of MPhe and organic contaminants and, to a lesser extent to heavy PAHs  
529 ( $p = 0.059$ ). When analyzed in detail, this genotoxicity endpoint showed significant positive  
530 correlation with fluoranthene and triphenylene+chrysene concentrations ( $r$ -values equal to 0.82;  
531  $p < 0.05$ ). Finally, craniofacial deformities proved to be significantly correlated with MPhe and  
532 total organic compounds whereas yolk sac resorption defects showed a positive correlation with  
533 total metallic contamination (Table 4), more specifically with Mn concentrations ( $r = 0.84$  with  $p$   
534 = 0.036; data not shown).

535

536 **Table 3** Pearson's correlation coefficients between physico-chemical, contamination and biological endpoints

	ES	LS	HS	TH	CA 6dpf	CA 7dpf	BL	HSz	Tot. D	Tail DNA	H. cells
ES	-	0.400	<b>0.986***</b>	0.096	-0.291	0.522	<b>0.821*</b>	<b>0.833*</b>	-0.348	-0.676	-0.604
LS	0.400	-	0.460	<b>-0.856*</b>	-0.795	0.281	0.129	0.539	-0.653	-0.436	-0.486
HS	<b>0.986***</b>	0.460	-	0.010	-0.411	0.538	<b>0.840*</b>	<b>0.849*</b>	-0.449	-0.767	-0.671
TH	0.096	<b>-0.856*</b>	0.010	-	0.800	-0.039	0.196	-0.229	0.635	0.198	0.175
CA 6dpf	-0.291	-0.795	-0.411	0.800	-	-0.379	-0.385	-0.631	<b>0.940**</b>	0.724	0.499
CA 7dpf	0.522	0.281	0.538	-0.039	-0.379	-	0.615	0.584	-0.615	-0.736	<b>-0.833*</b>
BL	<b>0.821*</b>	0.129	<b>0.840*</b>	0.196	-0.385	0.615	-	<b>0.868*</b>	-0.553	<b>-0.840*</b>	-0.526
HSz	<b>0.833*</b>	0.539	<b>0.849*</b>	-0.229	-0.631	0.584	<b>0.868*</b>	-	-0.726	-0.788	-0.497
Tot. D	-0.348	-0.653	-0.449	0.635	<b>0.940*</b>	-0.615	-0.553	-0.726	-	<b>0.824*</b>	0.576
Tail DNA	-0.676	-0.436	-0.767	0.198	0.724	-0.736	<b>-0.840*</b>	-0.788	<b>0.824*</b>	-	0.805
H. cells	-0.604	-0.486	-0.671	0.175	0.499	<b>-0.833*</b>	-0.526	-0.497	0.576	0.805	-
dO2	0.148	0.737	0.277	<b>-0.816*</b>	<b>-0.987***</b>	0.360	0.294	0.524	<b>-0.934**</b>	-0.675	-0.461
GPN	0.202	-0.696	0.154	<b>0.918*</b>	0.685	0.032	0.200	-0.245	0.584	0.046	-0.099
ΣMTE	-0.506	0.070	-0.534	-0.194	0.373	-0.566	<b>-0.903*</b>	-0.735	0.600	0.748	0.320

ΣLPAHs	-0.721	-0.110	-0.792	-0.192	0.361	-0.286	<b>-0.813*</b>	-0.598	0.370	0.774	0.515
ΣHPAHs	-0.465	-0.176	-0.484	-0.181	-0.037	-0.498	-0.185	-0.047	-0.030	0.367	0.794
ΣMPhe	-0.663	-0.180	-0.703	-0.225	0.112	-0.527	-0.484	-0.291	0.132	0.604	<b>0.835*</b>
DBT	-0.752	-0.135	<b>-0.819*</b>	-0.215	0.315	-0.366	-0.775	-0.551	0.326	0.776	0.634
ΣOC	-0.689	-0.179	-0.738	-0.223	0.157	-0.492	-0.541	-0.336	0.167	0.648	<b>0.814*</b>

537 Significant coefficients are mentioned in bold and asterisks indicate the significance level (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

538 ES, embryonic survival; LS, larval survival; HS, hatching success; TH, time to hatch; CA 6pdf, cardiac activity at 6 dpf; CA 7pdf, cardiac activity at 7 dpf; BL,  
539 total body length; HSz, head size; Tot. D, percentage of deformed larvae; Tail DNA, percentage of tail DNA; H. cells, percentage of hedgehog cells; dO<sub>2</sub>,  
540 dissolved oxygen.

