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Developmental toxicity of PAH mixtures in fish early life stages. Part II: adverse effects in Japanese medaka

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Abstract:

In aquatic environments, polycyclic aromatic hydrocarbons (PAHs) mostly occur as complex mixtures, for which risk assessment remains problematic. To better understand the effects of PAH mixture toxicity on fish early life stages, this study compared the developmental toxicity of three PAH complex mixtures. These mixtures were extracted from a PAH-contaminated sediment (Seine estuary, France) and two oils (*Arabian Light* and *Erika*). For each fraction, artificial sediment was spiked at three different environmental concentrations roughly equivalent to 0.5, 4, and 10 µg total PAH g^{-1} dw. Japanese medaka embryos were incubated on these PAH-spiked sediments throughout their development, right up until hatching. Several endpoints were recorded at different developmental stages, including acute endpoints, morphological abnormalities, larvae locomotion, and genotoxicity (comet and micronucleus assays). The three PAH fractions delayed hatching, induced developmental abnormalities, disrupted larvae swimming activity, and damaged DNA at environmental concentrations. Differences in toxicity levels, likely related to differences in PAH proportions, were highlighted between fractions. The Arabian Light and Erika petrogenic fractions, containing a high proportion of alkylated PAHs and low molecular weight PAHs, were more toxic to Japanese medaka early life stages than the pyrolytic fraction. This was not supported by the toxic equivalency approach, which appeared unsuitable for assessing the toxicity of the three PAH fractions to fish early life stages. This study highlights the potential risks posed by environmental mixtures of alkylated and low molecular weight PAHs to early stages of fish development.

Keywords: Developmental abnormalities ; Comet assay ; Micronucleus assay ; Larvae locomotion ; Oil extract ; Pyrolytic extract ; Medaka embryo-larval assay

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Abbreviations:

PAH : Polycyclic Aromatic Hydrocarbon
BSD : Blue Sac Diseas
dpf : days post fertilization
MELAc : Medaka Embryo-Larval Assay with sediment contact exposure
dw : dry weight
ERS : Egg Rearing Solution
ELS : Early Life Stages
MEM : Minimum Essential Medium
MN : Micronucleus; PY: pyrolytic
HO :Heavy Oil
LO : Light Oil
TEF : Toxic Equivalent Factor
TEQ : Toxic Equivalency

1. Introduction

While environmental contamination by polycyclic aromatic hydrocarbons (PAHs) is steadily increasing, assessing the actual risk posed by this contamination is problematic. In fact, PAHs occur mostly in complex mixtures in aquatic environments. Different methods have been proposed to assess the combined effects of these compounds. Assuming dose additivity, the concept of toxic equivalency was initially developed to evaluate the risk of dioxin-like compounds (Van den Berg et al. 2006; Safe 1993). This method consists of grading the toxic potency of different compounds based on a single reference toxicant. Toxic equivalent factor (TEF) values based on dioxin assume that effects are mediated by binding to the aryl hydrocarbon receptor (AhR), a cytosolic transcription factor (Barron et al. 2004b). TEF values, using benzo[*a*]pyrene as a reference, focus on the carcinogenic properties of the studied compounds (Nisbet and LaGoy 1992). However, some recent studies have reported toxicity of some weak AhR agonist PAHs that was independent of AhR activation (Incardona et al. 2006). This calls into question complex PAH mixture assessment based on toxic equivalencies. Furthermore the weak solubility of PAHs is also problematic to assess their toxicity to aquatic organisms. Indeed, waterborne exposure to these hydrophobic compounds is limited, and does not reflect their actual bioavailability in natural conditions (Hollert et al. 2003; Kiparissis et al. 2003).

The European regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) calls for the replacement, reduction, and refinement of the use of animals for scientific purposes (EC 2006). Fish embryo larval assays fulfill ethical requirements, providing an alternative to non-mammalian models (Embry et al. 2010; Strähle et al. 2012). Moreover, the transparency of the chorion enables direct and non-invasive observations during embryogenesis. Toxic effects of PAHs on fish early life stages (ELS) generally include both genotoxic and developmental effects. A common syndrome reported in dioxin and PAH-exposed fish embryos is Blue Sac Disease (BSD), which is characterized by pericardial and peritoneal edema, spinal curvature, craniofacial abnormalities, or altered heart development (Rhodes et al. 2005; Kim and Cooper 1999). It has previously been shown that pyrolytic PAH mixtures from contaminated sediment in the Seine River induced BSD — as well as mutations and tumors — in the transgenic Japanese medaka Lambda *cII* (Cachot et al. 2007). Petrogenic PAH mixtures also led to BSD, along with genetic damage and mortality among different species of fish embryos. (Couillard 2002; Carls et al. 1999; Colavecchia et al. 2004).

The MELAc (Medaka Embryo Larval Assay with sediment contact exposure) was selected in this study to improve and complement the risk assessment of hydrophobic compounds. (Vicquelin et al. 2011). The objective of our work was firstly to evaluate and compare the toxicity of three PAH fractions with different compositions: (i) a pyrolytic fraction (PY) from a contaminated sediment, (ii) a petrogenic PAH fraction from a heavy oil *Erika* (HO), (iii) and a second petrogenic PAH fraction from a type of light crude oil, *Arabian Light* (LO). Several endpoints were recorded, including survival, hatching delay, hatching success, abnormalities, larvae swimming activity, and DNA damages (comet and micronucleus assay). The micronucleus assay was carried out for the first time on cells extracted from the entire larvae in order to evaluate DNA damages in the whole body and not in a specific tissue.

Strong relationship has been shown between the results of acute toxicity tests in adults and in embryos for several fish model species (reviewed by Belanger et al., 2013). It is thereby questionable if the results obtained in this study can be extrapolated to other species. This is an important question since different model species are used in fish ELS assays (see for instance OECD's guidelines 212). Therefore an attempt was made in this study to compare ELS toxicity patterns and sensitivity between Japanese medaka (the present study) and another model fish, rainbow trout (Le Bihanic et al. this issue, *in revision*).

2. MATERIALS & METHODS

2.1 PAH fraction samples

The pyrolytic PAH fraction (PY) was extracted from contaminated sediment in the Seine River, sampled in March 2010 in Oissel (Normandy, France). CEDRE (Center of Documentation, Research and Experimentation on Accidental Water Pollution) provided both the *Arabian Light* crude Oil (LO) and the *Erika* Heavy Oil (HO). The PAH fraction from the sediment sample was extracted using microwaves with dichloromethane (Acros Organics, Thermo Fisher Scientific, Geel, Belgium). The two oils were dissolved in pentane to induce asphaltene precipitation. Both the sediment extract and two pentane extracts were then concentrated using a Vacuum Evaporation System (Rapidvap, Labconco, Kansas city, USA). All three PAH samples were then purified using alumina columns (preparative liquid chromatography) and eluted with dichloromethane (Acros Organics, Thermo Fisher Scientific, Geel, Belgium). Following a further concentration step, aliphatic fractions obtained after elution on silica columns with pentane were discarded, and aromatic fractions obtained using pentane/dichloromethane (65/35, v/v) as solvents. Atlantic Labo (Bruges, France) provided the pentane solvent. Finally, samples were concentrated, with the final fractions kept in dichloromethane (stored at -20 °C) for spiking experiments. Each step in the process was gravimetrically controlled to prevent PAH losses and allow results to be expressed in terms of quantity of the original substance (i.e. sediment or oils).

