Parental trophic exposure to three aromatic fractions of polycyclic aromatic hydrocarbons in the zebrafish: Consequences for the offspring

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Abstract :

In recent decades, PAH emissions due to extensive anthropogenic activities have risen sharply causing considerable pollution of aquatic ecosystems. This pollution represents a threat for organisms, among them are fish. Consequently, prenatal stress can have important repercussions, and may impact survival and population recruitment. To investigate this point, eggs were collected from zebrafish exposed during 6 months by trophic route to three aromatic fractions from two different origins, pyrolytic (PY) and petrogenic (light (BAL) and heavy (HFO) fractions) sources. Chronic dietary exposure of the parents was performed at environmentally relevant concentrations (0.3 ×, 1 × and 3 ×; 1 × represents an environmental concentration measured in French estuary). In order to explore the consequences of parental exposure for the next first generation, toxic responses were studied in both embryos and larvae using a multiscale approach. Toxic effects were assessed by looking at hatching success, developmental abnormalities, photomotor response and heartbeat. The level of PAH metabolites and EROD activity in fish larvae were measured to assess exposure to PAHs. Egg production of parents was significantly reduced compared to the Control; hence little information was available for BAL and HFO offspring. The size of larvae from PY parents was found to increase despite a reduced yolk sac compared to Control larvae. Furthermore, a high level of behavioral stress was observed in larvae originating from parents exposed to three-fold the environmental concentration. The cardiac activity was reduced in a concentration-dependent manner for the PY exposure group. No effect was however observed on biotransformation markers (cyp1a, EROD), nor on the level of DNA damage for all PY, BAL and HFO offspring. The absence of significant differences in metabolite levels may indicate a potential early depuration of transferred compounds or no PAH-transmission. The disruptions observed at the individual level in the next generation could impact on the longer-term, surviving population

Highlights

► We examine the toxicity of organic pollutants in a temporal dynamic (multigenerational study). ► We examine the consequences of parental trophic exposure to the first offspring generation at different biological level with exposures at environmentally relevant concentrations. ► We highlight the alteration of two vital functions in fish: the swimming and cardiac performances. ► Increasing information in the next generation will increase the risk prediction. ► The multigenerational studies should be widely undertaken to evaluate the potential for exposed-population to maintain in the future.

Abbreviations

- BAL, Brut Arabian Light;
- EROD, ethoxyresorufin-O-deethylase;
- dpf, days post-fertilization;
- HFO, heavy fuel oil;
- HMW, High Molecular Weight;
- hpf, hours post-fertilization;
- LMW, Low Molecular Weight;
- PAHs, polycyclic aromatic hydrocarbons;
- PY, pyrolytic;
- PMR, PhotoMotor Response;
- RT-PCR, real-time polymerase chain reaction

Keywords : Polycyclic aromatic hydrocarbons, Parental transmission, Fish early-life stages, PhotoMotor Response, Cardiac performance, PAH metabolites, Genotoxicity

1. Introduction

In recent decades, increasing industrialization and urbanization has resulted in significant chemical pollution of the aquatic environment (Shen et al., 2013). Among organic pollutants, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and semi-persistent pollutants in the environment (Laflamme and Hites, 1978). They are lipophilic compounds with two or more fused benzene rings. PAHs can be divided into two groups: the Low-Molecular-Weight (LMW) PAHs

(PAHs with 2 or 3-benzenic rings) and the High-Molecular-Weight (HMW) PAHs (PAHs presenting more than 3-benzenic rings). PAHs are produced from three pathways: i) pyrolytic processes such as incomplete combustion of organic matter from natural or anthropogenic sources (pyrogenic origin), ii) diagenesis of sedimentary organic matter giving form to fossil fuel (petrogenic origin), iii) natural precursor transformations during early diagenesis processes (diagenetic origin) (Neff, 1979). Although they may be produced naturally, their predominant emission is from anthropogenic sources, including burning of fossil fuel, coal tar, wood, waste incineration, urban runoff, maritime transport discharges and petroleum spills (Neff, 1979). Depending on their origin, PAHs are generally present in complex mixtures that can be characterized by their molecular fingerprint. Indeed, a predominance of HMW compounds associated with the presence of less or non-alkylated PAHs is representative of a pyrolytic mixture. Conversely, a petrogenic mixture is characterized by a majority of LMW PAHs and the presence of methylated compounds (Abrajano et al., 2003; Sakari, 2012).

Due to their physico-chemical properties, most PAHs exhibit hydrophobic and lipophilic properties making them relatively insoluble in water. They have a high capacity to adsorb onto suspended particles or non-polar matrices which respectively favors their accumulation in sediments and in the lipid tissue of organisms. Fish have the ability to biotransform PAHs to more water-soluble metabolites, hence reducing bioaccumulation and transfer efficiency in higher levels of marine food chains (Meador et al., 1995). Nonetheless, benthic invertebrate preys represent important pathways of PAH exposure for demersal fish. Indeed, many invertebrate species such as mollusks can bioaccumulate high PAH concentrations in their fatty tissues (Peterson et al., 2003; Varanasi et al., 1985) due to the lower biotransformation capability (Bustamante et al., 2012; Lee et al., 1972; Palmork and Solbakken, 1981). PAH toxicity in fish can be directly linked to biotransformation by enzymatic systems such as those from cytochrome

P450, which metabolize PAHs into reactive metabolites (Meador et al., 1995; van der Oost et al., 2003). Some of the metabolites produced are more toxic than the parent compounds, presenting, for example, a high reactivity for the DNA leading to genotoxic effects (Le Dû-Lacoste et al., 2013; Wessel et al., 2012, 2010). However, DNA damage is not necessary linked to activation of enzymatic systems such as ethoxyresorufin-O-deethylase (EROD) activity. A case in point is fluoranthene which is not a potential inducer of EROD activity, while its produced-metabolites exhibit genotoxic effects (Wessel et al. 2012). Furthermore, PAH metabolization can also lead to the production of reactive oxygen species (ROS) which may induce an oxidative stress (Frenzilli et al., 2009).

In aquatic organisms, exposure to PAHs can thus lead to detrimental defects from a developmental, physiological and behavioral point of view (Bihanic et al. 2015; Incardona et al. 2011; 2004; Perrichon et al. 2014). PAHs' impact on reproductive success is particularly studied due to its consequence on population recruitment. Indeed, PAHs can act as endocrine disruptors that interfere and thus lead to adverse reproductive and developmental effects (Cooper and Kavlock 1997; Hawliczek et al. 2012; Monteiro et al. 2000a; Monteiro et al. 2000b). Previous studies showed a reduction of the plasma level of gonad hormones in fish after PAH exposure (Monteiro et al., 2000a; Thomas and Budiantara, 1995). Thomas and Budiantara (1995) highlighted a blockage of sexual maturation, impairments of ovarian recrudescence and induction of oocyte atresia in female Atlantic croaker (*Micropogonias undulatus*) following exposure to water-soluble fractions of diesel fuel and naphthalene. Similarly, a delay in gonad maturation has been reported in female English sole from sites with high concentrations of PAHs (Casillas et al., 1991; Johnson et al., 2002, 1988) as well as in female Atlantic croaker exposed in the laboratory to benzo[a]pyrene (Thomas, 1990). An exposure to a PAH-contaminated sediment has been shown to reduce spawning in sole (Johnson et al., 2002). PAHs can hence have an effect on the

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parents themselves but also on their offspring. The physiological condition of a female at the time of vitellogenesis was reported as a modulation source for the offspring generation. Prenatal stress (i.e. chemical contamination) could thus affect the optimal development of the early life stage in the future generation (Eriksen et al., 2006; Gagliano and McCormick, 2009; Mccormick, 1999). Uptake of pollutants can occur during ovogenesis and consequently accumulate in the vitellin reserve of the embryos. Relatively few studies reported potential parental transmission of PAHs in offspring generation. Following maternal exposure, the transfer of anthracene in the vitellus of fathead minnows eggs has been shown by Tilghman Hall and Oris (1991). More recently, Pollino et al. (2009) demonstrated a positive correlation between the occurrences of abnormal larvae in offspring with naphthalene-exposed female rainbowfish (*Melanotaenia fluviatilis*). The transfer of PAHs to the embryos was associated with mortality, hatching delay and growth alterations (Hose et al., 1981; Nye et al., 2007; Tilghman Hall and Oris, 1991).

This study aimed to explore the consequences of parental exposure (F_0) to PAHs for the next first offspring generation (F_1) under chronic dietary exposure conditions. Parents were chronically exposed during their whole lifecycle, through diet, to three aromatic fractions of pyrolytic and petrogenic origin, at concentrations which were relevant from an environmental point of view. This study focused on the early life stages of offspring. Multiscale approach was undertaken in order to assess a wide range of fish toxic response, using phenotypic markers (hatching success, developmental abnormalities), behavioral (PhotoMotor Responses), physiological (e.g. heartbeat) and cellular/subcellular markers (DNA damage, apoptosis, oxidative stress). In addition to these effect markers, the degree of exposure to PAHs was evaluated with the level of EROD activity, *cyp1a* induction and PAH-metabolites in offspring larvae.

2.   Materials and Methods

2.1.   Chemicals

The *Arabian light* crude oil (BAL 100) and the heavy fuel oil from the *Erika* fuel oils (No. 2) used for this study was provided by *Cedre* (Centre of Documentation, Research and Experimentation on Accidental Water Pollution, France). PAH spiking of food and chemical analysis were performed with dichloromethane solvent from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). The ethyl-4-aminobenzoate (benzocaïne) was purchased from Sigma-Aldrich (St Quentin Fallavier, France).

