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Toxicity assessment of water-accommodated fractions from two different oils using a zebrafish (*Danio rerio*) embryo-larval bioassay with a multilevel approach

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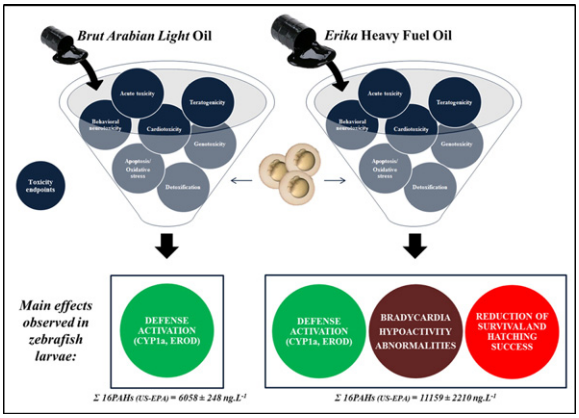
HIGHLIGHTS

- The toxicity of dissolved fractions from two different oils: *Brut Arabian Light* and *Erika* fuel oil, was examined in early life stages of zebrafish.
- A phenotypic map of the deleterious effects of these both oils-derived polycyclic aromatic hydrocarbons is provided.
- Two vital functions are primarily affected by oil: the swimming and cardiac performances.
- Critical impact on the vital functions might have disastrous consequences for survival population.

GRAPHICAL ABSTRACT



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ABSTRACT

Petroleum compounds from chronic discharges and oil spills represent an important source of environmental pollution. To better understand the deleterious effects of these compounds, the toxicity of water-accommodated fractions (WAF) from two different oils (brut *Arabian Light* and *Erika* heavy fuel oils) were used in this study. Zebrafish embryos (*Danio rerio*) were exposed during 96 h at three WAF concentrations (1, 10 and 100% for *Arabian Light* and 10, 50 and 100% for *Erika*) in order to cover a wide range of polycyclic aromatic hydrocarbon (PAH) concentrations, representative of the levels found after environmental oil spills. Several endpoints were recorded at different levels of biological organization, including lethal endpoints, morphological abnormalities, photomotor behavioral responses, cardiac activity, DNA damage and exposure level measurements (EROD activity, *cyp1a* and PAH metabolites). Neither morphological nor behavioral or physiological alterations were observed after exposure to *Arabian Light* fractions. In contrast, the *Erika* fractions led a high degree of toxicity in

Abbreviations: BAL, brut *Arabian Light*; ELS, early life stage; EROD, ethoxyresorufin-*O*-deethylase; hpf, hours post-fertilization; HFO, heavy fuel oil; HMW, high molecular weight; hpf, hours post-fertilization; LMW, low molecular weight; PAHs, polycyclic aromatic hydrocarbons; PMR, photomotor response; RT-PCR, Real-Time Polymerase Chain Reaction; WAF, water-accommodated fractions.

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Teratogenicity
Swimming performance
Bradycardia
Detoxification pathway

early life stages of zebrafish. Despite of defense mechanisms induced by oil, acute toxic effects have been recorded including mortality, delayed hatching, high rates of developmental abnormalities, disrupted locomotor activity and cardiac failures at the highest PAH concentrations ($\sum$TPAHs = 257,029 ± 47,231 ng·L^{-1}). Such differences in toxicity are likely related to the oil composition. The use of developing zebrafish is a good tool to identify wide range of detrimental effects and elucidate their underlying foundations. Our work highlights once more, the cardiotoxic action (and potentially neurotoxic) of petroleum-related PAHs.

1. Introduction

Oil pollution by liquid petroleum (crude oil and refined products) represents an important source of pollution in marine ecosystems. Petroleum compounds originating from oil spills and chronic discharges from maritime transport (oiled-ballast water, cargo washing, wreckage), offshore exploitation (drilling operations) and natural seepage may lead to disastrous ecological consequences when released into the environment (GESAMP, 2007; GESAMP, 1993). The risk assessment of petroleum discharges in aquatic ecosystems remains complicated as they usually occur as complex mixtures of organic compounds. Crude oils are composed of tens of thousands of compounds with 75% are hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs) (OSPAR, 2004). These persistent pollutants exhibit different levels of toxicity and induce different kinds of effects in aquatic organisms (Incardona et al., 2004; Lee and Anderson, 2005) depending on their physico-chemical properties and the speed of microbial transformation (Heintz et al., 1999). The low molecular weight (LMW) PAHs with 2 to 3 rings are usually rapidly dispersed in the water column (Log K_{ow} < 5) after oil spills and are less persistent in the environment because of their high volatility. In contrast, high molecular weight (HMW) PAHs (e.g., 4 rings and more) are more hydrophobic (Log K_{ow} > 5) and therefore less water soluble and more persistent (Black et al., 1983; Rice et al., 2001). Oil toxicity is mostly related to the aromatic fraction made of both LMW and HMW PAHs. As well, dissolved fractions of petroleum appear to be more bioavailable to aquatic organisms.

Oil-derived PAH contamination is responsible for notable environmental damage, exhibiting variable levels of toxicity (from the molecular to population levels) (NRC, 2003). Fish early-life stages (ELS) are known to be particularly sensitive to these compounds (Lammer et al., 2009) that can dramatically impact the survival and recruitment of fish populations (Heintz et al., 2000; McGurk and Brown, 1996; Muhling et al., 2012). DNA damage (strand breakage and adduct formation), mutations and tumors have been frequently observed in marine (Le Dû-Lacoste et al., 2013; Wessel et al., 2012) and freshwater (Cachot et al., 2007) fish species exposed to PAH-contaminated sediment and dissolved fractions of oils. Numerous studies have identified physiological disruptions, including blood disorders and cardiac defects after exposure to PAH mixtures. Reduction of heart rate was found in the embryonic stages of Inland silverside fish (*Menidia beryllina*) after being exposed to water-soluble fractions of Alaska North Slope crude oil (Middaugh et al., 1996). Heart contraction disruptions were recorded in cardiomyocytes of juvenile bluefin and yellowfin tunas after exposure to Deepwater Horizon crude oil (Brette et al., 2014). With a similar oil, Incardona et al. (2014) have demonstrated serious defects in heart development with circulatory disruption and irregular atrial arrhythmia in three pelagic fish embryos: bluefin tuna (*Thunnus thynnus*), yellowfin tuna (*Thunnus albacares*) and amberjacks (*Seriola dumerili*). For several years, these same authors have also documented cardiac detriments in blood circulation (anemia, atrial arrhythmia) and morphological defects (edemas) following exposure to PAH mixtures from crude oil in zebrafish (*Danio rerio*) and Pacific herring (*Clupea pallasi*) (Incardona et al., 2009, 2012, 2013). Various investigations have also found dissolved fractions of oil (*Ekofish* crude oil) responsible for modifying the swimming and feeding behavior, with a significant reduction in the ability to capture prey, in the early larval stages of cod (Tilseth et al., 1984). At larger biological scales, crude oil and refined products have been described to alter the growth and cause anatomical malformations, including induction of edemas along with craniofacial and spinal abnormalities (Carls and Thedinga, 2010; González-Doncel et al., 2008; Hose et al., 1996; Kerambrun et al., 2012; Marty et al., 1997; Norcross et al., 1996; Pollino and Holdway, 2002). Such disruptions in development stages that might be critical for later stages to ensure key physiological functioning (swimming, reproduction) compromise survival and maintenance of future generations. However, individuals exposed to oil-derived PAHs are not without defense and are able to metabolize much of the PAHs. Once assimilated by the fish, PAHs are biotransformed into more water soluble metabolites through phase I biotransformation activities, predominantly catalyzed by cytochrome P450 1A (CYP1A) (Meador et al., 1995; Varanasi et al., 1985). Activation of these mechanisms via binding to the aryl hydrocarbon receptor (AhR) modulates the expression of a battery of genes involved in PAH biotransformation and detoxification pathways. However, such processes can produce metabolites potentially endowed with a higher toxicity than the native compounds they originate from, becoming highly toxic to fish embryo development and the organism's health.

The present study aimed to explore the toxic effects of two water-accommodated fractions (WAF) of varying compositions, from a light crude oil, brut *Arabian Light* (BAL) and a heavy fuel oil, *Erika* (HFO), on fish embryos and larvae. Zebrafish (*Danio rerio*) was selected as the test species because it is easily bred in the laboratory and eggs are available in high numbers throughout the year. The transparency of both chorion and larvae enable direct and non-invasive morphological observations. Using a multidisciplinary approach by coupling biology and chemistry, we monitored a wide range of fish toxic responses using phenotypic (survival, hatching success and abnormalities), behavioral (swimming activity), physiological (heartbeat) and cellular/subcellular markers (DNA damage, apoptosis and oxidative stress). In addition, the degree of exposure to PAHs was evaluated through the level of EROD activity, *cyp1a* induction and PAH metabolites in larvae.

2. Materials and methods

2.1. Maintenance and egg production of zebrafish

Zebrafish (TU strain, Tübingen, Germany) were maintained according to Perrichon et al. (2014) in flow-through 10 L tanks in hatching groups of ten males and ten females. Maintenance standard conditions were characterized by the following parameters: pH 7.5 ± 0.5, conductivity 300 ± 50 µS·cm^{-1}, temperature 27 ± 1 °C and an oxygen saturation ≥ 80%. The photoperiod was set as 14 h light/10 h dark. Zebrafish were fed twice daily ad libitum with commercial granulates (INICIO Plus, BioMar, France) and live nauplius larvae of *Artemias* sp. (Ocean Nutrition, Belgium) once a day, occasionally supplemented with red sludge worms (Boschetto-Frozen fish food). Eggs were obtained by random pairwise mating of zebrafish. A couple of zebrafish was placed together in spawning boxes (AquaSchwarz, Germany) the evening before eggs were required. Spawning and fertilization took place within 30 min after the onset of light in the morning. Fertilized and normally developed eggs were then selected at 8-cell stage (1h15 post-fertilization) from a mix of five spawns for the exposure.

2.2. Experimental procedure

2.2.1. WAF preparation

The water-accommodated fraction of oil was prepared according to guidelines established by the Chemical Response to Oil Spills–Ecological Effects Research Forum (CROSSERF) (Singer et al., 2000). The WAF is a laboratory-prepared medium derived from low energy (no vortex) mixing of a poorly soluble test petroleum product which is essentially free of particles of bulk material (Aurand and Coelho, 1996; Coelho and Aurand, 1997). Two kinds of oils were used: an *Arabian Light* crude oil (BAL 110) and an *Erika* heavy fuel oil (HFO, no. 2). Center of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE) provided the both oils. Briefly, the WAF was prepared with 1.7 L of artificial water (0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 5 mM NaCl and 0.17 mM KCl) in 2 L glass aspirator bottle (equipped with a glass tap), closed by a lid to avoid the evaporation of light compounds. Oil concentrations introduced gently at the water surface were 103 mg oil·L^{-1} water and 55 mg oil·L^{-1} water for BAL and HFO oils respectively. These values were selected since they represent realistic concentrations after an oil spill release in static condition (Bado-Nilles et al., 2009a, 2009b; Tronczynski et al., 2004). A low mixing energy method (no vortexing) using magnetic stirrer was carried out for 24 h in order to favor solubilization of the hydrosoluble molecules from the oil to the water column. Following 24 hour mixing period, WAF was settled 30 min before aliquot collection (500 mL) in glass bottle. The sample was collected from the tap located at the bottom of the bottle. To maintain WAF equilibrium, procedure was set in an air-conditioned room (21 °C) and in the darkness. During exposure duration (96 h), WAF stock was stored at 4 °C in the dark to avoid compound degradation or photo-activation.