2.2 Artificial sediment spiking

The artificial sediment was composed of 92.5 % silica sand, 2.5 % dw *Sphagnum* blond peat and 5 % kaolin clay (Le Bihanic et al. this issue, *in press*). For each PAH fraction, the sediment was spiked to achieve 0.3X, 1X and 3X concentrations, with dichloromethane as a solvent (Biosolve, Valkenswaard, the Netherlands). 1X concentration refers to the sum of PAHs in sediments from the upper Seine estuary of 10 µg g^{-1} (Cachot et al. 2006). In fact, due to the relatively moderate sorption efficiency of PAHs on particles, the actual PAH concentrations in sediments were much lower. Measured PAHs included 21 non substituted PAHs (naphthalene, acenapthylene, acenaphthene, fluorene, dibenzo[*bd*]thiophene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, triphenylene, chrysene, benzo[*b*]naphto[2,1-*d*]thiophene, benzo[*b*+*k*+*j*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, indeno[1,2,3-*cd*]pyrene, dibenzo[*ah*]anthracene, dibenzo[*ac*]anthracene, benzo[*ghi*]perylene), methyl naphthalenic and methyl phenanthrenic compounds. PAHs spiked onto sediment were extracted using microwaves. PAHs were analyzed by gas chromatography coupled with mass spectrometry. Spiking and PAH analysis methods are detailed in (Le Bihanic et al. this issue, *in press*).

111

2.3 Embryo exposure

Gis-Amagen (INRA, Jouy-en-Josas, France) provided eggs at early gastrula. One day post fertilization (dpf) embryos were placed onto the spiked sediment surface throughout embryonic development and until hatching. The exposure unit, replicated 3 times for each treatment, contained 25 embryos, 3 g dw of sediment and 3 mL of Egg Rearing Solution (ERS). Experiments took place in climate chamber (Snidjers Scientific, Tilburg, Netherlands) at 26±0.3 °C with a photoperiod light: dark of 12h:12h, 5000 lx white light. Dissolved oxygen concentration in ERS medium was measured daily using a Fibox 3 fiber-optic oxygen mini-sensor (PreSens Precision Sensor, Regensburg, Germany). Exposure was stopped at the peak of hatching, when more than half of individuals hatched in the three replicates of a single condition (11 dpf for experiment 1 and 12 dpf for experiment 2). After hatching, yolk-sac larvae were observed and transferred into glass beakers containing 20 mL of a water mixture (dechlorinated water mixed with distilled water 1:2 v/v aerated for 24h). After exposure, non-hatched embryos were transferred into new plastic Petri dishes containing 3 mL ERS without sediment. Yolk-sac larvae were not fed during the experiment. Three days after the peak hatching period, the experiment was stopped by euthanizing all remaining larvae and embryos with a lethal dose of 120 mg L^{-1} ethyl 4-aminobenzoate (Sigma-Aldrich, St Quentin Fallavier, France). For convenience, toxicity tests on PAH fractions were divided into two experiments. The first experiment assessed PY PAH fraction toxicity, while the second focused on LO and HO PAH fraction toxicity. The two experiments were conducted under identical conditions.

2.4 Phenotypic effect measurements

Dead embryos and larvae were recorded daily and immediately removed to avoid alteration of the medium. Embryos that died within the first 24 h of the test (0 to 4 %) were not taken into consideration for our calculations, as their death was likely to have been due to transportation stress. Heartbeat measurements were taken at room temperature (23±1 °C) from 7 dpf embryos. Heartbeats were measured over three 20-second periods for the same embryo, using a Leica (Nanterre, France) MZ7.5 25x microscope and cold light source (Intralux® 4100, Volpi AG, Schlieren, Switzerland). Values were summed to obtain cardiac activity measurements in beat min^{-1} for each embryo. Five individuals were analyzed per replicate. The time for 50 % hatching was reported as hatching $T_{1/2}$. Head length, total body length, and yolk sac area were measured between 0 and 24 h after hatching, on 15 randomly selected larvae per condition. Yolk sac area was measured as the

140 abdominal cavity excluding the swim bladder area and the pericardial area. Swim bladder inflation was also
141 reported. These measurements were taken using a Leica MZ7.5 stereomicroscope with Leica Microsystems
142 software v3.8 (Nanterre, France).

143 At hatching, fifteen randomly selected larvae per replicate were individually examined to record morphological
144 abnormalities and lesions. Observed larvae were photographed at 25X magnification using a Leica MZ7.5
145 stereomicroscope coupled with a Leica DFP420C CCD camera (Nanterre, France). Five types of abnormalities
146 and lesions were scored: edema (peritoneal and pericardial); body (scoliosis, lordosis, kyphosis, and fin erosion);
147 craniofacial (jaw and skull abnormalities); ocular (missing eye, cyclopia and dystrophy); cardio-vascular
148 (anemia, hemorrhage, ventricle size, blood circulation, heart position). Abnormalities were scored on a scale of 1
149 to 5 (i.e. 1 point per type of abnormality). One larva could exhibit several abnormality types and/or several
150 abnormalities of the same type (counted once). The percentage of abnormal larvae represented the number of
151 larvae presenting at least one abnormality compare to the total number of examined individuals.

152

153 *2.5 Genotoxicity assays*

154 Comet and micronucleus assays (MN) were performed on the same cell suspension obtained from 10 larvae per
155 replicate at 3 days post-hatching (dph). Cell isolated from larvae exposed to solvent sediment were used as
156 negative controls for both assays. Cells were isolated based on a slightly modified version of the method
157 developed by Morin et al. (2011) to enable sufficient number of isolated cells for both assays. Larvae were
158 entirely minced with razor blades, and then digested at 37 °C for 45 min, under slight agitation (150 rpm) with
159 1.25 mg mL^{-1} dispase *II* from *Bacillus polymyxa* (Roche, Meylan, France) in Minimum Essential Medium Eagle
160 (MEM) (Sigma-Aldrich, St Quentin Fallavier, France). Following centrifugation at 1000 rpm for 10 min at room
161 temperature, pellets were rinsed in 1 mL of MEM. Cell viability was assessed under a microscope, using the
162 Trypan blue exclusion protocol. Cell suspension viability was above 95 % in all samples, while cell density
163 varied between 1300 – 4900 cell µL^{-1}.

164 For the comet assay, the protocol of Morin et al. (2011) was followed. Slides were covered with normal melting
165 point agarose 1 % w/w. Two 50 µL gels were laid on slides. The gels were composed of cell suspension of about
166 500 cell µL^{-1} and 1 % w/w low melting point agarose. After gel hardening and 1 h lysis at 4 °C, slides were
167 placed on an electrophoresis tray and covered with a freshly prepared electrophoresis buffer for 10 min, to allow

168 the DNA to unwind. Electrophoresis was carried out at 25 V, 300 mA for 10 min. After rinsing, slides were
169 dehydrated in absolute ice-cold ethanol for a further 20 min. Before reading, slides were stained with ethidium
170 bromide (20 µg mL^{-1}). Comet analysis was performed at X400 magnification using an Olympus epi-fluorescent
171 microscope (Olympus, Rungis, France) and a grayscale CCD camera (Zeiss, Germany). DNA damage was
172 measured at 100 nuclei per sample using Comet Assay IV (Perceptive instruments, Bury St Edmunds, UK) using
173 blind review analysis. Data was expressed as percentage tail DNA.

