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Transcriptional responses and embryotoxic effects induced by
pyrene and methylpyrene in Japanese medaka (*Oryzias latipes*)
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1 **Abstract**

2 Japanese medaka (*Oryzias latipes*) embryos were exposed to sediments spiked to environmental
3 concentrations (300 and 3,000 ng/g dry weight) of pyrene (Pyr) and methylpyrene (MePyr) throughout
4 their development. Embryotoxicity, teratogenicity and transcriptional responses (qRT-PCR) were
5 analyzed in embryos and newly hatched larvae. The genotoxicity of the two polycyclic aromatic
6 hydrocarbons (PAHs) was also tested in prolarvae using the comet assay. Exposure to each compound
7 had a clear impact on embryonic development and resulted in many teratogenic effects, including
8 cardiovascular injuries, reduced absorption of yolk sac reserves and jaw and spinal deformities.
9 Interestingly, the overall toxic effects of Pyr and MePyr considerably overlapped those induced following
10 dioxin exposure. qRT-PCR analysis revealed the transcriptional induction of genes involved in
11 mitochondrial energetic metabolism (*coxI*), xenobiotic biotransformation (*cyp1a*) and cell cycle
12 regulation (*wnt1*) by the two PAHs. MePyr also activated cell cycle arrest (*p53*), oxidative DNA damage
13 repair (*ogg1*) and retinoid-mediated (*raldh2* and *rara1*) gene transcription. Moreover, DNA damage was
14 found to increase in a concentration-dependent manner following Pyr and MePyr exposure. The lack of
15 significant genotoxic effect in comparison to control might be the consequence of the efficient onset of
16 DNA damage repair mechanisms as suggested by *ogg1* gene transcription upregulation. Results reported
17 in the present study have brought new insight into the modes of action of Pyr and the effects of MePyr
18 exposure have been investigated in fish ELS for the first time.

19

20 *Keywords:* PAH spiked sediments, fish early life stage, gene expression, embryotoxicity, teratogenicity,
21 genotoxicity

22

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that are primarily introduced into the environment through anthropogenic activities such as the incomplete combustion of organic matter, fossil fuels, oil and wood. Firstly released in the aquatic environment through land-based runoff, industrial and urban effluents and atmospheric deposition, they get trapped in sediments due to their hydrophobic properties (Dupree and Ahrens 2007; Ineris 2006). Since many fish species use sediments as spawning substrate, PAHs can represent a long-term threat for aquatic ecosystems due to their persistence and their ability to pass through biological membranes and to accumulate in organisms.

For the past few decades, many studies have demonstrated the high sensitivity of fish early life stages (ELS) to PAH exposure (e.g.: Li et al. 2011; Cachot et al. 2007; Farwell et al. 2006; Rhodes et al, 2005; Incardona et al. 2004; Carls et al. 1999). A wide range of developmental defects have been reported following exposure to PAHs including pericardial and yolk sac edema, jaw deformities, spinal curvature and various cardiovascular injuries. Among PAHs, certain congeners proved to be AhR (aryl hydrocarbon receptor)-agonists and teratogens with symptoms highly comparable to those of the blue sac disease (BSD) syndrome induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure, suggesting similar modes of action (Barron et al. 2004; Incardona et al. 2004; Brinkworth et al. 2003; Billiard et al. 1999). However, unlike dioxins, PAHs can be rapidly metabolized by vertebrates (Billiard et al. 2008). Indeed, PAHs have the particularity to induce their own metabolism by activating the transcription of genes encoding for phase I drug/xenobiotic cytochrome P450 enzymes, phase II conjugation enzymes and phase III transporters through AhR pathway activation (Feng et al. 2013; Denison and Heath-Pagliuso 1998). Paradoxically, it is nowadays well established that a variety of unstable and reactive intermediates, sometimes more toxic than the parent compound, as well as reactive oxygen species (ROS) can be generated during PAH metabolism (Shimada 2006; Morel et al. 1999). Consequently, PAHs can exert their developmental toxicity involving a wide range of mechanisms dependent on their chemical structure and rate of metabolism. For instance, Incardona et al. (2004; 2005) reported that primary toxicity of phenanthrene and dibenzothiophene was AhR-independent in zebrafish (*Danio rerio*) embryos. In contrast to these congeners but similarly to TCDD, the defects in cardiac function and morphogenesis induced by

1 benz[a]anthracene proved to be AhR2-dependent and CYP1A-independent in zebrafish embryos
2 (Incardona et al. 2006).

3 The AhR pathway is involved in various signaling pathways covering multiple physiological aspects such
4 as cell proliferation and differentiation, apoptosis, gene regulation and angiogenesis (Feng et al. 2013).
5 Thus, many AhR-dependent toxic effects of PAHs are likely to result from continuous and inappropriate
6 expression of specific genes in susceptible cells (Denison and Heath-Pagliuso 1998).

7 Moreover, several enzymes involved in the metabolism of PAHs also participate in the metabolism of
8 retinoids such as, CYP1A, UDP-glucuronosyl transferase and glutathione-S-transferase (Boily et al.
9 2004). Retinoids are crucial for many vital processes such as growth and development. Both excess and
10 deficiency of retinoids can result in embryotoxicity and/or teratogenicity in vertebrates (Novak et al.
11 2008). Alterations in retinoids have also been associated with exposure to PAHs and the embryotoxic and
12 teratogenic potencies of those compounds make them suspected to interfere with retinoid signaling
13 (Noval et al. 2008; Rolland 2000).

14 Despite careful analyses and consistent findings across species, the precise mechanisms leading to PAH-
15 associated malformations and sub-lethal effects in fish ELS are still unclear. In the present study, we
16 propose a modified version of the medaka embryo-larval assay with sediment-contact exposure (MELAc)
17 (Barjhoux et al. 2012; Vicquelin et al. 2011) to simultaneously analyze the phenotypic and molecular
18 effects of pyrene (Pyr) and methylpyrene (MePyr) in medaka (*Oryzias latipes*) ELS. Pyr has been found
19 at very high concentrations in sediments, sometimes exceeding 10 µg/g of sediment (see Table 1) from
20 areas heavily impacted by PAHs. Indeed, Pyr is often amongst the most abundant of PAHs in sites
21 affected by creosote or pyrogenic inputs (Nagy et al. 2013; Kimbrough and Dickhut 2006; Wang et al.
22 2001). Moreover, Pyr has been reported to greatly accumulate and to be efficiently metabolized by phase
23 I and phase II enzymes in fish ELS (Honkanen et al. 2008; Petersen and Kristensen 1998). In spite of its
24 weak potency as an AhR agonist (Barron et al. 2004), Pyr exposure induced developmental defects in *D.*
25 *rerio* embryos that considerably overlap those reported for TCDD (Incardona et al. 2006; 2005). These
26 studies also highlighted the crucial role of CYP1A enzyme activity in the underlying mechanism of Pyr
27 toxicity. MePyr is also present in sediments from PAH affected areas (Notar et al. 2001). MePyr proved
28 to be bioaccumulable in various aquatic species including teleost fish (Pancirov and Brown 1977) and is

1 considered to be genotoxic and a potent carcinogen in mammals (Glatt et al. 2008; Monien et al. 2008),
2 but to our knowledge, no study is available on the effects of MePyr in fish ELS.
3 To characterize the potential impact of environmental concentrations of these PAHs in fish ELS, medaka
4 embryos were exposed to Pyr or MePyr-spiked sediments at 300 (C1) and 3,000 (C2) ng/g dry weight
5 (dw) throughout their whole embryonic development. Several non-invasive markers of acute and
6 sublethal toxicity were recorded during the course of the exposure. In parallel, qRT-PCR analysis of
7 transcription levels of target genes was carried out in embryos and newly hatched larvae in order to obtain
8 an overview of the modulations in metabolic pathways following Pyr and MePyr exposure. Finally, the
9 potential genotoxicity of Pyr and MePyr was investigated in 2-day old larvae using the comet assay.

10

11 **2. Material and Methods**

12 *2.1. Experimental design*

13 Japanese medaka (*Oryzias latipes*) embryos of the CAB strain were purchased from GIS Amagen (Gif-
14 sur-Yvette, France). Embryos at one day post-fertilization (dpf) were exposed to five different spiked
15 sediments: a control group at 0 ng/g dw of sediment; a Pyr-C1 group at 300 ng Pyr/g dw; a MePyr-C1
16 group at 300 ng MePyr/g dw; a Pyr-C2 group at 3,000 ng Pyr/g dw; and a MePyr-C2 group at 3,000 ng
17 MePyr/g dw (nominal concentrations). Each treatment consisted of six replicates of 70 embryos each. The
18 first three replicates of each treatment were dedicated to sampling at the embryonic stage (T7) and the
19 three remaining ones kept for larval stage samplings (T9).

20 Medaka embryos remained in direct contact with the sediment up to sampling time, i.e. for 7 dpf (T7) or
21 up to hatching time (9 dpf, T9), depending on the replicate under consideration. For the duration of the
22 experiment, embryos and larvae were maintained at 26 °C with a 12 h:12 h photoperiod and under static
23 exposure conditions. After T9 sampling (corresponding to the hatching peak) the remaining embryos and
24 larvae were kept in a clean medium, up to the 11th dpf to assess the genotoxic impact of the exposure on 2
25 days post-hatching larvae (dph).

26 Sampled embryos/ larvae were divided into pools of adequate size to perform gene expression analysis by
27 qRT-PCR and DNA damage measurement using the Comet assay.

1 To complete this genetic aspect of the study, and as described in previous works from our laboratory
2 (Barjhoux et al. 2012; Vicquelin et al. 2011), a wide range of non-invasive phenotypic endpoints were
3 also studied. They included acute toxicity markers as embryonic survival and hatching success, and
4 markers of sublethal effects such as cardiac activity, biometric measurements, time to hatch and the
5 occurrence and spectrum of developmental abnormalities.
6 Finally, the time-course of Pyr and MePyr contamination was followed in the aqueous phase (i.e. in egg
7 rearing solution, ERS) at T0 (beginning of exposure) and at each sampling time dedicated to molecular
8 analysis (T7 and T9).

9

10 *2.2. Reference sediment characterization*

11 The reference sediment was collected in March 2010 in a pristine gravel pit near Yville-sur-Seine (Seine-
12 Maritime, France). This site has been shown to be marginally contaminated by heavy metals and organic
13 pollutants (Cachot et al. 2006). Moreover, previous studies demonstrated that Yville-sur-Seine sediment
14 is an adequate substrate for medaka embryonic development without any toxic impact on medaka ELS
15 (Vicquelin et al. 2011; Cachot et al. 2007).

16 The reference sediment was stored at -20 °C, then freeze-dried and lightly crushed using a mortar and a
17 pestle to eliminate larger particles and homogenize the grain size before use.

