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Comparative study of different exposure routes on the biotransformation and genotoxicity of PAHs in the flatfish species, *Scophthalmus maximus*

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Abstract In this study, laboratory experiments were carried out in order to come to a better understanding of the fate of polycyclic aromatic hydrocarbons (PAHs) in the marine environment and especially on their bioaccumulation, biotransformation and genotoxic effects in fish. Juveniles of turbot (*Scophthalmus maximus*) were exposed to PAHs through different routes via (1) a mixture of dissolved PAHs, (2) a PAH-polluted sediment and (3) an oil fuel elutriate. Fish were exposed 4 days followed by a 6-day depuration period. In each experiment, PAH concentrations in the seawater of the tanks were analysed regularly by gas chromatography coupled with mass spectrometry. Muscle and liver samples were also analysed for parent PAH levels and PAH bioconcentration factors were calculated. Biotransformation was evaluated by measuring the levels of PAH metabolites in fish bile. Genotoxicity was assessed by the alkaline comet assay. Regardless of exposure route, the parent PAH concentrations in the liver and muscle showed a peak level 1 day after the beginning of the exposure, followed by a decrease up to the background level towards the end of the experiment, except for the exposure to dissolved PAHs for which levels were relatively low throughout the study. As a consequence, no bioaccumulation was observed in fish tissues at the end of the experiment. In contrast, regardless of exposure routes, a rapid production of biliary metabolites was observed throughout the whole exposure experiment. This was especially true for 1-hydroxypyrene, the major metabolite of pyrene. After 6 days of recovery in clean water, a significant decrease in the total metabolite concentrations occurred in bile. Fish exposed through either route displayed a significant increase in DNA strand breaks after 4 days of exposure, and significant correlations were observed between the level of biliary PAH metabolites and the level of DNA lesions in fish erythrocytes. Overall results indicate that exposure to either a mixture of dissolved PAHs, a PAH-contaminated sediment or a dispersed oil fuel elutriate leads to biotransformation and increase in DNA damage in fish. The quantification of PAH metabolites in bile and DNA damage in erythrocytes appear to be suitable for environmental monitoring of marine pollution either in the case of accidental oil spills or sediment contamination.

Keywords PAHs · Biotransformation · Metabolites · DNA strand breaks · Marine fish · Mesocosm · Multi-route exposure

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants released into the environment primarily through the incomplete combustion of organic matter (pyrolytic processes) (Walker et al. 2005) including both

anthropogenic sources (Budzinski et al. 1997) and natural sources (forest fires) (Whyte et al. 2000). PAHs and alkylated PAHs are also naturally present in crude oils resulting from the diagenetic and catagenetic transformation of organic matter. Certain PAHs belong to the list of priority pollutants established by the Environmental Protection Agency (EPA, USA) due to their mutagenicity and carcinogenicity. Aquatic systems including marine environments appear to be a repository for PAHs by way of terrestrial runoffs, atmospheric depositions and petroleum release (Latimer and Zheng 2003). Petroleum releases into the marine environment are mainly occurring in the case of oil tanker transportation (operational discharges at terminals, deliberate releases of oiled ballast water, wreckage), offshore petroleum exploitation and natural seeps (GESAMP 1993). Sediments of freshwater and marine harbours are also frequently contaminated with PAHs as a consequence of spilled fuel, street runoff and natural releases from, e.g. creosote sources (Gómez-Gutiérrez et al. 2007).

Except for lower trophic levels, most organisms are able to metabolise PAHs. As a consequence, measurement of parent PAHs in animal tissues does not provide an adequate assessment of PAH exposure (Meador 2003). Moreover, parent PAHs can be extensively biotransformed into more toxic metabolites (Varanasi and Stein 1991). Therefore, the study of PAH biotransformation in marine organisms, and more particularly in fish, appears to be necessary in order to better access bioavailability and toxicity. In this study, the teleost flatfish turbot (Scophthalmus maximus) was selected as a bioindicator species because it is a commercially important fish that is widely distributed along the European coast that lives in close association with sediment and associated organic contaminants. Its benthic feeding preferences make it particularly vulnerable to contaminants in sediment (Kilemade et al. 2009). Flatfish have been commonly used in biomarker studies in relation to exposure to PAHs (Kirby et al. 2007; Wessel et al. 2010), and recently, Kerambrun et al. (2012) demonstrated the relevance of turbot for marine biomonitoring.

The biomarker approach is suggested as a suitable strategy for studying sublethal effects of pollutants, providing an early indication of possible adverse effects in organisms (Van der Oost et al. 2003; Al-Subiai et al. 2011). Biochemical markers of exposure, most of them being enzymes involved in biotransformation processes, have been used extensively to evaluate PAHs exposure in fish (e.g. Nahrgang et al. 2010; Santos et al. 2010; Trisciani et al. 2011). In the last years, biliary metabolites of PAHs have also been used as a particularly efficient biomarkers of exposure in fish exposed to PAHs in their environment (Van der Oost et al. 2003; Vuorinen et al. 2006; Kamman 2007; Tairova et al. 2012), including oil spills (Budzinski et al. 2004; Baršiene et al. 2006; Kreitsberg et al. 2010).

Interestingly, Budzinski et al. (2004) found no increase in PAH levels in sole (Solea solea) muscle following the Erika oil spill of 1999 but demonstrated high levels of PAH metabolites in the bile of the same fish indicating a significant uptake and biotransformation of PAHs in fish.

In parallel to biomarkers of exposure, some biological markers of pollutant effects have been proposed, among them biomarkers of genotoxicity. Genotoxic effects included different structural DNA lesions such as strand breaks, DNA base modifications, cross-linkages and DNA adducts that are associated with exposure to a wide number of genotoxic contaminants, among them certain PAHs and their metabolites (Xue and Warshawsky 2005). The genotoxic effects of PAHs in fish have been evaluated in a large number of studies (e.g. Kilemade et al. 2004; Goanvec et al. 2008). However, studies presenting results on both PAH metabolites and genotoxic effects are limited (Aas et al. 2000; Brinkmann et al. 2010; Wessel et al. 2010). DNA strand breaks are one of the most integrative measurements of genotoxicity and are used as early markers to recent PAHs exposure and effects even if they are reversible lesions. DNA strand breaks can be measured with the comet assay that has been widely applied in fish and molluscs to assess pollutant genotoxicity in both laboratory and field studies (Akcha et al. 2004; Cachot et al. 2006; Frenzilli et al. 2009; Wessel et al. 2010).

The purpose of this study was to compare the potential effects of different routes of PAH exposure on the biotransformation and genotoxic effects of this major class of marine pollutants. A contaminated sediment from Arcachon harbour (France) and a petrol elutriate prepared from oil fuel Erika #2 were used to expose hatchery-reared juveniles of turbot (S. maximus) under controlled laboratory conditions. In addition to an unexposed assay group, a separate group of turbot was also exposed to a dissolved mixture of seven PAHs as a positive control. Another objective was to investigate the relationship between PAH metabolites and DNA strand breaks with the aim to discuss their use as biomarkers of both PAH exposure and effects in this species. This study may also provide useful information regarding the impact different types of exposure to PAHs can have regarding the genotoxic risk in fish.

Materials and methods

Chemical, solvents and reagents

Hydroxylated PAHs: 1-hydroxynaphtalene (1-OHN) and 2-hydroxynaphtalene (2-OHN) were purchased from Lancaster (Bischeim, France); 2-hydroxybiphenyl (2-OHBi), 9-hydroxyfluorene (9-OHFe) and 1-hydroxypyrene (1-OHP) were obtained from Sigma-Aldrich (Saint Quentin Falavier,

France) and 9-hydroxyphenanthrene (9-OHPhe), 1-hydroxychrysene (1-OHC) and 3-hydroxybenzo[a]pyrene (3-OHBaP) from Promochem (Molsheim, France). 1-Hydroxypyrene-d9 (1-OHPd9) was obtained from Cambridge Isotope Laboratories (Cluzeau Info Labo, Ste Foy La Grande, France). Concerning parent PAHs, the standard reference SRM 2260, constituted of about 20 aromatic compounds, was provided by the U.S. National Institute of Standards and Technology (Gaithersburg, MD, USA). Perdeuterated aromatic compounds were obtained from MSD Isotopes (Montreal, Canada). All the standards and solvents used were of analytical grade (>98 %). Dichloromethane was provided by Acros Organics (Noisy-Le-Grand, France). Methanol Supersolv was provided by Merck and acetic acid 100 % Normapur (analytical reagent grade, Scharlau) was obtained from ICS (Belin-Beliet, France). Sodium acetate trihydrate 99 % (purity>99 %) and β-glucuronidase-aryl-sulfatase mixture from Helix pomatia (100,000 and 7,500 U mL^{-1}, respectively) were provided by Sigma-Aldrich. 2-Mercaptoethanol and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Acros Organics (Noisy-Le-Grand). Deionised water was obtained with a Milli-Q system (Millipore, Molsheim, France). Two solid-phase extraction cartridges were used: the Strata-X cartridge (3 cc, 200 mg) from Phenomenex (Le Pecq, France) and the HF Bond Elut-PSA cartridge (3 cc, 500 mg) from Varian. Cartridges were obtained from Atlantic Labo (Eysines, France). Aluminium oxide (150 basic type T; 0.063–0.2 mm) and silica gel (0.063–0.2 mm) were provided by VWR (Strasbourg, France). RPMI 1640 medium, dimethyl sulfoxide, 0.4 % trypan blue solution, ethidium bromide (BET), foetal bovine serum (SVF), normal and low melting point agarose, N-sarcosinate and Triton X-100 were purchased from Sigma-Aldrich Chemicals.