2.2.   Preparation of PAH-contaminated food

Four sizes of food were prepared (i.e. ≤ 125 µm, 125-315 µm, 315-500 µm, ≥ 500 µm) from ground commercial food INICIO Plus 0.5 mm (Biomar, France), in order to adapt the food to the fish morphology throughout the lifecycle (from larvae to adult stages). Each food lot was contaminated by the PAH aromatic fractions as described hereafter. The aromatic fractions were a pyrolytic fraction (PY) extracted from sediments collected in the highly polluted site of Oissel (Seine estuary, France) and two petrogenic fractions extracted from *Brut Arabian Light* oil (BAL) and heavy *Erika* fuel oil (HFO). Extraction and quantification of these aromatic fractions based on the 16 priority PAHs listed by US-EPA were performed according to procedures described in Budzinski et al. (1997) and Cachot et al. (2007). PAH and alkylated PAH concentrations were reported in Vignet et al. (2014) and given in Fig. 1 Supplementary data. Dry food was hence spiked with the three PAH fractions according to Vicquelin et al. (2011). Briefly, the amount of

food required for the experiment was mixed with dichloromethane (Solvent Control) and each tested aromatic extract in a round-bottom glass flask. Then, solvent was completely evaporated with a rotary evaporator (RV10 Basic VWR International) equipped with a heating water bath (HB10 Basic, VWR International) at 45 °C and until total evaporation. Finally, spiked-food was stored overnight in darkness at room temperature under a fume hood to ensure complete residual solvent elimination. For each aromatic fraction, three concentrations were prepared: 0.3X, 1X and 3X. The nominal environmental concentration 1X represents the total PAH concentration measured in mollusks from the Seine estuary corresponding to 5 µg PAHs g^{-1} of dry food. PAH and alkylated PAH concentrations measured in contaminated-food were also reported in Vignet et al. (2014) and given in Table 1 Supplementary data.

2.3. Fish exposure

2.3.1. *Parental dietary exposure (F_0 Generation)*

Wild-type zebrafish (TU strain, Tübingen, Germany) were used for the experiment. The parental dietary exposure was performed during the whole 9 months of the zebrafish lifecycle (larvae, juvenile, and adult). The parental generation was produced from fifty viable eggs per condition, provided by healthy spawning of zebrafish. Then, the eggs were transferred to Petri dishes (100 mm) filled with 30 mL of artificial water (0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 5 mM NaCl and 0.17 mM KCl) and incubated at 28 ± 0.5 °C until exposure started. Maintenance of the standard conditions of zebrafish was already described in Perrichon et al. (2014). The trophic exposure was then performed from 5 days post-fertilization (dpf) larvae which correspond to mouth opening stage. The procedure was already described in Vignet et al. (2014). Briefly, larvae

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were transferred in 1 L tanks in the breeding room and fed *ad libitum* with aromatic fraction-spiked food twice a day. In order to improve growth, larvae were then transferred to 3 and 10 L tanks from 12 dpf and 3 months post-fertilization (mpf) respectively. From this latter stage, diet rations distributed were set at 2% of the total biomass. During the 9 months of dietary exposure, a distribution of non-contaminated food with living *Artemias sp.* nauplii (INVE, Belgium) was given in supplement once a day. Breeding exposure was performed in a constant aeration and flow water systems with daily water renewal set at 40% in order to maintain optimal water quality. Water and room temperature were kept at 27 °C with a 14 h light/10 h dark photoperiod.

2.3.2.   *Offspring production (F$_1$ Generation)*

Eggs were obtained according to two distinct methods. Three spawning boxes (1 L, AquaSchwarz, Germany) were placed directly into the breeding tank the evening before spawning day. In addition, random pairwise mating was performed. One male and one female were placed together in a spawning box on the evening preceding egg production. With both methods, spawning and fertilization took place within 30 min after the onset of light in the morning. They were alternatively used in order to collect eggs twice a week. Fertilized eggs were selected by microscopic exam and viable eggs were distributed in batches of 30 into plastic Petri dishes (100 mm diameter) filled with 30 mL of artificial water and incubated at 28 ± 0.5 °C until analysis.

2.4.   Biological analysis

2.4.1.   *Hatching and developmental abnormalities*

Sublethal endpoints were recorded as described in Lammer et al. (2009) and Perrichon et al. (2014). Hatching success was monitored daily. Biometric analysis (standard length, whole body and yolk sac surface) and abnormalities were recorded in each Solvent Control and PAH-exposed group. Five categories of abnormalities were scored: 1- Edemas formation (brain, pericardia, yolk sac); 2- Skeletal deformities (scoliosis, lordosis, tail bud deformities); 3- Craniofacial deformities (jaw, development of eyes, head deformities); 4- Cardiac deformities (anemia, hemorrhage, atrium/ventricle size, blood circulation heart position); 5- Yolk sac malabsorption. Moreover, a scoring matrix (score of 0-3) was applied following occurrence and severity of these abnormalities: (0) healthy larvae, (1) one abnormality or mildly-affected larvae, (2) two abnormalities or moderately affected larvae, (3) three abnormalities or more or severely affected larvae. Results were expressed in percentage.

2.4.2. *Impact on behavioral: swimming activity*

PhotoMotor Response on 120 hpf larvae were monitored, following the procedure described by Péan et al. (2013) with modifications by Perrichon et al. (2014). Briefly, the locomotor activity of larvae was recorded during a light/dark change challenge in a temperature-controlled box (28 ± 0.5 °C). Recording included three periods of 5 min: 5 min Light On (1), 5 min Dark (D) and 5 min Light On (2). Video analyses were performed with Ethovision XT 8.5 software (Noldus, The Netherlands). The behavioral performances of larvae were assessed by measuring the swimming distance (cm) during these three periods. This distance was calculated considering the center point of the larvae between two consecutive X-Y coordinates summed over a 5-min periods.

2.4.3. Effects on cardiac activity

Cardiac activity was recorded at 28 ± 0.5 °C on 120 hpf larvae. Larvae were introduced in 1 mm wide lines molded in agarose (2%), immobilized with 3% methylcellulose and positioned in a lateral view. An acclimation of 2 hours, in a temperature-controlled environment (28 ± 0.5 °C), was performed before analysis in order to stabilize heartbeat. The heart area of each fish was recorded under an inverted microscope (Olympus SZX9, 40x) coupled to a camera (DMK 31AU03, The Imaging Sources, Germany) and to IC Capture 2.2 software (The Imaging Sources, Germany). Larvae were placed under the microscope for two minutes before recording their heartbeat in order to acclimatize them to the light microscope. For each larvae, three successive videos of 30 seconds were acquired. Video analysis was performed in three successive processing steps: i) A video conversion was made in images stack to greyscale using VirtualDub 1.9.11 software (http://www.virtualdub.org/). A total of 450 images were extracted per 30 seconds of video; ii) An images analysis was performed using ImageJ software (Schneider et al., 2012), focusing on two ROIs (Regions Of Interest) manually delimited and corresponding to the atrium and ventricle, both cardiac chambers. The integrated analysis of grey intensity was performed for each image in both ROI previously converted in order to calculate the alternate circulation between both chambers; and iii) Calculation of cardiac frequency for both chambers from a Single Spectrum (Fourier) analysis was made on integrated data of grey intensity with Statistica 9.0 software (StatSoft, USA). Cardiac frequencies were then corrected to obtain data expressed in beat.min^{-1}.

2.4.4. EROD activity

EROD activity as an indicator of phase I biotransformation activity was determined following the procedure described by Perrichon et al. (2014). To summarize, 96 hpf larvae were incubated in 7-ethoxyresorufin for 5 hours. Levels of produced resorufin into gastrointestinal cavity were observed by fluorescence microscopy (excitation/emission, 560 nm/580 nm) and quantified by imaging analysis software ImageJ (Schneider et al., 2012) coupled with HeatMap Histogram *plugin* (Péan S., http://www.samuelpean.com/heatmap-histogram/). Fluorescence results were expressed in integrated density of pixels (arbitrary unit).

2.4.5.   *DNA integrity of the offspring*

DNA strand break measurements by the alkaline comet assay were performed on 24 hpf embryos. Pools of five dechorionated embryos for four samples per concentration were digested with 1 mg.mL^{-1} of phosphate-buffered saline 1X/collagenase IV from *Clostridium histolyticum* (PBS 1X, 137 mM NaCl · KCl 2.7 mM · Na$_2$HPO$_4$ 10 mM · KH$_2$PO$_4$ 1.8 mM, pH 7.4, Sigma-Aldrich, Germany) solution during 45 minutes at room temperature. Cell suspension was filtered through 48 μm gauze into a 1.5 mL reaction tube in order to separate individual cells from tissue debris. Following a centrifugation for 10 minutes at 2300 rpm at room temperature, the cells pellet was resuspended with 30 μl of PBS. The viability of the dissociated embryo cells was evaluated by a Trypan blue exclusion test. Then, the comet assay was performed as described by Akcha et al. (2003). Two slides were prepared per sample. DNA was stained with 70 μL of GelRed™ solution (1/10000) for one hour at 4 °C in the dark. Slides were analyzed using a fluorescence microscope (Olympus BX60, 400x) coupled to a Luca-S EMCCD camera (Andor™ technology, Northern Ireland) and imaging analysis software (Komet 6.0, Andor™ Technology,

Northern Ireland). Genotoxic assessment was assessed by measuring the DNA percentage in the comet tail (50 cells per slide).

2.4.6. Gene expressions

Larvae were sampled at 96 hpf and stored in RNA Later (Sigma-Aldrich) at -20 °C until used. Total RNA were extracted on 20 pooled larvae using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions with chloroform/ethanol purifications. Total RNA concentration extracted was quantified by spectrophotometry at 260 nm. Furthermore, purity of RNAs was verified by measuring the A260/A230 nm and A260/A280 nm ratios and by electrophoresis on a 1.8% agarose gel with ethidium bromide staining. To avoid the possibility of genomic DNA contamination, the RNA sample was digested by RNase-free DNase I (Promega Madison, USA) and then purified.

Then, a reverse transcription of RNA was performed. First-strand cDNA was synthetized from total 1 μg RNA using a reaction mix including 500 ng of oligo(d_T)15, 250 ng of random hexamer primers (Promega, Madison, USA), 10 mM of deoxyribonucleotide triphosphates (dNTPs) solutions to a final volume of 10 μl. The reaction was initiated using M-MLV Reverse Transcriptase 5X (Promega, Madison, USA) following manufacturer's instructions. Reaction was incubated for 1 h at 42 °C in an Eppendorf Mastercycler and inactivated by heating for 15 min at 70°C. The cDNA mixture was stored at – 20°C until a real-time PCR analysis.

