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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

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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-

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 (NH₄) 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 ^a	PEC ^a
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 ₄ (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 ^b	3.2	85.4	127	2,603	214	15,649		
ΣHPAHs ^c	9.4	1,114	788	21,585	814	9,352		
ΣMPhe ^d	1.7	39.3	46.2	984	75.5	849		
ΣPAHs ^e	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 ^f	1.0	8.9	14.4	0.8	3.3	3.7	59.8	676
ΣPBDEs ^g	<dl	0.1	<dl	<dl	0.1	0.4		
ΣOHCs ^h	1.1	9.0	14.4	0.8	3.4	4.1		
ΣOC ⁱ	15.2	1,252	979	25,286	1,114	26,163		
Mean qPEC1 ^j	0.10	0.16	0.18	0.18	1.27	1.38		
Mean qPEC2 ^j	0.06	0.11	0.13	0.68	0.74	1.88		
Mean qPEC-based ranking ^k	#1	#2	#3	#4	#5	#6		
MELAc-based ranking ^l	#1 (0; reference)	#3 (4)	#5 (7)	#3 (4)	#2 (3)	#6 (11)		

193 ^a values taken from MacDonald et al. (2000)

194 ^b sum of low molecular weight PAHs (LPAHs; three or fewer aromatic rings) includes naphthalene,
195 acenaphthene, acenaphthylene, fluorene, phenanthrene and anthracene

196 ^c 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 ^d sum of methylphenanthrenes (MPhe) includes 1-, 2-, 3-, and 9-methylphenanthrene and 1-
201 methylantracene

202 ^e sum of PAHs includes Σ LPAHs, Σ HPAHs and Σ MPhe

203 ^f sum of PCBs includes congeners 50, 28, 52, 101, 118, 153, 138 and 180

204 ^g sum of PBDEs includes congeners 47, 119, 99 and 153

205 ^h sum of organohalogenated compounds (OHCs) includes Σ PCBs, Σ PBDE and lindane

206 ⁱ sum of organic contaminants (OC) includes Σ PAHs, DBT and Σ OHCs

207 ^j values calculated according to MacDonald et al. (2000) recommendations

208 ^k 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 ^l 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 Σ 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 CaCl₂; 1.36 mM MgSO₄, 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 CaCO₃, 0.025 mg/L NO₂; 1.5 ppm PO₄; 5 mg/L NO₃;
243 <0.1 mg/L NH₄). 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, NH₄ 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, NH₄ 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, NH₄ 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 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 ^a	1.28 ± 2.22 ^a	5.13 ± 2.22 ^a	0.00 ± 0.00	0.00 ± 0.00	8.89 ± 5.54 ^a	0.00 ± 0.00 ^a
Bdx	61.0 ± 3.83^{bc}	0.00 ± 0.00 ^a	22.4 ± 7.20^{bc}	1.23 ± 2.14	0.00 ± 0.00	49.0 ± 10.4^{bc}	1.23 ± 2.14 ^{ab}
LR	70.0 ± 7.96^c	15.7 ± 6.40^b	23.8 ± 5.01^{bc}	1.28 ± 2.22	1.28 ± 2.22	59.5 ± 10.5^c	13.1 ± 5.80 ^{ab}
LT	43.6 ± 5.57^b	3.96 ± 0.23 ^a	13.3 ± 2.98 ^{ab}	6.89 ± 8.70	0.00 ± 0.00	32.9 ± 0.77^b	4.11 ± 4.17 ^{ab}
Bo	65.2 ± 8.58^c	2.57 ± 2.23 ^a	11.7 ± 4.03 ^{ab}	6.57 ± 8.38	1.23 ± 2.14	56.1 ± 10.3^c	13.0 ± 6.23 ^{ab}

Jo **72.3 ± 7.88^c** **15.0 ± 1.84^b** **24.6 ± 3.30^c** 7.97 ± 6.43 0.00 ± 0.00 **62.8 ± 5.70^c** **16.2 ± 10.7^b**

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

ΣLPAHs	-0.721	-0.110	-0.792	-0.192	0.361	-0.286	-0.813*	-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	0.835*
DBT	-0.752	-0.135	-0.819*	-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	0.814*

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; dO₂,
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	-0,949***	-0,175	-0,309	-0,913**	-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	0,797*
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	0,774*	-0,463	0,108	0,224
DBT	0,544	0,411	0,714	-0,421	0,341	0,497
ΣOC	0,352	0,239	0,771*	-0,487	0,149	0,269
Tot. D	0,569	0,838**	0,435	0,461	0,996***	0,772*