Fish

Juvenile turbots (S. maximus) were purchased from a fish farm (France Turbot, Groupe Adrien, Noirmoutier Island, France). They were housed in fish tanks filled with seawater (salinity at 30 ‰) at 16 °C and acclimated for a minimum of 25 days. The light regime was also controlled and was set at 6 h light per day (from 8 to 14 h). Fish were not fed during the acclimation period and during the experiments. The mean fish weight and size were 101±1 g and 152±4 mm (n=255 fishes) at the beginning of the experiment.

Exposure design and sample collection

After acclimation, 30 fish were placed in each 100 L fish tank. The control group was only exposed to solvent (ethanol) used in the preparation of the dissolved PAH mixture. Three groups of fish were either exposed to a mixture of dissolved PAHs, a natural sediment from Arcachon harbour (South West Atlantic Coast, Arcachon Bay, France) or a petrol elutriate prepared from oil fuel Erika #2.

The dissolved PAH mixture was prepared with seven PAH compounds (gravimetrically weighted) and dissolved in ethanol. The compounds selected for this experiment were naphthalene (N), phenanthrene (Phe), anthracene (A), fluoranthene (Fluo), pyrene (Pyr), chrysene (Chrys) and benzo[a]pyrene (BaP). BaP and Fluo were selected since they have been already shown to be genotoxic in fish (Woo et al. 2006); the other compounds have been chosen because firstly they are frequently found in sediment and oil (Mazeas and Budzinski 2002), and secondly, they belong to two-ring to five-ring PAHs, exhibiting different physico-chemical properties and thus different behaviours in organisms. The raw solution of PAH mixture was then diluted in seawater and continuously added to the fish tank by peristaltic pumps (Pump Analog Reglo® 4/8 from ISMATEC, Fisher Bioblock, France) at a flow rate of 1 mLmin^{-1} during 4 days of exposure. PAH concentrations in this solution ranged from 3.3 to 15.5 mg L^{-1} (Table 1). The elutriate solution was prepared from 60 g oil fuel #2 (PAH: nd (LOD=0.001)–614 μgg^{-1}; Table 1), firstly dissolved in acetone and magnetically stirred overnight and then diluted in seawater (4 L; 15 °C, salinity 30 ‰). This final diluted solution called petrol elutriate was continuously added to the fish tank by peristaltic pumps at a flow rate of 0.8 mLmin^{-1} during 4 days of exposure. Nominal PAH concentrations in the elutriate solution ranged from 0.7 to 9.2 mg L^{-1}. The sediment was homogenised and allowed to settle for 2 h before the introduction of fish in the tank. PAH concentrations in the sediment used (2–465 ngg^{-1} dry weight) are given in Table 1. The PAH and petrol solutions and the sediment used for experimentation were previously characterised and analysed by gas chromatography coupled with mass spectrometry (GC/MS; according to protocols described in 'PAH analysis').

Fish were exposed to PAHs for 4 days, and a subset of five animals was removed and euthanised each day (days 0, 1, 2, 3 and 4, called T0, T1, T2, T3 and T4). In parallel, 100 mL water samples were collected at each sampling day from each tank to control for exposure concentrations in each treatment group. After 4 days, the five remaining fish were placed in clean water up to day 10 (T10) for 6 days of depuration. At each sampling time and for each exposure group, blood (for comet assay) and bile (for PAH metabolite determination) were sampled from each of the five sampled individuals. The bile was stored at −80 °C. The blood was sampled with a heparinised syringe, recovered in a freezing medium and stored at −80 °C prior to the application of the comet assay as previously described (Akcha et al. 2003). The muscle and the liver samples (for PAH body burden measurement) were pooled (n=5) and stored at −20 °C until their freeze-drying and homogenisation with a blender.

Table 1 PAH concentrations in natural sediment (microgramme per gramme dry weight), oil fuel (microgramme per gramme) and PAH mixture (milligramme per litre) used for the experiments

Only concentrations of the seven PAHs used in the waterborne PAH exposure are given. Total PAH comprises the sum of all 19 measured PAHs

nd not detected

Compounds	PAH concentration		
	Sediment (μg g^{-1} dw)	Oil fuel #2 Erika (μg g^{-1})	PAH solution (mg L^{-1})
Naphtalene	0.002	nd	10.6
Anthracene	0.096	90	10.2
Phenanthrene	0.018	614	7.5
Fluoranthene	0.362	46	15.3
Pyrene	0.281	218	3.3
Chrysene	0.201	324	15.5
Benzo[*a*]pyrene	0.190	96	5.2
Total PAH	2.561	1388	67.5

PAH analysis

Heavy fuel oil was analysed for PAHs according to the protocol described by Mazeas and Budzinski (2002). For this purpose, fuel oil was first dissolved in methylene chloride. For the determination of PAHs in the aqueous phase, water was first passed through a 0.7-μm GFF filter (VWR, Strasbourg, France) to separate dissolved PAHs from PAHs adsorbed on particulate matter. Dissolved PAHs were extracted by passing water samples through an octadecyl speedisk (Baker, Atlantic Labo, Eysines, France) that retains PAHs. Analytes were then recovered by successive elution with methylene chloride and methylene chloride/ethyl acetate (1:1, *v/v*). Trace amounts of water were then removed by passing the extract through sodium sulfate. For solid samples, PAHs were extracted from freeze-dried and ground samples (sediment, liver, muscle) by microwave-assisted extraction (10 min, 30 W) with methylene chloride followed by filtration with clean glass cotton (Budzinski et al. 1999). Several perdeuterated PAHs used as internal standards were added to the samples prior to the extraction step for PAH quantification (Baumard and Budzinski 1997). Extracts of the different matrices were then purified on aluminium oxide (Al$_2$O$_3$) and silica (SiO$_2$) columns and analysed by GC/MS (Mazeas and Budzinski 2002; Budzinski et al. 1999).

Analyses were performed on a HP 6890 gas chromatograph (GC) coupled to an HP 5973 mass selective detector (MSD) (Agilent Technologies, both provided by BIOS Analytique, L'Union, France), fitted with a HP5-MS capillary column (30 m×0.25 mm×0.25 μm; BIOS Analytique). Samples were injected (1 μL) into the GC in pulsed splitless (25 psi; 1 min) mode at 270 °C. The carrier gas was ultrapure helium 6.0 (Linde Gas, Bassens, France). The GC temperature programme was 70 °C (2 min hold) to 300 °C (5 min hold) at a rate of 10 °C min^{-1}. The MSD was operated in electron impact mode at 70 eV under the selected ion monitoring mode (SIM) using the molecular ion of each compound.

Different perdeuterated polycyclic aromatic hydrocarbons were used as internal standards for each class of aromaticity (Baumard and Budzinski 1997). The response factors of PAHs relative to perdeuterated PAHs were determined for each GC/MS sequence by injecting a mixture containing a solution of PAH (SRM 2260, National Institute of Science and Technology, Gaithersburg, MD, USA) and the solution of perdeuterated PAHs used for spiking samples. The monitored PAHs are, in ascending order of molecular weight, N*, acenaphthylene* (Ay), acenaphthene* (Ae), fluorene* (Fe), Phe*, A*, Fluo*, Pyr*, benz[*a*]anthracene* (BaA), chrys*+triphenylene (Chrys+T), benzo[*b*]fluoranthene*+benzo[*j*]fluoranthene+benzo[*k*]fluoranthene* (BbF+BjF+BkF=BF), BaP*, indeno[1,2,3-cd]pyrene* (IP), benzo[*g,h,i*]perylene* (BPer) and dibenz[*a,h*]anthracene*+dibenz[*a,c*]anthracene (DB[ah]A+DB[ac]A=DBA). Compounds marked with a '*' are the 16 PAHs of the US EPA. In this study, the sum of those compounds is referred as 19 PAHs because of the coelution of three other PAHs by GC/MS. The method for biota was periodically checked with the analysis of a certified muscle tissue (SRM 1974a, NIST, Gaithersburg, MD, USA) for which the PAH recoveries were 90±16 % with a coefficient of variation always less than 10 % (*n*=3). The detection limits in the fish tissues were in the range of 0.1–1 ng g^{-1} dry weight (dw), depending on the compounds.

Bioconcentration factors (BCFs) were calculated for muscle and liver tissues for each PAH at each sampling time for each exposure route as the ratio of PAH concentration in muscle or liver (nanogramme per gramme dry weight) divided by PAH concentration in seawater (microgramme per litre). BCF values were only determined when individual PAH levels in muscle or liver of fish exposed to PAH were significantly higher than those of control fish.