541

542 **Table 4** Pearson's correlation coefficients between physico-chemical, contamination and teratogenicity  
 543 endpoints including edemas (Ed), spinal (Sp) and craniofacial (Cf) deformities, ocular (Oc) abnormalities,  
 544 cardiovascular anomalies (Cv) and yolk sac malabsorptions (Ys)

	Ed	Sp	Cf	Oc	Cv	Ys
dO2	-0,527	<b>-0,949***</b>	-0,175	-0,309	<b>-0,913**</b>	-0,552
GPN	0,343	0,597	-0,421	0,661	0,572	0,275
ΣMTE	0,361	0,274	0,666	0,271	0,651	<b>0,797*</b>
ΣLPAHs	0,583	0,457	0,638	-0,366	0,397	0,553
ΣHPAHs	0,080	0,011	0,642	-0,435	-0,075	-0,017
ΣMPhe	0,297	0,180	<b>0,774*</b>	-0,463	0,108	0,224
DBT	0,544	0,411	0,714	-0,421	0,341	0,497
ΣOC	0,352	0,239	<b>0,771*</b>	-0,487	0,149	0,269
Tot. D	0,569	<b>0,838**</b>	0,435	0,461	<b>0,996***</b>	<b>0,772*</b>

545 Significant coefficients are mentioned in bold and asterisks indicate the significance level (\* p <0.1; \*\* p  
 546 <0.05; \*\*\* p <0.01).

547

548 **4. Discussion**

549 Sediments are of major concern in environmental risk assessment strategies as they alternatively  
550 act as a sink or a source of contamination for aquatic biota. However, the heterogeneity of the  
551 matrix and the numerous interactions existing between sediment particles, pore-water and water  
552 column that can govern bioavailability of pollutants make sediment toxicity assessment a real  
553 challenge for scientists. The present study proposes an experimental evaluation of sediment  
554 toxicity using the MELAc without any extraction/treatment of sediment to get as close as  
555 possible to environmental conditions of exposure. Such bioassay is particularly relevant as it  
556 allows toxicity to be assessed for the bioavailable fraction of overall chemicals present in  
557 sediments. A limited number of sediment-contact bioassays using fish ELS have been proposed  
558 and successfully applied to sediment toxicity assessment on zebrafish, medaka and salmonids  
559 (e.g. Barhoumi et al., 2016; Bartzke et al., 2010; Hollert et al., 2003; Vehniäinen et al., 2015).  
560 We supplemented this approach by a comprehensive analysis of physico-chemical properties and  
561 contamination levels in the studied sediments.

562  
563 The six sediments collected in the Lot and Garonne Rivers were ranked according to their  
564 contamination level (using their mean qPEC value) and their toxicity (using the number of  
565 significant effects observed on Japanese medaka ELS; Table 1). Given the calculated mean  
566 qPEC values, a toxic impact was probable following exposure to LT (in relation to high heavy  
567 PAH content such as fluoranthene, pyrene and benzo[a]anthracene), Bo (associated to the strong  
568 presence of MTE including Cd, Pb and Zn) and Jo (due to the same metallic pressure as in Bo  
569 but higher level of light PAHs such as phenanthrene and anthracene). In a way consistent with its  
570 marginal contamination, exposure to Mrc sediment did not result in either lethal or sublethal  
571 noticeable effects in medaka ELS which confirmed its suitability as reference sediment for the  
572 MELAc. Likewise, exposure to the other Lot-Garonne sediments did not induce any significant

573 acute toxicity but several sub-acute endpoints were modulated in comparison to the reference. As  
574 the most impacted site by POPs and MTE, Jo was expectedly classified as the most harmful  
575 sediment, according to both mean qPEC- and MELAc-based classifications. Whereas MTE and  
576 organic contaminant levels respectively in Bo and LT stations forecasted pronounced impacts on  
577 medaka ELS, exposure to these sediments only conducted to limited adverse effects, mainly  
578 affecting cardiac morphology and activity. Conversely, while the mean qPEC values calculated  
579 for Bdx and LR sites did not predict any particular toxicity (values < 0.5, ranked 2<sup>nd</sup> and 3<sup>rd</sup>  
580 respectively), both sediments led to noticeable developmental defects (3<sup>rd</sup> and 5<sup>th</sup> position in the  
581 MELAc-based classification, Table 1). In view of these unexpected results, multivariate analyses  
582 were performed to clarify (i) how the variability between sites could be jointly or independently  
583 explained by biotic (biological responses) and abiotic (physico-chemical parameters and  
584 contamination levels) endpoints, and (ii) how these parameters could be associated with each  
585 other.