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 2nd and 3rd
580 respectively), both sediments led to noticeable developmental defects (3rd and 5th 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, NH₄ 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, NH₄ 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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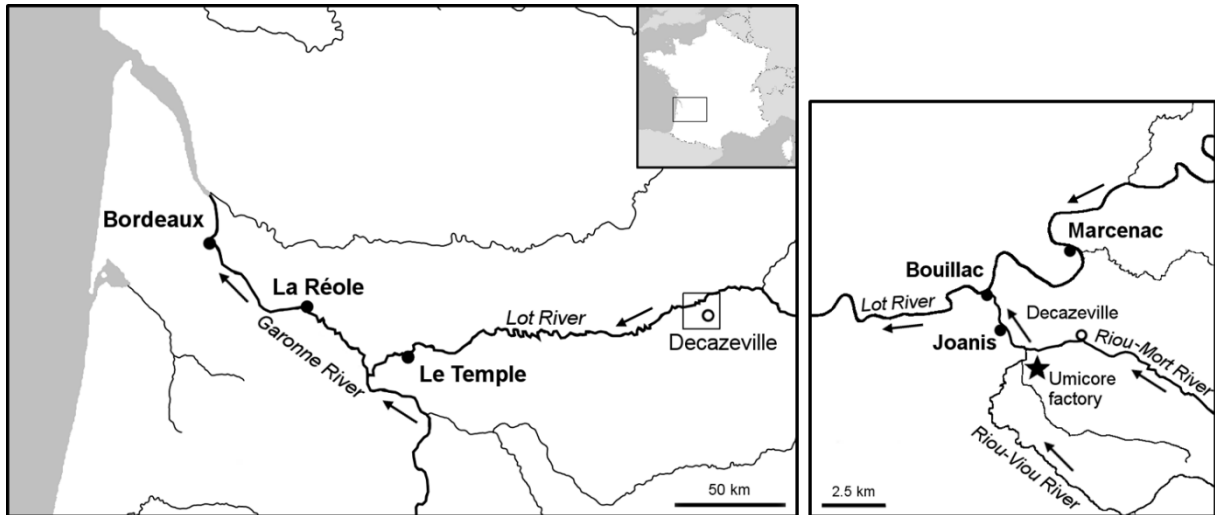
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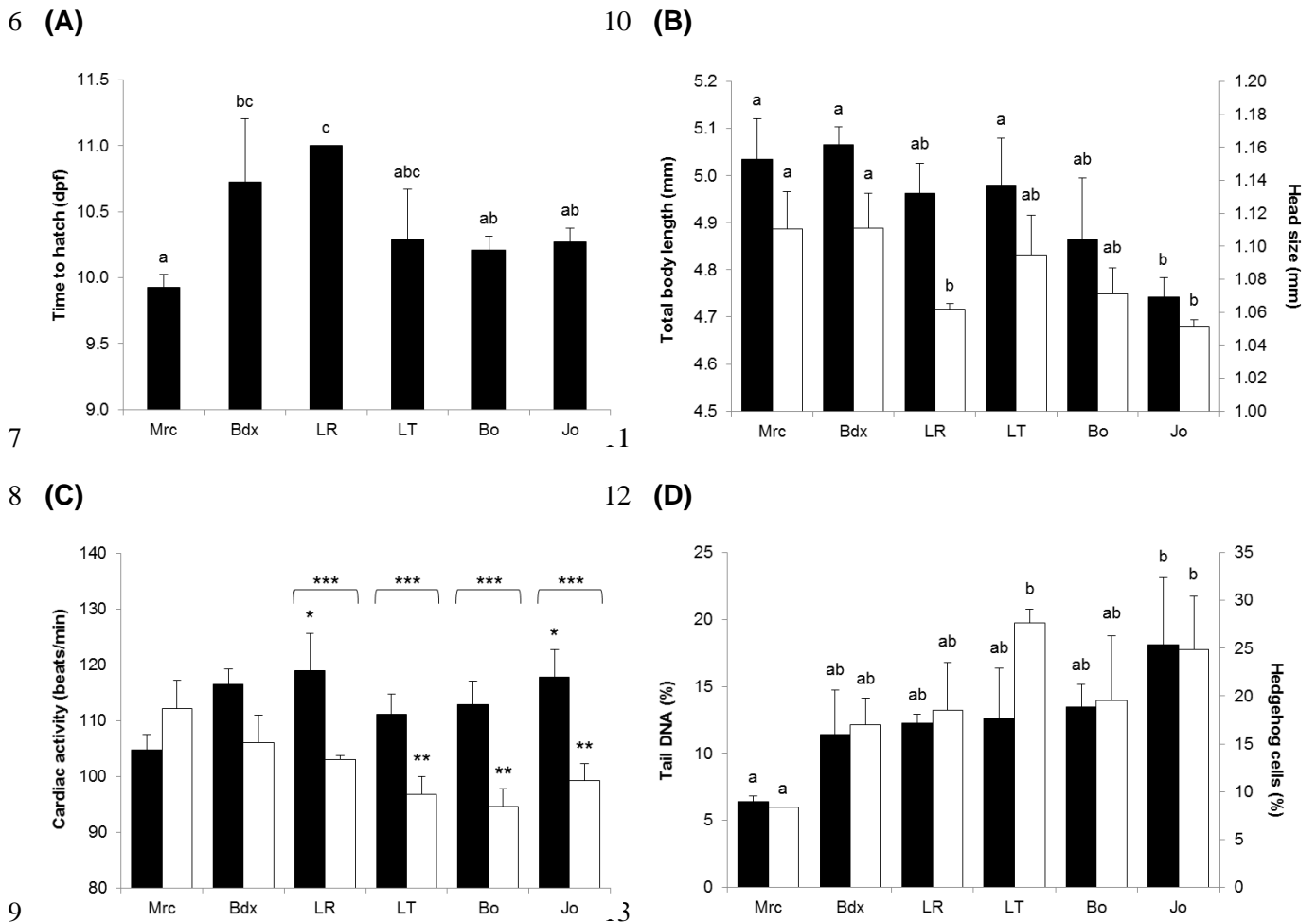
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2 **Fig. 1** Location of the study area and the six sampling stations (solid black dots) along the Lot-