18 The physico-chemical characteristics of the sediment were analyzed using the processes described by
19 Barjhoux et al. (2012) and Vicquelin et al. (2011). As shown in Table 2, Yville-sur-Seine sediment can be
20 classified as fine sand with a low carbon organic content. Chemical analyses of this sediment showed a
21 very marginal presence of trace metallic elements and persistent organic pollutants.

22

23 *2.3. Sediment spiking procedure*

24 The freeze-dried (-20 to -40 °C, at 0.1 mbar for about 24 hours) reference sediment was spiked with Pyr
25 or MePyr to obtain two different nominal concentrations of 300 (C1) and 3,000 ng/g dw (C2) for each
26 compound.

1 The consensus-based probable effect concentration (PEC) established for Pyr by MacDonald et al. (2000;
2 Table 1) was doubled to obtain the C2 nominal concentration. This PEC value was derived from the
3 analysis of sediment quality guidelines published previously and is defined as a threshold concentration
4 above which harmful effects on aquatic organisms are expected to occur frequently (MacDonald et al.
5 2000). The C2 concentration can thus be considered as ‘toxicological’, however such levels of Pyr
6 contamination can be achieved or exceeded in sediments from some particularly highly contaminated
7 sites (e.g. Boston harbor sediments; Table 1). The C1 concentration was set ten times lower than C2
8 concentration. This treatment corresponded to Pyr concentrations that can be measured in contaminated
9 sediments from areas moderately affected by anthropogenic activities (e.g. old waste sites, harbor areas;
10 Table 1). The C1 concentration is also close to the mean threshold effect concentrations (TECs) reported
11 in Table 1 (including ERL, TEL and consensus-based TEC), below which adverse effects are not
12 expected to occur. In order to compare the toxicity of Pyr and MePyr the same nominal concentrations
13 were used for both compounds.

14 The spiking procedure was adapted from the protocol described by Vicquelin et al. (2011). Briefly, an
15 adequate amount of freeze-dried and crushed sediment from Yville-sur-Seine was placed in a 250 ml
16 round-bottomed glass flask and immersed in dichloromethane (DCM, 2 ml/g dw of sediment). Then, 2 ml
17 of isooctane solution containing the required amount of the tested compound (Pyr or MePyr, purchased
18 from Sigma-Aldrich, Lyon, FR) was added to reach the target final concentration. Solvent evaporation
19 was performed using a rotavapor (RV10 Basic, VWR International, Strasbourg, FR) equipped with a
20 heating water bath (HB10 Basic, VWR International) set at 45 °C until the sediment was seen to be
21 completely dry. Any potentially remaining traces of organic solvent were eliminated by leaving the
22 sediment overnight at room temperature, in darkness, under an extractor hood. Finally, spiked sediment
23 from each treatment was divided into six aliquots of 12 g dw (for embryo exposure) and one additional
24 aliquot of 5 g dw dedicated to chemical analysis of Pyr and MePyr.

25

26 2.4. *Medaka embryo exposure*

1 Each aliquot of 12 g dw of spiked sediment was laid in a 55 mm-diameter plastic Petri dish and immersed
2 by adding 7 ml of ERS (17.11 mM NaCl; 0.4 mM KCl; 0.36 mM CaCl₂; 1.36 mM MgSO₄; pH 7.0). The
3 resulting system was then maintained at 26 °C for a 4-5 h equilibration period before the beginning of the
4 experiment. Upon receipt of 24 hours post-fertilization (hpf) medaka eggs, healthiness and developmental
5 stage synchronism of the embryos were checked using a stereomicroscope (Leica MZ75, Leica
6 Microsystems, Nanterre, FR) and cold light source (Intralux® 4100, Volpi AG, Schlieren, CH).
7 Immediately after sorting, 70 embryos per replicate (six replicates per treatment) were randomly placed
8 on a Nytex® mesh (mesh opening 1000 µm, Sefar Filtration Inc., Depew, NY, US). The Nytex® grid was
9 then slightly sunk into the sediment. Afterwards, embryos remained exposed to the sediment in a climate
10 cabinet (Economic Delux, Snijders Scientific, Tilburg, NL) at 26 ± 0.3 °C with a 12 h:12 h photoperiod
11 (5000 lx white light) until hatching time (9 dpf) and then placed in clean ERS medium up to the end of
12 experiment at 11 dpf (comet assay performed on 2-day old larvae). The remaining embryos or larvae were
13 euthanized using MS222 (Sigma-Aldrich) solution at 1 g/L. During the exposure period, the level of ERS
14 was checked daily and re-adjusted in case of evaporation. Dissolved oxygen was also checked daily at the
15 water-sediment interface using an oxygen optical microsensor (NeoFox® Foxy probe, Ocean Optics
16 sensors, Idle Fibres optiques, Lannion, FR). This measurement confirmed good oxygenation of the
17 medium with values always higher than 80% saturation (data not shown).

18

19 2.5. *Phenotypical endpoints*

20 The different procedures performed for phenotypic endpoint assessments have been previously detailed
21 by Barjhoux et al. (2012). Viability was checked daily for all individuals and all conditions. Cardiac
22 activity was monitored in 6 and 7 dpf-embryos (5 randomly selected individuals per replicate). Biometric
23 measurements (total body length, head size and head/body length ratio) and developmental anomalies
24 (spinal, craniofacial, ocular, cardiovascular, yolk-sac and edema) were observed in 15 randomly selected
25 newly hatched larvae per replicate. All these observations were done in an air-conditioned room at 23
26 ± 1 °C using a stereomicroscope (MZ75, Leica Microsystem) equipped with a color CCD camera (Leica

1 DFC 420C), connected to an image analysis software program (Leica Application Suite v2.8.1.) and a
2 cold light source (Intralux® 4100, Volpi AG).

3

4 2.6. Gene transcription analysis

5 Gene expression analysis was conducted on three samples of 8 pooled individuals per replicate, and on
6 three replicates per treatment at each sampling time (T7 and T9).

7 A panel of 12 genes was selected for transcriptional analysis due to their involvement in xenobiotic
8 metabolism, antioxidant defenses, mitochondrial metabolism, DNA repair, cell cycle regulation and
9 apoptosis. The response to oxidative stress was studied through cytoplasmic (*sod(Cu/Zn)*) and
10 mitochondrial superoxide dismutase (*sod(Mn)*) gene transcription. Activation of Pyr and MePyr phase I
11 metabolism was studied through *cyp1A* gene transcription. The impact of Pyr and MePyr on the
12 mitochondrial electron transport chain was investigated using cytochrome C oxidase subunit I (*coxI*)
13 transcripts. The *ogg1* (8-oxoguanine glycosylase 1) was selected for its involvement in oxidative DNA
14 damage repair. PAH-induced apoptosis was studied through Bcl-2 associated X protein (*bax*) and *p53*
15 gene transcription levels. The wingless integration site 1 (*wnt1*) gene was also selected for its key role in
16 cell differentiation and proliferation. Finally, four genes involved in retinoid metabolism (retinaldehyde
17 dehydrogenase type 2, *raldh2*) and retinoic acid signaling pathway (retinoic acid receptor alpha 1, *rara1*;
18 retinoic acid receptor gamma 1, *rarg1*; retinoid X receptor, *rxra1*) were also studied.

19 Each pool of embryos or larvae was entirely immersed in RNase-free microtubes containing 200 µL
20 RNA Later® (Qiagen, Manchester, UK) and quickly frozen by dipping in liquid nitrogen. Samples were
21 then stored at -80 °C until RNA extraction.

22 Total RNA extraction was performed using the Absolutely RNA® Miniprep kits (Agilent, Les Ulis, FR)
23 according to the manufacturer's instructions, with an additional phenol-chloroform-isoamyl alcohol
24 (25:24:1, v/v) purification step. The quality and the quantity of the extracted RNA were determined by
25 spectrophotometry at 260 and 280 nm.

26 First-strand cDNA was synthesized using the AffinityScript™ Multiple temperature cDNA Synthesis kits
27 (Agilent). Briefly, 1 µL of oligo dT (1 µM), 1 µL of random primers (1µM), 0.8 µL of dNTPs (10 mM)

1 and 2 μL of AffinityScript™ RT buffer (10X) were mixed together with 14 μL of the previously
2 extracted RNA (approximately 5 μg). The mixture was then incubated in a thermocycler (MasterCycler
3 pro™, Eppendorf, Le Pecq, FR) for 5 min at 65 °C. cDNA synthesis was performed by adding 1 μL of
4 reverse transcriptase (1 U) and 0.5 μL of RNase block ribonuclease inhibitor (0.5 U) and then by
5 incubating the mixture at 42 °C for 1 h. cDNA samples were then stored at -20 °C until quantitative real-
6 time PCR was performed.

7 The coding sequences of the 13 selected genes (Table 3) were obtained from the GenBank (PubMed –
8 NCBI) and HGNC (Ensembl, EMBL – EBI) databases. The accession number of each coding sequence is
9 reported in Table 3. For each gene, specific primer pairs were determined using the LightCycler probe
10 design software (v1.0, Roche, Meylan, FR) and are mentioned in Table 3. Primers were purchased from
11 Sigma-Aldrich (Easy Oligo™).

12 The amplification of cDNA was monitored using the DNA intercalating dye SyberGreen I. Real-time PCR
13 reactions were performed using an Mx3000P™ system (Stratagene, Agilent) and Brilliant III Ultra-Fast
14 SYBR® Green QPCR Master Mix kits (Agilent) according to the manufacturer's instructions.

15 PCR reactions were prepared in 96 well-microplates adding 10 μL of SYBR® Green QPCR master mix
16 (2X), 7 μL of ultra-pure water (nuclease-free PCR-grade water), 2 μL of primer-pair mix (2 μM) and
17 1 μL of cDNA. Afterwards, PCR reactions consisted of an activation cycle (10 min at 95 °C) followed by
18 50 amplification cycles (30 s at 95 °C, 40 s at 60 °C and 30 s at 72 °C).

19 The specificity of each amplification cycle was determined from the dissociation curve of the PCR
20 product. These dissociation curves were obtained by following the SyberGreen fluorescence level during
21 a gradual heating of the PCR products from 65 to 95 °C (0.1 °C/s). Relative quantification of each gene
22 transcription level was normalized according to *β -actin* and *rpl7* genes using the $2^{-\Delta C_t}$ method described
23 by Livak and Schmittgen (2001), where ΔC_t represents the difference between the cycle threshold (Ct) of
24 a specific gene and the mean Ct of the housekeeping genes (*β -actin* and *rpl7* genes in the present study).