586  
587 As a first observation, no significant correlation was found between physico-chemical properties  
588 (<63 µm, NH<sub>4</sub> and POC) and contamination levels (data not shown). Strikingly, a more  
589 comprehensive analysis showed a positive correlation between some organic micro-pollutants  
590 (heavy PAHs and total organic contamination level) and the size fraction >500 µm (data not  
591 shown). The highest PAH concentrations (>25 µg/g dw) were observed in LT and Jo which were  
592 also the coarsest sediments presenting low POC contents. These results are surprising as PAHs  
593 are generally associated to fine particle size fraction and/or rich organic matter content (Ghosh et  
594 al., 2000). Moreover, no such high PAH levels have been recorded in sediments from the  
595 WFD/SDAGE stations close to LT and Jo stations (≤8 µg/g dw at most for 18 PAHs analyzed;  
596 WFD/SDAGE data from the Adour-Garonne basin Water Information System, [http://adour-  
597 garonne.eaufrance.fr/](http://adour-garonne.eaufrance.fr/); ‘Viviez bas’, ‘Clairac’ and ‘Casseneuil’ stations) within the 2005-2013

598 period. Some wide spatio-temporal variability in the sediment characteristics and point source of  
599 contamination can be at the origin of (i) differences in PAH concentrations from the  
600 WFD/SDAGE data and the present study, (ii) the unexpected correlation between >500 µm grain  
601 size fraction and heavy PAH concentrations. This correlation may thus likely be a ‘coincidental’  
602 association rather than a real causal relationship. The contamination profile was different  
603 between these two PAH-contaminated sediments with a predominance of heavy PAHs in LT and  
604 light PAHs in Jo stations, representing a respective 2300-fold and 4800-fold increase in  
605 comparison to Mrc reference site. The limited biological responses following exposure to LT  
606 sediment as regards its level of contamination can be explained by a contamination particularly  
607 marked by heavy PAHs that are known to be poorly water soluble and strongly bounded to  
608 organic matter, which may limit their bioavailability to embryos exposed to this sediment.  
609 However, the source of these unforeseen PAH contaminations remained unknown and further  
610 investigations are needed to clarify their origin. Conversely, the highest metal contents were  
611 observed in Jo and Bo sites consistently with their vicinity of the now-abandoned zinc factory in  
612 Decazeville. The metal enrichment was particularly marked for Cd, Zn, Ag, Pb and Cu in  
613 agreement with previous works which identified these compounds as the main MTE released in  
614 the Lot-Garonne system from the former mining site (Audry et al., 2004).

615  
616 These extremely high levels of metallic and PAH contamination are most likely responsible for  
617 the adverse effects observed in medaka ELS as supported by multivariate analysis results.  
618 Indeed, the first component derived from the PCA explained more than 50% of the data  
619 variability and can be interpreted as the discriminating axis for the biological responses  
620 associated to micro-pollutant contamination. According to the multivariate analysis, high  
621 pollutant contents including DBT, light PAHs, and in a quite lesser extent, MTE and MPhe were  
622 clearly associated to adverse effects such as genotoxicity, teratogenicity, acute toxicity



623 (embryonic survival and hatching success), impairment of 7 dpf-cardiac activity and reduced  
624 body length and/or head size.

625 Hatching success was inversely correlated to DBT and phenanthrene concentrations. In turn,  
626 larvae body length showed similar relationship with several MTE (Cd, Cu, Pb, Zn etc.) and light  
627 PAHs (naphtalene, fluorene, anthracene etc.). Reduced hatching success or growth retardation  
628 have also been reported in fish ELS exposed to DBT (Rhodes et al., 2005), PAH mixtures  
629 containing the above-mentioned compounds (Incardona et al., 2004; Sundberg et al., 2005) and  
630 MTE (Cao et al., 2009; Nguyen and Janssen, 2002). The lack of noticeable effects on hatching  
631 success in our study could be explained by a lower bioavailability of pollutants in natural  
632 sediments in comparison to laboratory studies. Moreover, growth parameters have been already  
633 identified as more sensitive than survival or hatching success in fish embryo-larval assays  
634 (Nguyen and Janssen, 2002). In agreement, the positive correlation between embryonic survival,  
635 hatching success and biometric endpoints suggests that larvae body length and head size could  
636 represent good predictors for acute toxicity as they allow a better discrimination between sites  
637 with less inter-replicate variability.

638 Genotoxicity endpoints were differentially correlated to contaminants. The percentage of  
639 hedgehog cells was correlated to MPhe and total organic micro-pollutant contents whereas the  
640 Tail DNA was rather associated to light PAH and MTE levels. These observations suggested that  
641 the induction of DNA strand breaks and heavily DNA damaged cells could result from the  
642 impact of various potent genotoxicants and are thus complementary markers of genotoxicity. The  
643 comet assay already proved its reliability in genotoxicity testing of PAH- and MTE-  
644 contaminated sediments or extracts in fish ELS (Kammann et al., 2004; Kosmehl et al., 2008).