2.2.2. Zebrafish exposure

WAF solutions were diluted daily in three different concentrations with the artificial water. The WAF concentrations analyzed were 1%, 10% and 100% from BAL WAF stock solution and 10%, 50% and 100% from HFO WAF stock solution. A Control with only artificial water was also used. Exposures were carried out in five replicates in static conditions (Fig. 1). 30 embryos (8-cell stage) and 10 mL of solution (WAF or artificial water) were placed into 20 mL glass dishes. Glass dishes were covered with Parafilm in order to prevent evaporation and incubated at 28 ± 0.5 °C for an exposure period of 96 h. The photoperiod was similar to the rearing room. During exposure, WAF solutions and artificial water were renewed daily. Following exposure, surviving larvae were transferred to clean water until 15 days post-fertilization (dpf). For the both oils, four consecutive experiments were done in order to analyze the all chemical and toxicity parameters and to test the experiment reproducibility. During experiments, zebrafish larvae were kept unfed.

This study was conducted with the approval of the Animal Care Committee of France under the official license of Marie-Laure Bégout (17-010).

2.3. Chemicals analysis

2.3.1. PAHs analysis in water

WAF samples were stored at −20 °C before chemical analysis. The five replicates for each condition were pooled and water analyses were performed on two consecutive experiments for both oils. PAHs were extracted by SPME (Solid-Phase MicroExtraction) and then quantified by GC/MS (Gas Chromatography coupled Mass Spectrometer). During the whole exposure procedure, water (old and added new) was sampled every day in 10 mL SPME vials. Internal deuterated standard solutions (naphthalene-d_8, acenaphtylene-d_8, dibenzo[*b*,*d*]thiophene-d_8, acenaphtene-d_{10}, fluorene-d_{10}, phenanthrene-d_{10}, anthracene-d_{10}, fluoranthene-d_{10}, pyrene-d_{10}, chrysene-d_{12}, benzo[*a*]anthracene-d_{12}, benzo[*b* + *j* + *k*]fluoranthene-d_{12}, benzo[*e*]pyrene-d_{12}, benzo[*a*]pyrene-d_{12}, perylene-d_{12}, indeno[1,2,3*cd*]pyrene-d_{12}, benzo[*g*,*h*,*i*]perylene-d_{12}, dibenzo[*a*,*h*] + [*a*,*c*]anthracene-d_{14}), diluted in ethanol, were added to 8 mL of water sample prior to extraction. Extractions were entirely automated with a Multipurpose Sampler (Gerstel® MPS2XL, Switzerland) using a PolyDiMethylSiloxan (PDMS) fiber (Supelco, Sigma-Aldrich, South Africa) following the procedure described by Kanan (2012). Briefly, the fiber (100 μm) was immersed into the vial with 1 h stirring period at 250 rpm. After extraction, the fiber was thermally desorbed into the GC/MS system (Agilent GC 7890A/Agilent MSD 5975C, Agilent Technology, California) for 10 min at 270 °C. A blank analysis was carried out to ensure the absence of contamination prior and during analysis.

The 40 detected compounds are listed in Table 1. Among these target compounds, 16 priority PAHs selected by the US EPA (Environmental Protection Agency), 2 additional parent PAHs (benzo[*e*]pyrene and perylene), 2 sulphurated aromatics (dibenzo[*b*,*d*]thiophene and 2,1-dibenzonaphthothiophene) and 20 methylated-PAHs associated were identified.

2.3.2. PAH-metabolites analysis in larvae

Larvae were sampled at the end of the exposure period (96 hpf) and stored at −80 °C until used. Metabolites (hydroxyphenanthrenes, 3-hydroxyfluoranthene, 1-hydroxypyrene, 1-hydroxychrysene, hydroxybenzo[*a*]pyrenes) were extracted from 50 pooled larvae and three replicates per WAF concentrations. Larvae were manually homogenized in 3–4 mL of sodium acetate buffer (pH 5.0). An enzymatic deconjugation was performed during 16 h at 37 °C after addition of beta-glucuronidase enzyme from *Helix pomatia*-2 (≥100,000 units.mL^{-1}, Sigma-Aldrich, Germany) using methods adapted to Mazéas and Budzinski (2005). Prior to PAH-metabolites extraction, internal standard solutions (i.e. 1-hydroxypyrene-d_9) were added to samples. Metabolites

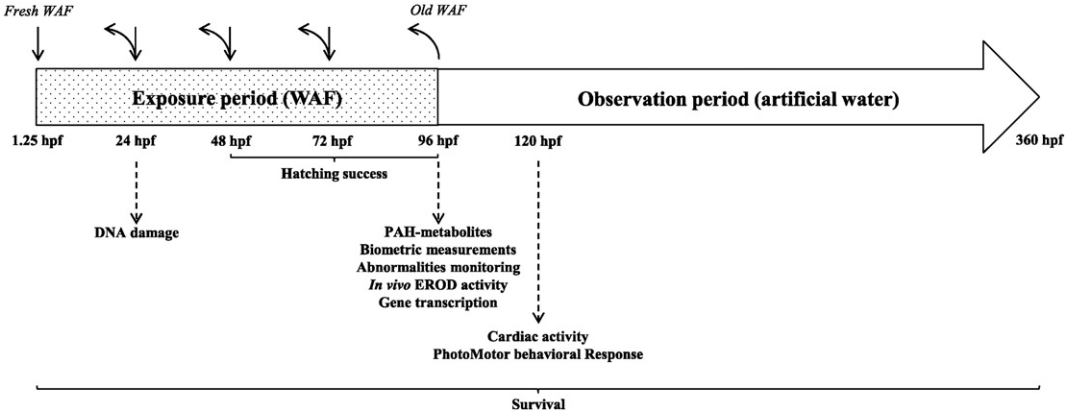

Fig. 1. Experimental design of zebrafish embryo-larval assay.

Table 1
Target compounds ($ng \cdot L^{-1}$) measured in water-accommodated fractions from brut *Arabian Light* oil and *Erika* heavy fuel oil during zebrafish exposures. Values are means ± SEM of both chemical replicates.

Compounds	Molecular weight ($g \cdot mol^{-1}$)	Concentration ($ng \cdot L^{-1}$) in brut *Arabian Light* oil WAF				Concentration ($ng \cdot L^{-1}$) in *Erika* heavy fuel oil WAF			
		Control	1%	10%	100%	Control	10%	50%	100%
Naphthalene[a]	128	2.2	159.8 ± 66.2	683.4 ± 28.5	5,187.2 ± 173.3	1.4	561.4 ± 29.4	2,916.2 ± 488.7	5,394.1 ± 146.4
1-Methylnaphthalene	142	1.8	803.8 ± 357.7	3,472.0 ± 12.9	25,624.6 ± 1,241.6	1.1	2,278.7 ± 172.1	10,583.3 ± 2,587.4	45,176.9 ± 18,810.7
2-Methylnaphthalene	142	1.8	636.1 ± 296.5	2,898.0 ± 55.9	22,302.2 ± 1,049.1	0.7	3,753.0 ± 265.3	17,989.0 ± 4,373.2	34,014.2 ± 791.3
∑ Dimethylnaphthalene	156	29.5	2,400.3 ± 1,023.0	12,074.8 ± 110.8	62,915.5 ± 4,526.2	n.d.	9,924.9 ± 436.0	48,633.3 ± 11,918.8	88,902.8 ± 3,404.2
∑ Trimethylnaphthalene	170	51.1	1,546.1 ± 638.7	7,201.5 ± 7.0	39,626.7 ± 3,530.4	n.d.	5,211.6 ± 253.3	26,215.0 ± 5,908.9	49,234.8 ± 2468.4
∑ Tetramethylnaphthalene	184	n.d.	317.9 ± 124.6	1,851.3 ± 20.9	8,971.7 ± 1,195.3	n.d.	1,388.5 ± 128.2	6,540.5 ± 1,437.5	12,949.0 ± 572.9
Acenaphthylene[a]	152	n.d.	n.d.	n.d.	n.d.	n.d.	8.4 ± 0.6	22.2 ± 1.8	86.1 ± 31.3
Acenaphthene[a]	154	0.8	n.d.	11.8 ± 1.5	64.3 ± 6.1	n.d.	90.2 ± 9.0	437.9 ± 103.0	1,302.4 ± 449.6
Fluorene[a]	166	0.6	14.7 ± 5.7	64.7 ± 1.1	398.5 ± 32.5	0.6	77.0 ± 7.1	375.7 ± 79.6	634.4 ± 59.1
Phenanthrene[a]	178	2.6	22.8 ± 8.1	67.4 ± 0.2	383.4 ± 36.6	1.7	566.6 ± 277.0	941.9 ± 168.3	3,531.0 ± 1,504.8
1-Methylphenanthrene	192	2.3	30.6 ± 12.4	34.6 ± 1.7	183.6 ± 15.1	0.9	307.2 ± 157.9	326.2 ± 50.8	1,402.0 ± 570.9
2-Methylphenanthrene	192	0.4	8.2 ± 3.5	22.7 ± 1.8	156.3 ± 14.9	0.7	118.3 ± 28.1	570.4 ± 83.8	2,454.7 ± 989.2
3-Methylphenanthrene	192	0.3	7.0 ± 3.1	20.7 ± 0.3	116.3 ± 1.6	0.3	292.1 ± 142.4	484.9 ± 70.2	2,078.1 ± 835.6
∑ Dimethylphenanthrene	206	5.5	68.3 ± 26.7	79.5 ± 1.3	419.7 ± 28.7	n.d.	382.2 ± 104.5	869.5 ± 219.2	4,062.6 ± 1,593.8
∑ Trimethylphenanthrene	220	3.8	n.d.	23.5 ± 1.9	128.5 ± 15.7	n.d.	473.3 ± 278.2	384.2 ± 115.6	1,266.1 ± 504.7
Anthracene[a]	178	n.d.	n.d.	17.2 ± 10.1	7.2 ± 0.8	n.d.	26.5 ± 3.0	118.9 ± 18.5	169.9 ± 2.3
2-Methylanthracene	192	0.3	n.d.	n.d.	n.d.	n.d.	77.5 ± 39.5	99.9 ± 12.3	430.3 ± 165.3
[9+4]-Methylphenanthrene + 1-methylanthracene + 4,5-mphenanthrene	192	0.7	5.6 ± 0.2	48.2 ± 0.9	318.2 ± 25.5	0.3	233.3 ± 114.7	366.8 ± 49.8	1,604.1 ± 651.6
Dibenzo[*b*,*d*]thiophene	184	0.3	51.5 ± 19.9	240.0 ± 0.2	1,503.7 ± 133.5	0.1	78.8 ± 7.5	368.7 ± 73.6	627.5 ± 61.5
1-Methyldibenzothiophene	198	n.d.	29.8 ± 11.6	150.3 ± 2.0	952.3 ± 77.3	n.d.	53.4 ± 4.6	246.3 ± 37.0	472.0 ± 2.9
3,2-Methyldibenzothiophene	198	n.d.	30.3 ± 12.2	137.5 ± 1.7	885.4 ± 67.5	n.d.	69.1 ± 6.0	305.4 ± 42.0	574.9 ± 6.8
1-Methyldibenzothiophene	198	n.d.	21.9 ± 8.7	98.4 ± 2.6	642.0 ± 48.5	n.d.	30.1 ± 2.7	129.5 ± 20.4	241.4 ± 3.7
2,1-Dibenzonaphthothiophene	234	n.d.	n.d.	1.1 ± 0.2	4.0 ± 1.0	n.d.	8.9 ± 3.9	19.7 ± 6.8	24.7 ± 5.8
Fluoranthene[a]	202	0.5	1.7 ± 0.2	2.1 ± 0.6	7.8 ± 2.5	0.5	5.9 ± 0.5	21.9 ± 2.0	40.3 ± 2.2
Pyrene[a]	202	0.3	1.3 ± 0.2	1.9 ± 0.5	5.2 ± 0.7	0.1	23.3 ± 3.3	93.2 ± 12.6	164.3 ± 6.2
Benzo[*a*]anthracene[a]	228	n.d.	n.d.	n.d.	2.2 ± 0.6	n.d.	13.7 ± 8.0	11.6 ± 3.5	14.9 ± 1.3
Chrysene + triphenylene[a]	228	0.2	1.3 ± 0.3	0.6 ± 0.01	2.6 ± 0.2	n.d.	10.9 ± 3.1	31.7 ± 10.1	45.3 ± 5.0
∑ Methylchrysene	242	n.d.	n.d.	n.d.	n.d.	n.d.	34.3 ± 7.4	89.4 ± 43.6	114.9 ± 36.4
Benzo[*b*+*j*+*k*]fluoranthene[a]	252	n.d.	n.d.	n.d.	n.d.	n.d.	1.5 ± 0.3	2.3 ± 1.2	2.5 ± 0.9
Benzo[*e*]pyrene	252	n.d.	n.d.	n.d.	n.d.	n.d.	1.5 ± 0.7	3.9 ± 2.4	3.5 ± 1.6
Benzo[*a*]pyrene[a]	252	n.d.	n.d.	n.d.	n.d.	n.d.	0.9 ± 0.4	2.3 ± 1.5	1.6 ± 0.8
Perylene	252	n.d.	n.d.	n.d.	n.d.	n.d.	0.7 ± 0.0	1.9 ± 0.0	1.0 ± 0.5
Indeno[1,2,3-*cd*]pyrene[a]	276	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[*g*,*h*,*i*]perylene[a]	276	n.d.	n.d.	n.d.	n.d.	n.d.	2.0 ± 0.0	3.8 ± 0.0	5.3 ± 0.0
Dibenzo[*a*,*h*]+[*a*,*c*]anthracene[a]	278	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2 ± 0.0