PAH metabolite analysis

The analytical procedure used to quantify PAH metabolites was adapted from Mazéas and Budzinski (2005). Whole bile was thawed and then homogenised in precooled buffered water (sodium acetate buffer at pH 5.0 with acetic acid) (100

μL of bile in 2 mL buffer). A mixture of the surrogate standard 1-hydroxypyrene-d9, 20 μL of mercaptoethanol (used as an antioxidant) and 20 μL of β-glucuronidase and arylsulfatase was added to the samples. The samples were then hydrolysed at 37 °C in an oven for 18 h. After deconjugation, bile samples were ultrasonicated and centrifuged (10 min, 5,000 rpm, 20 °C). Supernatants were loaded on Strata X cartridges and washed with 2 mL buffered water and then 2 mL water/methanol (70:30, v/v). Traces of water were then removed by applying vacuum to the cartridges for 45 min. Metabolites were then eluted with 5 mL of methanol. Extracts were reduced to dryness under nitrogen stream and redissolved in 1 mL methanol/methylene chloride (20:80, v/v) before purification with solid-phase HF PSA Varian cartridges. Extracts were reduced to dryness under a nitrogen stream and redissolved in 50 μL dichloromethane and 30 μL of a derivatising agent (BSTFA) was finally added. The derivatisation was then completed by incubation at 65 °C during 30 min. After derivatisation and before GC/MS analysis, a solution of deuterated internal standard (pyrene-d10, 20 μL) was added to the sample for recovery determination.

The analyses were carried out with an HP GC 6890A gas chromatograph (Agilent Technologies) coupled to an HP MSD 5973 Network mass selective detector (Agilent Technologies). The separation was performed on an Agilent Technologies HP5-MS capillary column (length 30 m; internal diameter 250 μm; stationary phase thickness 0.25 μm; phase 5 % diphenyl-, 95 % dimethyl-siloxane), with the following oven parameters: from 70 (2 min) to 180 °C (1 min) at 5 °C min^{-1}, and then from 180 to 290 °C (1 min) at 10 °C min^{-1}. The injection volume was set at 1 μL (automated PTV pulsed splitless injection; 25 psi, 1.5 min), while the injector temperature was set at 250 °C and the interface temperature at 280 °C. The purge flow was set at 60 mL min^{-1} during 1.5 min, and helium 6.0 (Linde, Bassens, France) was used as the carrier gas (constant flow 1 mL min^{-1}). Acquisition was performed in selected ion monitoring (SIM), with 1.53 scan/s and a dwell time of 50 ms for each ion. The source temperature was set at 150 °C, and the electron multiplier voltage set at 2,000 V. The compounds were quantified in SIM mode using the ions below: 1-OHN (m/z=201), 2-OHN (m/z=216), 2-OHBi (m/z=211), 9-OHFe (m/z=165), 9-OHPhe and isomers (m/z=266), 1-OHP (m/z=290), 1-OHC (m/z=316), 3-OHBaP (m/z=340), 1-OHP-d9 (m/z=299) and pyr-d10 (m/z=212). 1-OHP-d9 was used as surrogate standard and pyr-d10 was used as internal standard. A standard solution (model and surrogate compounds) prepared in dichloromethane and derivatised was injected at least every 10 real samples to monitor any drift in the instrumental performance (sensitivity and contamination) and to assure the accuracy of the quantification (determination of response factors). The recoveries of metabolites evaluated by triplicate

analyses of fish bile spiked with 10, 100 and 1,000 ng g^{-1} bile were in the range of 72–99 % with a coefficient of variation always less than 11 %. The limits of detection (LODs) obtained in bile samples when considering a signal-to-noise ratio above 3 from 100 μL of bile and 1 μL injected were in the range between 0.5 (1-OHP) and 2.2 (1-OHC) ng g^{-1} bile, except for 3-OHBaP (8.2 ng g^{-1} bile).

Comet assay

Blood samples were slowly defrosted at ambient temperature and diluted in PBS buffer before analysis. The comet assay was performed as previously described (Akcha et al. 2003). In order to obtain permanent preparations, the slides (two slides per sample) were immersed for 10 min in absolute ethanol for dehydrating and allowed to dry at room temperature. Just before analysis, 75 μL of BET at 20 μg mL^{-1} was pipetted on the slide and spread using a cover glass. Slides were placed for at least 1 h at 4 °C for coloration. Slides were analysed using an optical fluorescence microscope (Olympus BX60, ×40) fitted with a CDD camera (Olympus, France) and an image analysis system (Komet 4, Kinetic Imaging Ltd, UK). Several parameters were calculated for each observed nucleus (75 nuclei/slide), including the percentage of DNA present in the comet tail.

Statistical analysis

Data normality was tested with the Shapiro–Wilk test. To normalise data distribution, raw comet data were mathematically transformed by considering the square root value of the data. The effect of the depuration period on the comet parameter was studied by t test comparisons for each PAH-exposed group.

The data obtained for the control and the PAH-exposed groups were subjected to a main effects ANOVA analysis (factors: 'individual', 'treatment', 'experimental time') using Statistica 6.0 software (Statsoft, France). When a significant effect was observed, significant differences among means were determined using Tukey's post hoc test. Pearson correlations between endpoints were calculated. All tests were regarded as statistically significant when $p < 0.05$.

Results

PAH concentrations in water

Total PAH concentrations (18 PAHs, except for the standard mixture) were 2.56 mg kg^{-1} dry weight (dw), 1,388 μg g^{-1} and 67.5 mg L^{-1} in the sediment, oil fuel #2 Erika and PAH mixture used for the experiments, respectively (Table 1). In Table 1, only the individual concentrations of the seven

PAHs used in the standard mixture are presented for the three exposure routes. The concentration of dissolved PAHs in seawater showed different patterns depending on the exposure route. During the 4-day exposure period, total PAH concentrations ranged between 21 and 388, 204 and 538, 6,602 and 69,285 and 368 and 116,244 $ng\,L^{-1}$ in the control, sediment, petrol elutriate and dissolved PAHs exposure tanks, respectively (Fig. 1). Regardless of exposure route, the highest PAH concentrations were measured during the first day of exposure and decreased consistently after that. The distribution of individual PAHs varied among experimental groups and sampling time points (Fig. 2). For the petrol elutriate exposure, Phe (22 to 34 % of the total PAHs) and Chrys (17–22 %) were the most dominant PAH compounds in the seawater, whereas N (30–80 %) was the major compound in the control (except at day 4), sediment (except at day 1) and the PAH mixture exposure groups. In addition, the percentage contribution of individual PAHs was relatively constant during the 4 days of exposure to the petrol elutriate and the dissolved PAH mixture, compared to what was observed for the sediment exposure.

PAH concentrations in fish muscle and liver

There were statistical significant differences of PAH concentrations in white muscles and livers of juvenile turbots among treatment groups ($p<0.01$ and 0.05 for muscle and liver, respectively; Fig. 3). PAH concentrations were 10- to 100-fold and 5- to 30-fold higher in the muscles and the livers of fish exposed to the dissolved PAH mixture compared to fish exposed to sediment and petrol elutriate, respectively. Regardless of the route of exposure, Phe was the predominant PAH in muscle and liver (40–60 %), followed by Fluo and A (10–25 %); Pyr and BaP accounted for less than 10 and 3 %, respectively.

PAH concentrations in white muscles ranged between 30 and 52, 33 and 310, 38 and 432 and 688 and 12,682 $ng\,g^{-1}$ dw in fish from the control, sediment, petrol elutriate and dissolved PAH exposure groups, respectively. Regardless of the route of exposure, maximum concentrations were observed at day 1 and rapidly decreased after that. Except during the first 24 h exposure, PAH concentrations in fish exposed to sediments were similar to those observed in control fish. After the 6-day depuration period, PAH concentrations in muscles had returned to control levels, except for the dissolved PAH mix exposure (15-fold higher). Concentrations of PAHs in livers of fish were higher than in muscles and were between 80 and 277, 104 and 456, 697 and 2,105, and 602 and 28,221 $ng\,g^{-1}$ dw for the control, sediment, petrol elutriate and dissolved PAH exposure groups, respectively. Maximum concentrations were also observed after the first 24 h exposure, except for fish exposed to sediment for which concentrations in liver varied little (about 1.5-fold control levels). While concentrations decreased rapidly in fish exposed to the PAH mix, they remained relatively constant in the petrol elutriate experiment, even after the 6-day depuration period. In addition, PAH concentrations in livers did not return to control levels for both the dissolved PAH mix and the petrol elutriate experiments (6-fold higher).