174 For the MN assay, cell suspensions were fixed in a mixed solution of acetic acid: methanol 1:4 v/v, and smeared
175 onto slides for a final concentration of 5000 cell μL^{-1}. Slides were dried overnight at room temperature. Just
176 before reading, slides were stained with an acridine orange solution (0.003 % in PBS) and cover with a glass
177 slip. Readings were obtained using an epifluorescent microscope at X400 magnification (Olympus, Rungis,
178 France). The frequency of micronucleated cells was manually recorded for 1000 cells per slide, using blind
179 review analysis by a single observer. A cell was considered micronucleated if the MN was round-shaped, distinct
180 from the main nucleus, its size was less than a third of the main nucleus, and with a similar green staining to the
181 main nucleus (Hayashi et al. 1998). Cells that were non-isolated, stacked, more than bi-nucleated, or orange-red
182 stained were excluded from the test. The MN assay performed on the whole body of larvae and used in the
183 present study is under the rights of the French patent No.1058505 established 19/10/2010.

184

185 *2.6 Locomotion assay*

186 Our locomotion assay consisted of visual motor response measurements at 4 dph on 12 larvae per treatment
187 replicate. The procedure was adapted from Emran et al. (2008) and Vignet at al. (2013). Larvae were randomly
188 selected and placed into 48-well microplates. Each microplate well contained 1 larva in 500 µL of mixed water.
189 Larvae were first acclimated for 1h in the dark at room temperature (23±1 °C). Coordinates were then recorded
190 using an IR digital video camera (Ikegami Electronics, Neuss, Germany) for a 50 min analysis cycle in a
191 Daniovision chamber (Noldus, Wageningen, Netherlands). This 50 min cycle included three periods of 20 min
192 dark, 10 min light, and 20 min dark. Measurements were performed at 23±1 °C using Ethovision 9.0 (Noldus,
193 Wageningen, the Netherlands). Dynamic subtraction methods were applied with a sampling rate of 25 per sec,
194 dark contrast 16-255, current frame weight 4, subject size 2-12500, and no subject contour dilation. To limit
195 background noise, an input filter of minimal distance moved was set at 10 % of the total body larva equivalent to
196 0.4 mm. Mean velocity, distance swum, and mobility were calculated for each larva and for each light and dark

period. Mobility refers to the time period for the totality of the larva area being modified even if the central point did not change. When an area of more than 60 % changed for a given larva, that specimen was considered highly mobile. All microplates were analyzed at identical temperature, detection and acquisition settings. Larvae unable to swim because of morphological abnormalities were not taken into consideration for the locomotion assay.

2.7 Statistics

Each condition treatment was identically replicated three times. Each replicate was considered as an independent sample. Since only individuals from two replicates hatched for the 1X LO treatment, N = 2 for endpoints measured on 1X LO-exposed larvae. Normality of the data distribution was tested on data residues using the Shapiro-Wilk test ($p < 0.01$). Variance homogeneity was evaluated using the Levene test ($p < 0.05$). In case of homogenous variance and normalized data, one-way Anova analysis was performed, followed by the Tukey post-hoc test ($p < 0.05$). In converse cases, data were analyzed using the Kruskal-Wallis non-parametric test ($p < 0.05$). Since hatching rate data distribution of petrogenic-exposed individuals was platykurtic, data underwent an arcsine transformation prior to statistical analysis (Legendre and Legendre 1998). Statistical analyses were performed with Statistica software v7.1 (StatSoft, Maisons-Alfort, France).

2.8 Toxic equivalency

TEQ were calculated regarding two different reference compounds: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) as reviewed by (Barron et al. 2004b) and benzo[*a*]pyrene as described by (Nisbet and LaGoy 1992) and reviewed by (INERIS 2003). In the case of mixtures, TEF values are summed and weighted by the proportion of the compound in a given mixture. Only the 16 USEPA PAHs were taken into consideration for this calculation. Concentrations of benzo[*j*]fluoranthene, dibenzo[*ah*]anthracene and triphenylene were also included in calculation because they co-eluted with US EPA PAHs.

3. RESULTS

3.1 PAH concentrations in spiked sediments

223 Spiking efficiencies ranged between 17 and 43 % (Table 1). Some PAH molecules were lost during the spiking
224 process, particularly naphthalene and methylnaphthalenic compounds. PAH concentrations in spiked sediments
225 were comparable between fractions, and ranged from 0.5 to 0.8 µg g^{-1} for 0.3X, from 3.6 to 4.0 µg g^{-1} for 1X, and
226 from 7.3 to 11.9 µg g^{-1} for 3X. In contrast, PAH were detected at a very low concentration in control sediment.
227 Alkylated PAH represented about 60 % of measured PAH in LO sediment, 40 % in HO sediment and less than
228 10 % in PY sediment. Dissolved oxygen concentration was in average over 85 % in all exposure units.
229

230 *3.2 Acute toxicity*

231 No significant embryonic or larval mortality was observed during exposure to any of the treatments tested (Table
232 2). For solvents, 0.3X LO, 0.3X HO and all PY conditions, hatching rate was above 88 %. However, only 28 %
233 of embryos exposed to 1X of the LO fraction hatched and less than 2 % of 3X LO, 1X HO and 3X HO hatched
234 during the experiment. Those last three treatments were therefore not considered for the following observations.
235

236 *3.3 Developmental effects*

237 No treatment effects were observed on embryonic heartbeat or swim bladder inflation in larvae at hatching
238 (Table 2). The two oil fractions did not affect the kinetic of embryo development, although 3X PY treatment
239 significantly delayed larvae $T_{1/2}$ hatching up to 1.1 day. There was no significant difference in yolk sac area
240 between the exposed larvae and the solvent control group. In contrast, exposure to PAH fractions significantly
241 reduced the head length and total length of exposed individuals. Total length was reduced by 0.2 mm for 3X PY,
242 0.3 mm for 0.3X LO and 0.3X HO, and by 0.7 mm for 1X LO. The control larvae abnormality rate ranged
243 between 9 and 18 %. All three PAH fractions induced teratogenicity in medaka ELS. On average, half of 0.3X
244 LO-exposed larvae and all larvae exposed to 1X LO were abnormal. Although not significantly different from
245 the control group, about a third of individuals exposed to 0.3X HO exhibited developmental anomalies. The
246 frequency of deformed larvae increased from 11 to 62 %, while PY fraction increased from 0.3X to 3X. The
247 spectrum of abnormalities highlighted craniofacial abnormalities as appearing the most frequently. 3X PY larvae
248 presented significantly higher rates of edemas, craniofacial, and cardiovascular abnormalities than control larvae.
249 Larvae exposed to 1X LO displayed significant increases in edemas, spinal, craniofacial, and cardio-vascular
250 abnormalities when compared to the control group. Although PAH concentrations in sediment for a given dose
251 were similar for the three fractions, occurrences of abnormalities were different. Figure 1 presents photographs

252 of the different developmental defects observed in medaka larva exposed to PAH fractions. Larvae exposed to
253 0.3X petrogenic fractions presented several developmental defects, including changes in jaw and spinal
254 curvature. This was comparable to 1X PY treatment effects. Edemas and cardio-vascular anomalies were induced
255 by 1X LO. This was comparable to 3X PY treatment effects.