25

26 2.7. Comet assay

1 DNA damage induced by Pyr and MePyr exposure was evaluated on a pool of five 2 dph-larvae (one pool
2 sampled per replicate on three replicates per treatment) using the comet assay. Cell dissociation and
3 comet assay procedures were carried out following the protocol of Morin et al. (2011). Briefly, pools of
4 larvae were digested in a MEM-Collagenase IV 0.125% (w/v) medium, and cell viability was checked
5 using a trypan blue exclusion test (only cell suspensions with viability superior to 80% were used). Once
6 embedded in a 1% low melting point agarose gel, cells were lysed (2.5 M NaCl, 0.1 M EDTA,
7 0.01 M Tris; pH = 10; at 4 °C for 1 h) and immersed in an electrophoresis buffer (0.3 M NaOH, 1 mM
8 EDTA; pH>13) for 15 min to allow the DNA to unwind. Then, electrophoresis was carried out at 25 V,
9 300 mA for 15 min. Ethidium bromide (20 mg/L) was used as DNA fluorescent tag and all coded-slides
10 were randomly analyzed for 75 nuclei per gel (two gels per experimental replicate) using an Olympus epi-
11 fluorescent microscope (400 x magnification) equipped with a grayscale CCD camera (Zeiss, DE) and the
12 Komet 5.5 software program (Kinetic Imaging, Liverpool, UK). As recommended by Hartmann et al.
13 (2003), % tail DNA (percentage of DNA which migrates from the nucleus i.e. the head of the comet) was
14 the selected parameter used for the measurement of DNA damage. Heavily DNA-damaged nuclei
15 displaying a small or inexistent head and a large diffuse tail, known as 'hedgehog cells', were not taken
16 into account in the comet measurement, according to the recommendations of Kumaravel et al. (2009).
17 However, the percentage of hedgehog cells, which have also been reported as apoptotic or necrotic cells
18 (Olive and Banath 1995), was visually scored on a total of 100 cells per gel.

19

20 2.8. *Pyr and MePyr analysis*

21 2.8.1. Sediment analysis

22 Pyr and MePyr extraction and analysis were performed according to the procedures described in previous
23 studies (Devier et al. 2005; Letellier et al. 1999). In brief, spiked sediments were extracted using
24 microwave-assisted extraction (30 W for 10 min, in a Maxidigest 350, ProLabo, Fontenay-sous-Bois, FR)
25 with 30 mL of DCM and 30 µL of β-mercaptoethanol. Depending on the expected PAH concentration,
26 the extracted quantity of freeze-dried sediment varied from 0.5 g (for C2 treatments) and 1 g dw (control
27 sediment and C1 spiking level). One extraction blank (complete procedure without matrix) was included

1 in each series of extraction. Deuterated fluoranthene (Fluod10) was used as an internal standard and
2 gravimetrically added prior to the extraction procedure in each sample and blank.

3 After extraction, the samples were reconcentrated using a Vacuum Evaporation System (RapidVap,
4 Labconco, Fort Scott, KS, US; 900 mBars, 50 °C). The concentrated extracts were then purified on a
5 micro-column containing activated copper and alumina and reconcentrated in isooctane. Afterwards,
6 purified samples were loaded onto a micro-column containing silica. The aliphatic fraction was discarded
7 by elution with pentane whereas the aromatic fraction containing the PAHs was subsequently eluted with
8 a mixture of DCM-pentane (65:35 v/v). The aromatic extracts were reconcentrated in DCM and analyzed
9 using gas chromatography-mass spectrometry (GC-MS). Deuterated pyrene (Pyr10) was used as syringe
10 standard and gravimetrically added to each extract and blank just before injection.

11 The accuracy and the validity of the quantification method were tested during each analysis series using
12 two standards solutions containing known amounts of Pyr, MePyr and deuterated standards (Fluod10 and
13 Pyr10). The first standard solution was used to evaluate the response factors (Pyr/Fluod10,
14 MePyr/Fluod10 and Fluod10/Pyr10). The second independent standard solution was used to test the
15 efficiency of the quantification method, which varied between 103 % and 105 % for Pyr, between 100 %
16 and 101 % for MePyr and between 98 % and 99 % for Fluod10.

17

18 2.8.2. Aqueous phase analysis

19 At each sampling time, approximately 4 mL of ERS was sampled from three replicates and pooled
20 together for the same treatment (except for T0 sampling for which 20 ml of clean ERS was analyzed).
21 Aqueous samples were separated from sediment particles with a 15 min-centrifugation at 3 500 g at 15 °C
22 and stored at -20 °C in the dark until analysis.

23 Pyr and MePyr extraction was performed by SPME (Solid Phase MicroExtraction) and analyzed by
24 GC/MS as described by de Perre et al. (submitted). The same internal and syringe standards as mentioned
25 for sediment analysis were used. The efficiency of the quantification method has been evaluated to be
26 116% for Pyr and 126 % for MePyr.

27

2.9. *Statistical analysis*

The data is expressed as mean \pm standard deviation (SD). Statistical analyses were conducted using Statistica 7.1 software (Statsoft, Maisons-Alfort, FR). Results were initially tested for normality (Shapiro-Wilk's test on residues with 1% risk) and homoscedasticity (Levene's test, 5% risk). When necessary, data was transformed to fulfill normality and homoscedasticity criteria. Afterwards, significant differences between treatments were tested with a one-way or two-way ANOVA analysis followed by post-hoc Tukey's test ($p < 0.05$). If data transformation was not sufficient to perform parametric analysis, then the non-parametric Kruskal-Wallis' test followed by Bonferroni-Dunn's post-hoc test were used ($p < 0.05$). Finally, relative gene transcription data was statistically analyzed using the t test for independent samples (only pairing comparisons with the control treatment were performed).

3. Results and discussion

In previous studies, the MELAc proved to be a reliable, sensitive and integrative bioassay specifically designed for toxicity assessment of sediment bound chemicals including metals and hydrophobic substances (Barjhoux et al. 2012; Vicquelin et al. 2011). The present work proposes an adaptation of this assay for a combined analysis of embryotoxicity, teratogenicity and genetic disturbances induced by Pyr and MePyr in medaka ELS. During the time-course of the exposure to spiked sediments, several phenotypical and non-invasive markers of toxicity and embryonic development impairment were investigated in embryos and larvae. This approach was completed by the analysis of target gene transcription levels by qRT-PCR and DNA damage measurement using the comet assay.

3.1. *Pyr and MePyr contamination in the spiked-sediments and the water column*

Chemical analysis revealed a clear concentration-dependent increase of Pyr and MePyr content in Pyr- and MePyr-spiked sediments, respectively (Table 4). The spiking efficiencies, calculated by comparison between Pyr or MePyr contents in sediment at T0 and the expected concentration based on the amount of compound introduced during the spiking procedure, were satisfying and varied between 83% and 92% for

1 Pyr, and between 70% and 88% for MePyr. The resulting exposure concentrations in sediments were
2 close to the targeted values.

3 Pyr and MePyr concentrations in the aqueous phase of the control treatment were low and varied from
4 1.48 ng/L to 12.5 ng/L for Pyr and from 0.16 ng/L to 3.5 ng/L for MePyr (Table 4). Pyr concentration in
5 the ERS buffer from the different treatments showed a trend of increasing over the time-course of the
6 experiment to reach maximal values of 630 ng/l and 3,125 ng/L at T9 in C1 and C2 treatments,
7 respectively. The same tendency was observed for the MePyr-C2 treatment for which MePyr
8 concentration peaked at 1,830 ng/L at the end of exposure. Surprisingly, the aqueous phase from the
9 MePyr-C1 treatment was more contaminated by MePyr at T7 (98 ng/L) than at T9 (53.5 ng/L) (Table 4).
10 The contamination of the water column is probably due to the desorption of Pyr or MePyr labile fraction
11 from the sediment particles and the diffusion of these compounds from the sediment pore-water.
12 According to some authors, PAHs bioavailability (and thus, toxicity) is highly governed by their
13 concentration in the aqueous phase and more particularly in the pore-water of contaminated soils or
14 sediments (Geffard et al. 2003; Sverdrup et al. 2002). In the present work, measured concentrations of Pyr
15 and MePyr in the ERS buffer were relatively low and only reached a few $\mu\text{g/L}$ in the most highly
16 contaminated treatments. However, it can be hypothesized that dissolved PAHs may have been locally
17 more concentrated in sediment pore-water during the exposure. Sverdrup et al. (2002) proposed a method
18 for the calculation of PAH concentration in pore-water as a function of PAH content in spiked sediments
19 and their soil organic carbon-water partitioning coefficient (K_{oc}). When applied to Pyr and MePyr
20 sediment contents measured in the present work, concentrations in pore-water could reach 2.7 and
21 30 $\mu\text{g/L}$ for Pyr, and 1.16 and 14.9 $\mu\text{g/L}$ for MePyr, for C1 and C2 treatments, respectively (calculations
22 carried out using an *n*-octanol-water partitioning coefficient, $\log K_{ow}$, equals to 5.2 for Pyr according to
23 Sverdrup et al. (2002) and to 5.48 for MePyr as predicted by US-EPA EPI suite™). Concentrations
24 measured in the aqueous phase at the end of the exposure were well below these predicted values.

25

26 3.2. *Only Pyr induces low mortality in embryos exposed to spiked-sediments.*

1 MePyr exposure did not induce any significant impact on survival rate in exposed embryos; the mean
2 viability was above 95% in both treatments (Table 5). Conversely, Pyr exposure resulted in a slight
3 concentration-dependent reduction of embryos survival, which was significant for the Pyr-C2
4 concentration when compared to the control ($p<0.03$). However, mean embryonic survival in this
5 treatment remained high (>90%) and only represented a decline of 5% in comparison to unexposed
6 embryos (Table 5).

7 According to these observations and considering that only dissolved PAHs are biologically available for
8 fish embryos (Carls et al. 2008), a lethality threshold of 3 $\mu\text{g/L}$ of Pyr in the water column can be
9 established for medaka embryos exposed to spiked-sediment. These results are in agreement with the
10 LOEC (lowest observed effect concentration) value of 2.5 $\mu\text{g/L}$ reported for the survival of turbot larvae
11 (*Psetta maxima*) exposed to Pyr during 96 h (Mhadhbi et al. 2010). Li et al. (2011) also observed a 5% to
12 10% mortality in *Sebastiscus marmoratus* larvae exposed to 1 $\mu\text{g/L}$ of Pyr. On the contrary, *Cyprinodon*
13 *variegatus* exposure to 20 $\mu\text{g/L}$ of Pyr did not induce significant reduction of the embryonic survival rate
14 (Hendon et al. 2008). It has also been reported that Pyr-induced lethality following embryonic exposure
15 mainly occurred at the larval stage, several days after hatching (Hendon et al. 2008; Incardona et al.
16 2004). It is thus possible that additional mortality could occur in medaka ELS if the exposure was
17 prolonged later at the larval stage. Based on the above mentioned data, it seems that medaka ELS have
18 similar sensitivity to *P. maxima* larvae but are clearly more sensitive than *C. variegatus* embryos when
19 exposed to Pyr in the water column. Nonetheless, it must be emphasized that, as discussed above (see part
20 3.1), Pyr could be more concentrated in sediment pore-water (up to 30 $\mu\text{g/L}$, according to Sverdrup et
21 al.'s (2002) calculation method), thus leading to higher exposure concentrations when fish embryos
22 develop at the surface or in the sediment. Additionally, other studies suggested that particle-bound PAHs
23 could also contribute to the toxic effects acting as a secondary source of contamination in embryos
24 exposed to contaminated sediments (Hollert et al. 2003; Kocan et al. 1996). Consequently, it seems
25 relevant to specify Pyr concentrations in sediment when establishing toxicity threshold values using a
26 sediment-contact bioassay. The present study thus revealed the relatively low acute toxicity of Pyr in

1 medaka embryos with a lethality threshold equal to 2.8 $\mu\text{g/g}$ dw in the sediment and 3 $\mu\text{g/L}$ of Pyr in the
2 water column.