BCFs were also higher in livers compared to muscles (Table 2). The highest values were obtained in the sediment group (300–6,700), followed by the PAH mixture group (2–1,100) and then the petrol elutriate group (4–300), except for Chrys for which BCFs were slightly higher in the petrol group (30–70) than in the PAH mixture (<15). Regardless of the route of exposure, compound-specific BCFs, when determined, were generally in the descending order: A, Phe and Fluo > Pyr > Chrys and BaP, the two latter ones being extremely low (<15), except for BaP at T1 and T2 in the

Fig. 1 Total PAH concentrations (microgramme per litre) in water of the experimental tanks (control, sediment, petrol elutriate and dissolved PAH mixture tanks) measured each day during the 4-day exposure period (T0 to T4) and at the end of the 6-day depuration period (T10: 10 days)
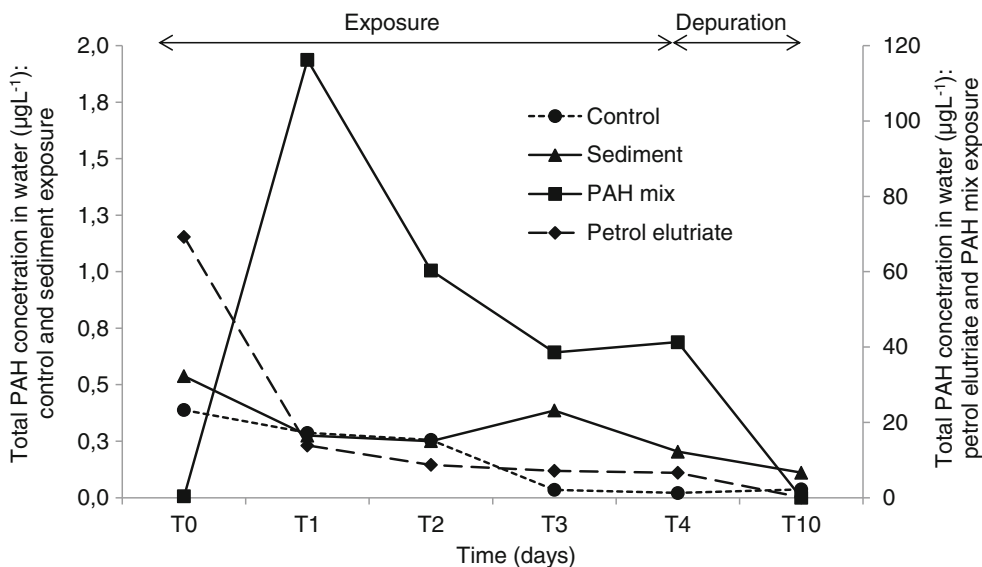
Fig. 2 Relative distributions (percent) of PAHs in water of the tanks during the 4 days of exposure (T0 to T4) from a control, b sediment, c petrol elutriate and d waterborne PAH mixture experiments. Abbreviations of PAH names are as follows: *N* naphthalene, *Phe* phenanthrene, *A* anthracene, *Fluo* fluoranthene, *Pyr* pyrene, *Chrys+T* chrysene +triphenylene, *BaP* benzo[a] pyrene, *Others* the sum of the 11 other PAHs investigated (see 'PAH analysis')
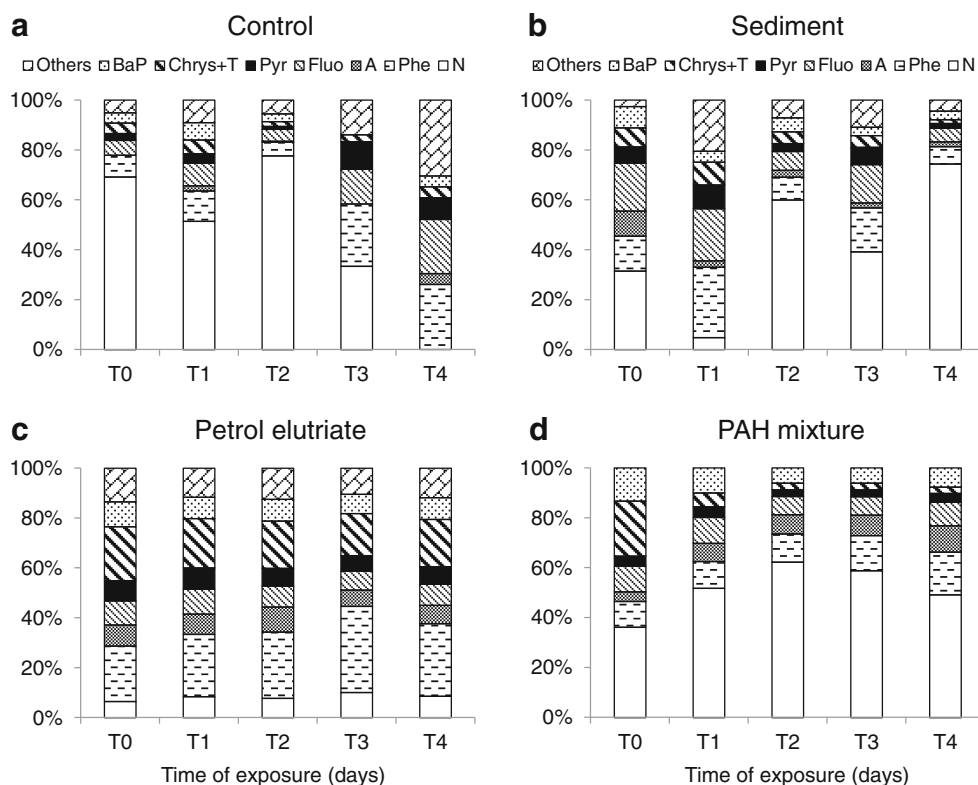
sediment group (276–980). BCFs for N could only be calculated for day 1 in livers. In the sediment exposure, BCFs could only be calculated for the first day of exposure in muscle, except for BaP (2 days), whereas liver BCFs of the lower molecular weight PAHs (Phe, A and Fluo) increased with exposure time (from T2 to T4). A very different pattern was obtained in the petrol elutriate and dissolved PAH mixture groups. BCFs were observed during the 4 days of exposure, except for the highest molecular weight PAHs (Chrys and BaP) during the two last days, as well as for Pyr in the petrol elutriate group. In addition, a continuous decrease (from day 1 to day 4) in BCF values for Phe was observed in muscle and liver of fish exposed to the PAH mixture, a decrease that was also evidenced at day 4 for A and Fluo, as well as for Pyr (liver only).

PAH metabolites in fish bile

In the control group, OH-PAH concentrations ranged from 60 to 170 $ng\,g^{-1}$ bile, except at the end of the experiment (day 10) for which higher concentrations were observed (337 $ng\,g^{-1}$ bile). Following exposure to the dissolved mixture of PAHs, a significant increase in the total biliary metabolite concentrations was observed during the exposure phase ($p < 0.01$), followed by a significant decrease with depuration ($p < 0.001$) (Fig. 4d). At the end of the depuration period, concentrations of PAH metabolites decreased to 51 % of the maximum concentration measured at the end

of exposure (276 $\mu g\,g^{-1}$). However, differences in kinetics were observed among compounds. Two scenarios can be distinguished. In the first scenario, the molecule was quickly biotransformed and excreted in the bile during the exposure period and was eliminated during the depuration period in an almost linear manner ($r^2 = 0.93$). This was the case for 1-OHP concentrations which decreased by 54 %, from 179 to 83 $\mu g\,g^{-1}$ between T4 and T10 (depuration phase). In the second scenario, the rate of biotransformation in the liver and the excretion in the bile were slower during the first 2 days of exposure before increasing between T2 and T4. This kinetic was observed for 3-OHBaP and OHPhe isomers as well. BaP and Phe metabolite concentrations exhibited differences in their depuration rates. The decrease in concentrations corresponded to 34 and 55 % for OHPhe and 3-OHBaP metabolites, respectively.

In the sediment exposure group, the production of biliary metabolites was slower than in the case of the two other routes of exposure (Fig. 4b), except for metabolites from the lowest molecular weight PAHs (OHNs, 2-OHBi and 9-OHFe) that reached their maximum levels after 2 days. Overall, a significant production of metabolites occurred in the bile only after the third day of exposure. The maximum increase in total metabolite concentrations was observed at the last day of exposure (6.26 $\mu g\,g^{-1}$). For example, 1-OHP metabolite excretion in the bile during the sediment experiment was slower than during the PAH mixture exposure from T0 to T3 (48 to 503 $ng\,g^{-1}$) but increased rapidly between T3 and T4 (more

Fig. 3 Total PAH concentrations (nanogramme per gramme dry weight) in **a** muscle and **b** liver of control fish and fish exposed to sediment, petrol elutriate and waterborne PAH mixture measured each day during the 4-day exposure period (T0 to T4) and at the end of the 6-day depuration period (T10). For each sampling time, concentrations were determined on a pool of five individuals. Concentrations in tissues of fish exposed to the waterborne PAH mixture are plotted using the secondary vertical axis
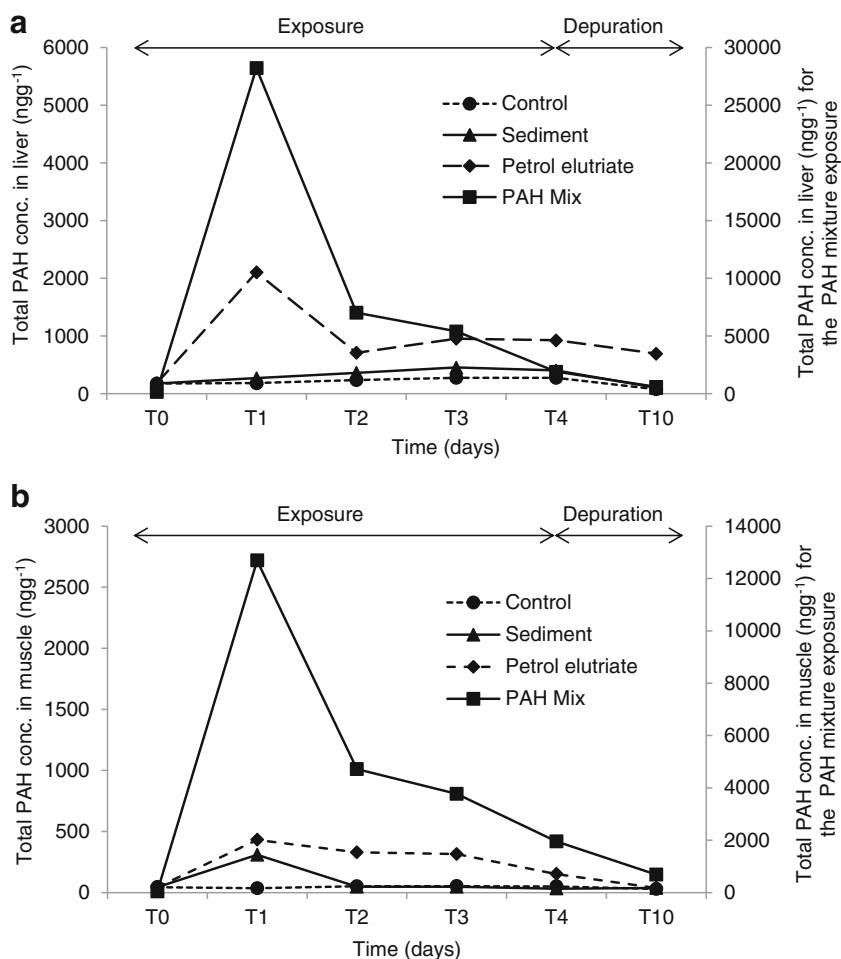
than 5 μgg^{-1}). In addition, OH-PAH concentration decreased by only 15 % after the 6-day depuration period. During the depuration time, a decrease in 1-OHP concentrations was observed (23 %) but to a lesser extent than in the case of exposure to dissolved PAHs.