n.d. < detection limit.
[a] Priority contaminants selected by the US Environmental Protection Agency (EPA).

Table 2
Specific primer sequences for the 12 target genes measured by real-time PCR.

Target gene	Accession number	Primer (5′ → 3′)
gapdh	BC083506	GTGGAGTCTACTGGTGTCTTCa
		GTGCAGGAGGCATTGCTTACAb
eef1	NM_131263.1	CAGCTGATCGTTGGAGTCAAa
		TGTATGCGCTGACTTCCTTGb
g6pd	BM_182602	GTCCCGAAAGGCTCCACTCa
		CCTCCGCTTTCCTCTCb
βactin	NM_131031	CCCAGACATCAGGGAGTGATa
		CACAATACCGTGCTCAATGGb
cyp1a	BC094977	GACAGGCGCTCCTAAAACAGa
		CTGAACGCCAGACTCTTTCCb
ahr2	NM_131264	GCCTGGGATAAAGGAGGAAGa
		CAGCTCCACCTGTCCAAATb
cat	NM_130912	CGCTTCTGTTTCCGTCTTTCa
		CCCTGAGCATTGACCAGTTTb
sod(Cu/Zn)	BC055516	GTTTCCACGTCCATGCTTTTa
		CGGTCACATTACCCAGGTCTb
sod(Mn)	CB923500	GGCCAAGGGTGATGTGACa
		ACGCTTTATGGCCTCCAACb
gpx4	NM_001007282.1	AGGATCCAAGTGTGGTGGAGa
		GGGGTTTCCAAACAAACCTTb
p53	NM_131327.1	GCTTGTCACAGGGGTCATTTa
		ACAAAGGTCCCAGTGGAGTGb
bax	AF231015	GGAGATGAGCTGGATGGAAAa
		GAAAAGCGCCACAACTCTTCb

a, forward primer.
b, reverse primer.
gapdh: glyceraldehyde-3-phosphate dehydrogenase; eef1: eukaryotic elongation factor; g6pd: glucose-6-phosphate dehydrogenase; βactin: beta-actin; cyp1a: cytochrome P4501A; ahr2: aryl hydrocarbon receptor 2; cat: catalase; sod(Cu/Zn): Cu/Zn-superoxide dismutase; sod(Mn): Mn-superoxide dismutase; gpx4: glutathione peroxidase 4; p53: bcl-2-binding component 3; bax: bcl-2-associates X protein.

were extracted by Solid-Phase Extraction (SPE) procedure using octadecyl cartridge (500 mg, 3 cm^3, Bakerbond C_{18}-SPE) and eluted with 100% methanol solvent. After re-concentration of organic extracts with gas nitrogen, samples were purified by SPE using amino-column (500 mg, 3 cm^3, Supelco NH_2-SPE) and eluted with dichloromethane/methanol solution (80/20, v/v). After another re-concentration step with gas nitrogen, PAH-metabolites extracts were analyzed by Liquid Chromatography coupled to tandem Mass Spectrometry (LC/MS/MS) analysis as described in Le Dû-Lacoste (2008).

2.4. Biological analysis

2.4.1. Lethal and morphological analyses

Lethal and sublethal morphological endpoints were recorded as described in Lammer et al. (2009). Briefly, mortality and hatching success were daily monitored during zebrafish exposures. Survival monitoring was prolonged until 15 dpf. After an exposure of 96 h, biometric analysis (standard body and head lengths, whole body and yolk sac surfaces) and abnormalities were recorded following methods reported in Perrichon et al. (2014). Five categories of abnormalities were scored: 1 - Edemas (brain, pericardial, yolk sac); 2 - skeletal deformities (scoliosis, lordosis, tail bud); 3 - craniofacial deformities (jaw, development of eyes, head); 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 mild-affected larvae, (2) two abnormalities or moderately affected larvae, (3) three abnormalities or more abnormalities or severely affected larvae. Results were expressed as percentages of surviving larvae.

2.4.2. Swimming ability

Photomotor behavioral response (PMR) on 120 hpf larvae was monitored, following the procedure described by Péan et al. (2013) with slight modifications (Perrichon et al., 2014). Briefly, swimming activity of larvae was recorded during a light/dark change challenge in a temperature-controlled box (28 ± 0.5 °C). This challenge consists of three successive alternating light cycles of 5 min: *Light On* (1), *Light Off* and *Light On* (2). Video analysis was performed with Ethovision XT 8.5 software (Noldus, The Netherlands). The behavioral activity was assessed by measuring the total swimming distance (cm) during all this light/dark challenge. This distance is calculated considering the center point of the larvae between two consecutive X-Y coordinates summed over a 5-min cycles.

2.4.3. Cardiac activity

Cardiac activity was recorded at 28 ± 1 °C on 120 hpf larvae. Larvae were introduced in 1 mm wide lines molded in agarose (2%), immobilized with 3% methylcellulose (diluted in artificial water) and positioned in a lateral view. An acclimation of 2 h in the system was then performed before analysis in order to stabilize heartbeat. Heartbeats were recorded under dissecting microscope (Olympus SZX9, 40×) coupled to a camera (DMK 31AU03, The Imaging Sources, Germany) at 15 frames per second and IC Capture 2.2 software (The Imaging Sources, Germany). Larvae were placed under dissecting microscope two minutes before recording their heartbeats in order to acclimatize them to the light microscope. For each larva, three successive movies of 30 s were acquired. Video analysis was then performed with the Heartbeat detector function of Ethovision XT 9.0 software (Noldus, The Netherlands), which is designed for analyzing frequencies of both cardiac chambers contractions (atrium and ventricle). Heart activity was expressed in beats per minute.

2.4.4. In vivo EROD activity

EROD activity as indicator of phase I biotransformation activity was determined individually following procedure described by Perrichon et al. (2014). To summarize, these activity measurements were performed on 96 hpf surviving larvae. Zebrafish larvae were incubated in 7-ethoxyresorufin solution (CYP1A substrate, 21 $\mu g \cdot L^{-1}$) for 5 h at

Table 3
Number of total individuals sampled for different chemical and biological analyses. The number into brackets corresponds to the number of replicates used.

Sampling point	Toxicity endpoints	BAL WAF				HFO WAF			
		Control	1%	10%	100%	Control	10%	50%	100%
0–15 dpf	Survival	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)
48–96 hpf	Hatching success	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)	150 (5)
96 hpf	Morphological analyses	47 (5)	44 (5)	46 (5)	45 (5)	37 (5)	55 (5)	50 (5)	45 (5)
120 hpf	PMR analyses	43 (5)	35 (5)	40 (5)	39 (5)	46 (5)	44 (5)	43 (5)	20 (5)
120 hpf	Heartbeat	16 (5)	15 (5)	19 (5)	16 (5)	18 (5)	20 (5)	20 (5)	23 (5)
96 hpf	EROD activity	25 (5)	25 (5)	25 (5)	25 (5)	25 (5)	25 (5)	25 (5)	25 (5)
24 hpf	DNA damages	5 (3)a	5 (3)a	5 (3)a	5 (3)a	5 (3)a	5 (3)a	5 (3)a	5 (3)a
96 hpf	Gene transcription	20 (5)ab	20 (5)ab	20 (5)ab	20 (5)ab	20 (5)ab	20 (5)ab	20 (5)ab	20 (5)ab
120 hpf	PAHs metabolites	50 (3)ab	50 (3)ab	50 (3)ab	50 (3)ab	50 (3)ab	50 (3)ab	50 (3)ab	50 (3)ab

a, individuals pooled for analysis.
b, the replicate number corresponds to replicates of experiment.