256

257 *3.4 Genotoxicity*

258 The comet assay did not reveal any significant DNA damage to cells from larvae treated with PAH fractions (Fig
259 2A). Cells from the control larvae presented 2 and 8 % of tail DNA. In contrast, the MN assay highlighted a
260 significant increase in DNA damage to larvae exposed to PY fractions at all concentrations, and in 0.3X LO-
261 treated larvae (Fig 2B). Exposure to 0.3X HO tended to increase the MN frequency for treated larvae, although
262 this was not significant. MN cell frequency in controls ranged between 2.3 and 3.7 ‰.

263

264 *3.5 Locomotion effects*

265 Figure 3 shows the effects of PAH fractions on larvae velocity under dark and light challenges. All larvae
266 followed a similar pattern. After a slight decrease from 0 to 5 min, larvae velocity stabilized and was constant
267 during the first dark period (0 to 20 min). When the light was turned on for a 10 min period (20 to 30 min),
268 larvae activity was slightly increased. In contrast, when the light was extinguished after 30 min, all larvae
269 reacted with a 3-5 fold increase in velocity. Larva velocity then progressively decreased back down to the level
270 of the first dark period. Larvae exposed to PAH fractions swam faster and over longer distances than control
271 larvae. Indeed, 3X PY larvae swam 1.9 times more than control larvae during the first dark period (Fig. 4A).
272 Larvae exposed to 0.3X LO swam 1.9 times more during the first dark period and 1.3 times more during the light
273 period (Fig. 4B). And 0.3X HO larvae swam 1.6 times more during the first dark period. Based on results
274 relating to velocity and distance swum, PAH-exposed larvae were more mobile than control larvae. This was
275 significant for 3X PY larvae, which were 2.5 times and 1.9 times more mobile than control larvae during the first
276 dark and light periods respectively (Fig. 4C). 0.3X LO larvae were 2.6 and 1.6 times more mobile than control
277 larvae during the two dark periods (Fig. 4D).

278

3.6 Toxic equivalency

Table 3 presents the TEQs of the three PAH mixtures regarding both benzo[*a*]pyrene and dioxin. In contrast to the observed toxicity on fish ELS described above, these two methods of calculation display the PY fraction as being the most toxic to fish. The PY TEQ value was twice (BaP) and four times (dioxin) as high as the HO fraction. The LO fraction would therefore appear to be the least potent toxic fraction, with a TEQ value nine times (BaP) and five times (regarding dioxin) lower than the HO fraction.

4. DISCUSSION

In our study, the toxicity of the three different PAH mixtures was evaluated in ELS of Japanese medaka. The experiments satisfied the recommendations set out in the OECD's guidelines for embryo-larval assays in terms of dissolved oxygen concentration (OECD 1992). Survival, heartbeat, biometry, developmental abnormality rate, DNA damages and larval locomotion of control individuals were within a similar range to that observed in previous studies (Morin et al. 2011; Vicquelin et al. 2011). Variability between experiment 1 and 2 of abnormal larvae and tail DNA percentages in controls could be explained by natural biological variations between the different batches of eggs. This natural variability was already reported and discussed (Lebihanic et al. this issue, *in press*). Exposure to the two petrogenic fractions induced lethal effects by preventing individuals from hatching before the end of the experiment at 1X and 3X concentrations. Dimethyl-PAHs and weathered crude oil were also shown to prevent hatching of Japanese medaka embryos and pink salmon embryos (Rhodes et al. 2005; Carls and Thedinga 2010). Numerous developmental effects, such as reduced growth, edemas, anemia, hemorrhages, cessation of blood flow and body axis deformities were recorded. The spectrum of effects observed was very similar to the BSD syndrome previously reported in medaka embryos exposed to various PAHs (Rhodes et al. 2005; Vicquelin et al. 2001). Kiparissis et al. (2003) reported edemas, craniofacial deformities, and impaired circulation following embryonic exposure to retene. Mummichog embryos exposed to crude oil contaminated sediment also exhibited reduced body length, pericardial edema, hemorrhages, and spinal deformities (Couillard 2002). BSD syndrome was demonstrated to be triggered by different non-specific mechanisms, such as narcosis, or via interactions with specific receptors such as the AhR or cardiotoxicity (Billiard et al. 2008). This syndrome evidenced only part of the mode of action of PAH mixtures. The MN assay has been in use for several decades to assess genotoxicity in various fish contaminated with PAHs (Baršienė et al. 2006; Rocha et al. 2009). In this study, the MN assay was carried out for the first time on cells from entire

308 larvae. This assay allowed global assessment of genotoxic damages in the whole body and not only in a specific
309 tissue, as currently performed. It is thus a more valuable endpoint to assess the global health status of a given
310 organism exposed to genotoxicants. MN was also analyzed on entire larvae. Data obtained highlighted
311 irreversible DNA damage caused by two out of three PAH fractions, PY and LO. Similarly, *Oreochromis*
312 *niloticus* exposed to petroleum refinery effluent exhibited micronuclei as well as bi-nucleate gill epithelial cells
313 and erythrocytes, lobed nuclei, and notched nuclei that complemented MN scoring (Çavaş and Ergene-Gözükara
314 2005). PAH fractions also activated larvae locomotion both after stabilization of the larvae in dark and under
315 light challenges. Irons et al. (2013) illustrated that fish larvae locomotion in response to chemicals depends on
316 the compound and concentration. A possible explanation for changes to locomotion functions is that PAHs affect
317 the neurotransmitter pathways (Gesto et al. 2009). Endocrine disruptor activities of PAHs have also already been
318 reported (Barron 2002). Interferences of PAHs or their metabolites with hormone system could partly explain
319 hyperactivity in contaminated larvae. Locomotion assays carried out on fish larvae represent a newly-expanding
320 method which serves to complement toxicity analysis by providing an integrative response to broad
321 physiological alterations, such as disruption of the central nervous system, vision, skeleton and muscles (de Esch
322 et al. 2012).