645 Interestingly, 7dpf-cardiac activity was negatively correlated to the percentage of hedgehog cells  
646 and close to significant threshold with Tail DNA, but no significant relationship was found with  
647 physico-chemical or contamination variables. Bradycardia was observed in 7 dpf-embryos

648 exposed to LT, Bo and Jo sediments, respectively impacted by heavy PAHs, MTE and both MTE  
649 and light PAHs. Such reduction of heart beats has been reported in fish ELS exposed to PAHs  
650 (Incardona et al., 2004), MTE such as Cd and As (Cao et al., 2009; Li et al., 2009) and sediment  
651 organic extracts (Hallare et al., 2005). We could thus hypothesize that the inhibition of 7dpf-  
652 cardiac activity in the present study is the result of PAHs and/or MTE contamination depending  
653 on the considered sediment but with a non-linear relationship to the concentration levels.

654

655 While contamination levels could easily explain hazardous effects reported in medaka ELS  
656 following exposure to the most contaminated stations, sediments from Bdx and LR stations were  
657 not supposed to induce any harmful responses according to their level of contamination. Actually  
658 these sediments conducted to non-negligible deleterious effects in exposed organisms, especially  
659 in embryos and larvae exposed to LR sediment which exhibited a toxicity spectrum almost as  
660 broad as for Jo station. Höss et al. (2010) also reported some toxic responses in several model  
661 organisms including fish embryos exposed to environmental sediments with mean qPEC values  
662 below 0.3 and qualified as lowly to moderately contaminated. The authors stated that even if a  
663 chemical pollution-mediated impact could not be excluded in the case of certain sediments,  
664 several geochemical inherent properties could also influence the response of various test  
665 organisms exposed to native sediments. Many physico-chemical characteristics, such as organic  
666 matter content, ammonia, particle grain size and dissolved oxygen at the water-sediment  
667 interface are known to govern bioavailability of sediment-bound chemicals and/or act as  
668 confounding factors in toxicity testing procedures (Chapman and Wang, 2001). Our results –  
669 consistent with these observations – strongly suggest that the physico-chemical status of  
670 sediments could drive the emergence of some of the toxic effects recorded during the MELAc.  
671 In fact, the second component derived from the PCA analysis (27% of the total variability)  
672 associated ‘bad’ physico-chemical properties (i.e. high contents of <63µm particles, NH<sub>4</sub> and

673 POC illustrated by the GPN variable, and low dissolved oxygen levels) to specific toxic  
674 responses including delayed hatching events, an increased 6 dpf-cardiac activity and low larval  
675 survival rates. Among these biological effects, a strong correlation was shown between time to  
676 hatch and larval survival. Considering that larval survival failed to show statistically significant  
677 modulations during the experiment whereas time to hatch did so, this latter endpoint could be  
678 considered as more sensitive and as a potential predictor for acute effects at the larval stage.  
679 However, delayed hatching time also proved to be strongly associated to low GPN and dissolved  
680 oxygen values. Strong hypoxic conditions (around 1 or 2 mg/L i.e. 12-24% sat.) may rapidly  
681 arise at the water-sediment interface and within the 500 µm-layer just above during sediment-  
682 contact bioassays using natural whole sediments rich in organic matter, even if dissolved oxygen  
683 concentration in the test medium remained high (Strecker et al., 2011). As this zone is exactly  
684 where embryos develop during the bioassay, this local oxygen depletion could result in toxic  
685 effects overlaying chemical expected impacts (e.g. developmental retardations, spinal curvatures,  
686 altered heart beats, vascular system development impairment, reduced larval length etc.), directly  
687 or via synergetic interactions with contaminants, thus leading to biased results in whole sediment  
688 testing (Hassell et al., 2008; Küster and Altenburger, 2008; Strecker et al., 2011). High organic  
689 content in sediment could also amplify hypoxia phenomenon by activating on microbial aerobic  
690 degradation of organic matter (Braunbeck et al., 2005; Strecker et al., 2011). Similarly, fine  
691 particles can stick onto the egg chorion and cover its micropores, disrupting oxygen supply to  
692 embryos and causing physiological and morphological impacts as well as hypoxia (Kemp et al.,  
693 2011). Fine particles remobilization and/or organic matter degradation could also increase the  
694 bioavailability, and thus the toxic effects of contaminants (Bartzke et al., 2010; Chapman and  
695 Wang, 2001; Kemp et al., 2011).