3

4 3.3. *Pyr and MePyr impact in ovo development of medaka embryos*

5 Hatching success and time to hatch of embryos was recorded over a 11 dpf-period (Table 5). However not
6 significant, a concentration-dependent decrease of hatching success was observed following Pyr
7 exposure, down to 71 % for the C2 treatment. The mean time to hatch in Pyr exposed treatments was
8 consistently delayed in a concentration-dependent way although not significantly different from the
9 control treatment ($p>0.05$ according to ANOVA results followed by Tukey's post-hoc test; Table 5). Only
10 46 % of the embryos exposed to MePyr-C1 concentration hatched during the 11 dpf-period which
11 resulted in a mean time to hatch of 10.8 dpf for this treatment (Table 5). This significant ($p<0.05$)
12 impairment of the hatching success and the time to hatch in comparison to the control indicates that
13 medaka embryonic development was noticeably delayed/halted by MePyr at concentrations of 202 ng/g
14 dw in sediment and up to 98 $\mu\text{g/L}$ in the water column. Surprisingly, exposure to the highest
15 concentration of MePyr did not noticeably impact hatching success, It could be interpreted as a hormesitic
16 effect of MePyr exposure on hatching events, however, further experiments are needed using a wider
17 concentration range to conclude.

18 Medaka *in ovo* growth was evaluated through biometric measurements acquisition on newly hatched
19 larvae. Results showed a concentration-dependent reduction of larvae total body length following
20 exposure to both Pyr and MePyr (Fig. 1). On average, larvae exposed to C2 treatments were significantly
21 4%- and 5%-shorter than control larvae in Pyr and MePyr treatments, respectively ($p<0.02$ for Pyr and
22 $p<0.01$ for MePyr). Nevertheless, no significant effect of Pyr and MePyr was highlighted on the average
23 head size of newly hatched larvae (Fig. 1) or on head/body length ratio (Data not shown). As a result,
24 analysis of general *in ovo* development endpoints (including hatching success; time to hatch and larval
25 length) demonstrated that Pyr significantly altered medaka growth at the highest concentration (2.8 $\mu\text{g/g}$
26 dw of sediment and up to 3.1 $\mu\text{g/L}$ in the aqueous phase) and that MePyr impacted on embryonic

1 development from the lowest concentration (0.2 µg.g dw of sediment and up to 0.1 µg/L in the aqueous
2 phase).

3 A concentration-dependent inhibition of hatching success was reported in *P. maxima* embryos exposed to
4 Pyr from 1.25 to 40 µg/L (Mhadhbi et al. 2010). Hatching rate (but not time to hatch) was also
5 significantly reduced in *C. variegatus* embryos exposed to 150 µg/L of Pyr and 18 dpf-larvae showed a
6 reduced body length following exposure to 20 µg/L (Hendon et al. 2008). Likewise, Rhodes et al. (2005)
7 reported a strong reduction of hatching success and larval hatching length in medaka ELS exposed to an
8 environmental extract characterized by a high content of alkyl-PAH. The authors assumed that PAH
9 metabolites could impair energy metabolism resulting in an inhibition of hatching success since escaping
10 the chorion is highly energy dependent. It could also be suggested that PAH biotransformation is energy-
11 consuming, therefore limiting the energy store available for growth and hatching. These hypotheses are
12 thus coherent with reduced growth (i.e. larval length) of newly hatched medaka larvae following exposure
13 to environmental PAH extracts (Farwell et al. 2006; Rhodes et al. 2005) and to Pyr and MePyr in the
14 present work. However, other observations seem to indicate that the reduction of larva body size
15 following PAH exposure is linked to the decreased absorption of yolk due to cardiovascular dysfunction
16 (Billiard et al. 1999; see part 3.5 for details).

17

18 3.4. *Pyr and MePyr are highly teratogenic in medaka ELS*

19 Observations of developmental abnormalities in newly hatched medaka larvae following embryonic
20 exposure to spiked-sediments revealed the high teratogenic potency of both Pyr and MePyr. Indeed, a
21 significant ($p < 0.001$, when compared to the control) and dramatic increase of the percentage of abnormal
22 larvae, above 80% in all treatments, was observed from the lowest concentrations of Pyr and MePyr, (Fig.
23 2). Such high percentages of affected larvae have been reported in medaka ELS exposed to phenanthrene
24 and alkyl-phenanthrenes up to 1000 µg/L (Turcotte et al. 2011) and in *C. variegatus* larvae following
25 exposure to 150 µg/L of Pyr (Hendon et al. 2008).

26 In the present study, exposed larvae were significantly affected by cardiovascular impairments (at all
27 tested concentrations of Pyr and MePyr), spinal deformities (in the MePyr-C2 treatment), yolk sac

1 resorption defects (in both Pyr treatments and at the highest concentration of MePyr) and cranio-facial
2 abnormalities (in the Pyr-C2 treatment) in comparison to the control group (Fig. 2). Most of these
3 developmental abnormalities have already been described in fish ELS following exposure to Pyr (Li et al.
4 2011; Mhadhbi et al. 2010; Incardona et al. 2004). Interestingly, many of these defects have also been
5 observed in fish ELS exposed to TCDD (Antkiewicz et al. 2005; Hornung et al. 1999; Elonen et al. 1998)
6 and are often assimilated to blue sac disease (BSD) symptoms.

7

8 *3.5. Pyr and MePyr exposure strongly affects the cardiovascular system of embryos*

9 The cardiovascular system is known to be particularly sensitive to PAH exposure in fish ELS (Carls et al.
10 2008; Incardona et al. 2004). In the present study, Pyr and MePyr exposure did not significantly affect
11 cardiac activity in 6 dpf- and 7 dpf-embryos (Data not shown). However, PAH exposure induced a
12 significant concentration-dependent increase of the percentage of larvae showing cardiovascular injuries
13 in all exposed treatments in comparison to the control (Fig. 2). This kind of developmental defect was the
14 most frequently observed and affected 76% to 87% of the larvae exposed to Pyr and 61% to 81% of the
15 larvae from MePyr treatments. These impairments included abnormal positioning of the heart chambers
16 in relation to each other and to the cephalo-caudal axis as well as heart hypo-, hyper- or dystrophies.

17 Antkiewicz et al. (2005) also observed altered heart looping resulting in abnormal relative positioning of
18 the heart chambers, hypo- and dystrophy of the heart chambers in zebrafish embryos exposed to TCDD.
19 Heart morphology impairments were associated with cardiac dysfunction and/or vascular circulatory
20 defects, even if both cause and effect relationship remained possible (Antkiewicz et al. 2005).

21 Similarities between Pyr and TCDD cardiotoxicity have been already reported in zebrafish ELS
22 (Incardona et al. 2006; 2004). However, in this species, Pyr mainly induced anemia syndrome, reduced
23 vascular peripheral circulation, pericardial and yolk sac edemas (Incardona et al. 2004). Interestingly, *D.*
24 *rerio* embryos exposure to phenanthrene and benzo[a]anthracene caused the appearance of similar
25 cardiovascular pathologies as described by Antkiewicz et al. (2005) and the present study.

1 Although not significant, pericardial edemas were observed in larvae following exposure to both Pyr
2 treatments (9% to 13%) and MePyr-C2 concentration (8%; Fig. 2) with a p value close to significance
3 threshold for Pyr-C2 treatment ($p=0.06$). Similar observations were reported in *C. variegatus* larvae
4 exposed to 150 $\mu\text{g/L}$ of Pyr (Hendon et al. 2008). According to Billiard et al. (1999), the appearance of
5 edemas in rainbow trout and zebrafish larvae exposed to retene suggested leakiness of the endothelial
6 vasculature and may be an overt sign of cardiovascular dysfunction. Considering that the vitelline vessels
7 supplying yolk to the embryo are critical for absorption of yolk nutriment, loss of vascular integrity
8 could interfere with release of yolk metabolites to vitelline circulation (Billiard et al. 1999). In the present
9 study, reduced yolk absorption was significantly induced in a concentration-dependent way in newly
10 hatched larvae exposed to both Pyr treatments and to MePyr-C2 treatment, in comparison to the control
11 group ($p<0.05$; Fig. 2). As described above, these effects were concomitant to a decrease of larval body
12 length and cardiovascular injuries in larvae exposed to Pyr and MePyr C2-treatments. Moreover,
13 cardiovascular dysfunction has been linked with decreased absorption of the yolk sac in medaka ELS
14 exposed to TCDD (Elonen et al. 1998). All these observations suggest that Pyr and MePyr-induced
15 inhibition of growth is the consequence of yolk malabsorption in exposed larvae, as a result of
16 cardiovascular dysfunctioning, as previously suggested for TCDD (Antkiewicz et al. 2005; Elonen et al.
17 1998). The reduced vascular peripheral circulation observed in *D. rerio* early larval stage exposed to Pyr
18 (Incardona et al. 2006, 2004) seems to corroborate this hypothesis.

19

20 3.6. *Skeletal deformities induction following embryonic exposure to Pyr and MePyr*

21 Although cardiovascular injuries were the main developmental abnormalities induced by Pyr and MePyr
22 exposure, skeletal deformities were also recorded in exposed larvae at hatching. Indeed, 26% to 29% of
23 Pyr-exposed larvae and 40% to 47% of MePyr-exposed larvae ($p<0.05$ for MePyr-C2 treatment when
24 compared to control, according to one-way ANOVA followed by Tukey's post-hoc test) showed
25 abnormal spinal curvature including lordosis, kyphosis and C-shaped larvae (Fig. 2). Additionally, 19%
26 and 25% of the larvae exposed to Pyr C1- and C2 treatments respectively, showed craniofacial
27 abnormalities primarily in the lower jaw (Fig. 2, $p=0.053$ and $p<0.05$ respectively). Similar

1 developmental impairments were also noticeable in 23% of larvae from MePyr C1-treatment ($p=0.053$ in
2 comparison to control; Fig. 2).