Table 4
Summarized of aromatic compounds (ng·L^{-1}) measured in water accommodated-fraction from Brut *Arabian Light* oil (WAF BAL) and *Erika* heavy fuel oil (WAF HFO) during zebrafish exposures. Values are means ± SEM of both chemical replicates.

		Concentrations (ng·L^{-1})							
		∑ TPAHs	∑ 16PAHs	∑ LMW PAHs	∑ HMW PAHs	∑ SulpPAHs	∑ MNaph	∑ MPhe	∑ MDBT	∑ MChrys
BAL	Control	105.8	7.1	6.2	0.9	0.3	84.1	13.0	n.d.	n.d.
	1%	6,158.5 ± 3,705.4	201.6 ± 75.0	197.3 ± 75.0	4.3 ± 0.1	51.5 ± 19.9	5,704.2 ± 2,440.4	119.7 ± 77.4	81.1 ± 32.7	n.d.
	10%	29,203.0 ± 23.3	849.0 ± 22.3	844.5 ± 29.9	4.5 ± 1.1	240.0 ± 0.2	20,997.6 ± 207.5	209.2 ± 7.9	387.3 ± 6.5	n.d.
	100%	170,808.9 ± 4,479.9	6,058.4 ± 248.3	6,040.6 ± 249.3	17.8 ± 1.1	1,503.7 ± 133.5	123,440.7 ± 11,542.6	1,322.5 ± 101.5	2,483.7 ± 194.3	n.d.
HFO	Control	8.4	4.3	4.3	3.7	0.1	1.8	2.2	n.d.	n.d.
	10%	26,105.4 ± 3,527.6	1,388.3 ± 341.7	1330.1 ± 324.8	58.2 ± 15.6	78.8 ± 7.5	22,556.7 ± 1,255.0	1,806.4 ± 825.8	161.5 ± 17.2	34.3 ± 7.4
	50%	119,207.2 ± 39,521.7	4,886.5 ± 890.8	4,812.8 ± 859.9	73.7 ± 30.9	368.7 ± 73.6	86,367.1 ± 26,225.8	3,001.9 ± 589.4	700.9 ± 106.2	89.4 ± 43.6
	100%	257,028.5 ± 47,230.7	11,158.8 ± 2,209.9	10,883.4 ± 2,193.5	275.4 ± 16.4	627.7 ± 61.5	230,277.6 ± 26,047.4	12,867.6 ± 5,145.8	1,313.0 ± 19.2	114.9 ± 36.4

∑ TPAHs: sum of total PAHs (including methylated PAHs); ∑ 16PAHs: sum of 16 PAHs listed priority by US-EPA; ∑ LMW PAHs: sum of low molecular weight PAHs based on the ∑ 16PAHs; ∑ HMW PAHs: sum of high molecular weight PAHs based on the ∑ 16PAHs; ∑ SulpPAHs: sum of aromatic sulphurated compounds based on the ∑ 16PAHs; ∑ MNaph: sum of methylnaphthalenes; ∑ MPhe: sum of methylphenanthrenes; ∑ MDBT: sum of methyldibenzothiophenes; ∑ MChrys: sum of methylchrysenes.
n.d. < detection limit.

28 ± 0.5 °C. Levels of produced resorufin into gastrointestinal cavity were observed by fluorescence microscopy (Rhodamine red filter, excitation/emission, 560/580 nm). The emitted-fluorescence was quantified using 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

DNA strand break measurements by the alkaline comet assay were performed on 24 hpf embryos. Pools of five dechlorinated embryos per replicate were digested with 1 mg·mL^{-1} of phosphate-buffered saline 1×/collagenase IV from *Clostridium histolyticum* (PBS 1×, 137 mM NaCl·KCl 2.7 mM·Na_2HPO_4 10 mM·KH_2PO_4 1.8 mM, pH 7.4, Sigma-Aldrich, Germany) during 45 min at room temperature. Cell suspension was filtered through a 48 μm gauze in order to separate individual cells from tissue debris. Following a centrifugation for 10 min at 2,300 rpm at room temperature, cells pellet was resuspended in 30 μL of PBS. Then, the comet assay was performed as described by Akcha et al. (2003). For each cell sample, two slides were prepared. DNA was stained with 70 μL of GelRed™ solution (1/10,000) for 1 h at 4 °C in the dark. Slides were analyzed using a fluorescence microscope (Olympus BX60, 400×) coupled to a Luca-S EMCCD camera (Andor™ technology, Northern Ireland) and imaging analysis software (Komet 6.0, Andor™ Technology, Northern Ireland). Genotoxicity was assessed by measuring the DNA percentage in the comet tail for at least 50 nuclei per slide.

2.4.6. Gene transcription

PCR primers were designed with Primer3 software (Rozen and Skaletsky, http://bioinfo.ut.ee/primer3-0.4.0/). The accession numbers of the 8 selected genes (*cytochrome P4501A*, *aryl hydrocarbon receptor 2*, *catalase*, *Cu/Zn-superoxide dismutase*, *Mn-superoxide dismutase*, *glutathione peroxidase 4*, *bcl-2-binding component 3*, *bcl-2-associates X protein*) and the corresponding primers are reported in Table 2.

Larvae were sampled at the end of exposure (96 hpf) and stored in RNA later (Sigma-Aldrich) at −20 °C until used. Total RNAs 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 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 genomic DNA contamination, RNA samples were digested by RNase-free DNase I (Promega Madison, USA) and then purified.

First-strand cDNA was synthetized from 1 μg total RNAs. Reaction mix included 500 ng of oligo$(d_T)_{15}$, 250 ng of random hexamer primers (Promega, Madison, USA), and 10 mM of deoxyribonucleotide triphosphate (dNTPs) for a final volume of 10 μL. The reaction was initiated using 2.5 μL of M-MLV Reverse Transcriptase 5× (Promega, Madison, USA) following manufacturer's instructions. Reverse transcription reaction was carried out 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 real-time PCR analysis.

Real-time PCR reactions were performed in sterile 96-wells PCR microplates with StepOnePlus™ instrument (Applied Biosystems®, Life Technologies USA) following the manufacturer's instructions. Quantification procedure was based on fluorescence of the reaction mixture which is related to the total amount of labeled double-stranded DNA. The reaction mixture contained 10 μL of Fast SYBR® Green Master Mix 5× (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 a denaturation step (95 °C, 15 s), a hybridization step (60 °C, 40 s) and an annealing step (72 °C, 30 s).

The specificity of the process has been verified after completion of the PCR run, by testing the nature of the amplified product with gel electrophoresis and melting curves.

Gene expression levels were quantified from the threshold cycle (CT) number and normalized with four housekeeping genes (*glyceraldehyde-3-phosphate dehydrogenase*, *eukaryotic elongation factor 1*, *glucose-6-phosphate dehydrogenase*, *beta-actin*).

2.5. Statistical analysis

The number of individuals sampled for chemical and biological analyses was reported in Table 3. Statistical analyses were performed using Statistica 9.0 software (StatSoft, USA) with significant level of 5%. Comet assay data were normalized using a logarithm transformation (Zar, 2010). Biological endpoints of fish exposed to the different concentrations of WAF were analyzed with nested-ANOVA (N-ANOVA), followed by a post hoc Tukey test. When the parametric assumption of normality (Shapiro-Wilk tests) and homoscedasticity of variance (Levene tests) were not respected, the non-parametric Kruskal-Wallis (KW) test and multiple pairwise comparisons were conducted. Behavioral data were analyzed using a one-way repeated measure ANOVA (R-ANOVA) with exposure concentrations as a dependent factor and light as a fixed factor. A post hoc Newman Keuls test was performed when a significant difference was detected. These results were expressed as mean ± standard error of mean (SEM). The survival, hatching and abnormalities data, expressed in percentage, were analyzed with Chi-square tests to detect significant differences between exposure conditions. 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 relative expression ratio compared to the control condition.

3. Results

3.1. Chemical analysis

During the experiment period, the WAF concentration renewal was stable for the tested concentrations (results not shown). In the control group, the background water level of PAHs was low (Tables 1 and 4).

The concentrations of PAHs linearly decreased with WAF dilutions (Table 4). The total PAH concentration ranged from 6,158.5 ± 3,705.4 ng·L^{-1} to 170,808.9 ± 4,479.9 ng·L^{-1} in WAF prepared with *Arabian Light* oil and from 26,105.4 ± 3,527.6 ng·L^{-1} to 257,028.5 ± 47,230.7 ng·L^{-1} in WAF prepared with *Erika* fuel oil. Regarding the 16 PAHs listed priority by US-EPA, the portion of LMW PAHs (from 197.3 ± 75.0 to 6,040.6 ± 249.3 ng·L^{-1}) were predominant compared to HMW PAHs (from 4.3 ± 0.1 to 17.8 ± 1.1 ng·L^{-1}) in BAL WAF (Table 4). Furthermore, the BAL WAF was characterized by a greater amount of aromatic sulphurated compounds (sulpPAHs and MDBT) compared to HFO WAF. Regarding the sum of TPAHs, the concentration of BAL_10% WAF appeared to be of same order of magnitude to that of the HFO_10% WAF. However, the portion of the 16 priority PAHs, was 1.6-fold higher in HFO_10% WAF than in BAL_10% WAF, which makes it difficult the direct comparison of these both dissolved fractions of oil, in terms of composition. A higher proportion of phenanthrene (parent and methylated molecules) seems to be identified in HFO WAF. Even if the HMW PAHs such as pyrene, fluoranthene, benzo[*a*]pyrene do not appear in significant concentrations, their presence, as well as that of methylchrysene (from 34.3 ± 7.4 to 114.9 ± 36.4 ng·L^{-1}), a compound undetected in BAL WAF, characterize the dissolved fractions of *Erika* oil.

3.2. Survival and hatching success

Survival and hatching rates measured following WAF exposures are shown in Fig. 2.