696 LR and Bdx sites were clearly discriminated from the others with regard to the second  
697 component axis which indicates that the toxic effects induced by these sediments are more likely

698 related to their 'bad' physico-chemicals characteristics than to their contamination levels. The  
699 toxicity spectrum observed in medaka ELS exposed to these sediments (delayed hatching,  
700 cardiac activity modulation, cardiovascular anomalies, spinal deformities and edemas)  
701 considerably overlaps the one induced by hypoxia or fine particles, as described above.  
702 However, it cannot be completely excluded that these biological responses are the result of  
703 additive or synergetic interactions between the physico-chemical components and the micro-  
704 pollutant contamination. Moreover, it must be kept in mind that sediment chemical analysis is  
705 never exhaustive and that hazardous pollutants – not analyzed here – could play a major role in  
706 the above-mentioned adverse effects.

707 The developmental impairments observed in organisms exposed to LR were very similar to those  
708 observed for Jo sediment, whose toxic effects were associated to contaminants levels according  
709 to the PCA results. On one hand, it could be interpreted as supporting the above-mentioned  
710 hypothesis of the increased toxicity of pollutants with 'bad' physico-chemical properties of  
711 sediment. On the other hand, similarities between the two teratogenicity spectra could indicate  
712 the non-specificity of the observed deformities. When the correlation matrix between physico-  
713 chemical characteristics, contamination levels and developmental impairments is examined,  
714 cardiovascular and spinal anomalies proved to be strongly inversely correlated to dissolved  
715 oxygen levels. As these deformities were the most commonly observed in exposed larvae, the  
716 percentage of abnormal larvae was also negatively correlated to the oxygenation level. However,  
717 certain type of less frequent developmental abnormalities proved to be associated to  
718 contamination variables. Craniofacial deformities were correlated to organic contaminant levels  
719 whereas yolk sac malabsorptions and cardiovascular injuries were associated to metal content.

720 These observations explained the intermediate position of the percentage of abnormal larvae  
721 between the two principal component axes albeit it proved to be more strongly associated to the  
722 first component traducing the biological impacts of pollutants.

723 Another unexpected result was the differential modulation of embryo cardiac activity at 6 dpf  
724 (tachycardia) and at 7 dpf (bradycardia). Such responses have been already reported in medaka  
725 embryos exposed to Cd-spiked sediments (Barjhoux et al., 2016). The authors also reported a  
726 positive correlation between 6 dpf-cardiac activity and cardiovascular injuries. They  
727 hypothesized that tachycardia at 6 dpf may be a first stress response and could be considered as  
728 an early marker of general injuries in exposed embryos whereas bradycardia at 7 dpf could likely  
729 reflect particular effect of the tested compound. These hypotheses are supported by the results of  
730 the present work where (i) 7 dpf-cardiac activity mainly contributed to the first component  
731 associating specific toxic responses to sediment contamination levels, and was correlated to  
732 craniofacial deformities and hedgehog cells, themselves correlated to several contaminants, (ii)  
733 6 dpf-cardiac activity significantly contributed to the second component traducing the biological  
734 responses associated to physico-chemical characteristics of sediments and was correlated to non-  
735 specific injuries such as the percentage of deformed larvae, cardiovascular injuries and spinal  
736 deformities. However, the underlying mechanisms and physiological repercussions of such  
737 modulation as well as the kinetics of these responses have to be clarified in further studies.  
738 It must be also emphasized that the correlations were calculated based on a limited number of  
739 sites including two ‘particular cases’ that LR and LT sediments are considering the mismatch  
740 between their contamination levels and the biological responses they induced.

741

742 To summarize, in the present study, more than a half of inter-site variability is explained by  
743 sediment contamination status and associated toxic impacts. Nonetheless, a non-negligible part  
744 of the toxicity can also be explained by the physico-chemical characteristics of sediments such as  
745 fine particle and POC contents and dissolved oxygen levels. The second component axis is thus  
746 extremely important in the conclusion drawn in terms of toxicity as it identified sediments whose  
747 inherent properties are inappropriate for medaka embryonic development and the biological