In the case of the exposure to elutriate of heavy fuel no. 2 Erika, a significant time effect ($p<0.001$) was observed for all metabolites. The quantity of metabolites produced was significantly higher than during the sediment exposure but less than that observed after exposure to the dissolved mixture of PAHs (Fig. 4b). A linear increase in the concentration was observed for 1-OHP between T0 and T2 ($r^2=0.99$) and T2 and T10 ($r^2=0.99$). A low decrease in biliary OH-PAH concentration occurred during the depuration period (27 %), whereas 1-OHP level increased by 30 %. Thus, regardless of the route of PAH exposure and the levels of PAHs in water, PAH metabolite concentrations did not return to basal levels after the 6-day depuration period. They only decreased from about 50 % to as low as 15 % (sediment exposure) and were still 20-fold (sediment exposure) to 600-fold (PAH mixture exposure) higher than those of the control group.

Whatever the route of exposure, 1-OHP was the major metabolite detected, except for one sampling day

in the control group (day 4) and in the sediment group (day 2). Its mean percentage contribution during the exposure period was the highest in the case of the exposure to the PAH mixture (80 %), followed by the petrol elutriate (60 %) and the sediment (40–50 %, similar to control) (Fig. 5). In addition, the relative contribution of 1-OHP decreased continuously during the exposure period in the petrol elutriate and the dissolved PAH mixture experiments in favour of an increasing proportion of 3-OHBaP (from 0 to 20 %) in the petrol elutriate experiment and of OHPhe (from 6 to 20 %) and an increasing proportion of OHN and 3-OHBaP (from 1 to 10 %) in the dissolved PAH mixture experiment. In contrast, the percentage contribution of 1-OHP increased during the exposure period (from 25 to 90 %) in the sediment experiment, while the low molecular weight PAHs (OHN, OHBi, OHFe and OHPhe) decreased.

Comet assay

There was significant inter-individual variability in the extent of DNA damage among control fish (ANOVA,

Table 2 Bioconcentration factors (BCFs) of PAHs in muscle and liver of fish exposed to sediment, petrol elutriate or PAH mixture, calculated each day during the 4-day exposure (T1, T2, T3 and T4)

| | | Route of PAH exposure | | | | | | | | | | | |
| | | Sediment | | | | Petrol elutriate | | | | PAH mixture | | | |
Exposure time (days)		T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
Muscle	Naphthalene	nd	nd	nd	nd	nd	nd	nd	nd		nd	nd	nd
	Phenanthrene	1,569				← 40–70 →				376	337	274	109
	Anthracene	6,725				← 70–120 →			34	← 290–360 →			110
	Fluoranthene	961				← 35–70 →				← 150–400 →			86
	Pyrene	618				28				← 60–220 →			
	Chrysene	nd	nd	nd	nd	11	6	5	4	8	6	nd	nd
	Benzo[a]pyrene	980	276	nd	nd	8	4	nd	nd	4	2		
Liver	Naphthalene	4,523				220				25			
	Phenanthrene	← 2,500–3,500 →				← 100–220 →				1108	445	463	138
	Anthracene	← 5000–6000 →				← 60–280 →				← 320–720 →			51
	Fluoranthene	← 800–1200 →				← 60–260 →				← 490–525 →			117
	Pyrene					← 10–60 →				← 80–130 →			16
	Chrysene	nd	nd	nd	nd	← 30–70 →				3	12	15	10
	Benzo[a]pyrene	nd	nd	nd	nd	4		nd	nd	2			

BCFs were calculated as the ratio of PAH concentration in muscle or liver (ngg^{-1} dw) divided by PAH concentration in the seawater (μgL^{-1}). PAH concentrations in muscle (or liver) were determined from a pooled muscle (or liver) sample and water concentrations from one sampling in each tank at each day of exposure. BCFs were only determined when individual PAH levels in muscle or liver of fish exposed to PAH were significantly higher than those of control fish (otherwise cells have been left empty)

nd compound not detected in liver or muscle

$p < 0.001$). In this group, comet assay parameters appeared to be stable during the experimental period, validating our experimental conditions for fish maintenance at the laboratory (Fig. 6).

Significant effects of exposure time and treatment group on the extent of DNA strand breaks (ANOVA, $p < 0.001$) were observed. A significant increase in DNA damage was observed after 4 days in fish exposed to the sediment or the dissolved mixture. Regardless of the route of exposure (petrol, sediment, dissolved phase), a significantly higher level of DNA damage was measured in fish exposed to PAHs when compared to the control group (Tukey test, $p < 0.001$; Fig. 7). No difference in the extent of the genotoxic response was observed as a function of PAH exposure route ($p > 0.05$).

For fish exposed to the contaminated sediment, a non-significant decrease in comet parameter was observed following depuration ($p = 0.09$). However, this decrease was significant for the fish exposed via the dissolved phase ($p < 0.001$) (Fig. 8a). For fish exposed to the petrol elutriate, the level of DNA damage was still increasing during the depuration phase ($p < 0.001$) (Fig. 8b).

Weak but statistically significant positive correlations were observed between the level of DNA damage as measured by the comet assay (percent tail DNA) and the level of 1-OHP, 3-OHBaP, OHPhe and OH-PAH (Spearman correlation coefficients of 0.286, 0.250, 0.365 and 0.325, respectively, for $N = 80$; $p < 0.05$).