3 Garonne system

4

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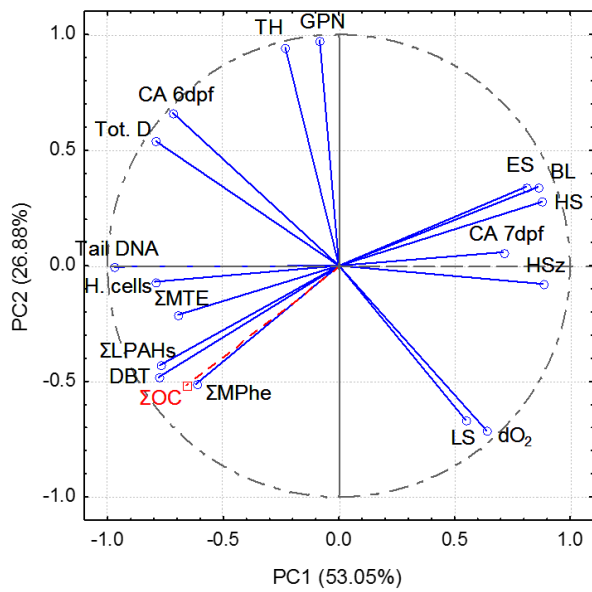


14 **Fig. 2** Phenotypical and genotoxic effects in medaka ELS exposed to six sediments collected along the
 15 Lot-Garonne continuum: time to hatch (A), biometric measurements (B) at hatching (total body length
 16 (black bars, left axis) and head size (black bars, left axis), cardiac activity (C) in 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.*

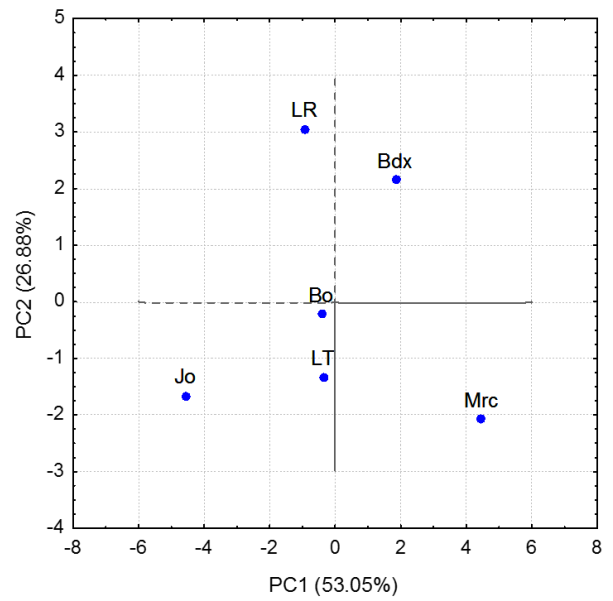
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28 (A)



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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 ΣMTE , $\Sigma LPAHs$, $\Sigma MPhe$, DBT and ΣOC
 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.*