PCR primers were designed with Primer3 software (Rozen and Skaletsky, http://bioinfo.ut.ee/primer3-0.4.0/). The accession numbers of the 11 genes used in our study and the corresponding primers are reported in Table 1. Real-time PCR reactions were performed in sterile 96-well PCR plates with StepOnePlus™ instrument (Applied Biosystems®, Life

Technologies USA) following the manufacturer's instructions. Quantification procedure was based on fluorescence of reaction mixture which related to double-stranded DNA. This reaction mixture included Fast SYBR® Green Master Mix 5X (Applied Biosystems®, USA), 2.4 µL of primers at 600 nM (Eurofins MWG Operon, Germany), 5.6 µL of Milli-Q water and 2 µL of cDNA. Thermal cycling conditions were: enzymatic activation during 10 min at 95 °C, followed by 40 cycles including denaturation (95 °C, 15 s), annealing (60 °C, 40 s) and synthesis (72 °C, 30 s). Gene expression levels were quantified from the threshold cycle (CT) number and normalized to three housekeeping genes: *eef1*, *βactin*, *gapdh*.

2.4.7. PAH-metabolites analysis in larvae

Larvae were sampled at 120 hpf and stored at -80 °C until used. Metabolites were extracted on 50 pooled larvae and three replicates per concentration. Larvae were manually homogenized in 3-4 mL of sodium acetate buffer (pH 5.0). An enzymatic deconjugation was performed for 16 hours at 37 °C after addition to 20 µL of beta-glucuronidase enzyme from *Helix pomatia-2* ($\geq$100 000 units.mL^{-1}, Sigma-Aldrich, Germany) using a method adapted by Mazéas and Budzinski (2005). Prior to PAH-metabolites extraction, internal standards solution was added to samples. Metabolites were extracted by a Solid-Phase Extraction (SPE) procedure using octadecyl cartridge (500 mg, 3 cc, Bakerbond C_{18}-SPE) and eluted in 100% methanol solvent. After re-concentration of organic extracts with gas nitrogen, samples were purified by SPE using amino-column (500 mg, 3 cc, Supelco NH_2-SPE) and eluted with dicholoromethane/methanol solution (80/20, v/v). After another re-concentration step with gas nitrogen, PAH-metabolites (hydroxy-PAH, OH-PAH) extracts were analyzed by Liquid Chromatography coupled to tandem

Mass Spectrometry (LC/MS/MS) analysis as described in Le Dû-Lacoste et al. (2013). PAH concentrations are reported as ng OH-PAH.g^{-1} larvae.

2.5.   Statistical analysis

Sampling procedure of chemical and biological analyses was reported in Table 2. Statistical analyses were performed using Statistica 9.0 software (StatSoft, USA). Results are expressed as means ± standard error of means (SEM) corresponding to experimental groups. A logarithm transformation was performed on comet assay data for normalization (Zar, 2010). The assumption of normality (Shapiro-Wilk tests) and homoscedasticity of variance (Levene tests) were verified. Biological data were analyzed by a one-way analysis of variance (ANOVA) in order to assess significant effects between tested concentrations for each treatment (PY, BAL, HFO). When only two concentrations were tested, a student t test was performed. For PAH-metabolites analyses, normality and homoscedasticity were not validated; therefore a non-parametric Kruskal-Wallis test and Mann-Whitney test were performed.

Behavioral data for PY treatment were analyzed using a one-way repeated measure ANOVA (R-ANOVA) with exposure concentrations as a dependent factor and light (Light On(1), Light Off and Light On(2)) as a within factor. A post-hoc Newman Keuls test was performed each time a difference was found to be significant. For behavioral data of BAL and HFO treatments, normality and homoscedasticity were not validated; therefore non-parametric Mann-Whitney tests were used. In order to test significant differences between three challenge periods, Kruskal-Wallis tests were conducted with multiple pairwise comparisons.

Gene expressions were analyzed with a Relative Expression Software Tool REST-2009©
(Qiagen, http://www.REST.de.com). The gene expression level was given in a relative expression
ratio compared to the control condition.

The significance of the results was ascertained at $\alpha=0.05$.

3.   Results and Discussion

Following 6 months of parents' exposure to the three aromatic fractions (PY, BAL and
HFO), PAHs-metabolites were detected in whole larvae of the F_0 generation showing that a
parental contamination had occurred (Vignet et al. 2014). The mean number of spawning events
and batch fecundity obtained from these exposed parents were also reported by these authors
(Vignet, 2014). Parents' reproduction of 3X concentrations for the three treatments was severely
impaired. Similar results were recorded in 1X concentrations for fish exposed to BAL and HFO
aromatic fractions. These complex mixtures have direct consequences on egg production with no
eggs or few eggs obtained for these concentrations. Consequently, analyses of the offspring were
not possible in the case of any egg production. For samples with an insufficient number of eggs,
statistical analyses have not been performed and thus the results were given on an indicative
basis.

3.1.   Morphological impairments in offspring larvae (F_1 generation)

No hatching delay was observed in offspring larvae whatever the nature and the
concentration of the PAH extract tested. Hatching rates in offspring larvae from PY and BAL
exposed parents were not significantly different whatever the tested concentrations (Fig. 1A, 1B)

(P>0.05). Statistical analyses revealed a decrease in the hatching rate (2.0-fold) in larvae from HFO-0.3X parents compared to the Solvent Control group (P<0.001) at the first hatching day (48 hpf) (Fig. 1C). However, no significant difference was recorded for the following days, with hatching rates averaging 100%. Regarding these latter results, it should be noted that this decrease observed at 48 hpf, probably did not lead to the PAH parental transmission but only to the hatching process unsynchronization in zebrafish (Kimmel et al., 1995). Only one spawning was obtained from HFO-1X exposed parents, consequently statistical analysis was not possible. Data are thus represented on an indicative basis. Therefore, our results did not show any alteration of hatching success in the first generation offspring from parents exposed to three PAH complex mixtures. In spite of the fact that relatively few studies reported results about PAHs transmission to offspring generation, Hose et al. (1981) have however demonstrated a reduction of hatching success in offspring eggs after BaP injection in the gonads of sexually mature flatfish. Similarly, Hall and Oris (1991) have reported that maternally transferred anthracene reduced hatching success in the offspring eggs of fathead minnows (*Pimephales promelas*). In addition, Nagler and Cyr (1997) have proved a significant decline in the number of hatched larvae because of sperm quality alteration in male American plaice (*Hippoglossoides platessoides)* exposed to contaminated sediment.

Biometric measurements in offspring larvae originating from PY, BAL and HFO fractions-exposed parents are presented in Table 3. Offspring larvae from PY-0.3X treatment were slightly longer than Solvent Control and PY-1X larvae (P<0.001 both). Yolk sac surface and ratio yolk sac/whole body surfaces of larvae from 0.3X and 1X concentrations were slightly reduced compared to Solvent Control larvae (P<0.001 both). Larvae originating from HFO-0.3X parents were slightly longer than Solvent Control larvae (P<0.001). No significant difference in yolk sac surface, in ratio yolk sac/whole body surfaces were detected in these offspring larvae (P>0.05). In

addition, Table 3 gives a summary of abnormal larvae proportions and details of abnormalities and their associated severity in larvae. No significant morphological abnormalities were recorded between tested concentrations compared to Solvent Control, whatever the parental treatment (P>0.05). Abnormal individuals (and mainly mild-affected) ranged from 3.34 to 11.1% for PY offspring larvae, from 21.9 to 25.9% for BAL offspring larvae and from 18.0 to 19.8% for HFO offspring larvae. Taken together, these results showed that PAHs transmitted from the parents induced some biometric impairment. However, the toxic action of PAHs transmitted following these treatments will be not so severe as to induce edemas, skeletal, craniofacial deformations in offspring larvae. These results are in accordance with those of Nye et al. (2007) who demonstrated that offspring larvae from contaminant-exposed mothers were longer with smaller yolk sacs than larvae produced by unexposed mothers. From those results, two hypothesis were proposed to explain these differences in size : i) the spawning event from contaminant-exposed mothers took place earlier or ii) a faster development in offspring embryos which will consume more yolk reserves compared to reference embryos. In the present study, development of offspring embryos was assessed during 96 hpf, from 30 min after spawning and no growth delay were recorded whatever the parental treatment considered. In addition, a greater number of hatched larvae would be then observed earlier. Therefore, the first hypothesis is unlikely. Thus, a greater consumption of nutritional reserves in offspring larvae from exposed parents is possible to offset potential physiological failure before hatching, leading to faster development, which would explain longer larvae at 96 hpf. Survival time in starved offspring larvae during 15 dpf was not assessed in the present study, but this parameter will be determinant to assess this potential consumption of reserves and therefore the resistance to starvation. A reduced ability by larvae to resist starvation could lead to a decline in the fish population.

3.2. Behavioral disruptions in offspring larvae (F_1 generation)

Behavioral responses were analyzed in order to assess the potential developmental neurotoxic effects of parental transmission. These responses are currently used to assess the toxicity of various compounds through the analysis of swimming capacity, stress-regulating systems and motor responses (perception and reaction) to stimuli (Champagne et al., 2010; Drapeau et al., 2002; de Esch et al., 2012; Linney et al., 2004). Figure 2 presents the measurements of distance moved per 5-min intervals in offspring larvae from PY, BAL and HFO-exposed parents following a light/dark challenge. Whatever the tested-aromatic fraction, response patterns of offspring larvae were similar, dissociating three swimming activity phases as reported in the literature: i) basal swimming activity phase (Light On(1)), ii) challenge phase (Light Off) leading to an increasing swimming activity and iii) the recovery phase (Light On(2)) when the light is turned on again, where swimming activity was comparable to that observed in the basal phase (Kokel et al., 2010; MacPhail et al., 2009; Padilla et al., 2011). The swimming activity of larvae from PY-3X parents was significantly higher than the other concentrations during both light periods ($P<0.05$) (Fig. 2A). Our results clearly demonstrated hyperactivity for 3X offspring larvae from PY-exposed parents during all behavioral challenges. The ability of these larvae to swim a greater distance could be due to the greater length and/or a possible neurotoxic stimulation of transferred-pyrolytic mixture. The significant lack of morphological data for this condition did not support the first hypothesis. Furthermore, results obtained in PY-0.3X offspring larvae did not reflect these findings. While these larvae had a high swimming activity, these results primarily demonstrate a high level of basic stress, a lower tolerance during the stress period and lower stress recovery. Regarding offspring larvae from BAL-0.3X parents (Fig. 2B), while no significant difference was observed in the basal swimming activity phase,