3 Comparable spinal and jaw deformities have been reported in several fish larvae following exposure to
4 Pyr (Li et al. 2011; Hendon et al. 2008; Incardona et al. 2006). Such developmental defects were also
5 induced in fish ELS exposed to TCDD (Teraoka et al. 2006, 2002; Elonen et al. 1998). The concomitant
6 induction of CYP1A (at the protein and mRNA levels) and occurrence of skeletal defects in *C. variegatus*
7 and *D. rerio* larvae suggested that CYP1A pathway is involved in the development of spinal deformities
8 in Pyr-exposed organisms (Hendon et al. 2008; Incardona et al. 2006). Moreover, spinal deformities
9 (mainly kyphosis or lordosis) were reported in Pacific herring (*Clupea pallasii*) larvae following exposure
10 to weathered crude oil (Carls et al. 1999). The authors also noticed that deformed larvae showed
11 difficulties to swim normally and that spinal curvature was the most important predictor of swimming
12 ability. As reduced swimming ability may also impact upon prey capture capacity or predator escape
13 performance, these observations suggested that additional mortality could occur in heavily affected
14 individuals after complete resorption of yolk reserves.

15 Wnt-1 signaling pathway is highly implicated in embryogenesis and morphogenesis through cell
16 differentiation and proliferation control (L'Allemain 2006). In particular, Wnt signaling (including Wnt-1
17 members) is involved in the dorso-ventral patterning of the spinal cord, promoting dorsal identities in
18 combination to Sonic hedgehog (Shh) pathway which induces ventral ones (Ulloa and Marti 2010).
19 Several lines of evidence indicate that antagonist interactions between these two signaling pathways,
20 highly conserved through evolution, would define the dorso-ventral patterning of the developing central
21 nervous system and direct myotome formation (Ulloa and Marti 2010; Münsterberg et al. 1995). In the
22 present study, *wnt1* transcription level was significantly increased in comparison to control in medaka
23 larvae following exposure to Pyr-C1 treatment ($p<0.05$) and both MePyr concentrations ($p<0.01$, Table
24 6). Supporting these observations, Fairbairn et al. (2012) reported that the exposure of *D. rerio* embryos
25 to phenanthrene and fluorene induced morphological abnormalities resulting from the disruption of
26 embryonic axis determination and a concomitant increase in the levels of nuclear β -catenin throughout the
27 embryo (suggesting the aberrant activation of Wnt/ β -catenin pathway). Similar observations were made in

1 sea urchin (*Lytechinus anemesis*) embryos exposed to creosote and PAHs (Pillai et al. 2003). According
2 to these studies, it seems that PAHs disrupt dorsal–ventral axis determination in the embryos via the
3 Wnt/ β -catenin signaling pathway (Fairbairn et al. 2012). It is thus possible that some of the spinal
4 deformities observed in the present study following exposure of medaka embryos to Pyr and MePyr could
5 result from Wnt signaling disturbance during somitogenesis as suggested for Cd (Chow and Chen 2003).
6 Similarly, exposure of *S. marmoratus* embryos to benzo[a]pyrene resulted in spinal and cranio-facial
7 deformities (including lower jaw deformities) in association to the inhibition of *shh* transcription levels
8 (He et al. 2011). Likewise, *D. rerio* embryos exposure to TCDD caused lower jaw deformities and the
9 *ahr2*-dependent down-regulation of *shh* genes transcription (Teraoka et al. 2006). The authors of these
10 studies concluded that craniofacial deformities induced by benzo[a]pyrene and TCDD could be the result
11 of Shh signaling pathway impairment, leading to a failure of cell proliferation (He et al. 2011; Teraoka et
12 al. 2006). Considering the antagonist interactions existing between Wnt and Shh pathways, it is possible
13 that some spinal and craniofacial deformities observed in the present study following medaka embryos
14 exposure to Pyr and MePyr are the result of Wnt signaling pathway disruption, as suggested by the
15 significant increase of *wnt1* transcription level (Table 6). Moreover, recent genomic analysis performed in
16 a zebrafish tissue regeneration model revealed functional cross talk between AhR and the Wnt/ β -catenin
17 signal transduction pathway (Lijoy et al. 2009). It thus can be hypothesized that the AhR pathway is
18 involved in Wnt signaling disruption as Shh pathway deregulation was shown to be AhR-dependent
19 following TCDD exposure (Teraoka et al. 2006).

20

21 3.7. Impact of Pyr and MePyr exposure on retinoid metabolism genes transcription

22 The impact of Pyr and MePyr exposure on retinoid metabolism was investigated through the analysis of
23 retinoic acid receptors (*rara1*, *raryl* and *rxra1*) and *raldh2* transcription levels. In 7 dpf-embryos exposed
24 to Pyr-C2 treatment, *rxra1* transcription level tended to increase in comparison to control treatment ($p=$
25 0.07, Table 6). Similarly, exposure to MePyr-C2 concentration significantly stimulated *rara1*
26 transcription in medaka embryos ($p<0.05$, according to *t* test when compared to control). These
27 modulations of retinoic acid receptor transcripts were simultaneous to *cypla* transcription induction

1 ($p < 0.05$, Table 6). The *rara1* transcription levels were still overexpressed when compared to control in
2 larvae exposed to both MePyr treatments ($p < 0.02$) and *rxra1* transcripts level in larvae was close to
3 significant threshold ($p = 0.08$) when compared to control in MePyr-C1 treatment. Moreover, *raldh2* gene
4 transcripts were also significantly up-regulated in newly hatched larvae exposed to the highest
5 concentration of MePyr. If also reflected at the protein level, the modulations of these genes could
6 indicate an increased transformation of retinoid reserves or retinol into retinoic acid and the activation of
7 the RXR/RAR pathway. It is consistent with the reduction of retinoid stores correlated to CYP1A or
8 EROD induction observed in fish exposed to PAHs (Arcand-Hoy and Metcalfe 1999; Besselink et al.
9 1998). Furthermore, a concentration-dependent increase of all-*trans*-retinoic acids concentration as well
10 as CYP1A1 activity and *cyp1a1* mRNA level were observed in TCDD-exposed rats, suggesting that
11 CYP1A pathway is involved in retinoic acid synthesis induced by dioxin (Schmidt et al. 2003). All these
12 observations suggest that, as for TCDD, the AhR/CYP1A pathway is involved in retinoid metabolism
13 deregulation induced by Pyr and MePyr. Molecular and physiological interactions between retinoid
14 signaling and AhR pathways have been investigated in medaka embryos (Hayashida et al. 2004). The
15 study demonstrated that both retinoic acid and its receptors RXR/RAR were required for AhR mRNA
16 expression. In particular, the study evidenced that (1) retinoic acid excess and activation of RXR/RAR
17 receptors led to *ahr* transcription up-regulation; (2) retinoic acid excess and AhR activation resulted in
18 vascular damage and body-axis malformations. Consequently, it is possible that retinoid metabolism and
19 signaling pathway deregulation following Pyr and MePyr exposure is involved in the appearance of at
20 least a part of skeletal and cardiovascular deformities in exposed fish ELS. However, other investigations
21 are needed to demonstrate if retinoid system disruption plays a critical role in Pyr and MePyr
22 teratogenicity.

23

24 3.8. *Pyr and MePyr exposure modulates transcription of genes involved in mitochondrial* 25 *metabolism*

26 Gene transcription analysis performed on embryos exposed to Pyr-C2 and both MePyr treatments
27 revealed a significant induction of *coxI* (cytochrome C oxidase subunit I) gene transcription ($p < 0.05$,

1 Table 6). *CoxI* gene product is a mitochondria encoded subunit of complex IV of the mitochondrial
2 respiratory chain. Significant increase of *coxI* transcripts level has been already reported in the kidney and
3 the liver of *O. mykiss* juveniles exposed to Pyr (Krasnov et al. 2005). The stimulation of its transcription
4 could be interpreted as a compensatory response of embryos to exposure to Pyr and MePyr. Indeed, as
5 COX is considered as the rate-limiting step for mitochondrial respiration, *coxI* transcripts overexpression
6 could indicate an attempt of the organism to restore a decrease in mitochondrial activity (i.e. ATP
7 synthesis) and to efficiently consume O₂, thus limiting ROS overproduction (Achard-Joris et al. 2006).
8 Considering that biologically activated PAHs could disrupt oxidative phosphorylation which may
9 interrupt the mitochondrial electron transport chain, a reduction in available ATP and subsequent energy
10 reserves have been proposed as a possible mechanism to explain the decreased hatching success in
11 medaka ELS exposed to PAH and alkyl-PAH (Rhodes et al. 2005). A depletion of available energy stores
12 during embryonic development could thus also be responsible for the decrease of larval growth (in Pyr-
13 and MePyr-C2 treatments) as well as the reduced and delayed hatching events (in MePyr-C1 treatment)
14 observed in the present study. However, it remains unclear if the impact of these PAHs on the energy
15 reserves is the result of an impairment of the mitochondrial electron transport chain functioning and/or of
16 an important allocation of available energy to biotransformation of xenobiotics. This latter hypothesis
17 seems to be supported by the concomitant overexpression of *cyp1a* and *coxI* genes transcripts in medaka
18 embryos exposed to the highest concentration of Pyr and MePyr (Table 6).

19

20 3.9. Induction of DNA damage by Pyr and MePyr

21 The potential induction of DNA damage by Pyr and MePyr was evaluated in 2 dph-larvae using the comet
22 assay. DNA strand breaks were not significantly induced in comparison to the control ($p>0.05$), although
23 a concentration-dependent increase trend of the percentage of tail DNA was observed (Fig. 3).
24 Conversely, the percentage of hedgehog cells (i.e. those with heavily DNA damaged nuclei) significantly
25 increased in larvae from Pyr-C1 and MePyr-C2 treatments when compared to the control ($p<0.01$, Fig. 3).
26 Moreover, *p53* transcripts were significantly overexpressed in larvae exposed to the two concentrations of
27 MePyr ($p<0.05$, Table 6). If reflected at the protein level, *p53* induction could result in cell cycle arrest

1 before the S phase to allow repair of DNA damage prior to DNA replication or the induction of apoptosis
2 in cases where DNA damage is too severe to be properly repaired (Basu and Haldar 1998). DNA damage
3 could be the result of ROS overproduction as suggested by *coxI* transcription induction (see part 3.8).
4 This hypothesis is supported by *ogg1* gene expression whose encoded protein is involved in the base
5 excision repair mechanism in the case of oxidative lesions of DNA. Indeed *ogg1* transcripts were
6 significantly up-regulated in larvae following exposure to MePyr-C1 treatments ($p<0.05$) and close to the
7 significant threshold ($p=0.05$ in comparison to the control; Table 6) in medaka larvae exposed to MePyr-
8 C2 concentrations. However, no significant induction of the antioxidant *sod(Mn)* and *sod(Cu/Zn)* gene
9 transcripts was noticeable to confirm ROS overproduction following exposure to Pyr and MePyr ($p>0.05$
10 in comparison to the control; Table 6).