748 markers that are more likely related to these ‘bad’ physico-chemical characteristics than to  
749 contamination levels. Depending on the position of each site in the component projection plan,  
750 we might conclude that Jo, Bo, LT and Mrc sediments represented adequate substrates for  
751 medaka ELS development and that the biological responses observed following exposure to  
752 these matrixes could be mainly interpreted in terms of contamination impacts. As a result, Mrc  
753 confirmed its reliability as a reference site whereas Jo station proved to be the more problematic  
754 site from the studied area with high levels of MTE and light PAHs, and a wide spectrum of toxic  
755 responses. Bo and LT occupied an intermediate position indicating that they induced only few  
756 impacts in exposed organisms despite their non-negligible respective contamination in MTE and  
757 heavy PAHs probably traducing a limited contaminant bioavailability in these sediments. On the  
758 contrary, LR and Bdx sediments induced unexpected developmental impairments in medaka ELS  
759 when considering their contamination status. The toxic responses observed (including delayed  
760 time to hatch, reduced head size, induction of edemas, spinal and cardio-vascular deformities)  
761 had to be carefully interpreted as they are more likely the result of sediments ‘bad’ physico-  
762 chemical properties (high organic content and fine particle fraction, reduced dissolved oxygen).  
763 Overall results clearly highlight the importance, relevance and complementarity of a biological  
764 and chemical combined approach in sediment toxicity assessments. On one hand *in vivo* toxicity  
765 testing using whole sediments such as the MELAc allows a more realistic toxicity evaluation  
766 since it integrates the total bioavailable fraction present in the sample which is directly translated  
767 as biological responses. One the other hand, physico-chemical analysis, as comprehensive as  
768 possible (i.e. including sediment matrix characteristics and pollutants levels), enables to crosslink  
769 biological responses to chemical data in order to (i) pinpoint contaminants (or class of chemicals)  
770 responsible for the observed toxic effects and (ii) potentially identify some ‘false positive’ results  
771 due to confounding factors as some intrinsic physico-chemical properties of natural sediments.

772

## 773 **5. Conclusion**

774 The present study proposes to widen the use of the MELAc to toxicity assessment of  
775 environmentally multi-contaminated sediments as it allows the investigation of the impact of the  
776 whole bioavailable fraction of chemicals bounded to sediment particles, with limited handling of  
777 the matrix.

778 According to the results of multivariate and correlation analyses, we could identify markers that  
779 are more reliable for toxicity evaluation thanks to their higher sensitivity to micro-pollutants and  
780 lower sensitivity to physico-chemical properties of the sediment. In the present study, it appeared  
781 that biometric measurements, genotoxicity endpoints, hatching success, embryonic survival,  
782 cardiac activity at 7 dpf, and some particular developmental impairments such as craniofacial  
783 deformities and yolk sac malabsorption, were the most appropriate and specific markers of  
784 pollutant-induced toxicity. Conversely, time to hatch, cardio-vascular and spinal deformities,  
785 cardiac activity at 6 dpf and larval survival (due to its correlation to time to hatch) might be  
786 carefully interpreted as they proved to be correlated to physico-chemical properties of sediment  
787 and dissolved oxygen level. These observations once again highlight the extreme importance of  
788 the measurement of some critical endpoints such as oxygenation level, organic matter content  
789 and fine particle fraction in natural sediments as potential confounding factors in sediment-  
790 contact bioassays.

791 Nonetheless, the present study demonstrates the relevance and the applicability of the MELAc  
792 and associated biomarkers to the evaluation of the toxicity of multi-contaminated and complex  
793 matrixes as sediments. Such an approach could be efficiently integrated to a battery of tests on  
794 various phyla and trophic levels as a meaningful tool to assess the toxicity of complex  
795 environmental matrix in an environment risk assessment strategy. This work also illustrated the  
796 importance and the appropriateness of a toxicity assessment strategy combining both biological

797 and physico-chemicals analyses to avoid misinterpretation of the results obtained from one or the  
798 other approach.

799

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806



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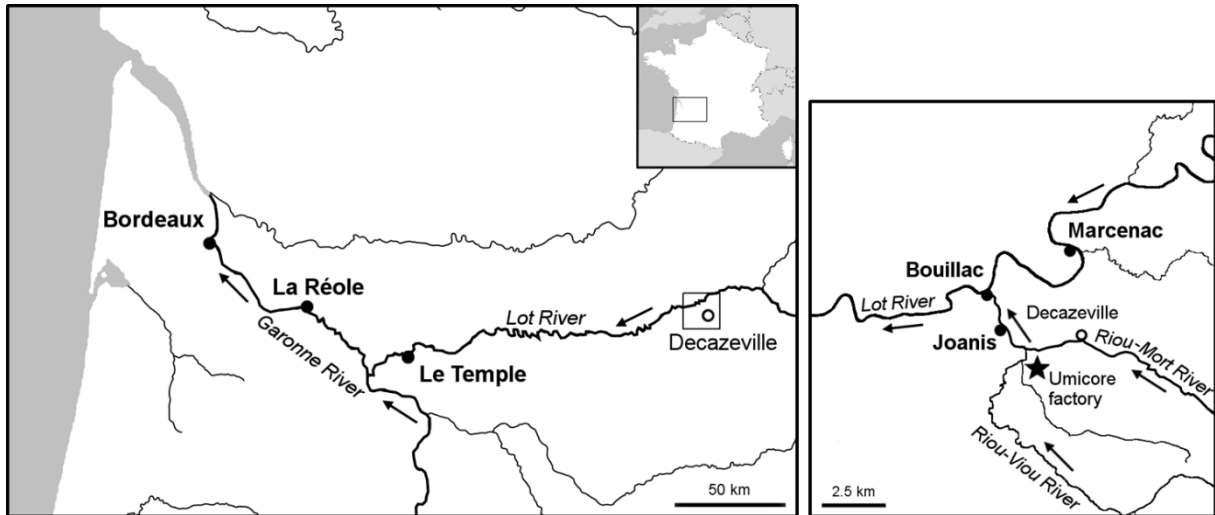