their PMR pattern tended to be similar to the PMR pattern in PY-3X larvae (P>0.05). Indeed, these larvae showed a significant increase in the distance moved during the recovery phase (Light On(2), P=0.01). Regarding HFO treatment (Fig. 2C), PMR of 0.3X offspring larvae was significantly reduced during the all light/dark challenge compared to Solvent Control larvae (Light On(1): P<0.001, Light Off: P=0.002, Light On(2): P<0.001). Their behavioral responses were in total opposition with those of PY and BAL larvae, where 0.3X offspring larvae displayed a significant early lethargic activity whatever the swimming activity phase. No data were available for 1X and 3X concentrations in either BAL or HFO offspring larvae. According to current understandings, this is the first study concerning behavioral assessment of complex PAHs mixtures in the offspring of contaminated fish and relatively few studies have dealt with the impact of organic pollutant on the fish PMR. Therefore, we cannot directly compare effects with those reported in the literature. However, using a similar approach, Péan et al. (2013) have reported stimulation of this PMR in offspring zebrafish larvae originating from fish contaminated with polychlorinated biphenyls. Their results proved that a potential transmission of organic pollutants may alter the neurotoxic development of offspring. Recently, our previous studies have reported a reduction of basal swimming activity and response to light stress in ELS of zebrafish and an increase in Japanese medaka following sediment contact exposures with two unique PAH, fluoranthene (3-benzenic rings PAH) and benzo[a]pyrene (5-benzenic rings PAH) respectively (Perrichon et al. 2014). A reduction in the swimming activity behavioral pattern was also recorded in Japanese medaka after exposure to benzo[a]anthracene (4-benzenic rings) (Bihanic et al. 2015). The present study demonstrated a parental influence in the neurotoxic behavior of their offspring. These modifications in swimming activity could be critical for vital functions like their visual acuity, their hunting behavior or their escape response to predation or for the spawning migrations, which could be significantly reduced (Gonçalves et al., 2008). However, it is

unknown whether any of the above observations translate to physiological defects and to fitness in surviving fish over the longer-term.

3.3.   Physiological disruptions in offspring larvae (F_1 generation)

Behavior impairments observed previously may be caused by physiological alterations. Therefore, cardiac activity in 120 hpf offspring larvae from parents exposed to three aromatic fractions was measured and represented in Fig. 3. While no morphological abnormalities (edemas, anemia, hemorrhages) were observed, a decrease in the cardiac activity was observed to be concentration dependent in larvae originating from PY parents, with respectively 159, 165 and 148 heartbeats.min^{-1} for 0.3X, 1X and 3X concentrations (P=0.01, P=0.03 and P<0.001). In contrast, heartbeat was not significantly different compared to the Solvent Control respectively in offspring larvae from BAL and HFO parents. No data available for HFO-1X and BAL/HFO-3X. Cardiac frequencies recorded in Solvent Control larvae were consistent with those reported in the literature at this development stage (Barrionuevo and Burggren, 2013).

Numerous studies have documented cardiac defects following PAH exposure. The toxic action of unique predominant PAHs (from 3 to 5-benzenic rings PAHs) in the cardiovascular system of zebrafish mainly leads to pericardial edema, blockage of blood circulation and bradycardia (Incardona et al., 2011, 2009, 2004). Reduction in heart rate has also been reported in herring embryos and zebrafish embryos after respectively water-soluble fraction exposure of Alaska crude oil (Middaugh et al., 1998, 1996) and after sediment eluates/extracts exposure (Strmac et al., 2002). Recently, Milinkovitch et al. (2013; 2012) recorded cardiac impairments in terms of contraction velocity and not contraction force following a water-soluble fraction exposure of crude oil in golden grey mullets (*Liza aurata)*. Similarly, the excitation-contraction

coupling in cardiomyocytes of juvenile bluefin and yellowfin tunas was disrupted after exposure

to Deepwater Horizon crude oil (Brette et al. 2014). For similar oil, Incardona et al. (2014) have

shown serious defects in heart development with circulatory disruption and irregular atrial

arrhythmia in three pelagic fish: bluefin tuna, yellowfin tuna and an amberjack. These

physiological responses (bradycardia) observed in PY offspring larvae could indicate metabolic

disruptions (e.g. respiratory distress) and thus alter fish fitness and survival (Claireaux et al.,

2005). Our results are in agreement with those obtained by Lucas et al. (2014) who assessed

aerobic metabolic performance in offspring fish from PY-3X parents. These authors have

recorded a decrease of active metabolic rate in 2 month-old fish originating from exposed

parents, thus reflecting earlier cardio-respiratory impairments. Taken together, the present results

are in agreement with those of Davoodi and Claireaux (2007) who demonstrated that cardiac

defects observed in sole (*Solea solea*) exposed to petroleum hydrocarbons induced a reduced

AMR. However, we cannot attribute the changes in swimming activity to the heart disruption.

A lack of data in other biological parameters excluded any specific conclusions as to the

potential PAH transmission and toxicity effects in offspring for BAL and HFO treatments.

3.4.   Genotoxic effects in offspring (F_1 generation)

Genotoxic effects on offspring embryos and larvae following the PAH exposure of parents

were investigated through the analysis of detoxification, oxidative processes and DNA damage.

Whatever the tested fractions (PY, BAL or HFO), no significant differences in levels of EROD

activity were observed in 96 hpf offspring larvae compared to respective Solvent Control

($P > 0.05$) (Fig. 4). Similarly, comet assay did not reveal any significant differences ($P > 0.05$) in

the levels of DNA strand breaks measured earlier in 24 hpf offspring embryos from contaminated parents, whatever the tested-concentrations for PY, BAL and HFO fractions (Fig. 5). Unfortunately, no data were available for all 3X concentrations and HFO-1X. Furthermore, relative expressions of target genes compared to respective Solvent Control are presented in Table 4. Data obtained in larvae offspring were normalized to three housekeeping genes: *eef1*, *βactin*, *gapdh*. No data were available for BAL-3X and HFO-3X. Regarding offspring larvae from parents exposed to PY aromatic fraction, no significant expression change was revealed between all concentrations for genes involved in the detoxification (*cyp1a*, *ahr2*), antioxidant defense (*gpx4a*, *cat*, *sod(Cu/Zn)*, *sod(Mn)*) and the apoptosis processes (*p53*, *bax*). Similarly, no significant differences in terms of detoxification (*cyp1a*, *ahr2*) processes were found at 0.3X and 1X concentrations for BAL and HFO compared to their respective Solvent Control ($P > 0.05$). With regards to *Sod(Mn)*, expression levels were 1.30-fold up-regulated in larvae offspring from BAL-1X parents ($P = 0.006$). Expression of gene encoding for *gpx4* was 0.59-fold repressed in offspring larvae from HFO-1X parents ($P = 0.018$). The expression levels of others genes encoding for antioxidant defenses were not significantly different compared to Solvent Control. However, apoptosis processes appeared slightly impacted in offspring larvae from BAL-0.3X parents, with expression levels 1.47-fold and 1.30-fold higher than Solvent Control for *p53* and *bax* respectively ($P = 0.03$ for both conditions). In contrast, no significant change in either genes was observed in 1X offspring larvae. For offspring larvae from HFO-1X parents, the *p53* level was significantly up-regulated ($P = 0.03$).

Taken together, these results showed no evidence of repairable or unrepairable DNA damage or potential activation of detoxification processes (*ahr2*, c*yp1a*, EROD) despite parental exposure to PAHs that are potential inducers of these activities. These results are in agreement with those directly found in the parental generation of our offspring larvae (Larcher et al. 2014).

Indeed, these authors have recorded a high incidence of tumors related to a refractory detoxification bioactivation or DNA damage (DNA strand breaks and micronuclei) via dietary exposure, whatever the aromatic fraction considered. In parallel, a kinetic of *cyp1a* expression related to the larval feeding condition was performed and had demonstrated that following a 24-hour starvation period, an early activation of *cyp1a* occurred after a new feeding event with contaminated food, but rapidly returned to background level. This might explain the absence of genotoxic effects in parental generation and consequently in the first generation of offspring in case of genetically-based response transmission.

Furthermore, fish have a high ability to biotransform PAHs, and are therefore able to eliminate a significant part of these compounds (Aas et al. 2000; Budzinski et al. 2004; Jonsson et al. 2004). PAH-metabolites (OH-PAH) concentrations were then measured in offspring larvae in order to identify parental contamination transmission. The hydroxynaphtalene (OH-N) and dihydroxybiphenyl (2-OH-Bi) were predominant metabolites produced in offspring larvae from PY, BAL and HFO fractions (Table 5). A relatively low level of 1-hydroxypyrene (1-OH-Pyr) metabolite was also present in larvae. However, the amount of metabolites was not statistically different between tested concentrations, from each origin fractions ($P > 0.05$), which could explain the absence of a genotoxic effect. Furthermore, among these PAH-metabolites measured, none are potentially genotoxic (Le Dû-Lacoste et al., 2013).

Regarding, toxicity associated with the three aromatic fractions, the HFO fraction appeared to have greater toxicity with an induced-mortality, growth and reproductive disruptions, which lead to a significant reduction in egg production, followed by BAL and PY fractions (Vignet 2014). Although HFO and BAL fractions were the most reprotoxic, only a narcotic effect appeared in offspring larvae of HFO-0.3X, which was not closely linked to cardiac activity or

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genotoxic impairments. Furthermore, the slight induction of *p53* and *bax* involved in pro-apoptotic processes for both of these fractions, lead us to believe that processes of cellular reparation were induced to offset the potential detrimental effects, which might also explain the absence of genotoxic effects. Finally, regarding biological responses, no conclusion can be drawn about contamination transmission or a stress transfer via neurotransmitters to the eggs.

4. Conclusion

Whereas early reproduction alteration was shown to lead to a significant reduction of produced eggs under environmentally relevant conditions, our study also demonstrated that long-term and chronic dietary exposures of zebrafish to PAH mixtures (from their first meal) led to impairments in their first offspring generation. Using a multiscale approach of toxicity endpoints, the present study reported some disruptions on the individual level. Larval offspring from parents exposed to PY fraction showed a higher degree of adverse effects than offspring from BAL and HFO parents. Indeed, mild morphometrical changes were recorded and bradycardias were significantly induced in larvae from PY parents. Behavioral disruptions were shown at the highest exposure concentration (3X), with basal hyperactive swimming and low reaction to second stress. From acquired data, behavioral changes (lethargy and hyperactivity post-stress) were also demonstrated in offspring from BAL and HFO parents. It appears surprising that these effects were not related to bioactivation of processes involved in detoxification, oxidative stress or DNA damage which might be expected to explain a major part of these disruptions. A supplementary focus on the underlying mechanisms of lipid peroxidation should be performed in order to identify potential disruptions in the energetic mechanisms. Similarly, metabolic hormones (e.g. cortisol) should be analyzed in order to validate these biological responses, which

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are potentially released into the nutritive yolk sac during ontogenesis closely associated with the female condition. Furthermore, the lack of data and experiments in the literature compels us to be cautious in our conclusions. Multigenerational studies have proven to be an integrated approach for the toxicity assessment of chemicals and strengthen the predictive effects. These ecotoxicological studies should be widely undertaken to evaluate the potential for the exposed population to maintain its numbers in the future.