In the both control groups, survival rates were 95.3 ± 1.7% after exposure to BAL (Fig. 2A) and HFO (Fig. 2B) WAFs (96 hpf). After 264 hpf, a basal mortality of unfed larvae was observed in both of control groups with a survival rate that decreased to 4.3 ± 3.3% and 7.3 ± 2.6% for BAL and HFO WAFs, respectively, at the experiments' end (360 hpf). Whatever the tested WAF concentration, BAL and HFO WAFs did not induce any significantly lethal effects on zebrafish embryos and larvae during the exposure period (from 1.5 to 96 hpf). The survival rates ranged from 93.3 ± 1.6% to 96.0 ± 1.2% for BAL WAF exposures (Fig. 2A) (Chi-square, p = 0.74) and from 89.3 ± 2.2% to 93.3 ± 1.7% for HFO WAF exposures (Fig. 2B) (Chi-square, p = 0.25). During the observation period (from 96 hpf) in control water, no significant mortality

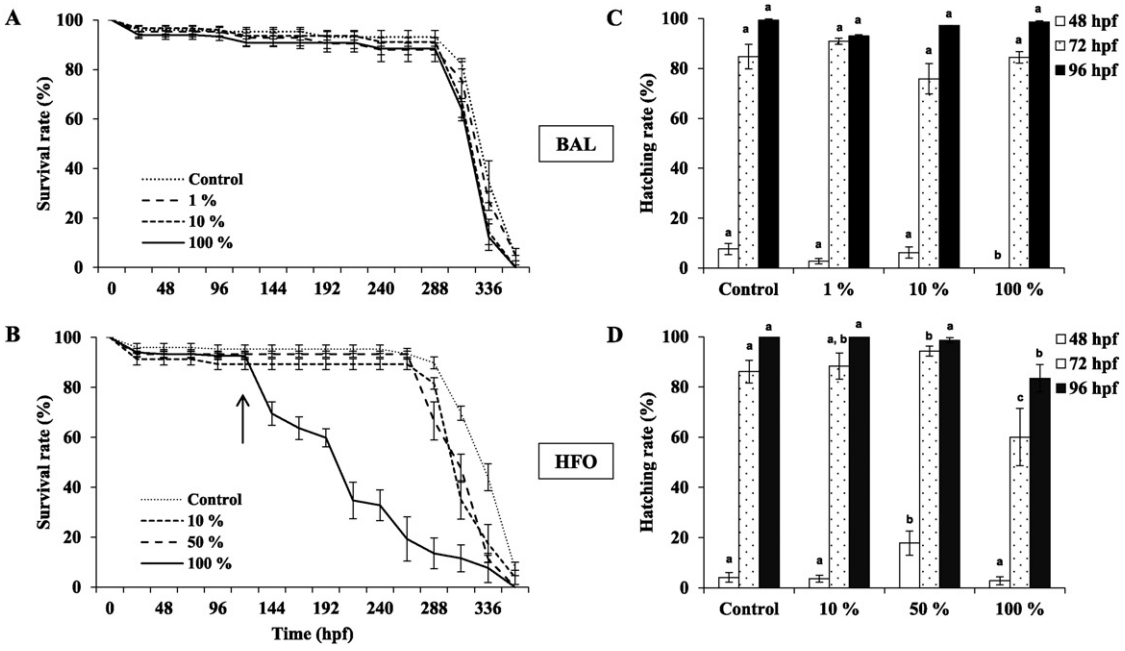

Fig. 2. Zebrafish embryo-larval viability and hatching rates following BAL WAF (A, C) and HFO WAF (B, D) exposures. Arrow indicates time point from which significant differences were observed between HFO_100% WAF and others concentrations. Letters denote significant differences between WAF concentrations at different exposure times (Chi-square, p < 0.05).

was brought about with any BAL WAF concentrations when compared to the control group (Chi-square, $p > 0.05$). The basal mortality of larvae contaminated by BAL WAF followed the same kinetics as the control group whatever the exposure concentration was (Fig. 2A). Following HFO WAF exposure (Fig. 2B), the highest concentration HFO_100% WAF caused a significant decrease in survival rate compared to other exposure concentrations, from 144 hpf until the experiments' end (Chi-square, $p < 0.001$).

Regarding hatching success, 7.6 ± 2.2% and 4.2 ± 1.9% at 48 hpf and 84.7 ± 4.9% and 86.1 ± 4.5% of the control zebrafish embryos hatched out of the chorion between 48 and 72 hpf. Hatching success reached around 100% at 96 hpf (99.3 ± 0.5% and 100 ± 0.0% for BAL and HFO WAFs, respectively) (Fig. 2C and D). No significant reductions in hatching rates were recorded in larvae exposed to BAL WAF versus the Control larvae at the end of exposure (Fig. 2C). Similar results were observed after HFO_10% WAF and HFO_50% WAF exposures with around 100% of hatched larvae at the end of exposure (Fig. 2D). However, the highest concentration of HFO WAF (HFO_100% WAF) induced a significant hatching delay with 60.0 ± 11.4% at 72 hpf and 83.5 ± 5.5% at 96 hpf (Chi-square, $p < 0.001$).

3.3. Developmental defects

Biometric measurements of 96 hpf larvae exposed to BAL and HFO WAFs are shown in Table 5. At the end of the exposure period, the standard lengths in control larvae were 3.67 ± 0.04 mm and 3.77 ± 0.02 mm for BAL and HFO WAFs experiments, respectively. Larvae exposed to BAL_1% (3.79 ± 0.02 mm) and BAL_10% (3.78 ± 0.03 mm) WAF were significantly larger in size than the control larvae (N-ANOVA, $p = 0.01$ and $p = 0.03$, respectively). BAL WAF did not evoke any significant modifications in other biometric parameters (head/

Table 5
Developmental endpoints for assessing toxicity and teratogenicity of both BAL and HFO WAFs on zebrafish larvae at the end of exposure (96 hpf). Biometric values are means ± SEM and abnormalities values are percentages of total effective.

	BAL WAF				HFO WAF			
	Control	1%	10%	100%	Control	10%	50%	100%
Standard length (mm)	3.67 ± 0.04a	3.79 ± 0.02b	3.78 ± 0.03b	3.77 ± 0.02a,b	3.77 ± 0.02a	3.82 ± 0.03a	3.77 ± 0.04a	3.57 ± 0.03b
Ratio head/standard length (%)	0.197 ± 0.003a	0.196 ± 0.001a	0.198 ± 0.001a	0.194 ± 0.001a	0.191 ± 0.001a	0.199 ± 0.001b	0.195 ± 0.002a,b	0.189 ± 0.003a
Yolk sac surface (mm^2)	0.27 ± 0.006a	0.29 ± 0.006a	0.29 ± 0.006a	0.29 ± 0.006a	0.32 ± 0.005a,b	0.30 ± 0.005a	0.30 ± 0.010a	0.36 ± 0.015b
Ratio yolk sac/whole larval surface	0.227 ± 0.007a	0.225 ± 0.004a	0.226 ± 0.003a	0.228 ± 0.003a	0.239 ± 0.003a	0.222 ± 0.004b	0.224 ± 0.009b	0.258 ± 0.008a
Abnormal individuals (%)	10.6a	9.1a	10.8a	20.0a	2.7a	1.8a	22.0b	91.1c
Severity of abnormality (scoring/3) (%)								
No affected (score 0)	89.4	90.9	89.1	80.0	97.3	98.2	78.0	8.9
Mild (score 1)	6.4	9.1	6.5	20.0	2.7	0.0	18.0	28.9
Moderate (score 2)	2.1	0.0	4.4	0.0	0.0	0.0	2.0	20.0
Severe (score 3 or more)	2.1	0.0	0.0	0.0	0.0	1.8	2.0	42.2
Abnormalities among abnormal individuals (%)								
Edemas	5.3	4.6	3.1	13.3	0.0	0.5	13.9	41.6
Axial skeleton	2.7	4.6	4.7	4.4	2.7	0.9	3.5	6.5
Craniofacial	0.0	0.0	0.0	2.2	0.0	0.0	2.3	16.5
Cardiovascular	0.0	0.0	1.6	0.0	0.0	0.0	2.3	19.4
Yolk sac malabsorption	2.7	0.0	1.6	0.0	0.0	0.5	0.0	7.2

Letters denote significant differences between WAF concentrations (N-ANOVA and Chi-square test for biometric measurements and abnormalities respectively, $p < 0.05$).

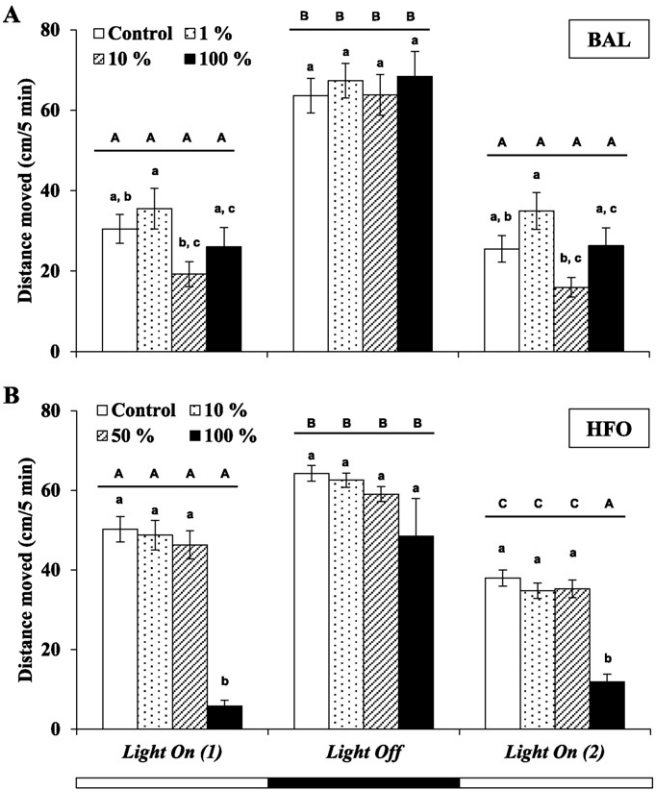

Fig. 3. PhotoMotor Response measured in 120 hpf zebrafish larvae after exposure to (A) BAL WAF and (B) HFO WAF. Means ± SEM distance moved of larvae for each 5-min period of light/dark challenge. Lower and upper cases denote significant differences between WAF concentrations and light/dark periods (*Light On* (1), *Light Off* and *Light On* (2)) respectively (R-ANOVA, $p < 0.05$).

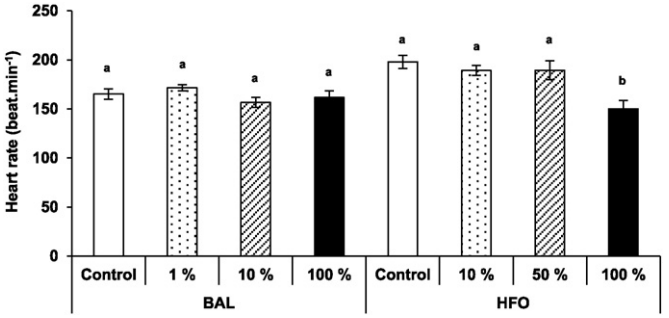

Fig. 4. Heartbeat measured in 120 hpf zebrafish larvae after exposure to both BAL and HFO WAFs. Values are expressed in beats per minute. No significant difference was observed after BAL WAF exposure (N-ANOVA, $p > 0.05$).

body length ratio, yolk sac surface and yolk sac/whole larval surface ratio) in zebrafish larvae. Regarding HFO WAF exposure, larvae exposed to HFO_100% WAF were 0.2 mm smaller than the Control group (N-ANOVA, $p < 0.001$). No significant differences, compared to the control larvae, were seen in the head/body length ratio, yolk sac length and yolk sac/whole larvae surface ratio in larvae exposed to the different concentrations of HFO WAF (N-ANOVA, $p > 0.05$).