323 One aim of this work was also to compare the sensitivity of two model species for the same three PAH fractions
324 (PY, LO, and HO) at 0.3X concentration (Table 4). The toxicity of these three fractions was initially tested on
325 Rainbow trout ELS (Le Bihanic et al. this issue, *in revision*), then tested on Japanese medaka (this study). PAH-
326 fractions and operators were identical, but comparison was limited by differences in exposure matrix, PAH-
327 spiked concentrations, time of exposure, rearing temperature, photoperiod, water renewal and salinity. This is
328 relevant because it has been proved that certain light radiations enhance PAH toxicity, temperature increases
329 dissolution of the compound, and salinity seems to raise PAH sorption (Barron et al. 2003; Turner and Rawling
330 2001; Faksness et al. 2008). Despite these differences, the PY fraction appeared to be the less toxic fraction for
331 the two species. The two petrogenic fractions LO and HO fractions were shown to induce the majority of the
332 effects on fish embryos and larvae. The PY fraction was mainly composed of 4-5 rings non-alkylated PAHs in
333 opposition with the two petrogenic fractions, mainly composed of alkylated PAHs known to drive the
334 developmental toxicity of PAH mixtures (Sundberg et al. 2005; Fallahtafti et al. 2012; Incardona et al. 2005).
335 Several studies documented alkylated PAHs as more toxic than their non-alkylated homologs (Barron et al.
336 2004a; Turcotte et al. 2011). Exposure to PAH fractions impacted biometry, morphology and DNA integrity of
337 both species. On one hand, medaka larvae exhibited DNA damages detected with the MN assay, which are

338 chromosomal aberrations caused by clastogenic and aneugenic events. On the other hand, trout larvae presented
339 only labile damages detected with the comet assay, such as single/double DNA strand breaks, alkali labile sites
340 and excision/repair DNA damages. Medaka individuals develop faster than trout, meaning that their cells present
341 a higher mitotic index. We can therefore hypothesize that DNA damages set quicker with medaka than it did with
342 trout.

343 Several previous studies have compared toxicity responses exhibited by Japanese medaka and rainbow trout. On
344 one hand relationship was identified between both species (Belanger et al., 2013), and on the other hand
345 sensitivity was shown to vary between compounds. For endocrine disruptors, trout was more sensitive than
346 medaka (Dobbins et al. 2008), while medaka appeared to be the more sensitive of the two when exposed to pulp
347 and paper mill effluents (Orrego et al. 2011). When considering the three PAH fractions tested, rainbow trout
348 ELS were significantly more sensitive to HO fraction than to LO, but this was not statistically evident for
349 Japanese medaka. The main difference between these two petrogenic fractions is their proportion in alkylated
350 naphthalene higher for LO, and alkylated phenanthrene higher for HO. Our study showed similar toxicity
351 gradients for PAH mixtures between medaka and trout ELS but slight differences in toxic potency. Compared to
352 studies performed on single model species, multi-species studies offer a more comprehensive and accurate risk
353 assessment of chemicals. Further supplementary experiments using similar exposure conditions for both species
354 would strengthen these results.

355 TEQ calculations were not consistent with the toxicity observed in fish ELS (Table 3). The PY fraction is
356 considered the most potent toxic fraction for both methods of TEQ calculation, while it was the least toxic from
357 the point of view of acute and sub-lethal toxicity on Japanese medaka and rainbow trout ELS. TEQ values
358 depend on the mode of action considered: carcinogenicity or AhR agonist (Barron et al. 2004b; INERIS 2003).
359 However, these methods do not consider other modes of action such as nonspecific toxicity or toxicity
360 independent of the binding to AhR. Moreover, PAHs are different in terms of structure and bioaccumulation to
361 dioxin (Billiard et al. 2008). In order to take into account PAH developmental toxicity on fish ELS, other TEF
362 values are needed. Considering complex mixtures of PAHs, the toxic potency is a function of the components
363 and their interactions. The TEQ approach to PAH mixture assessment is oversimplified, because it is based on
364 the hypothesis of potency additivity, and does not consider possible synergistic or antagonistic interactions
365 between molecules (Wassenberg &Di Giulio 2004, Wassenberg et al. 2005). The findings described in our study
366 do not support the TEQ approach. We may therefore assume that TEQ methods with the existing TEF values are
367 not suitable for the toxicity assessment of complex PAH mixtures to fish ELS.

CONCLUSION

Developmental toxicity, locomotion defects, and genotoxicity were all detected at PAH concentrations similar to those occurring in sediments from various European rivers and estuaries. Our results raise the question of the impact of PAH-contaminated sediments on survival and recruitment in fish ELS. The toxic equivalency approach was not suitable for predicting the developmental toxicity of PAHs mixtures. The range of induced toxicity differed between fractions tested. The LO and the HO petrogenic fractions, with high proportions in alkylated and low to medium molecular weight PAHs, were more toxic to Japanese medaka ELS than the PY fraction. This was consistent with a previous study carried out on rainbow trout ELS.

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FIGURE CAPTIONS

Figure 1. The PAH fractions induced teratogenicity in medaka larvae following embryo exposure to PAH-spiked sediment. A) No noticeable developmental abnormality in control larva; B) Jaw deformity and spinal curvature in 1X PY-exposed larva; C) Jaw and cranial deformities, pericardial and peritoneal edemas in 3X PY-exposed larva. White arrows indicate pericardial or peritoneal edemas. Black arrows indicate skeleton deformities either cranio-facial or spinal.

Figure 2. The PAH fractions induced genotoxicity following medaka embryo exposure to PAH-spiked sediments. Comet and micronucleus assays were performed on the same sample of cells obtained from the whole body of ten larvae. The different letters stand for significant differences between treatments (Mean ± SD, N = 3, Anova, $p < 0.05$).

Figure 3. Medaka larvae velocity during 50 min video tracked analysis: 20 min dark + 10 min light + 20 min dark for 0.3X treated individuals. Embryos were exposed to PAH-spiked sediments. Data was averaged over each 1min interval. (Mean ± SD, N = 3).

Figure 4. The PAH fractions disrupted medaka larvae locomotion. Data was averaged out across each dark or light interval. Embryos were exposed to PAH-spiked sediment. The different letters stand for significant differences between treatments (Mean ± SD, N = 3, Anova, $p < 0.05$).