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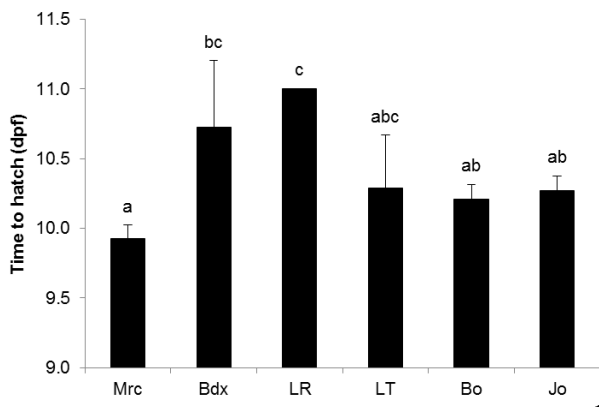
2 **Fig. 1** Location of the study area and the six sampling stations (solid black dots) along the Lot-

3 Garonne system

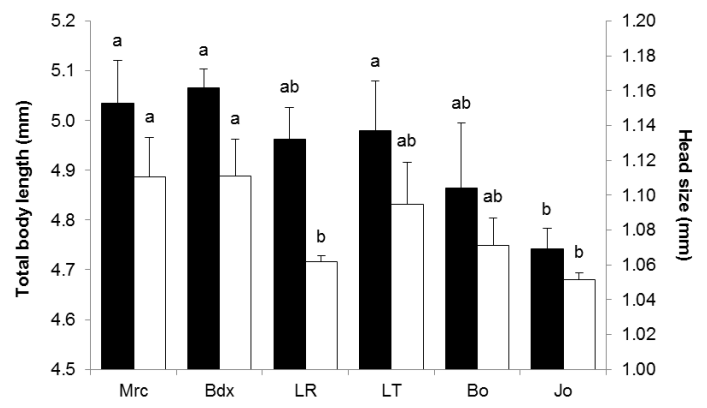
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6 (A)



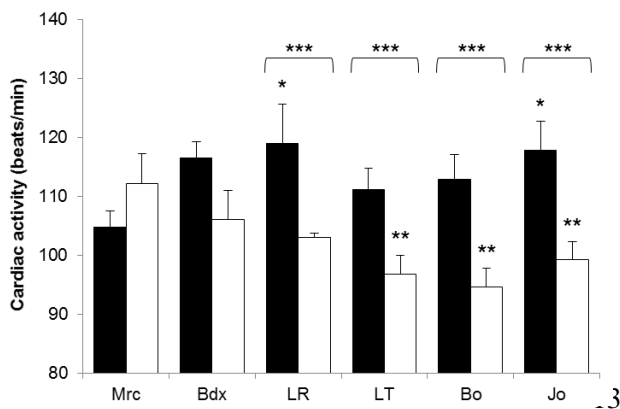
10 (B)



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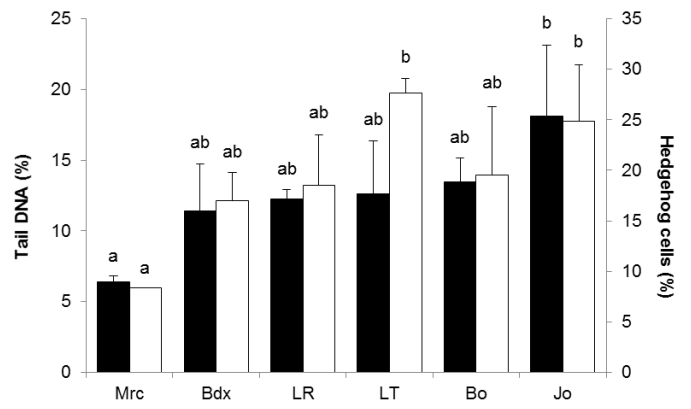
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8 (C)



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12 (D)