11 In summary, the present study demonstrated (1) no significant induction of DNA strand breaks (illustrated
12 by the percentage of tail DNA) by either Pyr or MePyr; (2) a significant increase in the percentage of
13 hedgehog cells by Pyr and MePyr; and (3) *p53* and *ogg1* transcription levels induction following MePyr
14 exposure. Further investigations are needed to conclude on the impact of Pyr exposure on DNA integrity
15 in medaka ELS. However, we could hypothesize that MePyr exposure induces oxidative DNA damage
16 that is fully repaired by DNA repair enzymes (such as Ogg1), except in the case of heavily DNA damaged
17 cells (i.e. hedgehog cells) in which apoptosis could be initiated through the *p53*-pathway. This latter
18 hypothesis is supported by the observation of cell death in the brain and the spinal cord of *D. rerio*
19 embryos exposed to Pyr (Incardona et al. 2004). Apoptotic cell death was also reported in medaka
20 embryonic vasculature following TCDD exposure (Cantrell et al. 1998).

21 Interestingly, *p53* transcription overexpression following MePyr exposure was simultaneous to *wnt1*
22 transcription level induction (Table 6). There is little evidence to suggest that an abnormal activation of
23 the Wnt pathway (including the *wnt1* gene) could deregulate the normal ontogenesis process by affecting
24 cell differentiation (L'Allemain 2006). Moreover, aberrant Wnt signaling has been reported to be
25 involved in some development of cancers which most likely results from inappropriate gene activation
26 mediated by stabilized β -catenin (Polakis 2000; L'Allemain 2006). Recently, an anti-differentiation
27 function of *p53* through direct regulation of the Wnt signaling pathway was highlighted in mouse

1 embryonic stem cells (Lee et al. 2010). The authors concluded that p53 could become ‘tumorigenic’ if the
2 induction of Wnt ligands by p53 is inherited aberrantly by the progeny cells of embryonic stem cells.
3 Further specific studies on the implication of p53 and Wnt pathways in ontogenesis mechanisms of fish
4 embryos could bring new insight into the potential carcinogenicity of MePyr.

5

6 3.10. Implication of CYP1A pathway in Pyr and MePyr toxicity

7 The overall toxic effects of Pyr and MePyr in medaka ELS highlighted in the present study overlapped
8 considerably with those induced following TCDD exposure. Such similarities between Pyr and TCDD
9 toxicity have been already reported and suggest the involvement of the AhR/CYP1A metabolism pathway
10 (e.g., Hendon et al. 2008; Incardona et al. 2006, 2005).

11 Supporting this hypothesis, *cyp1a* transcription levels were significantly induced when compared to the
12 control in embryos exposed to the highest concentration of Pyr and MePyr ($p<0.05$, Table 6). *Cyp1a*
13 induction factors were moderate with values equal to 1.8 and 2.5 for Pyr and MePyr respectively, which
14 seems to be in agreement with their relative AhR-agonist potency in comparison with other strong AhR-
15 agonists such as TCDD or benzo[a]pyrene (Barron et al. 2004). *Cyp1a* transcript induction was still
16 noticeable in larvae from the Pyr-C2 treatment with a p -value close to the significant threshold in
17 comparison to the control ($p=0.076$).

18 CYP1A induction was reported throughout the vascular endothelium and the liver of *D. rerio* larvae
19 exposed to Pyr (Incardona et al. 2006). The induction of this enzyme preceded and accompanied the
20 emergence of developmental defects (including dorsal curvature, anemia, edemas, cell death and reduced
21 peripheral circulation) and systemic toxicity induced by Pyr exposure. Moreover, Pyr-induced mortality
22 was time-coincident with alterations in the appearance of the liver (Incardona et al. 2006). Gene silencing
23 with *ahr2* antisense morpholino largely prevented teratogenicity and lethal effects of Pyr in *D. rerio*
24 embryos and also markedly reduced the levels of CYP1A induction (Incardona et al. 2005). Similarly, an
25 *ahr1a* morpholino injection partially improved embryos resistance to Pyr toxicity, including normal liver
26 appearance and absence of pericardial edemas and neural tube cell death. *Cyp1a* knockdown also reduced
27 defects in embryos exposed to Pyr (Incardona et al. 2005). Moreover, Pyr-induced lethality was prevented

1 or markedly delayed by several hours in *cyp1a*, *ahr1* and *ahr2* morphants (Incardona et al. 2006, 2005).
2 Evidence gathered in these studies indicates that: (1) the toxic effects of Pyr are clearly AhR/CYP1A-
3 dependent; (2) the metabolism of Pyr by CYP1A in the vascular endothelium probably contributes to the
4 emergence of some teratogenic effects, in particular dorsal curvature; (3) hepatic CYP1A activity strongly
5 contributes to the systemic toxicity of Pyr, probably through the formation of Pyr toxic metabolite(s)
6 (Incardona et al. 2006, 2005).

7 This last assumption is supported by the positive correlation between DNA damage and the level of
8 biliary hydroxylated metabolites (mainly 1-OH-Pyr) observed in juvenile flatfish (*Solea solea*) exposed to
9 a mixture of benzo[a]pyrene, fluoranthene and pyrene (Wessel et al. 2010). The authors concluded that
10 PAH-induced DNA strand breaks probably result from the induction of oxidative stress by pro-oxidant
11 PAH metabolites and the excision activity of DNA repair enzymes. Furthermore, it has been
12 demonstrated that ROS formation could occur during the CYP1A catalytic cycle or in a latter step of PAH
13 biotransformation, during quinones redox cycle (Baulig et al. 2003; Morel et al. 1999). To conclude, it
14 seems that the AhR/CYP1A pathway is strongly involved in Pyr and MePyr toxic effects as it could
15 contribute, directly or indirectly, to ROS overproduction, genotoxicity, teratogenicity and finally systemic
16 toxicity of both compounds.

17

18 **4. Conclusion**

19 The effects of environmental concentrations of Pyr and MePyr were investigated in medaka ELS exposed
20 by sediment-contact. Both compounds strongly impacted medaka embryonic development, with the
21 cardiovascular system as primary target of toxicity. Developmental abnormalities also included spinal and
22 jaw deformities observed in medaka larvae following Pyr and MePyr exposure considerably overlapped
23 those induced by TCDD. In addition, the transcription level of several genes involved in mitochondrial
24 metabolism, cell differentiation and proliferation control, cell cycle arrest and retinoid metabolism was
25 significantly deregulated. Significant increase in *cyp1a* transcript levels suggested that AhR/CYP1A
26 pathway is involved in several toxic effects induced by Pyr and MePyr exposure. Overall, the results

1 showed that exposure to the tested environmental concentrations of Pyr and MePyr in sediment has a
2 clear impact on the development of medaka embryos resulting in noticeable effects at both phenotypical
3 and transcriptional levels. These observations highlight the ecotoxicological risk presented by these
4 compounds in PAH impacted areas. The present study also showed new aspects of the mode of action of
5 Pyr and, for the first time, the effects of MePyr were investigated in fish ELS. With regards to the
6 efficiency of the MELAc and the relevance of the route of exposure, this approach could be applied to the
7 investigation of the toxico-kinetics and toxico-dynamics of various chemicals including particle-bound
8 pollutants in fish ELS.

9

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31

32

1

2 **Figure captions**

3

4 **Fig. 1** Total body length (grey bars) and head size (white bars) of medaka larvae at hatching following
5 exposure to Pyr- or MePyr-spiked sediments. Values represent the mean response (\pm SD) from three
6 replicates. Different letters indicate significant differences between treatments using one-way ANOVA
7 followed by Tukey's post-hoc test ($p < 0.05$; $df = 4$)

8

9 **Fig. 2** Developmental abnormalities in newly hatched larvae following Pyr or MePyr exposure. Values
10 represent the mean response (\pm SD) for three replicates. Statistical analysis was performed on each
11 endpoint independently. Asterisks indicate significant differences in comparison with the control
12 according to the results of one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$; $df = 4$) or non-
13 parametric Kruskal-Wallis ANOVA followed by Bonferroni-Dunn's post-hoc test ($p < 0.05$; $df = 4$) for
14 craniofacial deformities

15

16 **Fig. 3** DNA damage in 2 dph-larvae following exposure of medaka embryos to Pyr- or MePyr-spiked
17 sediments assessed with the comet assay. Values represent the mean response (\pm SD) from three
18 replicates for the percentage of Tail DNA (grey bars) and of hedgehog cells (white bars). Different letters
19 indicate significant differences between treatments using one-way ANOVA followed by Tukey's post-
20 hoc test ($p < 0.05$; $df = 4$)