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Captions for the figures

Fig. 1. Hatching kinetic in 120 hpf offspring larvae originating from (A) PY, (B) BAL and (C) HFO-exposed parents. Values are means ± SEM, nd = no data available. Letters denote significant differences between concentrations at different hatching times ($P<0.05$).

Fig. 2. Behavioral performance in 120 hpf offspring larvae originating from (A) PY, (B) BAL and (C) HFO-exposed parents following a light/dark challenge. Values are means distance moved ± SEM per 5-min intervals, nd = no data available. Lower and upper cases denote significant differences between concentrations and light/dark phases (Light On (1), Dark and Light On (2)) respectively.

Fig. 3. Cardiac activity in 120 hpf offspring larvae from PY, BAL, HFO-exposed parents. Values are means ± SEM, nd = no data available. Letters denote significant differences between concentrations ($P<0.05$).

Fig. 4. In vivo EROD activity in 96 hpf offspring larvae originating from PY, BAL and HFO-exposed parents. Values are expressed in response factor compared to respective Solvent Control, nd = no data available. No significant difference was observed ($P>0.05$).

Fig. 5. DNA strand break levels in 24 hpf offspring embryos originating from (A) PY (N=4), (B) BAL (N=3) and (C) HFO (N=3) exposed parents. Boxes denote SEM and vertical bars denote 95% confidence interval, nd = no data available. No significant difference was observed ($P>0.05$).

Fig. 1 Supplementary data. Concentrations (µg.g^{-1}) (A) and relative proportion (%) (B) of PAHs in the three aromatic fractions (PY, BAL, HFO).
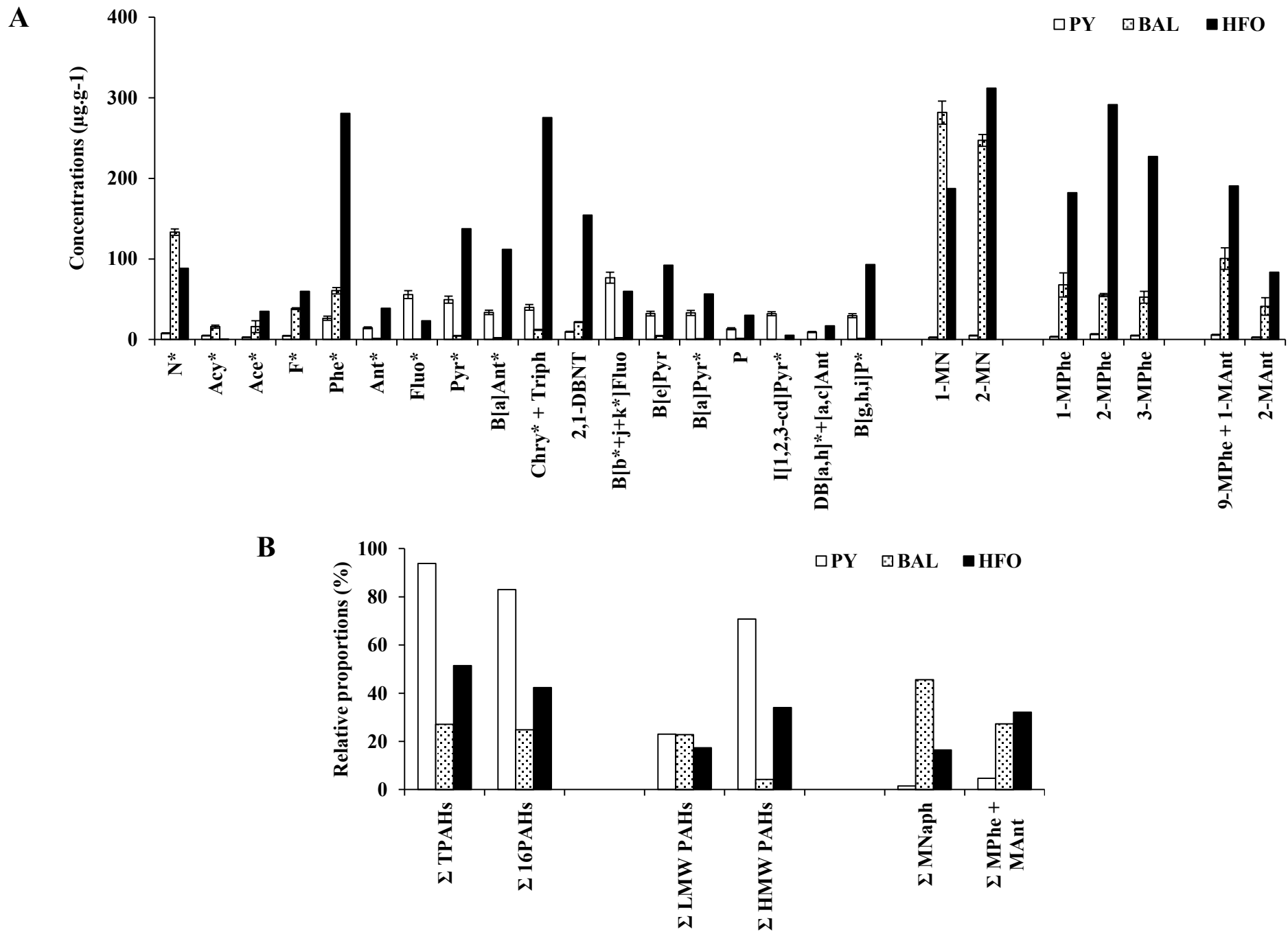
Figure 1 Supplementary data

Figure 1
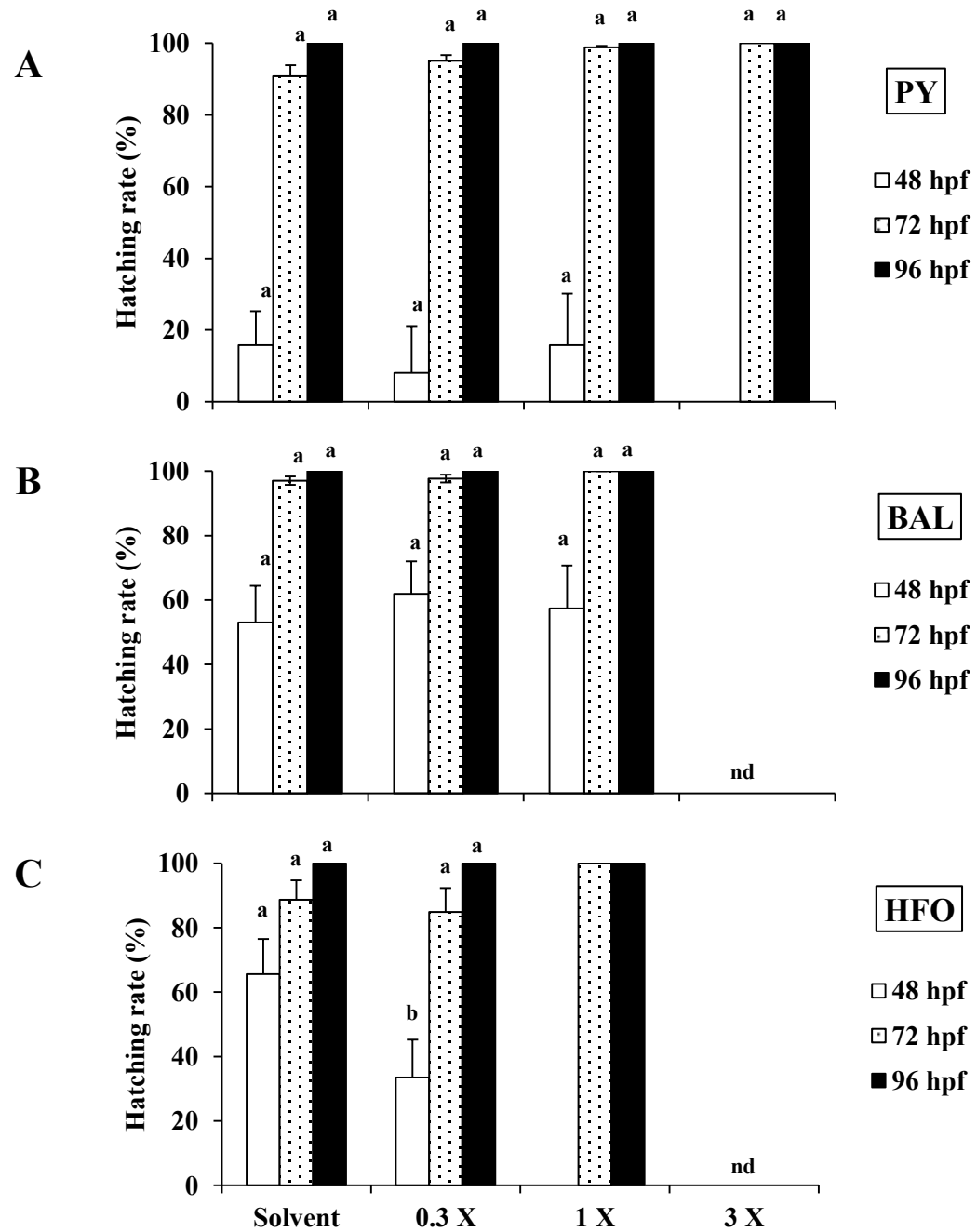
Figure 2
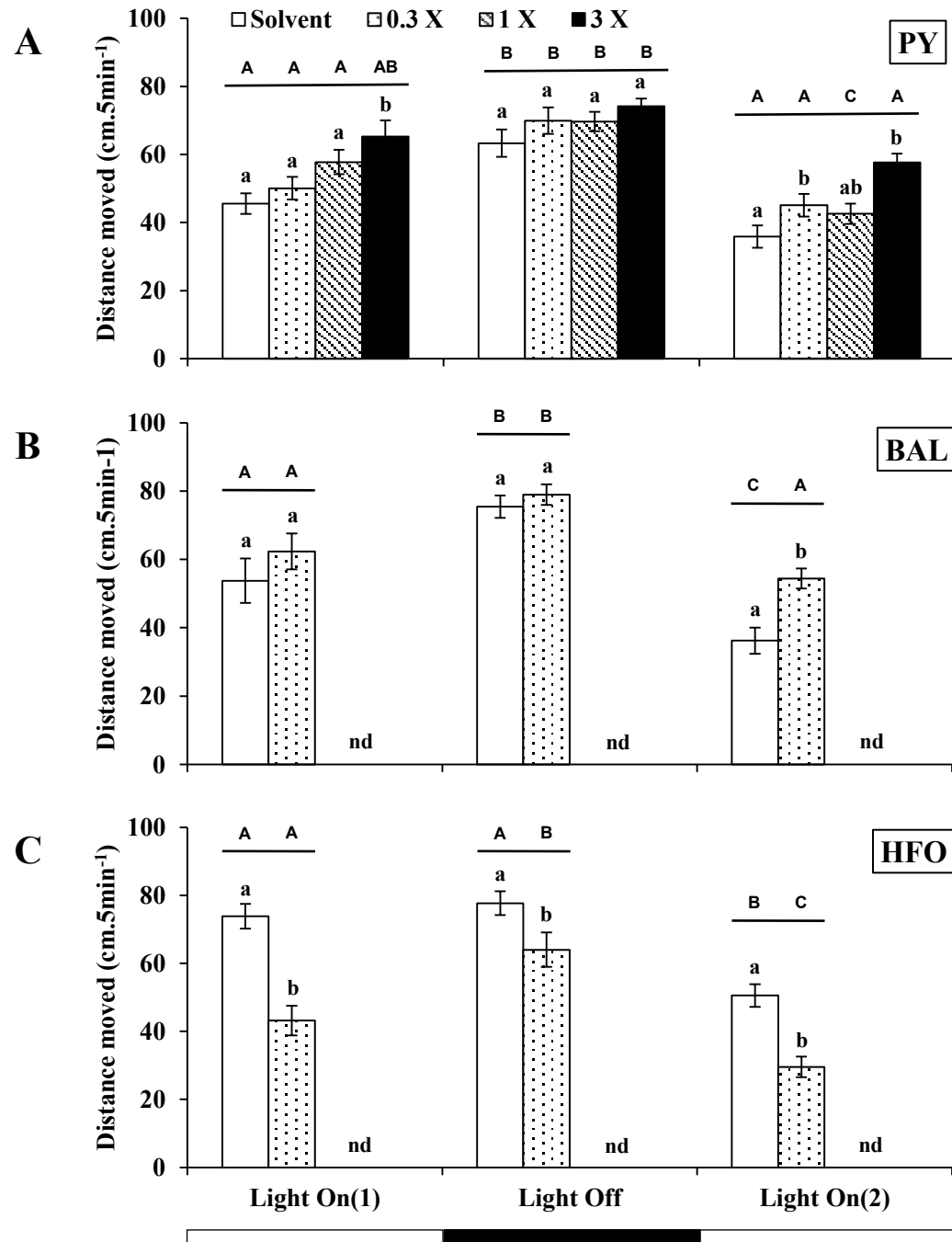
Figure 3