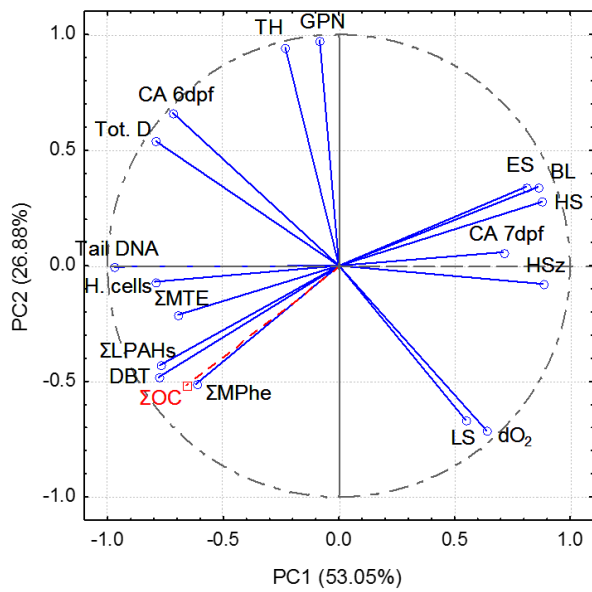


14 **Fig. 2** Phenotypical and genotoxic effects in medaka ELS exposed to six sediments collected along the  
 15 Lot-Garonne continuum: time to hatch (A), biometric measurements (B) at hatching (total body length  
 16 (black bars, left axis) and head size (black bars, left axis), cardiac activity (C) in 6 dpf- (black bars) and 7  
 17 dpf-embryos (white bars), and DNA damage (D) in 2 dph-larvae assessed with the comet assay showing  
 18 the percentage of tail DNA (black bars, left axis) and of the percentage of hedgehog cells (white bars,  
 19 right axis). Values represent the mean response ( $\pm$ SD) from three replicates. Different letters (A, B and D)  
 20 indicate significant differences between treatments using one-way ANOVA followed by Tukey's post hoc  
 21 test ( $p < 0.05$ ). For cardiac activity (C), statistical analysis was performed using two-way ANOVA analysis  
 22 followed by Tukey's post-hoc test. One asterisk indicates a significant difference ( $p < 0.05$ ) in comparison  
 23 to Mrc treatment at 6 dpf. Two asterisks indicate a significant difference ( $p < 0.05$ ) in comparison to Mrc  
 24 treatment at 7 dpf. Three asterisks indicate a significant difference ( $p < 0.05$ ) between 6 dpf- and 7 dpf-  
 25 values within the same treatment

26 *Color is neither needed in printed nor online version. This figure could be a 2-column fitting image.*

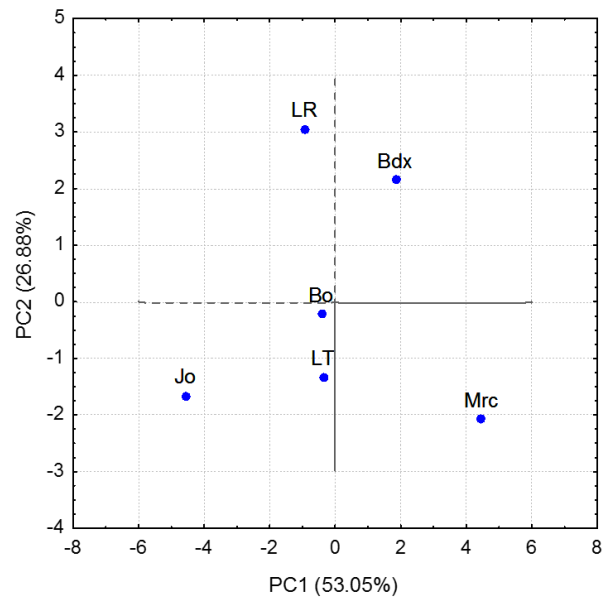
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28 (A)



29

30 (B)



31

32 **Fig. 3** PCA results for the two principal components produced by physico-chemical, contamination and  
 33 biological endpoints in sediments collected along the Lot-Garonne continuum. (A) Plot of variable  
 34 vectors: solid lines (ended by circles) represent active variables whereas illustrative (supplementary)  
 35 variable is shown as dotted line (ended by a square). (B) Plot of case factor coordinates for the  
 36 different sites. Physico-chemical variables included GPN variable (see part 3.1 for definition) and  
 37 dissolved oxygen ( $dO_2$ ). Contamination variables included  $\Sigma MTE$ ,  $\Sigma LPAHs$ ,  $\Sigma MPhe$ , DBT and  $\Sigma OC$   
 38 (see Table 1 for definition). Biological variables included embryonic (ES) and larval (LS) survival rates,  
 39 hatching success (HS), time to hatch (TH), cardiac activities (CA 6dpf and 7dpf), total body length  
 40 (TL), head size (HSz), the percentage of abnormal larvae (Tot. D), the percentage of Tail DNA and the  
 41 percentage of hedgehog cells (H. cells)

42

43 *Color is not needed in printed version, only in the online one. This figure could be a 1.5- or 2-column*  
 44 *fitting image.*