The percentage of abnormal individuals observed at 96 hpf as well as the various abnormalities and their severity are also summarized in Table 5. BAL WAF exposures did not induce significant increase of morphological abnormalities versus the Control group (Chi-square, $p = 0.39$) whereas the HFO WAF induced a concentration-dependent increase of abnormalities, accounting for 2.7, 1.8, 22.0 and 91.1% of abnormal individuals for the Control, HFO_10%, HFO_50% and HFO_100% groups, respectively (Chi-square, $p < 0.001$). Regarding the occurrence of abnormalities, 18.0% of abnormal larvae exposed to HFO_50% presented at least one kind of deformation (score 1). A major proportion of these abnormalities were comprised of edemas (13.9%). Larvae exposed to HFO_100% WAF were for 28.9% mildly affected (score 1), 20.0% moderately affected (score 2), 42.2% severely affected (score 3 or more) in terms of abnormalities. Among these deformations, it was observed that 41.6% were edemas (100% of pericardial edemas, 41.5% yolk sac edemas, results not shown), 19.4% were cardiovascular failures and 16.5% were craniofacial.

3.4. Swimming and cardiac activities

Fig. 3 portrays the PMR of zebrafish larvae (at 120 hpf) following exposure to both BAL and HFO WAFs. Response patterns of control larvae were similar during the three activity phases (*Light On* (1), *Light Off* and *Light On* (2)) of the challenge for the BAL and HFO WAF experiments. An increasing swimming activity was observed during the sudden darkness period (R-ANOVA, $p < 0.001$). Then, a recovery phase was observed, in which the second *Light On* period exhibited a similar (BAL WAF) or close (HFO WAF) response pattern to the first *Light On* period. Regarding the BAL WAF exposure (Fig. 3A), the response patterns were similar to the control group and no significant modifications in locomotor activity were found with the different concentrations (R-ANOVA, $p > 0.05$). Larvae exposed to the highest concentration of HFO WAF (HFO_100%) swam a significantly reduced distance than the Control and larvae exposed to HFO_10% and 50% (from 7.9- to 8.6-fold lower in the *Light On* (1) and from 3.0 to 3.2-fold in the *Light On* (2) phases, ANOVA, $p < 0.001$) (Fig. 3B). During the stress phase (*Light Off*), the distance moved by the larvae were not significantly different than the others conditions (ANOVA, $p > 0.05$).

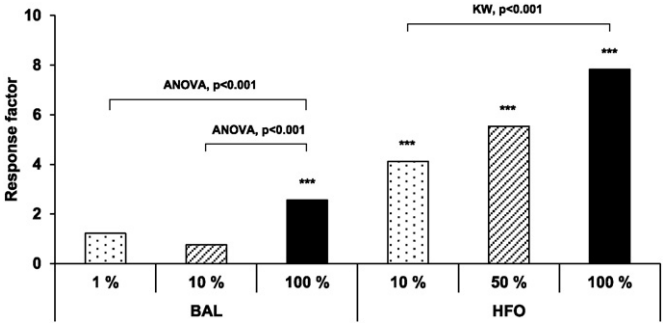

Fig. 5. In vivo measurement of EROD activity in WAF-exposed larvae at the end of exposure (96 hpf). Values are expressed in response factor compared to respective control group. Asterisks denote significant differences with respective control. Dark traits denote significant differences between WAF concentrations (N-ANOVA, ***$p < 0.001$).

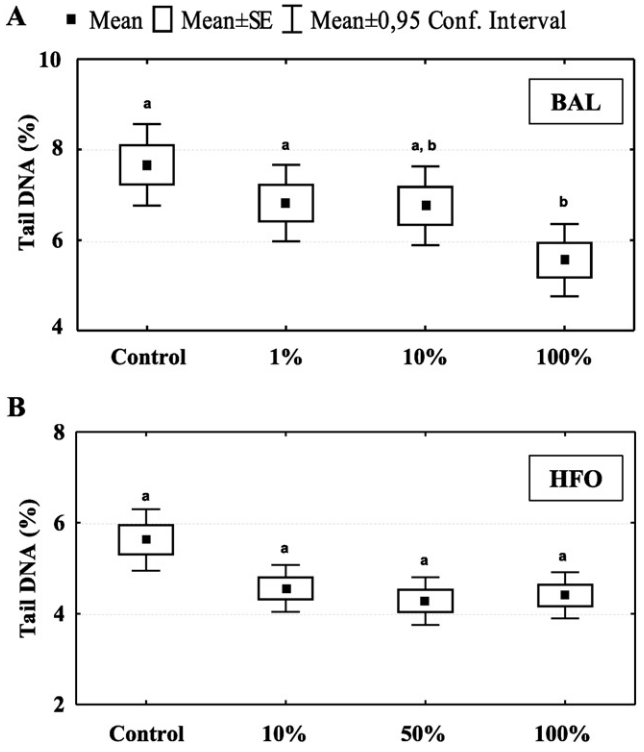

Fig. 6. DNA strand breaks (% tail DNA) measured in 24 hpf zebrafish embryos exposed to (A) BAL WAF and (B) HFO WAF by the comet assay. Values are means ± 95% confidence interval. Letters denote significant differences between BAL WAF concentrations (N-ANOVA, $p < 0.05$, $n = 3$ samples/300 nuclei).

With respect to cardiac activity, no significant differences were observed in heartbeat of 120 hpf larvae exposed to the various concentrations of BAL WAF (Fig. 4). On the contrary, a significant drop in heartbeats was measured in larvae exposed to HFO_100% WAF (KW, $p < 0.05$).

3.5. EROD activity

EROD activity in larvae exposed to both WAFs is described in Fig. 5. A significant increase in EROD activity compared to the Control (2.6-fold) was observed in larvae exposed to BAL WAF but only at the highest tested concentration (N-ANOVA, $p < 0.001$). A 3.4-fold increase in activity was also observed by raising the BAL WAF concentration from 10 to 100%. The HFO WAF exposure led to a concentration-dependent increase of EROD activity in zebrafish larvae, with inductions 4.1-, 5.5- and 7.8-fold higher than the Control group for HFO_10%, HFO_50% and HFO_100% WAF concentrations, respectively (KW, $p < 0.001$).

Table 6
Relative mRNA expression ratio from the control condition (standard error) of target genes in zebrafish larvae after 96 h of WAFs exposures.

	BAL WAF			HFO WAF		
	1%	10%	100%	10%	50%	100%
Detoxification						
cyp1a	1.166 (0.993–1.443)	1.266 (1.041–1.535)**	3.994 (3.492–4.678)**	2.041 (1.176–6.440)**	4.546 (2.370–14.780)**	8.308 (4.242–24.222)**
ahr2	1.073 (0.950–1.187)	0.944 (0.879–1.026)	1.053 (0.913–1.196)	1.826 (1.041–4.060)*	1.298 (0.644–3.656)	1.275 (0.773–3.364)
Oxidative stress						
gpx4a	1.081 (0.975–1.257)	1.257 (1.076–1.450)**	1.294 (1.107–1.508)*	1.242 (0.853–2.040)	1.374 (0.924–2.287)	1.609 (1.029–2.545)*
cat	0.978 (0.839–1.125)	0.988 (0.877–1.146)	1.126 (0.973–1.291)	1.362 (1.113–1.614)**	1.420 (1.217–1.649)***	1.252 (1.022–1.474)*
sod(*Cu/Zn*)	1.138 (1.023–1.258)*	1.183 (1.064–1.323)*	1.239 (1.101–1.410)*	1.204 (0.826–1.663)	1.388 (0.985–1.937)*	1.626 (1.038–2.575)*
sod(*Mn*)	1.058 (0.979–1.169)	1.113 (1.020–1.226)	1.191 (1.088–1.312)*	1.088 (0.932–1.294)	1.003 (0.839–1.261)	1.385 (1.106–1.932)*
Apoptosis						
p53	1.083 (0.999–1.181)	1.074 (0.952–1.220)	1.142 (1.069–1.210)**	0.863 (0.568–1.277)	1.075 (0.716–1.574)	1.236 (0.781–1.846)
bax	0.979 (0.932–1.023)	0.985 (0.932–1.106)	1.025 (0.979–1.076)	0.959 (0.808–1.104)	0.963 (0.881–1.060)	1.036 (0.873–1.252)

* $p < 0.05$, significant changes from respective controls.
** $p < 0.01$, significant changes from respective controls.
*** $p < 0.001$, significant changes from respective controls.

3.6. DNA integrity

At 24 hpf, the comet parameter, tail DNA, measured in the Control and WAF-exposed larvae was less than 10%. For both BAL and HFO WAF-exposed larvae, the levels of DNA strand breaks were not significantly different from that of Control larvae (Fig. 6A and B), except for BAL_100% WAF where the cell counts were significantly lower (N-ANOVA, $p = 0.001$). These results point to the absence of genotoxic effects from WAF exposure.

3.7. Gene transcription

The relative expressions of target genes compared to their respective controls are presented in Table 6. Data obtained for BAL and HFO WAFs were normalized using four housekeeping genes, *eukaryotic elongation factor 1* (*eef1*), *beta-actin* (*βactin*), *glucose-6-phosphate dehydrogenase* (*g6pd*) and *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*).

The gene encoding for *cytochrome P4501A* (*cyp1a*) was 1.27- and 3.99-fold upregulated for the two highest BAL WAF concentrations ($p = 0.004$ and 0.002, respectively). *Cyp1a* was upregulated from 2.04- to 8.31-fold for the three tested concentrations of HFO WAF ($p = 0.004$; 0.002 and 0.002, respectively). Concerning the transcription level of *aryl hydrocarbon receptor 2* (*ahr2*), it was only significantly increased following exposure to the lowest HFO WAF concentration ($p = 0.03$).