Figure 4

Figure 5

Legend: ■ Mean □ Mean±SE ⊺ Mean±0,95 Conf. Interval

A — PY, Tail DNA (%), categories: Solvent, 0.3 X, 1 X

B — BAL, Tail DNA (%), categories: Solvent, 0.3 X, 1 X
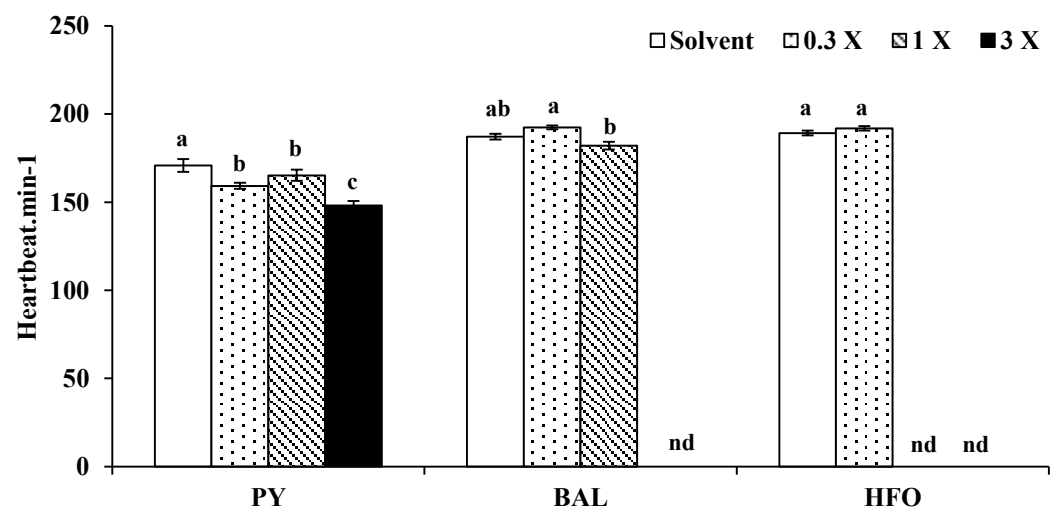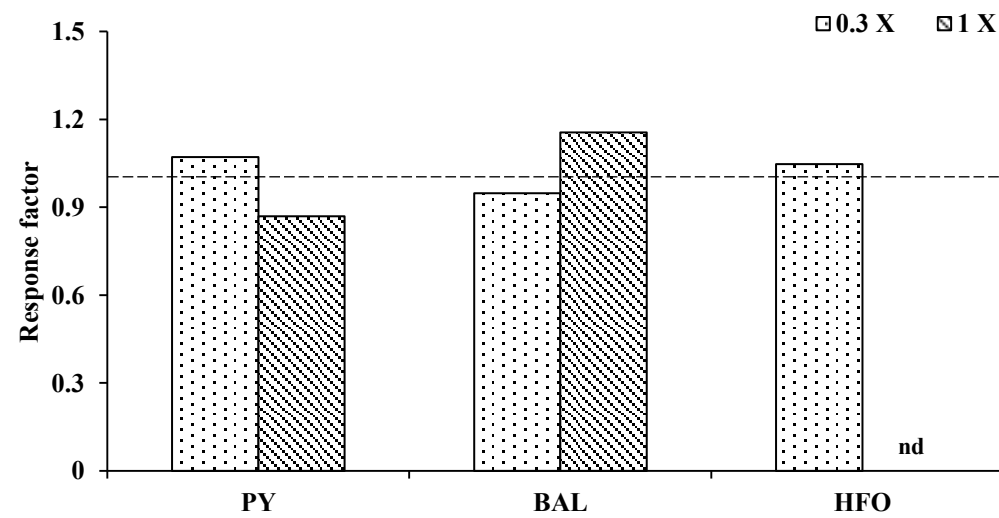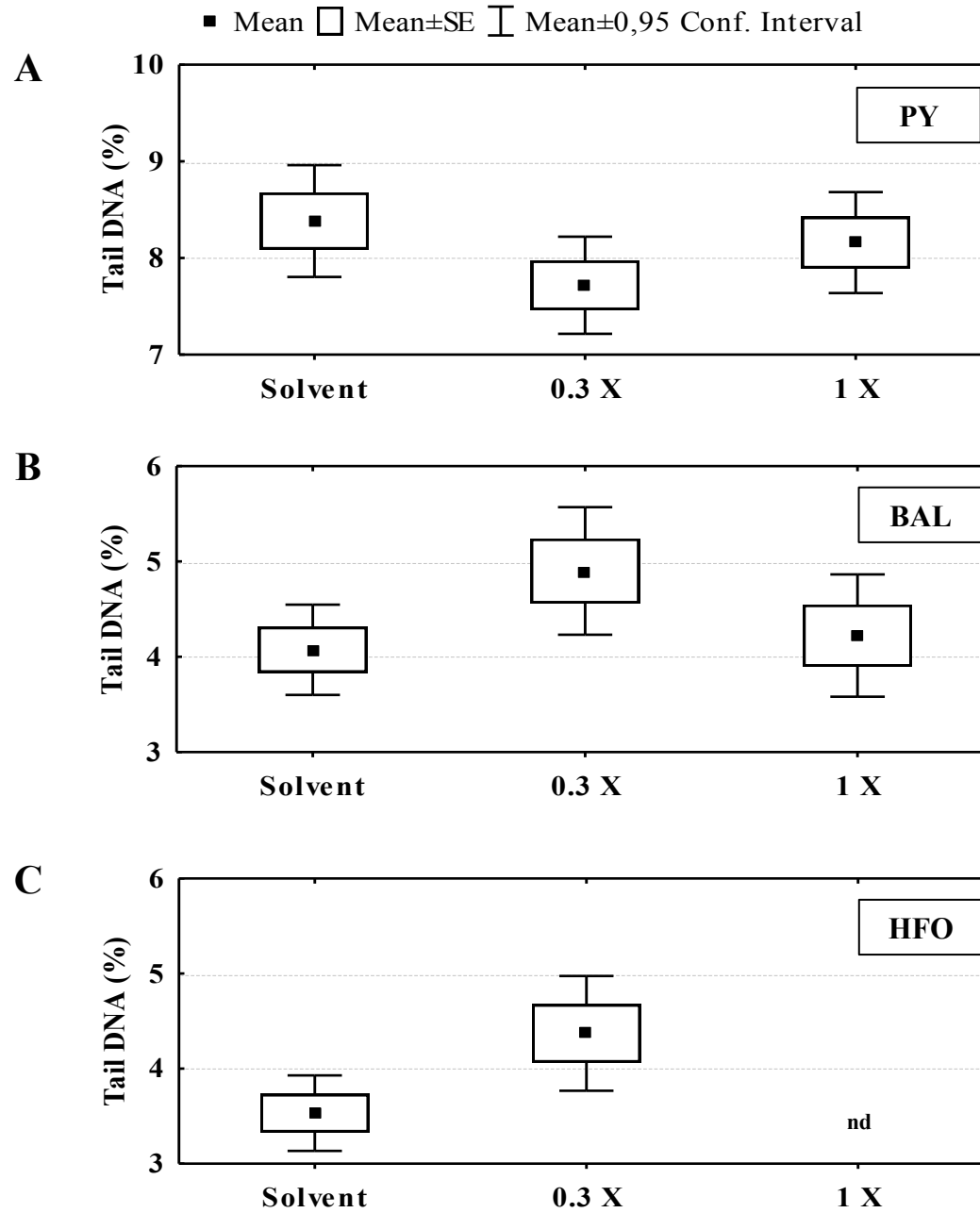
C — HFO, Tail DNA (%), categories: Solvent, 0.3 X, 1 X (nd)

Table 1 Supplementary data

Target compounds (ng.g^{-1}) measured in spiked-food with three aromatic fractions (PY, BAL, HFO) during parental exposures. Values are means ± standard deviation (SD) (N=4-7) and values without SD indicate the compound was identified in one sample.