Figures

Figure 1

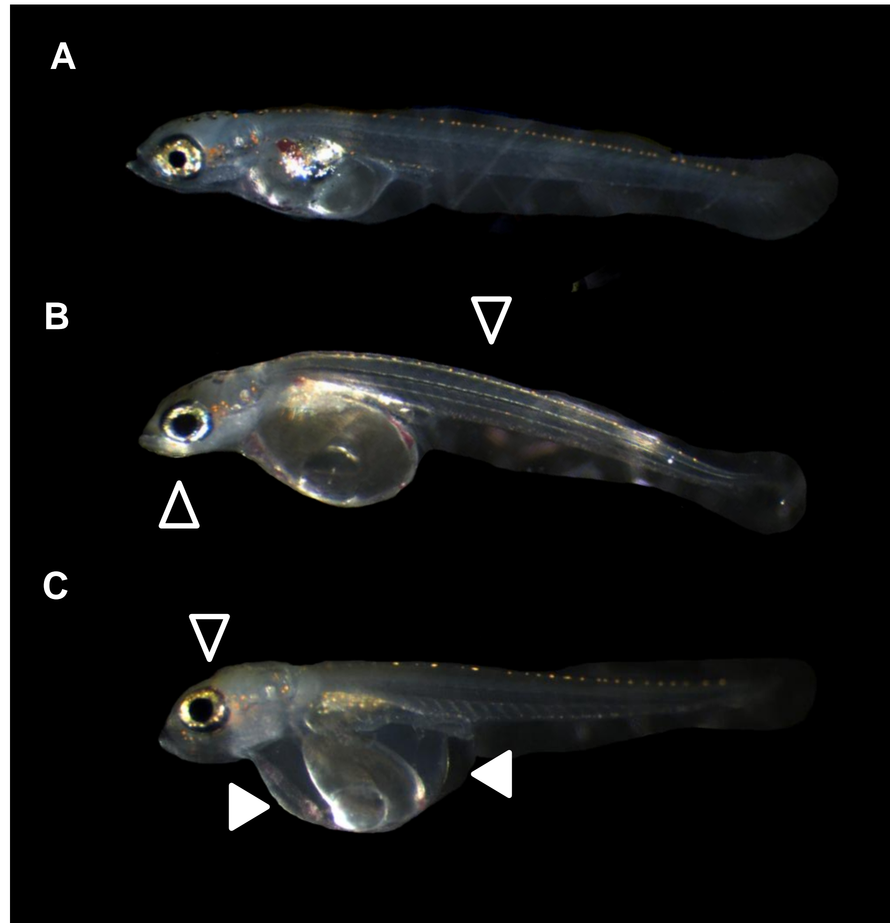
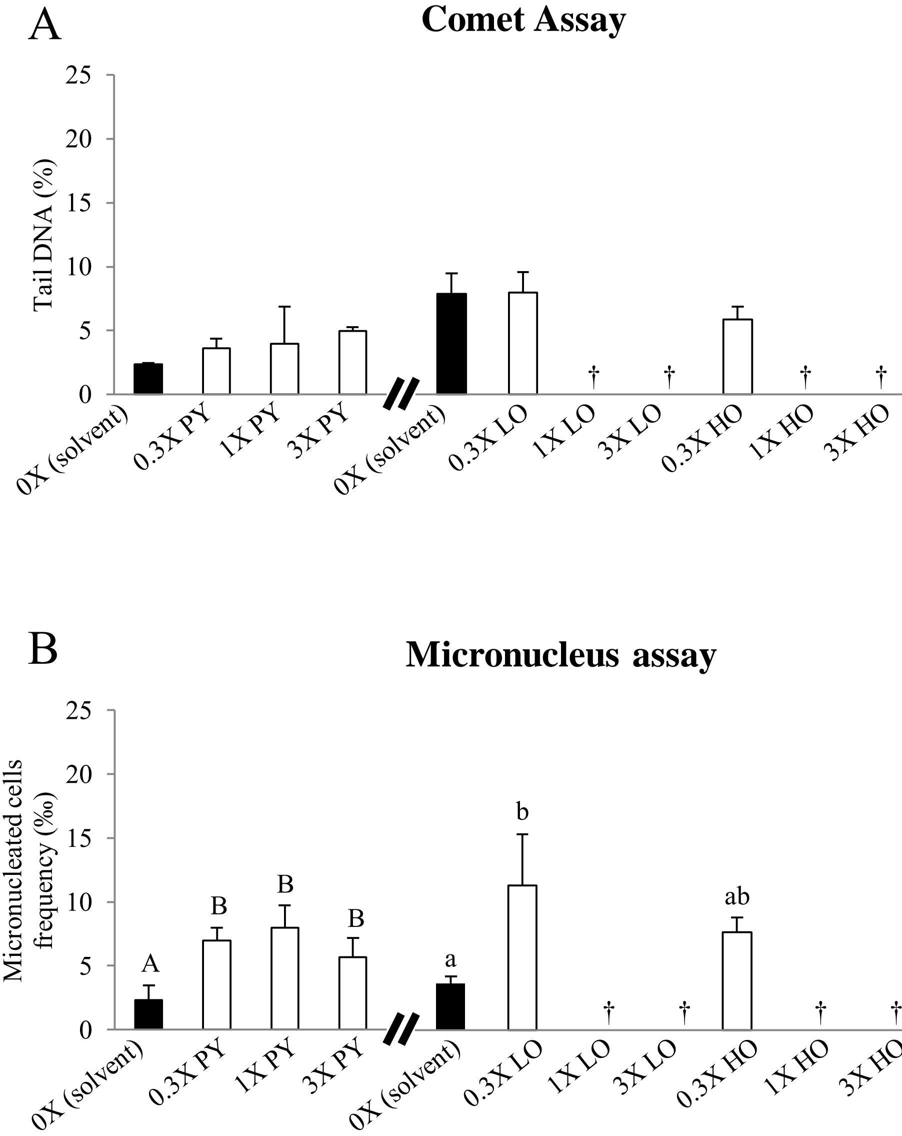