Discussion

PAH levels in exposure tanks

The total PAH concentration in the oil fuel no. 2 Erika was 1,338 μgg^{-1}. This value is comparable to that reported for the Erika heavy fuel oil where concentration of the 16 EPA PAHs was 5,211 μgg^{-1} (Ruiz et al. 2012). The resulting PAH concentrations in the water of the experimental tank were relatively high (6,602–69,285 ngL^{-1}), especially during the first day of exposure, compared to the values described in the literature after oil spills although a wide range of PAH concentrations were mentioned (Tronczyński et al. 2004; González et al. 2006). However, in similar studies where European seabass and Atlantic cod were exposed to weathered oil (Della Torre et al. 2012) and to dispersed crude oil (Aas et al. 2000), respectively, the levels of PAHs in the exposure tanks were close to our experimental condition (40,400 ngL^{-1} at 2 days of exposure in Della Torre et al. (2012) and from 1,140 to 7,810 ngL^{-1}, for a lowest nominal exposure concentration of 60,000 ngL^{-1}, in Aas et al. (2000)). PAH exposure levels in the spiked seawater experiment were also similar to those used in other studies (e.g. Jonsson et al. 2004). After 24 h of exposure, PAH concentration decreased approximately 10 and 2 times in the petrole elutriate and waterborne PAH experiments, respectively. This drop can be explained by the volatilisation of low
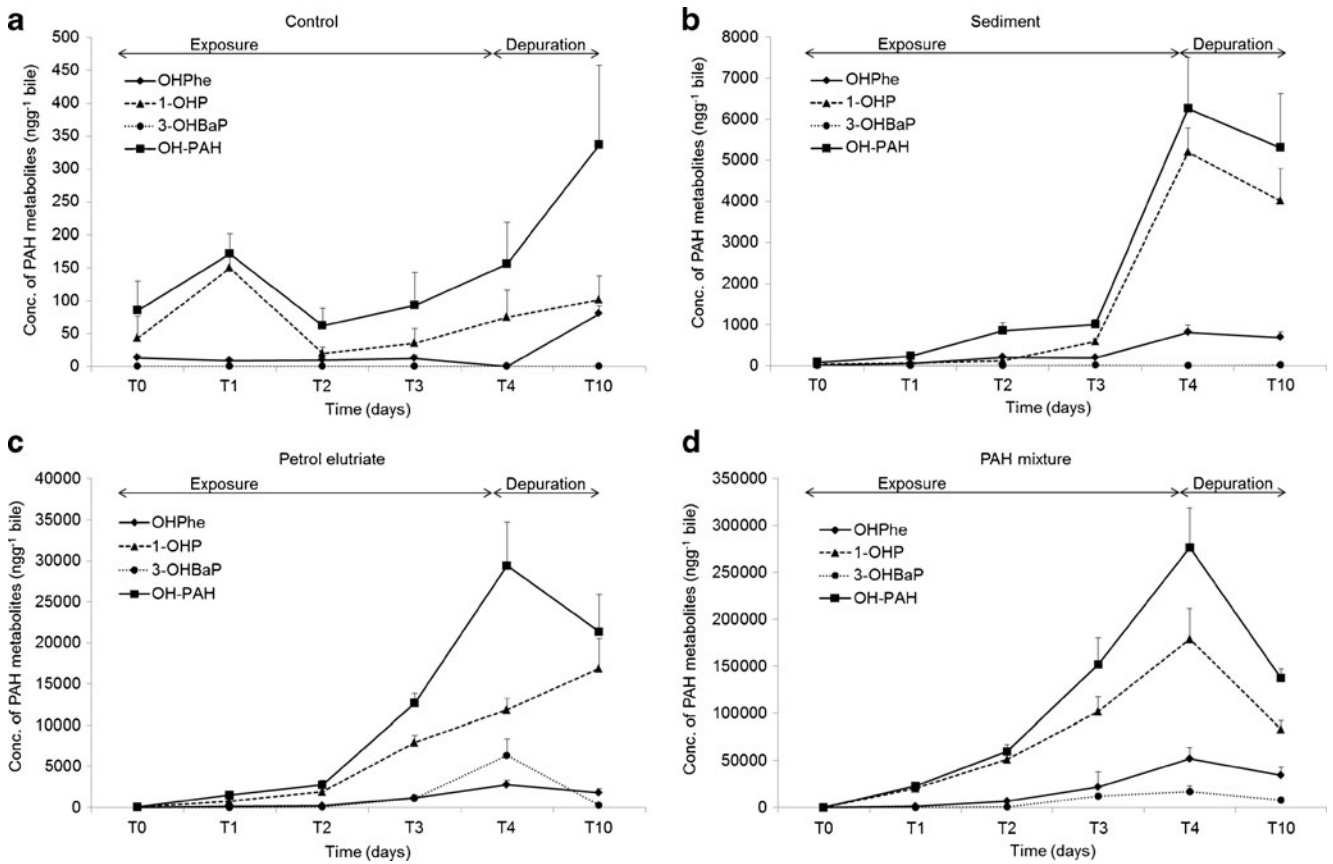
Fig. 4 PAH metabolite concentrations in bile (nanogramme per gramme of bile) **a** of control fish and fish exposed to **b** sediment, **c** petrol elutriate and **d** waterborne PAH mixture measured each day during the 4-day exposure period (T0 to T4) and at the end of the 6-day depuration period (T10). For each sampling time, concentrations were determined on five individuals, and standard deviations of the measurements ($n = 5$) are represented by the *upper error bars*. Abbreviations used for PAH metabolites are as follows: *OH-PAH*: sum of all the monohydroxylated metabolites investigated (12 compounds), *1-OHP* 1-hydroxypyrene, *OHPhe* sum of the 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene isomers *OHBaP* 3-hydroxybenzo[*a*]pyrene

molecular weight PAHs such as naphtalene, as well as PAH adsorption/absorption (exposure tank, pump, tube and fish) and photodegradation in the exposure system. Similar decreases were observed previously both in oil (Ruiz et al. 2012; Bilbao et al. 2010) and in waterborne PAH exposures (Budzinski et al. 2004). Therefore, PAH concentration in water during the 3 days following elutriate exposure corresponded to PAH levels reported in the seawater near oiled beaches after an oil spill (Short and Harris 1996) or in the vicinity of oil platforms (Stagg and McIntosh 1996).

The concentrations of total PAHs (2.56 $\mu g\,g^{-1}$ dw) in the sediment from Arcachon harbour were also comparable to, or were lower than, levels reported in other studies where juvenile turbot (*S. maximus*) were exposed to natural contaminated sediments (1.3–2.4 $\mu g\,g^{-1}$ dw) (Kerambrun et al. 2012) and rainbow trout (*Oncorhynchus mykiss*) to artificial sediment spiked with Phe, Pyr, Chrys and BaP (20 $\mu g\,g^{-1}$ dw) (Brinkmann et al. 2010). During the exposure period, low PAH concentrations were measured in seawater (individual PAH concentrations ranged from 1 to 170 $ng\,L^{-1}$, data not shown). Similar PAH concentrations were reported in the Greek Mediterranean sea (113 to 459 $ng\,L^{-1}$) (Valavanidis et al. 2008). Target values of PAHs in the test water were not reached during the experiment, and PAH concentrations in water rapidly decreased in the tanks although the PAH mixture solution and petrol elutriate were continuously added. This justified the necessity of chemical analysis to measure the exact level of PAH exposure.

PAH concentrations in fish

PAH concentrations were higher in the muscles and the livers of fish exposed to the dissolved PAH mixture compared to fish exposed to sediment and petrol elutriate. This can be explained by the high concentrations of PAHs measured in water compared to the other treatment groups. Regardless of the route of exposure, Phe was the predominant PAH in tissues (40–60 %), followed by A and Fluo, whereas the relative contribution of Pyr was relatively low (1–3 and 1–15 % in liver and muscle, respectively). PAH

Fig. 5 Relative distributions (percent) of PAH metabolites in bile **a** of control fish and fish exposed to **b** sediment, **c** petrol elutriate and **d** waterborne PAH mixture during the 4 days of exposure (T0 to T4). Abbreviations used for PAH metabolites are as follows: *OHN* sum of 1- and 2-hydroxynaphthalene isomers, *OHPhe* sum of the 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene isomers, *1-OHP* 1-hydroxypyrene, *3-OHBaP* 3-hydroxybenzo[*a*]pyrene, *Others* sum of the three other monohydroxylated metabolites investigated (2-hydroxybiphenyl, 9-hydroxyfluorene and 1-hydroxychrysene)
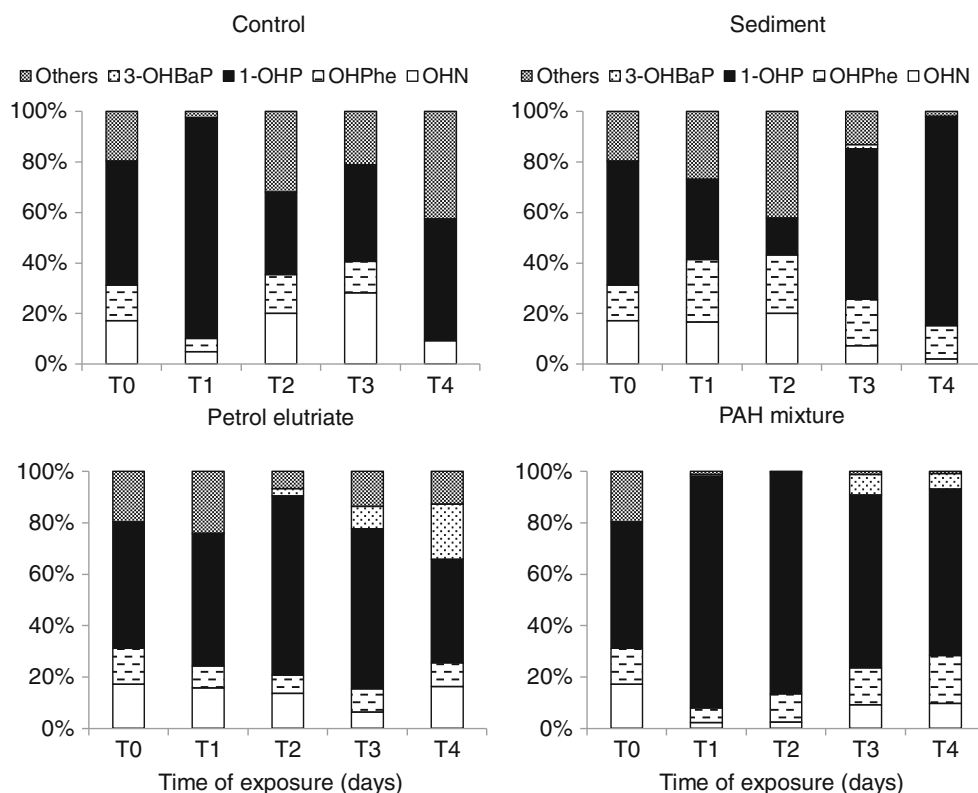

concentrations in muscle and liver of fish showed a rapid uptake, with maximum levels observed the day after the beginning of the exposure, followed by a reduction towards the end of the exposure period. These observations are in agreement with the results previously reported by Baussant et al. (2001), who reported maximum levels of two-, three- and four-ring PAHs in juvenile turbot tissue (*S. maximus*) after 3 days of exposure to dispersed crude oil. Total PAHs

in liver of Atlantic cod reached maximum values of about 100 and 20 $\mu g\,g^{-1}$ after 3 days of exposure when exposed to mechanically dispersed crude oil at 1 and 0.25 $mg\,L^{-1}$ (nominal concentrations), respectively (either total 16 EPA PAH concentrations in water in the range of 1–7 $\mu g\,L^{-1}$). Elimination, resulting from the combined processes of metabolism, excretion, and diffusive release, can be measured in fish as a decline in body tissue residues of parent PAHs (Meador et