21

22

Fig. 1

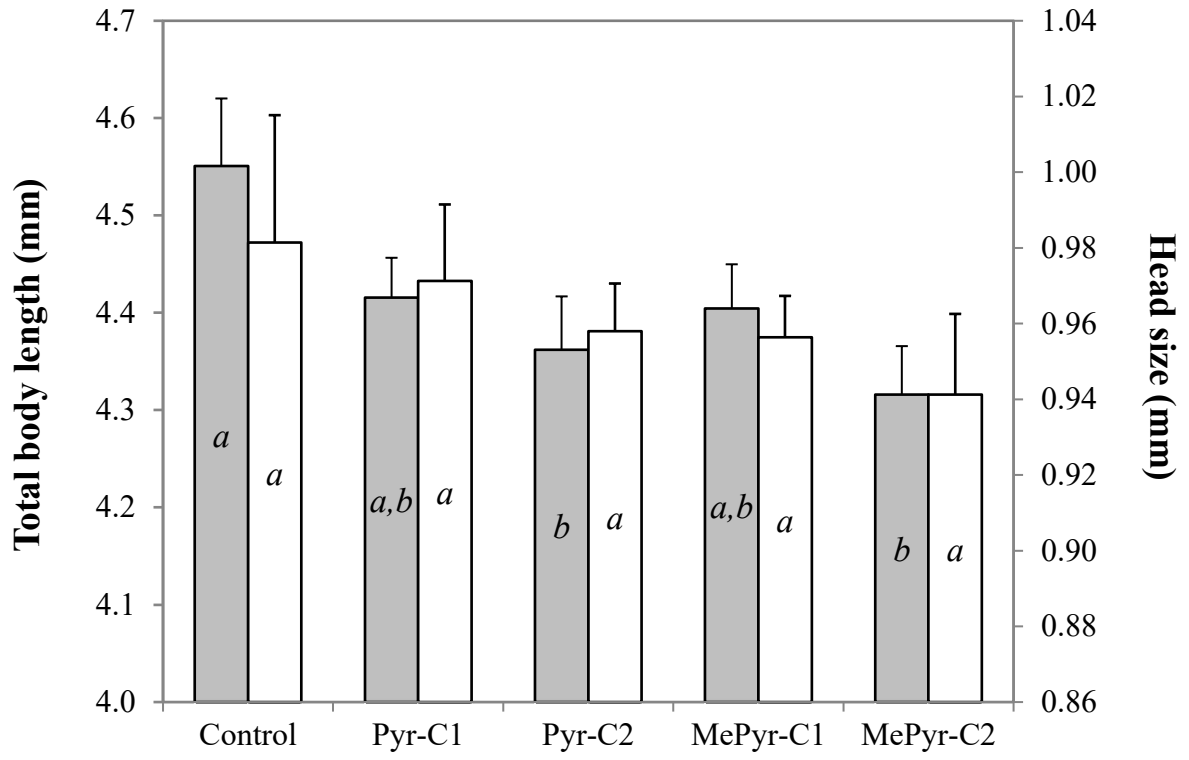


Fig. 2

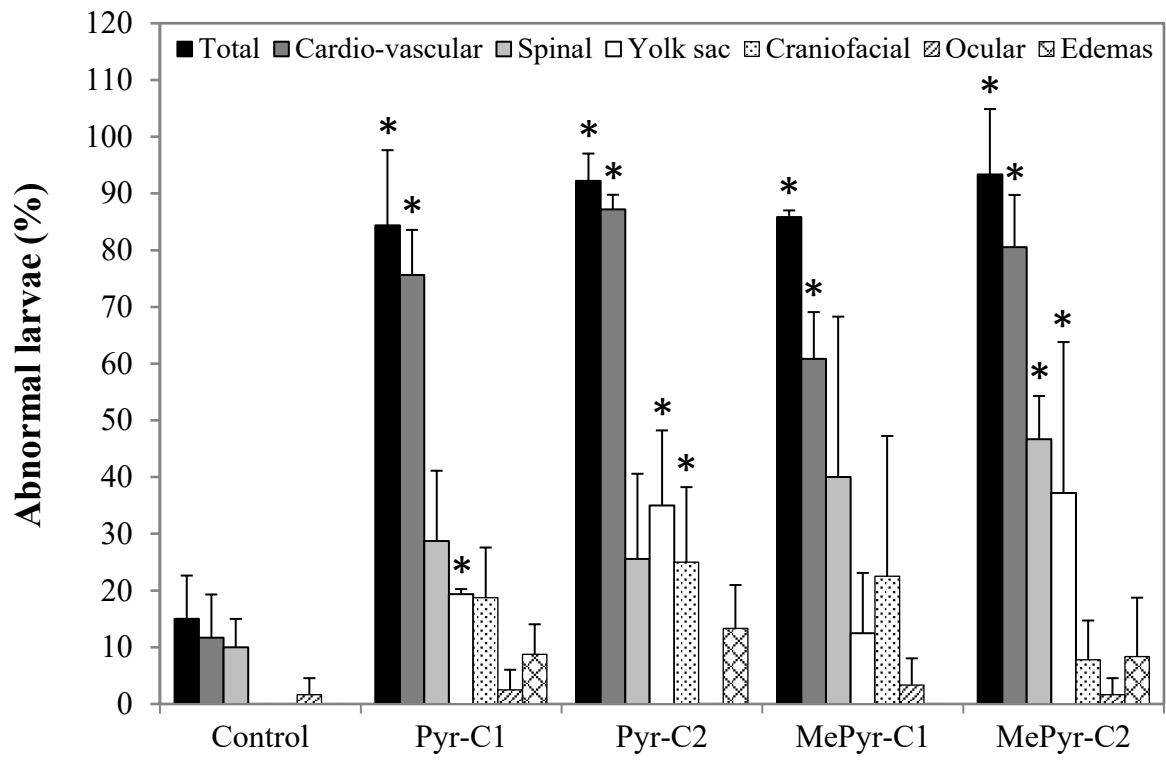
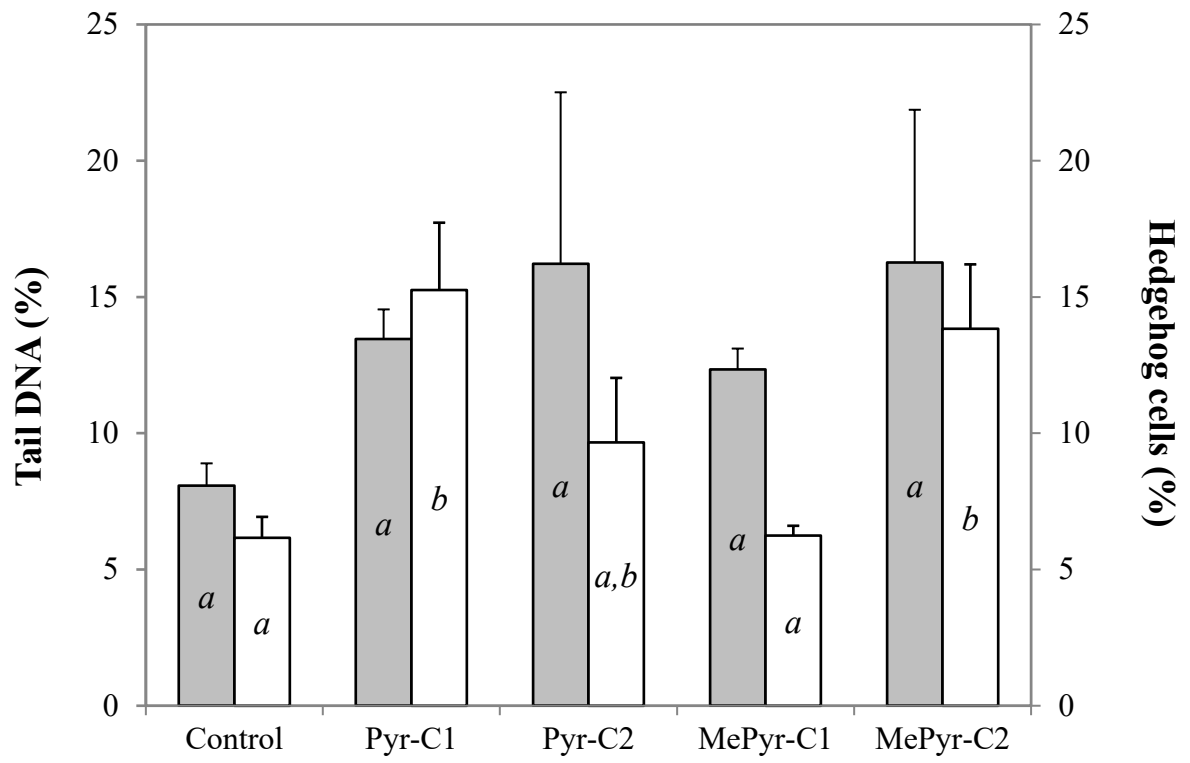


Fig. 3



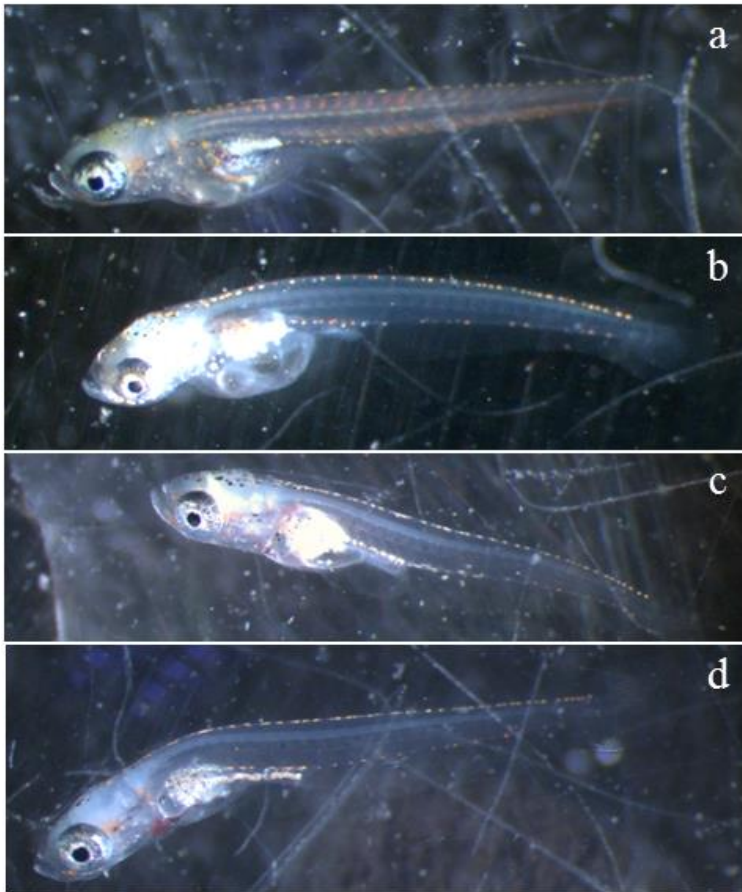


Fig. X. Examples of spinal deformities observed in newly hatched larvae following Pyr or MePyr exposure. (a) normal larvae from control treatment; (b) C-shaped larvae following Pyr-C1 exposure; larvae showing lordosis- (c) and kyphosis (d) -type deformities following MePyr-C2 exposure.

Table 1. Sediment quality guidelines for Pyr in coastal and freshwater sediments and examples of Pyr contamination in sediments for moderately and highly impacted sites

| Sediment quality guideline/Sampling site | Pyrene concentration (ng/g dw) | Reference |
|--|--------------------------------|----------------------------|
| ERL (Effect range low) | 665 | Long et al., 1995 |
| ERM (Effect range medium) | 2,600 | Long et al., 1995 |
| TEL (Threshold effects level) | 153 | MacDonald et al., 1996 |
| PEL (Probable effect level) | 1,398 | MacDonald et al., 1996 |
| Consensus-based TEC (Threshold effect concentration) | 195 | MacDonald et al., 2000 |
| Consensus-based PEC (Probable effect concentration) | 1,520 | MacDonald et al., 2000 |
| Island End River (Boston harbor, USA) | 10,548 – 42,600 ^a | Wang et al., 2001 |
| Fort Point Channel (Boston harbor, USA) | 1,258 – 10,042 ^a | Wang et al., 2001 |
| Mystic River (Boston harbor, USA) | 1,694 – 7,045 ^a | Wang et al., 2001 |
| Industrial discharge area (Cantabrian sea, Spain) | 3,723 | Sanchez-Avila et al., 2013 |
| San Vicente de la Barqueira (Cantabrian Sea, Spain) | 319 | Sanchez-Avila et al., 2013 |
| Urdaibai (Cantabrian Sea, Spain) | 225 | Sanchez-Avila et al., 2013 |
| Beerkanaal (port of Rotterdam, the Netherlands) | 1,130 | Heister et al., 2013 |
| Beneden Merwede River (port of Rotterdam, the Netherlands) | 200 | Heister et al., 2013 |
| Mecklenburg Bight dump site (western Baltic Sea, Germany) | 224 – 814 ^b | Liehr et al., 2013 |
| Oissel (Seine estuary, France) | 1,418 | Cachot et al., 2006 |
| La Bouille (Seine estuary, France) | 361 - 532 | Cachot et al., 2006 |
| Le Havre (Seine estuary, France) | 215 | Cachot et al., 2006 |

a, concentration range measured in different grain size fractions of sediment; b, concentration range measured between 2002 and 2004 at the same sampling site.