Figure 2



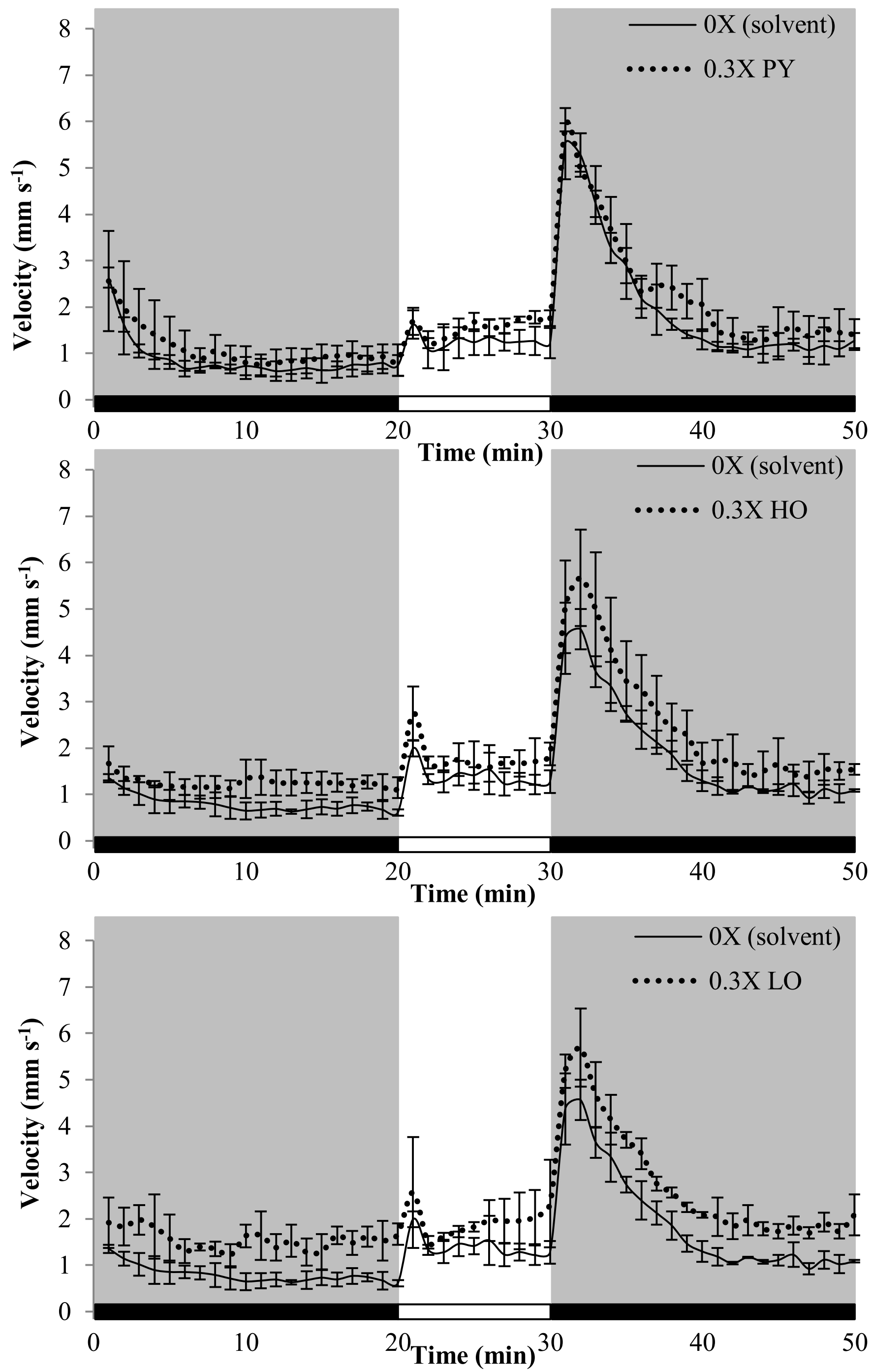
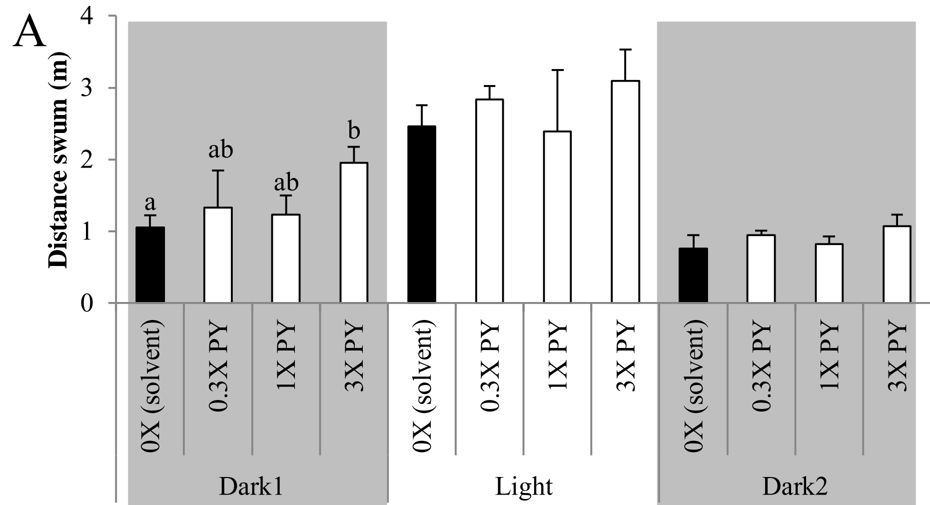
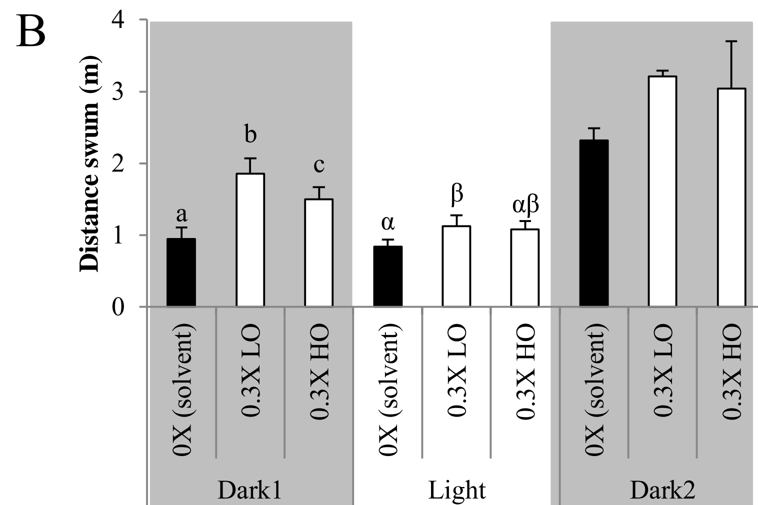
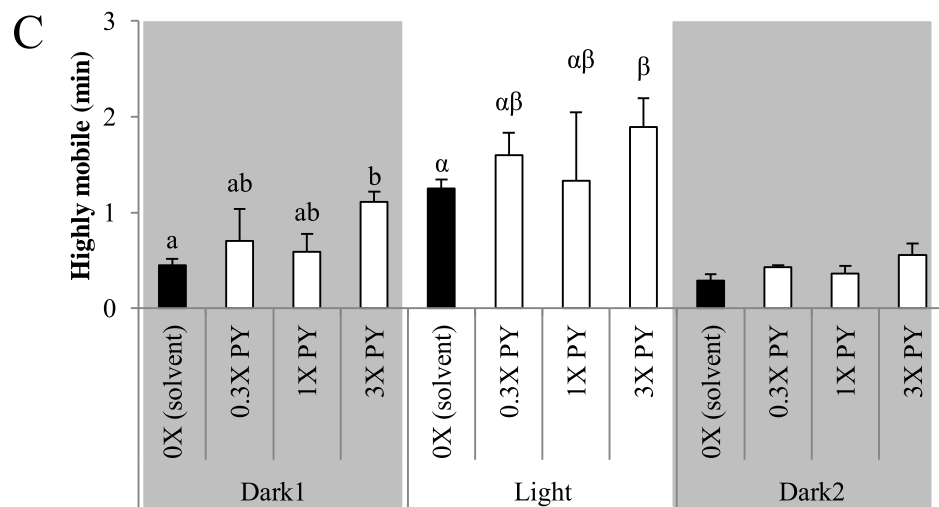


Figure 3

Figure 4







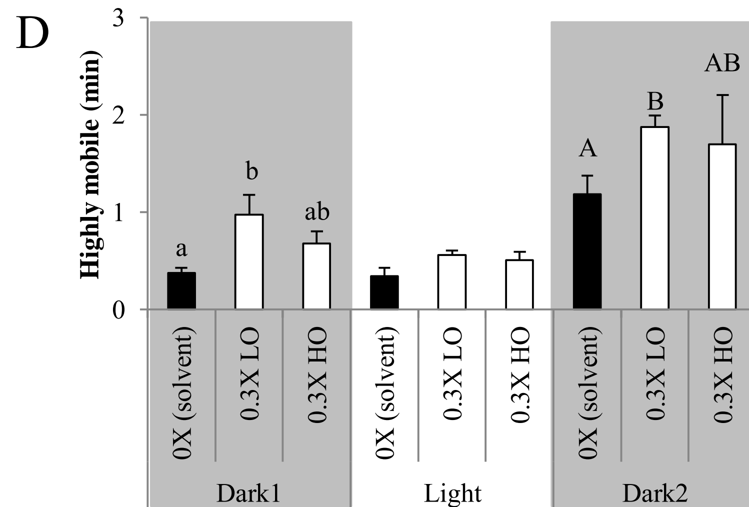


Table 1

Table 1. Measured PAH concentrations and dissolved oxygen in the assays

	PY				LO				HO		
	Solvent	**0.3X**	**1X**	**3X**	**Solvent**	**0.3X**	**1X**	**3X**	**0.3X**	**1X**	**3X**
PAH concentration (μg g^{-1}) (N = 1)	0.022	0.816	3.862	11.779	0.009	0.499	3.618	7.253	0.717	4.039	11.929
Alkylated PAH (%) (N = 1)	-	6	5	5	-	61	57	66	40	41	42
Spiking efficiency (%) (N = 1)	-	27	39	39	-	17	38	25	26	43	43
Dissolved O_2 (%) (Mean ± SD, N = 3)	90.0±2.4	89.9±1.0	88.3±2.1	87.7±2.0	87.2±1.3	87.3±0.7	85.9±0.7	88.1±0.5	86.9±0.5	88.9±0.2	88.0±0.9

Table 2

Table 2. Developmental toxicity of the three PAH fractions after medaka embryos exposure to PAH-spiked sediments. Different letters and bold characters refer to significant differences between treatments (Mean ± SD, N = 3, Anova, $p < 0.05$).