Regarding antioxidant defenses, genes encoding for *glutathione peroxidase 4* (*gpx4*), *Cu/Zn-superoxide dismutase* (*sod*(*Cu/Zn*) and *Mn-superoxide dismutase* (*sod*(*Mn*)) were slightly upregulated in larvae exposed to BAL WAF. *Gpx4* expression was also slightly increased in BAL_10% and BAL_100% WAF. *Sod*(*Cu/Zn*) expression was 1.12- to 1.24-fold

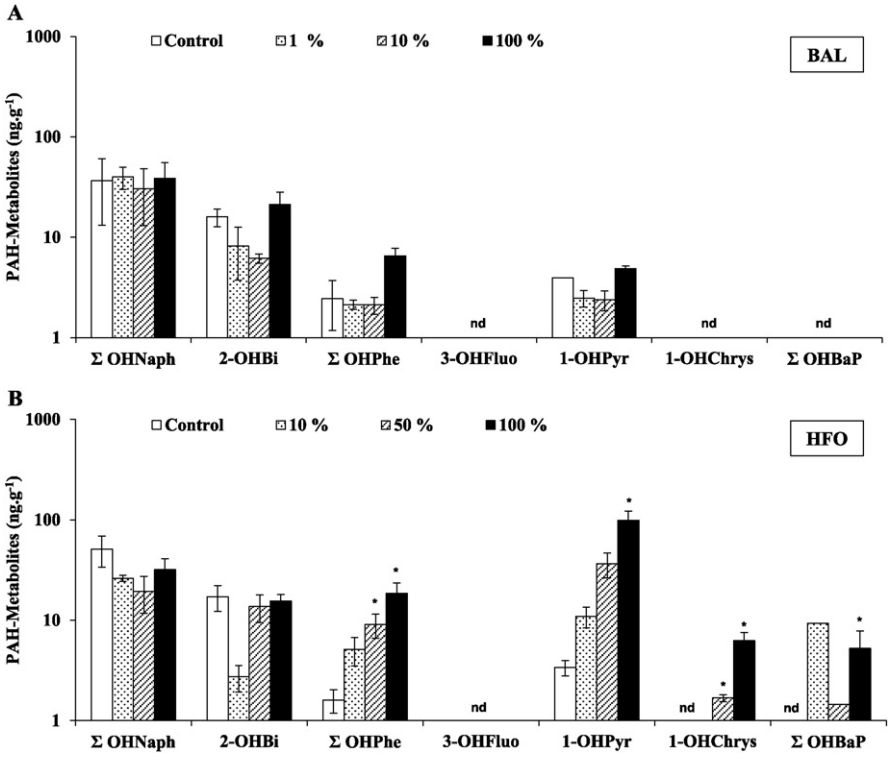

Fig. 7. Concentrations of PAH-metabolites (OHPAHs) measured in larvae at the end of exposure (96 hpf) for (A) BAL WAF and (B) HFO WAF. Values are means ± SEM. n.d.: not detected. Stars denote significant differences compared to the control. OHNaph: hydroxynaphthalenes; 2-OHBi: 2-hydroxybiphenyl, OHPhe: hydroxyphenanthrenes, 3-OHFluo: 3-hydroxyfluoranthene, 1-OHPyr: 1-hydroxypyrene, 1-OHChrys: 1-hydroxychrysene, OHBaP: hydroxybenzo[*a*]pyrene.

higher than in the Control group ($p < 0.05$). An increase in the expression level of the antioxidant enzyme *Sod*(*Mn*) was only revealed at BAL_100% WAF concentration ($p = 0.02$). No significant change in *catalase* (*cat*) mRNA levels was observed in larvae exposed to BAL WAF.

For HFO WAF exposures, *gpx4* expression was significantly upregulated at the highest concentration ($p = 0.03$). In contrast to BAL WAF exposure, a significant increase of *cat*-mRNA levels was found in larvae exposed to HFO WAF. Transcription levels were 1.25- to 1.42-fold above the Control group. *Sod*(*Cu/Zn*) was also significantly upregulated for the two highest concentrations ($p = 0.046$ and 0.02, respectively). *Sod*(*Mn*) *mRNA* was 1.39-fold higher at the highest concentration versus the control group ($p = 0.01$).

The apoptosis pathways (*bcl-2-binding component 3*, *p53* and *bcl-2-associates X protein bax*) were not impacted by either HFO and BAL WAFs, except for the *p53* gene which was upregulated at the BAL_100% WAF concentration ($p = 0.003$).

3.8. PAH metabolites

Quantification of hydroxylated PAH metabolites (OHPAHs) is shown in Fig. 7. In the control group, the total sum of OHPAHs was 59.1 ± 31.9 $ng \cdot g^{-1}$ larvae for BAL WAF and 73.3 ± 27.3 $ng \cdot g^{-1}$ larvae for HFO WAF. 3-OH-Fluo was not detected in larvae exposed to either WAF. The detected metabolites were not significantly different after exposure to BAL WAF (KW, $p > 0.05$) (Fig. 7A). The 1-OH-Chrys and $\sum$ OH-BaP were only detected in larvae exposed to HFO WAF (Fig. 7B). The HFO WAF initiated a concentration-dependent increase of 1-OHPyr and 1-OHChrys. The $\sum$ OH-BaP were present in the larvae exposed to the various concentrations of HFO WAF, significantly rising with HFO_100% WAF.

4. Discussion

The aim of this study was to assess the toxicity of WAF from two different oil compositions: i) a brut *Arabian Light* oil and ii) a heavy fuel oil from *Erika*, through an experimental system using a zebrafish embryo-larval assay.

4.1. Chemical assessment of water-accommodated fractions

In the context of hazard risk assessment, the use of dissolved fractions or WAF appeared suitable to evaluate the toxicity of petroleum compounds released into the environment through an oil spill (Martinez-Gomez et al., 2010). In our study, PAH concentrations in WAF decreased significantly (50-fold in mean) over the 24 h incubation at 28 °C (results not shown). For this reason, the tested solution was renewed every day during the experiments using the same WAF stock solution. When stored at 4 °C in dark, this solution remained stable within the 96 h exposure period, validating the experimental protocol. Such a decrease in PAH concentrations in WAF solutions has already been observed by Couillard et al. (2005), suggesting a possible degradation, volatilization and/or uptake of compounds by fish larvae. In the present study, the high temperature of incubation likely increased the loss of the dissolved fraction.

The concentrations used in the present study were chosen to cover a wide range of PAH concentrations found in the environment after oil spills. Indeed the concentrations for BAL WAF ranged from 6,159 ± 3,705 to 170,809 ± 4,480 $ng \cdot L^{-1}$ (ΣTPAHs) and for HFO WAF, they ranged from 26,105 ± 3,528 to 257,029 ± 47,231 $ng \cdot L^{-1}$ (Table 4). Law (1978a, 1978b) recorded concentrations from 2,200 up to 200,000 $ng \cdot L^{-1}$ in water sampled at a depth of 1 m after the *Amoco Cadiz* oil spill. This author also reported a concentration of aromatic hydrocarbons up to 1700 $ng \cdot L^{-1}$ after the *Ekofisk Bravo* blowout in 1977 (Law, 1978b). After the *Prestige* oil spill in 2002, PAH concentrations in seawater reached 28,800 $ng \cdot L^{-1}$ (Bado-Nilles et al., 2009b; González et al., 2006). The concentrations documented following the *Exxon Valdez* and *Erika* spills were, respectively, 600,000 $ng \cdot L^{-1}$ and from 23.5 to 54.9 $ng \cdot L^{-1}$ in coastal waters (Boehm et al., 2007; Tronczynski et al., 2004).

The composition of the prepared dissolved fractions are consistent with the specific PAH profile of brut *Arabian Light* and *Erika* heavy fuel oils described in the literature (Geffard et al., 2004; Saeed and Al-Mutairi, 2000; Tronczynski et al., 2004). Certainly, *Erika* fuel oil consists mainly of alkylated compounds of naphthalene, phenanthrene, pyrene and chrysene and HMW PAHs when compared to BAL oil. Conversely, BAL oil is characterized by a higher concentration of sulphurated PAHs and their homolog compounds with a large proportion of LMW PAHs.

4.2. Survival/hatching and developmental defects

BAL WAFs did not display any lethal effect or developmental disruption in zebrafish embryo-larval development whatever the tested concentration was (Fig. 2A, C and Table 5). On the contrary, WAF from *Erika* oil spills (HFO) was acutely toxic for larvae exposed at the highest concentration (Fig. 2B and D). This indicates that direct embryonic exposure may have important consequences on larval survival and may impact the fish recruitment under environmental conditions. This lethal body burden might be explained by the high incidence and severity of different morphological abnormalities observed in the larvae exposed to high concentration of HFO WAF (Table 5). Indeed, 40.0% to 91.1% of abnormal larvae were severely affected by edemas (41.6%) as well as cardiovascular (19.4%) and craniofacial (16.5%) disruptions. These deleterious effects were deemed more severe as the larvae were significantly smaller. Despite the body burden in larvae exposed to lowest concentrations of HFO WAF, no acute effect was recorded.

Our toxicological data are consistent with previous studies reporting acute and/or developmental effects of PAH mixtures on fish ELS (Carls and Thedinga, 2010; Cachot et al., 2007; Hose et al., 1996; Kammann et al., 2004; Kocan et al., 1996; Marty et al., 1997; Tilseth et al., 1984). However, a direct comparison is difficult due to differences in oil composition (mixture or oil) and concentrations plus exposure time between studies. Furthermore, it was shown in literature that the toxicity threshold for marine species are lower than they are for zebrafish, from 10 to 100 times lower (Incardona et al., 2014).

Lethal and sublethal effects observed in the present study might be associated with a reduction in the use of nutritional reserves, and therefore of the energy required for optimal physiological mechanism functioning (Billiard et al., 1999; Carls and Thedinga, 2010). This is one of the reasons why survival monitoring should be prolonged after the exposure period in order to assess the ability of starved larvae to draw upon their reserves and to survive (Örn et al., 1998).

4.3. Behavioral toxicity assessment

Based on motor responses (perception and reaction) to stimuli closely associated to swimming activity, the PMR of larval zebrafish were analyzed in order to evaluate the developmental neurotoxicity of spilled petroleum-related compounds (Fig. 3). Whichever the fraction considered, PMR patterns during the challenge were consistent with those found in the literature, dissociating three swimming activity phases: i) basal swimming activity phase (*Light On* (1)), ii) challenge phase or stress phase (*Light Off*) causing an increase in swimming activity and iii) the recovery phase during which swimming activity returns to the basal level in the new light phase (Kokel et al., 2010; MacPhail et al., 2009; Padilla et al., 2011).

The BAL WAF did not induce any swimming activity change in response to the light stress (Fig. 3A). However, an interesting response was demonstrated by larvae exposed to the highest concentration of HFO WAF (Fig. 3B). Indeed, compared to their basal swimming activity level, HFO_100%-exposed larvae swam, on average, 8.3-fold less distance-wise than other exposed groups (HFO_10%, HFO_50%) or

unexposed larvae. Despite an important interindividual variability, the larvae exposed to HFO_100% reacted to a second stress (*Light Off*) in a similar manner than other exposed- and Control larvae in terms of distance moved and swimming speed (results not shown). As well, the larvae exposed to HFO_100% returned to basal swimming activity levels when the light was switched back on. This notable reduction in swimming activity was closely linked with morphological impairments. Indeed, mildly to severely affected larvae engaged in a minimum of vital swimming for their survival, but in the case of sudden stress (e.g. predation), they would be unable to rapidly react. And, as previously observed, their survival was more limited than control larvae.