Table 2. Physico-chemical characteristics of the reference sediment (freeze-dried)

| Yville-sur-Seine sediment (Seine-Maritime, Haute-Normandie, France) | | | | | | | | | | |
|---|-----|-----|-----|------|------|------|------|--------------|------|---------|
| Particulate organic carbon | | | | | | | | | | 0.14 % |
| Dissolved ammonia (NH ₄ ⁺) ^a | | | | | | | | | | 40.7 μM |
| Dissolved sulfur (H ₂ S) ^a | | | | | | | | | | 17.6 μM |
| <i>Grain size distribution</i> | | | | | | | | | | |
| 10 th percentile diameter | | | | | | | | | | 41.3 μm |
| 50 th percentile diameter | | | | | | | | | | 230 μm |
| 90 th percentile diameter | | | | | | | | | | 391 μm |
| ≤ 65 μm fraction | | | | | | | | | | 15.8 % |
| <i>Trace metals levels (μg/g dw)</i> | | | | | | | | | | |
| Co | Mn | Ni | Zn | Cr | As | Ag | Pb | Cd | Cu | |
| 0.74 | 8.0 | 1.0 | 7.3 | 1.74 | 0.33 | 0.01 | 12.5 | 0.02 | 1.09 | |
| <i>Organic compounds levels (ng/g dw)</i> | | | | | | | | | | |
| Σ PAH ^b | | | | | | | | 16 | | |
| Σ PCB ^c | | | | | | | | 0.7 | | |
| Σ PBDE ^d | | | | | | | | Not detected | | |
| Σ OCP ^e | | | | | | | | 0.1 | | |

a, measurements performed on remoistened freeze-dried sediment

b, cumulative concentration of 21 analyzed polycyclic aromatic hydrocarbon compounds

c, cumulative concentration of 8 analyzed polychlorobiphenyl congeners

d, cumulative concentration of 4 analyzed polybrominated diphenylethers

e, cumulative concentration of 16 analyzed organochlorine pesticides

Table 3. Accession number and sequence of primer pairs for the thirteen *O. latipes* genes used in the present study

| Gene | Function | Accession number (EMBL or GenBank) | Primers sequences |
|-------------------|--|---------------------------------------|---|
| <i>β-actin</i> | Cytoskeletal gene (housekeeping gene) | S74868 | GTGACCCACACAGTGCC ^a GCGACGTAGCACAGCTTC ^b |
| <i>rpl7</i> | Ribosomal protein L7 (housekeeping gene) | NM_001104870 | AACGTGGCTACGGCAG ^a CGAGGTGACGACAGCTT ^b |
| <i>coxI</i> | Cytochrome c oxidase subunit I (complex IV of the mitochondrial respiratory channel) | NC_004387 (gene ID 805432) | TTCCCCAACACTTCTTAGGC ^a TGTGGCTGTTAGTTCGACTGA ^b |
| <i>p53</i> | Tumor suppressor gene P53 | AF212997 | TCTGGCACTGCAAAGTCTGT ^a CCTCGTTTTGGTGGTGGG ^b |
| <i>cyp1a</i> | Cytochrome P450 1A | AY297923 | CTCCCTTTCACAATTCCACACT ^a TGCAACGCCGCTTTC ^b |
| <i>wnt1</i> | Wingless integration site 1 (cell proliferation and somitogenesis) | AJ243208 | CCGCTTTGACGGAGCAT ^a TTGAACCCACGCCACAGC ^b |
| <i>sod(Mn)</i> | Mitochondrial Fe/Mn superoxide dismutase | ENSORLG00000013261 | ATGGCTGGGCTATGACAAAG ^a TGGCTATCTGAAGACGCTCAC ^b |
| <i>sod(Cu/Zn)</i> | Cytosolic Cu/Zn superoxide dismutase | ENSORLG00000008041 | GGGAAATGTGACCGCAGG ^a GCCAAACGCGCTCCAG ^b |
| <i>bax</i> | Bcl-2 associated X protein gene | ENSORLG0000000456 | TCTTCGCTCAGTCCCTCC ^a GCCAACGTCTGCCAGCCA ^b |
| <i>ogg1</i> | 8-oxoguanine glycosylase 1 gene (BER family) | ENSORLG00000010758 | CTCGTATTCAGGGCATGGT ^a ACCCGTGGCTGTCTAAG ^b |
| <i>raldh2</i> | Retinaldehyde dehydrogenase type 2 | NM_001104821 | GCCGCTCACCTGTCTCTAT ^a TCCCTGCCGCTCTTG ^b |
| <i>rara1</i> | Retinoic acid receptor alpha 1 | EF546452 | GCATCATCAAGACGGTGGAG ^a GGCGAAAGCGAAAACCAGG ^b |
| <i>rarg1</i> | Retinoic acid receptor gamma 1 | EF546454 | CTCGTGTCTACAAACCCTGC ^a ATGCCGACCTCGAAGC ^b |
| <i>rxra1</i> | Retinoid X receptor alpha 1 | EF537036 | GGGTGCCTTCGAGCCA ^a CCGGTAACCGCAGCAACAGT ^b |

a, upstream primer; b, forward primer

Table 4. Pyr and MePyr concentrations in spiked sediments and ERS buffer at T0- (beginning of exposure), T7- (7 dpf) and T9- (9 dpf) sampling times.

| Condition | Pyrene | | | Methylpyrene | | | Nominal concentration | Spiking efficiency (%)* |
|--|--------|-------|-------|--------------|-------|-------|-----------------------|-------------------------|
| | T0 | T7 | T9 | T0 | T7 | T9 | | |
| <i>Concentration in sediment (ng/g dw)</i> | | | | | | | | |
| Control | <0.1 | - | - | <0.1 | - | - | 0 | |
| Pyr-C1 | 250 | - | - | <0.1 | - | - | 300 | 82.8 |
| Pyr-C2 | 2,770 | - | - | <0.1 | - | - | 3,000 | 92.1 |
| MePyr-C1 | 62 | - | - | 202 | - | - | 300 | 69.7 |
| MePyr-C2 | <0.1 | - | - | 2,586 | - | - | 3,000 | 87.8 |
| <i>Concentration in ERS buffer (ng/L)</i> | | | | | | | | |
| Control | 1.48 | 12.5 | 3.17 | 0.16 | 0.39 | 3.50 | | |
| Pyr-C1 | - | 204 | 629 | - | 0.12 | 1.17 | | |
| Pyr-C2 | - | 2,488 | 3,125 | - | 0.19 | 1.10 | | |
| MePyr-C1 | - | 8.02 | 58.4 | - | 98.3 | 53.5 | | |
| MePyr-C2 | - | 9.56 | 5.21 | - | 1,698 | 1,831 | | |

* Spiking efficiency calculation was based on the exact amount of Pyr or MePyr introduced during the spiking procedure.

Table 5. Embryonic survival, hatching success and time to hatch following medaka embryos exposure to Pyr or MePyr-spiked sediments

| Condition | Embryonic survival (%) | Hatching success (%) | Time to hatch (dpf) |
|-----------|----------------------------|----------------------------|------------------------------|
| Control | 96.7 ± 2.24 ^a | 87.0 ± 8.74 ^a | 9.50 ± 0.26 ^a |
| Pyr-C1 | 94.0 ± 4.40 ^{a,b} | 77.2 ± 1.04 ^{a,b} | 9.82 ± 0.01 ^{a,b} |
| Pyr-C2 | 91.6 ± 1.33 ^b | 71.4 ± 14.4 ^{a,b} | 9.87 ± 0.57 ^{a,b} |
| MePyr-C1 | 96.0 ± 2.35 ^{a,b} | 46.1 ± 17.0 ^b | 10.8 ± 0.39 ^b |
| MePyr-C2 | 95.6 ± 2.58 ^{a,b} | 80.3 ± 12.1 ^{a,b} | 9.70 ± 0.1144 ^{a,b} |

Values represent the mean responses (\pm SD) from six replicates for embryonic survival and from three replicates for hatching success and time to hatch. For time to hatch calculations, the hatching day of unhatched embryos at the end of experiment was set at 12 dpf. Different letters indicate significant differences between treatments using one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$; degree of freedom (df) = 4).

Table 6. Variations in differential gene transcription levels as compared to control observed in medaka embryos (T7) and larvae (T9) after exposure to Pyr- or MePyr-spiked sediments

| | Mitochondrial metabolism | Xenobiotic metabolism | Oxidative stress defense | | Cell cycle arrest/apoptosis | | Development/embryogenesis | DNA repair | Retinoid metabolism | | | |
|-----------------------------|--------------------------|------------------------|--------------------------|-------------------|-----------------------------|------------|---------------------------|------------------------|---------------------|--------------|--------------|------------------------|
| | <i>cox I</i> | <i>cyp1a</i> | <i>sod(Mn)</i> | <i>sod(Cu/Zn)</i> | <i>p53</i> | <i>bax</i> | <i>wnt1</i> | <i>oggl</i> | <i>raldh2</i> | <i>rara1</i> | <i>rarg1</i> | <i>rxra1</i> |
| <i>Embryonic stage (T7)</i> | | | | | | | | | | | | |
| Pyr-C1 | 1.1 | 1.0 | 1.0 | 0.9 | 1.1 | 0.8 | 1.0 | 0.9 | 0.8 | 1.3 | 0.8 | 1.1 |
| Pyr-C2 | 1.5* | 1.8* | 1.1 | 1.0 | 1.2 | 1.4 | 1.8 | 1.2 | 0.8 | 1.5 | 1.0 | 2.0^a |
| MePyr-C1 | 1.4* | 1.1 | 1.1 | 1.0 | 1.4 | 0.9 | 0.8 | 1.0 | 0.7 | 1.4 | 0.8 | 1.5 |
| MePyr-C2 | 1.7* | 2.5* | 1.1 | 1.0 | 1.3 | 1.1 | 1.7 | 2.1 | 1.0 | 1.8* | 1.1 | 2.0 |
| <i>Larval stage (T9)</i> | | | | | | | | | | | | |
| Pyr-C1 | 0.9 | 1.7 | 1.1 | 0.8 | 1.4 | 0.9 | 2.6* | 1.1 | 1.4 | 1.5 | 1.0 | 1.2 |
| Pyr-C2 | 0.8 | 2.0^b | 0.9 | 0.8 | 1.3 | 0.9 | 1.6 | 1.3 | 1.0 | 1.4 | 0.9 | 1.2 |
| MePyr-C1 | 0.9 | 1.4 | 0.9 | 0.8 | 1.6* | 0.9 | 2.9* | 1.2* | 1.2 | 1.7* | 1.1 | 1.4^d |
| MePyr-C2 | 0.9 | 1.2 | 1.1 | 0.9 | 2.0* | 1.1 | 3.0* | 1.9^c | 1.4* | 2.0* | 1.2 | 1.5 |

Results are mentioned as control-relative gene induction (>1) or repression (<1) factors. The * sign indicates a significant difference in comparison to the control, according to *t* test for independent samples ($p < 0.05$; $df = 4$). a, $p = 0.069$; b, $p = 0.076$; c, $p = 0.054$; d, $p = 0.078$.