Fig. 6 Time course of the comet parameter tail DNA in fish exposed to PAHs by the three different routes (sediment, petrol elutriate and PAH mixture). Samplings were performed at the beginning of exposure (T0; *circle*) and after 1 (T1; *square*), 2 (T3; *diamond*) and 4 days (T4; *triangle*) of exposure
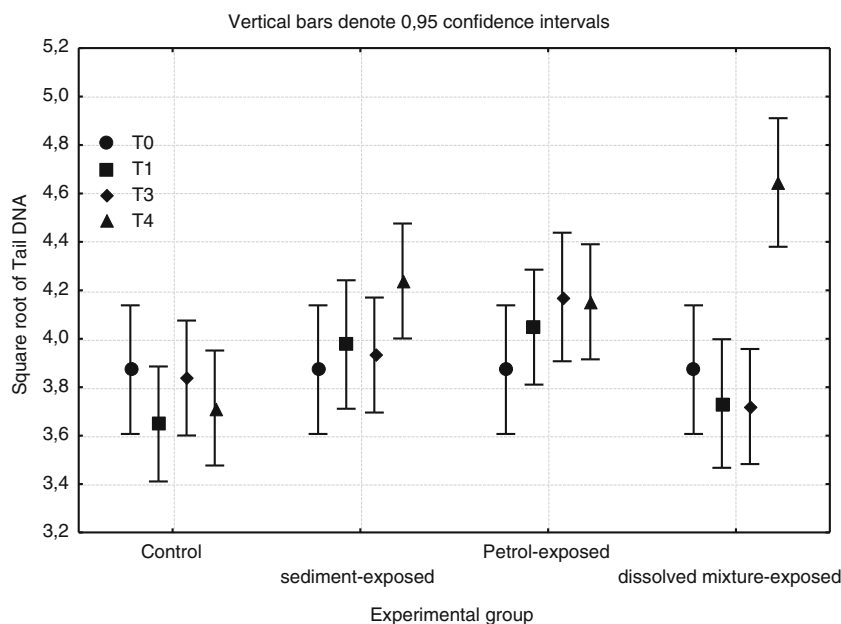
Fig. 7 Effects of the route of PAH exposure on the extent of DNA damage in fish erythrocytes. *Asterisks* indicate significant differences from the control ($p < 0.05$)
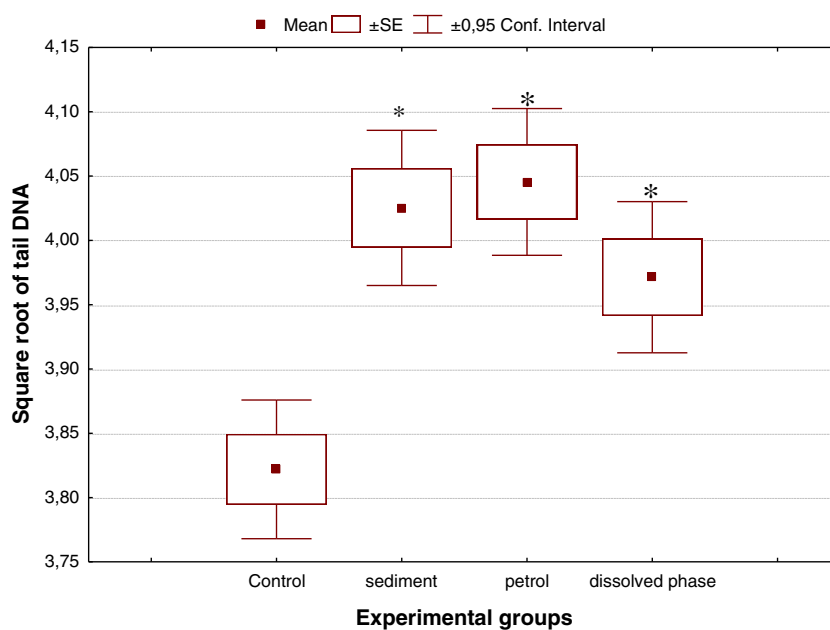

al. 1995). This decrease of PAH levels observed in tissue can be explained by the increased biotransformation of PAH, as indicated by the increased level of PAH metabolites in bile. This also indicated that the metabolic capacity to regulate in the organism did not seem to have been exceeded regardless of the route of contamination and the level of PAHs in water. After 6 days of depuration, PAH concentrations in fish tissues generally returned to background levels, demonstrating the high capability of the fish to eliminate a major part of these compounds, as also observed in other studies (Aas et al. 2000; Jonsson et al. 2004; Budzinski et al. 2004). The rapid metabolism and removal of parent PAH compounds in fish also demonstrates the relevance of measuring PAH metabolites.

The propensity of individual PAHs to accumulate in fish can be estimated from their bioconcentration factor. In the present study, BCF values calculated for muscle were significantly less than those for liver. This is likely due to the higher lipid contents in liver than in muscle. BCF values determined in turbots during the 4 days of exposure were relatively low but comparable to those calculated in sheepshead minnows (*Cyprinodon variegatus*) exposed to PAHs dissolved in water (7.6 and 72.3 $\mu g L^{-1}$) (Jonsson et al. 2004) or in juvenile turbot (*S. maximus*) (Baussant et al. 2001) and Atlantic cod (Aas et al. 2000) exposed to dispersed crude oil, although they were determined after longer exposure periods compared to the present study. However, 95 % clearance times for Phe and Pyr in fish tissue were estimated at less than 4 days in sheepshead minnows (Jonsson et al. 2004). In the present study, the highest BCFs were observed for the lower molecular weight PAHs (Phe, A and Fluo) in fish exposed to the sediment, although

PAH concentrations in water were 20- to 250-fold lower than those measured in the two other experiments, indicating that greater bioaccumulation occurred from sediment than petrol elutriate or water-only exposures. This observation can be explained by the longer time observed for the formation of biliary metabolites, probably due to the very low PAH levels in water and the differences in bioavailability of PAHs in the sediment experiment compared to the two other routes of exposure, as discussed in the section 'PAH concentrations in fish'. The higher BCFs observed for the three-ring PAH compounds (A and Phe) as compared with larger compounds might reflect a more efficient uptake of the smaller PAH molecules. However, a relatively more efficient metabolism of the higher molecular weight PAHs may also play a role (Varanasi and Gmur 1981), with this possibility being further supported by quantitative analysis of selected PAH metabolites in the present study. In addition, the bioaccumulation pattern of Fluo, a four-ring PAH, was much more like that observed for the three-ring PAHs Phe and A than that of pyrene which is much lower, probably indicating a particularly more efficient metabolism of pyrene, as discussed below. Interestingly, in the dissolved PAH mixture experiment, the continuous increase in the percentage contribution of OHPhe during the exposure period can be related with the continuous decrease observed in BCFs of Phe in liver and muscle of fish. In conclusion, the PAH body burden, defined as the internal and time-dependent exposure concentration of a contaminant in an organism, is the result of a dynamic and complex balance between phenomena of assimilation and elimination (Barron 1990). To further understand the importance of efficient metabolism for BCF values generated for PAHs from different exposure

Fig. 8 Effect of depuration on the level of DNA strand breaks (square root of tail DNA) in fish exposed to **a** waterborne PAH mixture (*upper graph*) and **b** petrol elutriate (*lower graph*). *Asterisks* indicate significant differences between before and after depuration (*p*<0.05)
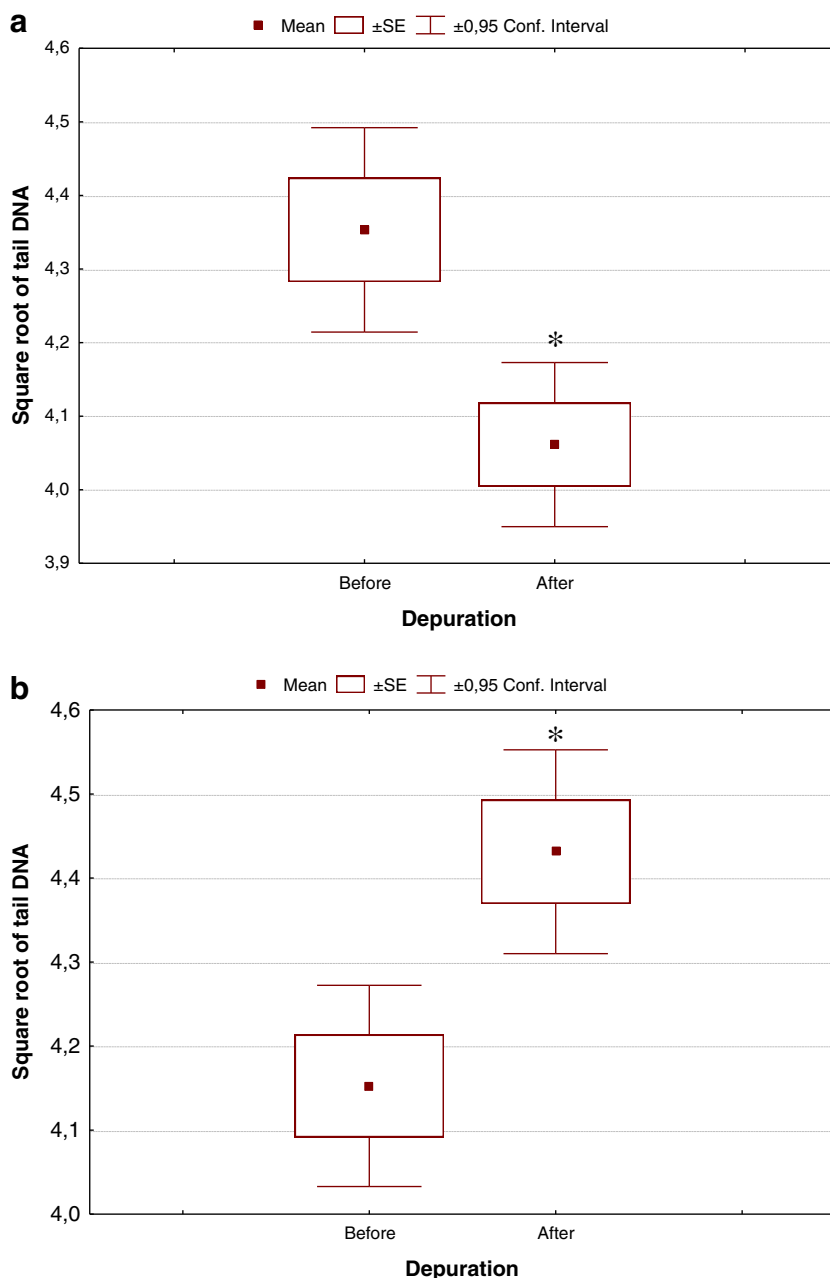
routes, selected OH-PAHs were quantified in bile throughout the experiments.