Information on fish PMR following exposure to pollutants is limited to only a few studies (Péan et al., 2013; Usenko et al., 2011) and to the best of our knowledge, no data about the impact of PAH mixtures is currently available in the literature, limiting comparisons. In zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) exposed to PAH-contaminated sediments, modulations in PMR were recorded dependent on the exposed compound. Fluoranthene (10 $\mu g \cdot g^{-1}$, 3-benzenic rings PAH) led to a reduction of distance moved by larval zebrafish whereas benzo[*a*]pyrene (10 $\mu g \cdot g^{-1}$, 5-benzenic rings PAH) increased swimming activity in Japanese medaka (Perrichon et al., 2014). Through the PMR analysis, both BAL and HFO WAFs did not elicit behavioral abnormalities in the larvae exposed whatever the tested concentration. We also note that these behavioral analyses did not assess the swimming performance (strict sense) of larvae but aimed to test their ability to rapidly react to a second stress, which could be a benefit when escaping a predator or a polluted area, or to capture prey. The behavioral consequences observed might be directly associated to the morphological alterations or representative of physiological impairments. The body burden observed in larvae exposed to oil might trigger a strong energy demand. Consequently, larvae might reduce their swimming activity to gain energy and allocate it for vital functions (cardiac, respiratory) to maintain a good fitness.

4.4. Cardiotoxicity assessment

Once more, the BAL fraction did not induce any cardiac disruptions at any of the tested concentrations (Fig. 4). However, the lethal and sublethal effects previously observed in larvae exposed to HFO_50% and HFO_100% WAFs could be the direct consequences of metabolism impairments induced by petroleum compound exposure (Billiard et al., 1999; Rhodes et al., 2005). Indeed, we observed defects in heartbeat in 120 hpf larvae exposed to the highest concentration of HFO WAF (Fig. 4). The observed bradycardia is consistent with the direct influence that was observed on cardiac morphogenesis. Among the abnormal larvae recorded in the present study, a major percentage of the malformations were of a pericardial and cardiovascular nature (e.g. anemia, hemorrhages, peripheral vascular defects, respective position of atrium and ventricle). However, no heartbeat difference was recorded with larvae exposed to HFO_50% WAF, though 22.0% of larvae were deformed.

Disruption in cardiac functioning will be primarily affected by oil exposure and may lead to important consequences for survival during later developmental stages and consequently could be deleterious for fish recruitment. Recent studies have documented the cardiotoxic action of PAHs in fish ELS, with bradycardia, irregular arrhythmia and morphogenesis disturbances (González-Doncel et al., 2008; Hicken et al., 2011; Incardona et al., 2004, 2005, 2006, 2009, 2010, 2011; Scott et al., 2011). Heartbeat was also decreased in Pacific herring (*Clupea pallasi*) and zebrafish (*Danio rerio*) embryos following a water soluble fraction (WSF) exposure of Alaska crude oil no. 2 ($\sum$TPAH = 9.3 $\mu g \cdot mL^{-1}$; Middaugh et al., 1996, 1998) and after sediment eluates/extracts exposure (Strmac et al., 2002). In 2010, Claireaux and Davoodi described a reduction of cardiac output (heart rate and stroke volume) in oil-exposed common sole (*Solea solea*). The ventricular contractility of developing zebrafish has been seen reduced after exposure to high energy WAF of Iranian and Alaska crude oils (Jung et al., 2013). Recently, Brette et al. (2014) have established that the physiological mechanism underlying to these cardiotoxic action previously observed, might be due to a blockade of the excitation-contraction coupling in cardiomyocytes. Further, the abundance of parent tricyclic PAHs and their alkylated homologs in weathered crude oil or PAH mixtures could be the etiology of cardiac dysfunction in fish embryos (Heintz et al., 1999; Incardona et al., 2004, 2005; Jung et al., 2013).

4.5. Genotoxicity assessment

Surprisingly, no significant difference in DNA damage levels was observed following exposure to both WAFs in 24 hpf embryos (Fig. 6). As a matter of fact, it has been shown that some PAHs and their metabolites have the ability to interact with DNA that leads to a number of structural DNA lesions, such as chromosomal modifications, cross-linkages, strand breaks or DNA adducts (Kosmehl et al., 2008; Nogueira et al., 2009; Xue and Warshawsky, 2005). In case of non-reparation, these DNA damages may affect development, growth dynamic, reproduction and therefore organism's fitness (Lawrence and Hemingway, 2003; Xue and Warshawsky, 2005). The early analysis (24 hpf) of these forms of damage may be an explanation for the lack of significant results. Recently, Le Dû-Lacoste et al. (2013) have demonstrated that genotoxic responses of turbot exposed to PAH mixtures ($\sum$TPAH = 6,602–69,285 $ng \cdot L^{-1}$) extracted from *Erika* fuel oil was only significant after four days of exposure. A minimal time to onset toxic mechanisms (enzymatic activation and metabolic system) could be necessary to reveal a positive response in the comet assay (Le Dû-Lacoste et al., 2013; Kammann et al., 2004). Consequently, we cannot definitively comment on genotoxic effects in zebrafish exposed to both oil fractions (BAL, HFO).

4.6. Detoxification and antioxidant defenses assessment

The induction of cytochrome P450 and its catalytic activity involved in phase I detoxification has been used in environmental biomonitoring as a sensitive biomarker of PAH exposure (van der Oost et al., 2003). An increase of *cyp1a* mRNA levels was observed in larvae exposed to both BAL_10% and BAL_100% WAFs (Table 6). This gene upregulation was not sufficient to induce a significant upregulation of ethoxyresorufin-*O*-deethylase (EROD) activity in BAL_10%-exposed larvae whereas EROD was significantly raised in BAL_100%-exposed larvae ($\sum$TPAH = 170,809 ± 4,480 $ng \cdot L^{-1}$) (Fig. 5). In the same way, EROD activity and *cyp1a* upregulation were rose linearly based on the concentration of HFO WAF. Our findings have revealed that even though few morphological, behavioral or physiological alterations were observed in BAL_10%–100% WAFs and HFO_10%–50% WAFs, defense mechanisms were activated to prevent potential damage from petroleum compounds. *Cyp1a* induction has been shown to play a protective role rather to enhance the toxicity of petrogenic PAHs in fish early life stages (Hicken et al., 2011). However, in our work, even if CYP1A and EROD activity inductions were obviously higher at the highest concentration of HFO WAF, the PAHs toxicity have counteracted this protective barrier, inducing morphological, behavioral and physiological disruptions.

Furthermore, the increase of these detoxification systems could be linked with the production of reactive oxygen species (ROS) or reactive PAH metabolites (Basu et al., 2001; van der Oost et al., 2003). Indeed, when the production of ROS exceeds the limit of antioxidant defenses within an organism, ROS can interact adversely with cellular components (DNA, proteins, membrane lipids), causing oxidative stress and ultimately cell death (Di Giulio and Meyer, 2008). In the present study, the transcription of genes involved in antioxidant defenses (*gpx*, *cat*, *sod*) was slightly increased with the WAF concentrations of brut *Arabian Light* and *Erika* heavy fuel oils (Table 6). This suggests a slight induction of oxidative stress in WAF-exposed larvae. This

oxidative stress appears to also be related to the induction of enzymatic detoxification systems.

Activation of antioxidant defenses could also play a protective role against petroleum compounds by preventing adverse effects in exposed larvae. However, while a slight upregulation of antioxidant genes was shown, their activation does not seem to stabilize the prooxidant-antioxidant system in HFO_50% and HFO_100% that both led to morphological damage and the subsequent lethal effects. Several studies have examined changes in oxidative stress in response to PAH exposure in aquatic organisms but most were concerned protein activity and not mRNA expression responses, focused exclusively on the exposure to single PAH compounds (Ferreira et al., 2005; Oliveira et al., 2008; Sun et al., 2006).

The modulation of antioxidant defenses appears to be a sensitive but highly variable marker and species-specific. For instance, phenanthrene (3-benzenic rings PAH) induced oxidative stress in golden grey mullet (*Liza aurata*) and in goldfish (*Carassius auratus*) (Oliveira et al., 2008; Sun et al., 2006). The concentrations used in these studies were relatively high. An increase of catalase activity was also reported in the liver of the African catfish (*Clarias gariepinus*) exposed for 196 h to crude oil (Achuba and Osakwe, 2004). In contrast, antioxidant enzyme activities were modulated in golden grey mullet exposed to chemically dispersed oil (Milinkovitch et al., 2011a; Milinkovitch et al., 2011b). Kerambrun et al. (2012) have also described the lack of modulation of catalase activity in sea bass (*Dicentrarchus labrax*) exposed to acute concentrations of crude oil. Throughout the literature, these antioxidant defenses appear less sensitive to pollutant exposure versus detoxification and biotransformation processes and little evidence are shown that they are important modes of toxicity of petroleum compounds.

The activation of detoxification systems and the potential production of ROS could be because of the production of reactive PAH metabolites, generally more toxic than their parent PAHs. While the results were not statistically different between WAF exposures for most PAH metabolites, a high proportion of OH-Pyr, OH-Chrys and OH-BaP were found in larvae exposed to HFO (Fig. 7B). Even still, we cannot directly associate these metabolites to the adverse effects observed in the present study, though they are known to induce physiological (e.g. cardiac dysfunction) and morphological (e.g. development abnormalities) impairments (Incardona et al., 2004, 2005, 2009). These metabolites (mainly OH-Pyr) are currently observed as the predominant compounds metabolized by fish after PAH exposure (Brinkmann et al., 2010; Le Dû-Lacoste et al., 2013). Additionally, OH-BaP is known to be a genotoxic metabolite and could be the origin of cellular damage causing adverse effects on fish morphology (Le Dû-Lacoste et al., 2013). While OH-Chrys was also present in HFO larvae as well as its parent and alkylated homologs in water, these compounds are not known to alter embryonic development (Incardona et al., 2004, 2009) but are high *Cyp1a*-inducers (Barron et al., 2004).

Evidence of exposure has been demonstrated in this study, but the observed effects cannot be directly linked to specific compounds constituting the WAF. Several investigations have found that alkylated compounds play an important role in oil toxicity (Fallahtafti et al., 2012; Knecht et al., 2013; Turcotte et al., 2011). An important proportion of alkyl PAHs (e.g. MPhe) are present in WAFs from crude oils but the observed-influence will probably result from a combination of compounds that will generate toxicity through different dependent- or independent-aryl hydrocarbon receptor pathways (*ahr2* was not induced in our study). The narcotic action of LMW PAHs (mainly by naphthalene) might play an important role in the observed developmental toxicity whereas the HMW PAHs (e.g. pyrene, benzo[*a*]pyrene and/or chrysene) may modulate certain enzymatic activities, such as MFO activity. Further alkyl-PAH toxicity should be further evaluated in order to elucidate their specific impact on fish ELS.

In the study presented here, a zebrafish embryo-larval bioassay was employed as a tool to provide a more comprehensive phenotypic map for assessing the effects of pollution on development. The dissolved fractions from HFO induced a high level of toxicity in zebrafish larvae. The use of zebrafish ELS as model of water risk assessment should be cautiously used because of the high resistance level of this fish compared to marine species. Our findings strengthen once more, that the petroleum compounds (PAHs) affect specifically the cardiac function and swimming activity of developing fish. Future research should focus on these both physiological and behavioral functions and to understand the underlying foundations of these detrimental effects. The use of a zebrafish assay could be the subject of further investigations to assess the impact of early oil exposure (mechanistic point of view) on later stages and the ability of fish to ensure future generations.

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