Compounds	Molecular Weight (g.mol^{-1})	PY Aromatic Fraction				BAL Aromatic Fraction				HFO Aromatic Fraction			
		Solvent	0.3 X	1 X	3 X	Solvent	0.3 X	1 X	3 X	Solvent	0.3 X	1 X	3 X
Naphthalene*	128	6 ± 6	15 ± 7	56 ± 14	157 ± 74	3 ± 1	161 ± 21	315 ± 183	1110 ± 472	4 ± 1	37 ± 6	120 ± 16	405 ± 73
1-Methylnaphthalene	142	4 ± 6	8 ± 2	22 ± 5	62 ± 21	2 ± 0	458 ± 19	1147 ± 102	3300 ± 739	2 ± 0	86 ± 8	261 ± 20	854 ± 84
2-Methylnaphthalene	142	8 ± 9	15 ± 3	43 ± 8	116 ± 39	4 ± 1	420 ± 19	1036 ± 96	2982 ± 725	4 ± 1	137 ± 13	396 ± 32	1259 ± 118
∑Methylnapththalene	142	12 ± 15	23 ± 5	65 ± 13	178 ± 60	5 ± 1	878 ± 38	2183 ± 197	6282 ± 1465	6 ± 2	223 ± 20	657 ± 52	2113 ± 201
Acenaphtylene*	152	1 ± 0	11 ± 2	35 ± 6	114 ± 23	1 ± 0	15 ± 1	46 ± 2	136 ± 19	1 ± 0	3 ± 1	3 ± 1	13 ± 13
Acenaphtene*	154	2 ± 1	14 ± 12	29 ± 7	89 ± 24	23 ± 21	37 ± 26	67 ± 68	90 ± 30	27 ± 26	46 ± 23	74 ± 19	190 ± 17
Fluorene*	166	2 ± 1	14 ± 3	42 ± 8	137 ± 28	2 ± 1	79 ± 1	232 ± 2	677 ± 55	2 ± 1	34 ± 2	99 ± 8	312 ± 25
Phenanthrene*	178	8 ± 4	95 ± 21	291 ± 60	895 ± 213	6 ± 4	178 ± 7	492 ± 10	1438 ± 86	6 ± 3	152 ± 7	418 ± 32	1279 ± 51
1-Methylphenanthrene	192	1 ± 0	12 ± 3	37 ± 11	100 ± 28	1 ± 0	122 ± 4	348 ± 14	956 ± 97	0 ± 0	73 ± 8	227 ± 15	617 ± 71
2-Methylphenanthrene	192	2 ± 0	19 ± 5	60 ± 13	175 ± 41	1 ± 0	121 ± 2	340 ± 16	915 ± 124	1 ± 0	149 ± 11	400 ± 28	1172 ± 166
3-Methylphenanthrene	192	2 ± 1	17 ± 3	47 ± 11	149 ± 31	1 ± 0	114 ± 14	319 ± 38	850 ± 156	1 ± 0	126 ± 24	326 ± 47	934 ± 160
∑Methylphenanthrene	192	7 ± 3	70 ± 15	218 ± 41	668 ± 157	2 ± 2	640 ± 35	1804 ± 67	4957 ± 731	2 ± 2	530 ± 34	1448 ± 144	4122 ± 581
Anthracene*	178	1 ± 0	49 ± 13	159 ± 41	482 ± 165	0 ± 0	2 ± 1	4 ± 1	42 ± 54	0 ± 0	22 ± 1	70 ± 2	220 ± 10
2-Methylanthracene	192	0 ± 0	8 ± 2	25 ± 6	78 ± 18	blq	blq	blq	32 ± 8	blq	blq	blq	409 ± 32
9-Methylphenanthrene + 2-Methylanthracene	192	1 ± 0	14 ± 4	49 ± 12	165 ± 56	1 ± 0	283 ± 29	798 ± 42	2226 ± 394	1 ± 0	137 ± 20	360 ± 85	990 ± 206
Dibenzo[b,d]thiophene	184	2 ± 3	11 ± 1	34 ± 5	102 ± 26	0 ± 0	443 ± 8	1161 ± 18	3489 ± 208	0 ± 0	54 ± 3	166 ± 11	546 ± 54
2,1-Dibenzonaphthothiophene	234	5 ± 3	52 ± 26	156 ± 70	472 ± 230	0	66 ± 1	194 ± 9	588 ± 30	0	56 ± 4	186 ± 8	573 ± 36
Fluoranthene*	202	3 ± 3	130 ± 28	523 ± 182	1782 ± 353	2 ± 1	2 ± 2	1 ± 1	15 ± 17	2 ± 1	17 ± 1	44 ± 2	145 ± 18
Pyrene*	202	3 ± 3	112 ± 24	447 ± 160	1496 ± 311	2 ± 0	13 ± 2	18 ± 2	73 ± 28	1 ± 0	80 ± 3	227 ± 19	709 ± 64
Benzo[a]anthracene*	228	1 ± 0	171 ± 75	581 ± 221	1671 ± 763	0	4 ± 1	14 ± 2	49 ± 28	0	57 ± 4	172 ± 11	543 ± 29
Chrysene + Triphenylene*	228	1 ± 0	215 ± 91	744 ± 290	2144 ± 1032	blq	30 ± 1	98 ± 4	320 ± 62	blq	108 ± 8	336 ± 21	1073 ± 79
Benzo[b+j+k]fluoranthene*	252	2 ± 1	273 ± 72	868 ± 220	2740 ± 674	2.4	6 ± 0	20 ± 1	66 ± 7	2.4	32 ± 2	110 ± 8	363 ± 18
Benzo[e]pyrene	252	1 ± 0	109 ± 29	346 ± 86	1084 ± 286	blq	17 ± 0	52 ± 1	160 ± 10	blq	56 ± 3	173 ± 11	536 ± 26
Benzo[a]pyrene*	252	1 ± 0	118 ± 33	373 ± 95	1168 ± 346	0 ± 0	blq	4 ± 1	17 ± 3	0 ± 0	blq	108 ± 6	342 ± 10
Indeno[1,2,3-cd]pyrene*	276	0 ± 0	123 ± 41	349 ± 89	1188 ± 265	blq	blq	blq	blq	blq	blq	blq	blq
Benzo[g,h,i]perylene*	276	0 ± 0	87 ± 27	268 ± 67	893 ± 191	0 ± 0	blq	12 ± 0	42 ± 12	0 ± 0	blq	146 ± 10	481 ± 12
Benzo[g,h,i]perylene*	276	1 ± 0	37 ± 9	121 ± 27	390 ± 83	0 ± 0	blq	5 ± 1	13 ± 1	0 ± 0	blq	56 ± 4	172 ± 14
Dibenzo[a,h]+[a,c]anthracene*	278	2 ± 2	32 ± 11	108 ± 43	301 ± 106	0.3	blq	3 ± 1	11 ± 1	0.3	blq	34 ± 2	113 ± 6
Sum of parent PAHs		34 ± 6	1670 ± 448	5532 ± 1383	17305 ± 4798	33 ± 26	1053 ± 63	2739 ± 231	8335 ± 854	39 ± 30	880 ± 28	2558 ± 169	8082 ± 305
Sum of total PAHs		55 ± 12	1763 ± 468	5816 ± 1433	18151 ± 4983	40 ± 29	2572 ± 96	6726 ± 278	19574 ± 1945	47 ± 33	1633 ± 71	4663 ± 360	14317 ± 813

* Priority contaminants selected by the US Environmental Protection Agency (EPA)

blq : below limit of quantification

Table 1

Specific primer sequences for the 11 target genes measured by real-time PCR.

Target gene	Name	Accession Number	Primer (5' → 3')
gapdh	*glyceraldehyde-3-phosphate dehydrogenase*	BC083506	GTGGAGTCTACTGGTGTCTTC[a]/GTGCAGGAGGCATTGCTTACA[b]
eef1	*eukaryotic elongation factor 1*	NM_131263.1	CAGCTGATCGTTGGAGTCAA[a]/TGTATGCGCTGACTTCCTTG[b]
βactin	*beta-actin*	NM_131031	CCCAGACATCAGGGAGTGATa/CACAATACCGTGCTCAATGGb
cyp1A	*cytochrome P4501A*	BC094977	GACAGGCGCTCCTAAAACAG[a]/CTGAACGCCAGACTCTTTCC[b]
ahr2	*aryl hydrocarbon receptor 2*	NM_131264	GCCTGGGATAAAGGAGGAAG[a]/CAGCTCCACCTGTCCAAAT[b]
cat	*catalase*	NM_130912	CGCTTCTGTTTCCGTCTTTC[a]/CCCTGAGCATTGACCAGTTT[b]
sod(Cu/Zn)	*Cu/Zn-superoxide dismutase*	BC055516	GTTTCCACGTCCATGCTTTT[a]/CGGTCACATTACCCAGGTCT[b]
sod(Mn)	*Mn-superoxide dismutase*	CB923500	GGCCAAGGGTGATGTGAC[a]/ACGCTTTATGGCCTCCAAC[b]
gpx4	*glutathione peroxidase 4*	NM_001007282.1	AGGATCCAAGTGTGGTGGAG[a]/GGGGTTTCCAAACAAACCTT[b]
p53	*bcl-2-binding component 3*	NM_131327.1	GCTTGTCACAGGGGTCATTT[a]/ACAAAGGTCCCAGTGGAGTG[b]
bax	*bcl-2-associates X protein*	AF231015	GGAGATGAGCTGGATGGAAA[a]/GAAAAGCGCCACAACTCTTC[b]

Forward primer[a]/Reverse primer[b]

Table 2

Number of total individuals sampled for different chemical and biological analysis. The number into brackets corresponds to the number of spawning events used as replicates. The Comet assay was realized from one spawning event, the replicate number corresponds to analysis replicates.