	Experiment 1				**Experiment 2**						
	Solvent	**0.3X PY**	**1X PY**	**3X PY**	**Solvent**	**0.3X LO**	**1X LO**	**3X LO**	**0.3X HO**	**1X HO**	**3X HO**
Acute Toxicity											
Embryonic survival (%)	94.7±2.3	97.2±4.8	95.9±4.2	100.0±0.0	97.0±2.0	93.3±5.0	96.0±3.3	97.3±1.9	96.0±3.3	98.7±1.9	96.0±0.0
Hatching rate (%)	93.3±2.3	95.8±7.2	94.6±2.5	88.0±12.0	**93.3±4.6**^{a}	**86.7±10.1**^{a}	**28.0±26.2**^{b}	**1.3±2.3**^{bc}	**96.0±4.0**^{a}	**1.3±2.3**^{bc}	**0.0±0.0**^{c}
Larval survival (%)	100.0±0.0	98.6±2.4	98.0±2.8	100.0±0.0	100.0±0.0	98.5±2.6	80.2±21.6	/	100.0±0.0	/	/
Sub-lethal toxicity											
Heartbeat (beat min^{-1})	112.6±3.2	112.3±4.8	116.6±3.1	120.5±3.3	114.1±4.0	117.2±8.2	118.1±7.9	109.4±4.5	116.1±4.4	117.8±2.9	105.5±8.0
Non-inflated swim bladder (%)	64.4±23.4	64.4±13.9	60.0±17.6	82.2±16.8	55.6±7.7	68.9±15.4	56.7±23.6	/	64.4±10.2	/	/
Hatching $T_{1/2}$ (day)	**10.8±0.1**^{A}	**11.2±0.3**^{AB}	**11.1±0.1**^{A}	**11.9±0.3**^{B}	10.2±0.1	10.2±0.2	10.4±0.1	/	10.4±0.1	/	/
Total length (mm)	**4.46±0.05**^{A}	**4.47±0.02**^{AB}	**4.46±0.06**^{AB}	**4.25±0.0**^{B}	**4.64±0.07**^{a}	**4.32±0.12**^{b}	**3.91±0.01**^{c}	/	**4.35±0.05**^{b}	/	/
Head length (mm)	0.92±0.02	0.92±0.02	0.91±0.02	0.92±0.02	**0.97±0.03**^{a}	**0.92±0.03**^{ab}	**0.89±0.05**^{b}	/	**0.93±0.01**^{ab}	/	/
Yolk sac area (mm^2)	0.48±0.03	0.45±0.02	0.44±0.04	0.43±0.02	**0.40±0.03**^{ab}	**0.39±0.02**^{ab}	**0.31±0.01**^{a}	/	**0.40±0.04** ^{b}	/	/
Deformed larvae (%)	**9.0±3.7**^{A}	**11.1±3.8**^{AB}	**22.2±3.8**^{B}	**62.2±7.7**^{C}	**17.8±7.7**^{a}	**48.9±16.8**^{b}	**100.0±0.0**^{c}	/	**33.3±6.7**^{ab}	/	/
Abnormalities (score /5)	**0.1±0.1**^{A}	**0.1±0.0**^{A}	**0.4±0.1**^{B}	**1.1±0.1**^{C}	**0.2±0.2**^{a}	**0.7±0.3**^{ab}	**3.1±0.3**^{b}	/	**0.4±10.2**^{ab}	/	/
Abnormalities (%)											
Edema	**0.0±0.0**^{A}	**0.0±0.0**^{A}	**2.2±3.8**^{A}	**17.8±0.1**^{B}	**0.0±0.0**^{a}	**2.2±3.8**^{a}	**60.6±8.6**^{b}	/	**3.2.2±3.8**^{a}	/	/
Spinal	2.2±3.8	4.4±7.7	13.3±6.7	20.0±7.7	**11.1±7.7**^{a}	**24.4±20.4**^{a}	**65.2±2.1**^{b}	/	**4.4±3.8**^{a}	/	/
Craniofacial	**4.6±4.0**^{A}	**6.7±6.7**^{A}	**13.3±0.0**^{A}	**46.7±17.6**^{B}	**8.9±10.2**^{a}	**24.4±7.7**^{ab}	**95.5±6.4**^{b}	/	**24.4±3.8**^{ab}	/	/
Eye	2.2±3.8	0.0±0.0	2.2±3.8	0.0±0.0	0.0±0.0	2.2±3.8	0.0±0.0	/	0.0±0.0	/	/
Cardio-vascular	**2.2±3.8**^{A}	**0.0±0.0**^{A}	**8.9±3.8**^{A}	**28.9±13.9**^{B}	2.2±3.8 a	**17.8±3.8**^{ab}	**90.9±12.9**^{b}	/	**8.9±7.7**^{ab}	/	/

Table 3

Table 3. Toxic equivalencies (TEQ) calculated for the three PAH fractions according to BaP or dioxin.

PAH fraction	TEQ / BaP [a]	TEQ / dioxin [b]
PY	13.3	0.032
LO	0.8	0.002
HO	7.5	0.009

[a](INERIS 2003) [b](Barron et al. 2004b)

Table 4

Table 4. Comparative toxicity of the three PAH fractions between Rainbow trout and Japanese medaka. Values refer to the lowest PAH sediment concentration causing a significant effect to the fish ELS (LOEC). PAH concentrations include the sum of 21 non-substituted PAHs, methyl naphtalenic and methyl phenanthrenic compounds.

	Rainbow trout (Le Bihanic et al. *submitted-a*)			**Japanese medaka** (This study)		
Parameters						
Concentrations tested	0.3X and 1X			0.3X, 1X and 3X		
Exposure matrix	Gravel			Artificial sediment		
Time of exposure	From eyed stage until hatching			From fecundation until hatching		
Temperature	10 °C			26 °C		
Photoperiod	Full dark			12:12		
Water renewal	No renewal			Daily renewal		
Salinity	0 PSU			5 PSU		
LOEC(μg g^{-1} dw)	**PY**	**LO**	**HO**	**PY**	**LO**	**HO**
Survival	-	-	-	-	-	-
Hatching success	-	-	4.8 (1X)	-	3.6 (1X)	4.0 (1X)
Larvae total length	-	0.7 (0.3X)	1.3 (0.3X)	11.8 (3X)	0.5 (0.3X)	0.7 (0.3X)
Abnormal larvae	4.2 (1X)	0.7 (0.3X)	1.3 (0.3X)	11.8 (3X)	0.5 (0.3X)	-
Comet assay	-	4.1 (1X)	1.3 (0.3X)	-	-	-
Micronucleus assay	-	-	-	0.8 (0.3X)	0.5 (0.3X)	