Sampling point	Endpoints	Treatment	Concentrations			
			Solvent Control	0.3X	1X	3X
48-96 hpf	Hatching success	PY	296(3)	323(5)	552(5)	20(1)
		BAL	2216(9)	521(7)	258(7)	nsa
		HFO	620(6)	596(6)	4(1)	nsa
96 hpf	Morphological analyses	PY	190(4)	172(5)	175(5)	nsa
		BAL	75(3)	60(3)	17(1)	nsa
		HFO	85(4)	85(4)	nsa	nsa
120 hpf	PMR analyses	PY	140(5)	100(4)	76(2)	39(3)
		BAL	37(3)	35(3)	nsa	nsa
		HFO	115(3)	90(3)	nsa	nsa
120 hpf	Heartbeat	PY	35(3)	52(3)	48(3)	26(1)
		BAL	21(2)	24(2)	22(2)	nsa
		HFO	15(2)	23(2)	nsa	nsa
96 hpf	EROD activity	PY	40(2)	32(2)	40(2)	nsa
		BAL	58(3)	47(3)	26(3)	nsa
		HFO	60(2)	34(2)	nsa	nsa
24 hpf	Comet assay	PY	5(4)*	5(4)*	5(4)*	nsa
		BAL	5(3)*	5(3)*	5(3)*	nsa
		HFO	5(3)*	5(3)*	nsa	nsa
96 hpf	Gene expression (RT-PCR)	PY	20(8)*	20(3)*	20(6)*	20(5)*
		BAL	20(8)*	20(8)*	20(4)*	nsa
		HFO	20(8)*	20(8)*	20(4)*	nsa
120 hpf	PAHs metabolites	PY	50(3)*	50(3)*	50(3)*	50(3)*
		BAL	50(3)*	50(3)*	nsa	nsa
		HFO	50(3)*	50(3)*	nsa	nsa

* Individuals pooled for analysis; nsa: no sample available.

Table 3

Developmental endpoints for assessing toxicity and teratogenicity of three aromatic fractions (PY, BAL, HFO) on 96 hpf offspring larvae from contaminated-genitors. Biometric values are means ± SEM.

	PY			BAL			HFO	
	Solvent N=4 ; n=190	0.3 X N=5 ; n=172	1 X N=5 ; n=175	Solvent N=3 ; n=75	0.3 X N=3 ; n=60	1 X N=1 ; n=17	Solvent N=4 ; n=85	0.3 X N=4 ; n=85
Standard length (mm)	3.58 ± 0.009^a	3.63 ± 0.009^b	3.57 ± 0.012^a	3.55 ± 0.018^a	3.57 ± 0.022^a	3.61	3.48 ± 0.023^a	3.59 ± 0.014^b
Yolk sac surface (mm²)	0.26 ± 0.002^a	0.24 ± 0.002^b	0.25 ± 0.002^c	0.27 ± 0.0026^a	0.27 ± 0.006^a	0.32	0.28 ± 0.005^a	0.29 ± 0.003^a
Ratio Yolk sac/Whole larval surface	0.229 ± 0.001^a	0.213 ± 0.001^b	0.216 ± 0.002^c	0.224 ± 0.004^a	0.224 ± 0.005^a	0.265	0.241 ± 0.004^a	0.236 ± 0.002^a
Abnormal individuals (%)	6.34 ± 1.58^a	11.1 ± 2.20^a	8.48 ± 3.03^a	21.9 ± 2.01^a	25.9 ± 2.26^a	0.00	19.8 ± 5.13^a	18.0 ± 3.50^a
Severity of abnormality (Scoring/3) (%):								
No affected (Score 0)	93.7 ± 1.58	88.9 ± 2.20	91.5 ± 3.03	78.1 ± 2.01	74.1 ± 2.26	100.0	80.3 ± 5.13	81.0 ± 4.25
Mild (Score 1)	5.24 ± 1.63	9.12 ± 2.07	5.28 ± 2.04	18.9 ± 0.86	17.4 ± 1.00	0.00	14.8 ± 3.88	19.0 ± 4.25
Moderate (Score 2)	1.10 ± 0.55	1.35 ± 0.72	2.90 ± 1.04	3.00 ± 1.15	6.15 ± 1.48	0.00	2.50 ± 1.25	0.00 ± 0.00
Severe (Score 3 or more)	0.00 ± 0.00	0.67 ± 0.48	0.30 ± 0.21	0.00 ± 0.00	2.30 ± 1.80	0.00	2.50 ± 1.30	0.00 ± 0.00
Abnormalities among abnormal individuals (%):								
Edemas	2.19 ± 1.09	1.10 ± 0.59	0.94 ± 0.67	8.67 ± 1.47	6.87 ± 2.65	0.00	14.4 ± 6.41	10.3 ± 5.14
Axial Skeleton	1.46 ± 1.10	8.47 ± 1.74	4.58 ± 1.80	7.29 ± 0.40	12.3 ± 6.81	0.00	1.44 ± 0.72	7.72 ± 2.39
Craniofacial	2.09 ± 0.73	0.79 ± 0.57	2.21 ± 0.92	2.50 ± 0.96	1.15 ± 0.88	0.00	1.44 ± 0.72	0.00 ± 0.00
Cardiovascular	0.00 ± 0.00	0.80 ± 0.40	0.40 ± 0.03	0.00 ± 0.00	2.30 ± 1.77	0.00	1.00 ± 0.75	0.00 ± 0.00
Yolk sac Malabsorption	0.60 ± 0.30	0.00 ± 0.00	0.37 ± 0.27	3.43 ± 2.64	3.24 ± 1.83	0.00	1.50 ± 1.13	0.00 ± 0.00

Letters denote significant differences between experimental group (P<0.05).

Table 4

Relative mRNA expression (Standard Error) of target genes in 96 hpf larvae offspring from parental contamination to three aromatic fractions (PY, BAL, HFO).

	PY			BAL		HFO	
	0.3 X	1 X	3 X	0.3 X	1 X	0.3 X	1 X
	N=3	N=6	N=5	N=8	N=4	N=8	N=4
Housekeeping genes							
eef1	1,122	1,038	1,039	0,912	0,879	0,905	0,944
ßactin	0,699	0,435	0,369	1,139	1,193	1,211	1,390
gapdh	1,225	1,101	1,011	1,044	0,839	0,869	0,869
Detoxification							
cyp1A	1,211 (0.829-1.846)	1,057 (0.720-1.465)	0,722 (0.471-1.062)	1,013 (0.735-1.311)	1,123 (0.804-1.687)	1,058 (0.749-1.858)	1,142 (0.909-1.486)
ahr2	1,075 (0.911-1.240)	0,790 (0.663-0.929)	0,790 (0.689-1.012)	0,984 (0.740-1.326)	0,818 (0.511-1.321)	0,965 (0.781-1.244)	1,064 (0.990-1.170)
Oxidative stress							
gpx4a	1,051 (0.881-1.218)	0,828 (0.683-1.034)*	0,895 (0.726-1.183)	1,147 (0.790-1.608)	1,329 (0.995-1.813)	1,075 (0.723-1.384)	0,588 (0.420-0.758)*
cat	1,068 (0.833-1.321)	0,901 (0.750-1.064)	0,909 (0.746-1.122)	0,992 (0.893-1.120)	1,074 (0.893-1.216)	1,174 (0.867-1.616)	1,067 (0.908-1.266)
sod(Cu/Zn)	1,149 (1.072-1.344)	0,937 (0.774-1.095)	0,982 (0.756-1.437)	1,132 (0.931-1.308)	1,072 (0.832-1.331)	1,328 (0.968-1.831)	0,845 (0.747-1.980)
sod(Mn)	1,203 (1.010-1.429)	0,921 (0.745-1.164)	0,944 (0.706-1.336)	1,142 (0.991-1.294)	1,303 (1.126-1.551)**	1,292 (1.011-1.574)	1,146 (1.030-1.303)
Apoptosis							
p53	1,034 (0.836-1.373)	0,765 (0.622-1.035)*	0,924 (0.547-1.447)	1,468 (1.036-2.027)*	1,371 (0.979-1.796)	1,607 (1.177-2.290)	1,665 (1.157-2.246)*
bax	1,142 (0.906-1.441)	0,972 (0.761-1.206)	0,999 (0.815-1.321)	1,302 (1.075-1.613)*	1,303 (1.013-1.694)	1,384 (1.106-1.790)	1,421 (1.121-1.796)

Asterisks denote significant changes from respective controls (* $p<0.05$, ** $p<0.01$). nd : no data available.

Table 5

Metabolites compounds (ng.g^{-1}) measured in 120 hpf offspring larvae. Concentration values are means ± SEM and values without SEM indicate the compound was identified in one sample.

Metabolites	PY				BAL		HFO	
	Solvent	0.3 X	1 X	3 X	Solvent	0.3 X	Solvent	0.3 X
Σ OH-Naphtalenes	22.9 ± 5.51	21.07 ± 1.89	8.47 ± 1.10	31.2 ± 7.5	65.0 ± 13.5	13.0 ± 3.43	87.0 ± 13.5	99.0 ± 13.6
2 OH-Biphenyl	22.7 ± 7.63	8.27 ± 0.92	9.22 ± 1.98	26.37 ± 4.74	14.4 ± 5.16	20.1 ± 4.13	23.1 ± 0.52	15.7
Σ OH-Phenanthrene	1.05	0.54 ± 0.19	blq	blq	0.2	blq	blq	blq
3 OH-Fluoranthene	blq	blq	blq	blq	blq	blq	blq	blq
1 OH-Pyrene	2.23 ± 0.08	3.61 ± 1.23	1.97 ± 0.68	1.65 ± 0.11	2.07 ± 0.40	blq	1.05	1.74 ± 0.34
1 OH-Chrysene	blq	blq	blq	blq	blq	blq	blq	blq
Σ OH-Benzo[a]pyrenes	blq	blq	blq	blq	blq	blq	blq	blq
Σ OH-HAP	48.3 ± 24.2	33.3 ± 4.19	19.7 ± 2.43	59.2 ± 12.2	80.8 ± 15.2	33.1 ± 5.98	102.7 ± 19.5	106.0 ± 17.9

No significant differences between experimental group ($P > 0.05$). Blq : below limit of quantification.