PAH metabolites in bile

In this study, regardless of the route of exposure, the presence of bile metabolites in fish exposed to PAHs after only 24 h indicated a rapid uptake and metabolism of PAHs, which proceeded during the 4-day exposure period. Rapid uptake and metabolism of PAHs after 48 h of exposure were also observed in other fish exposed to PAHs, regardless of the route of exposure, such as via oil (e.g. Camus et al. 1998; Aas et al. 2000; Della Torre et al. 2012), sediment

(e.g. Brinkmann et al. 2010; Kerambrun et al. 2012), waterborne PAHs (e.g. Budzinski et al. 2004) or spiked food (e.g. Reynolds et al. 2003; Wessel et al. 2010). However, in our experiments, if the levels of biliary PAH metabolites in fish decreased during the 6-day depuration period, they did not return to basal levels at the end of the depuration period as has been observed in other studies (Aas et al. 2000; Brinkmann et al. 2010; Wessel et al. 2010), and 1-OHP concentrations even increased in the petrol elutriate exposure. This could be due to the lack of feeding of fish in our study, which likely prevented normal elimination of the bile into the intestine. The level of PAH metabolites in the bile is, in addition to the level of PAH exposure, influenced by

the flow of bile through the gallbladder. The regulation of this flow is mainly a function of feeding status of the organism since bile is used as a digestion secretion (Collier and Varanasi 1991). Because the animals were not fed during the present experiments, the experimental conditions were different from that of the natural environment but allowed a higher amount of bile available for analysis. In addition, this made the results among fish more comparable as the variation to be expected among individuals as a function of different feeding status was somewhat controlled for. This observation is in accordance with our previous study in which at the end of the experiment a partial excretion of 1-OHP (less than 30 %) was observed in starved soles (*S. solea*) exposed for 1 week to waterborne Pyr (single dose, 367 ngL^{-1}), followed by 1 week of depuration (Budzinski et al. 2004).

Formation of bile metabolites took longest in the sediment experiment, which could be explained by the longer time required for the induction of biotransformation enzymes. Bioavailability plays a role in the activation of the biotransformation processes. The greater the quantity of PAHs in the aqueous phase, the faster it is anticipated that enzymatic systems involved in the production of PAH metabolites are induced. PAH concentrations in water from the sediment experiment were very low (from 4 to 50 ngL^{-1} for A, Pyr, Chrys and BaP and from 15 to 150 ngL^{-1} for N, Phe and Fluo), and the higher BCFs calculated in fish muscle and liver corroborated the hypothesis of a slower induction of biotransformation enzymes in fish exposed to the sediment. A significant increase in PAH metabolites measured by fixed wavelength fluorescence has been also observed in juvenile turbot (*S. maximus*) exposed to contaminated sediments exhibiting similar individual PAH concentrations (total concentration of 2.4 μgg^{-1} dw) than those of the sediment used in the present study (Kerambrun et al. 2012).

When comparing the exposure routes, higher PAH metabolite concentrations were observed for fish exposed to dissolved PAH mix, followed by those exposed to petrol elutriate (10-fold lower) and then by those exposed to sediment (40-fold lower). The levels of metabolites quantified in fish bile correlated with the levels of contamination of water. However, these trends did not follow the concentration ratios between parent PAHs in water and PAH metabolites found in bile. When considering differences in PAH concentrations in water between the three exposure routes, differences of the relatively same order between OH-PAH concentrations in bile were observed between the PAH mixture and the petrol elutriate experiments, but this was not the case between the two latter experiments and the sediment experiment. As an example, although individual PAH concentrations in water were from 100- to 400-fold higher in the PAH mixture experiment than in the sediment

experiment, PAH metabolite concentrations were only about 40-fold higher. The higher ratios observed in the sediment experiment may possibly indicate that particle-bound PAHs from sediment were also available and could account for a significant proportion in the PAH uptake for this flatfish species maintaining a close association with sediments, as demonstrated by Brinkmann et al. (2010).

Regardless of the route of exposure and the levels of individual PAHs in water, 1-OHP was the predominant metabolite in fish bile. This indicated that the low BCFs calculated for Pyr were likely the result of a very efficient metabolism rather than a pronounced reduced bioavailability. In the present study, biotransformation was shown to be more efficient for Pyr than for Phe. Other studies have also identified 1-OHP as the most abundant compound present in fish bile (Aas et al. 2000; Ruddock et al. 2003; Brinkmann et al. 2010). Compared to 1-OHP, metabolites of Phe, Chrys and BaP were detected at significantly lesser concentrations (Ruddock et al. 2003; Brinkmann et al. 2010). These observations corroborated the assumption that 1-OHP could be used as early biomarker of PAH exposure in biomonitoring studies (Kamman 2007).

Genotoxic effects

During this study, genotoxicity of PAHs for turbot was confirmed regardless of the tested route of exposure. The genotoxic effect was significant only after 4 days of exposure. This delay in response may be attributed to the time necessary for the production of a threshold amount of genotoxic metabolites required to cause a positive response in the comet assay. During this study, the differences observed between the routes of exposure and the profiles of PAH metabolites indicated probable differences in PAH bioavailability and biotransformation. Despite these differences, it is noteworthy that the genotoxic response was of the same magnitude regardless of the route and level of exposure. This finding can be explained by the lack of significant differences in the production of genotoxic metabolites among experimental groups. In fact, among the produced metabolites, only one compound was thought to be involved in the genotoxic response. In our study, 3-OHBaP was the only metabolite investigated that has been shown to be potentially genotoxic. BaP is initially oxidised to several arene oxides that may rearrange spontaneously to phenols (3-OH-, 6-OH-, 7-OH- and 9-OH-BaP). These latter metabolites can be further oxidised to pro-oxidant quinones but can also lead to the production of highly reactive diol epoxides by hydratation and oxidation catalysed by epoxide hydrolase and cytochrome P-450 monooxygenase, respectively (Siddens et al. 2012). In higher vertebrates, urinary 3-OHBaP has been hence proposed as a relevant biomarker of BaP genotoxic exposure for health risk assessment (Marie-

Desvergne et al. 2010). Different mechanisms of genotoxicity have been proposed for the formation of DNA strand breaks following PAH exposure in higher vertebrates. The incomplete repair of stable and depurinating DNA adducts by base and nucleotide excision repair systems can lead to DNA breakage (Frosina et al. 1996; Van Steeg 2001) that could account for a high amount of the strand breaks induced by PAH exposure (Speit and Hartmann 1995). The biotransformation of PAHs can also result in an oxidative stress via the production of reactive oxygen species (Flowers-Geary et al. 1992; Gelboin 1980; Bolton et al. 2000) that can lead to DNA fragmentation either directly or by the incomplete repair of the resulting oxidised DNA bases (Laval et al. 1998; Memisoglu and Samson 2000). In fish, the production of DNA strand breaks has been demonstrated in previous studies following PAH exposure. A significant increase in the level of DNA strand breaks and alkali-labile sites was also shown in turbots exposed to waterborne BaP (Peters et al. 1997), to PAH-contaminated sediment (Hartl et al. 2007; Kilemade et al. 2004) and to dispersed oil (Camus et al. 1998). Similar results were obtained in rainbow trout (Inzunza et al. 2006) and in flounder exposed to waterborne BaP (Woo et al. 2006) and in sole exposed to food spiked with a mixture of BaP, pyrene and fluoranthene (Wessel et al. 2010).

For fish exposed via sediment or the aqueous phase, the decrease with depuration observed in the level of DNA strand breaks likely indicates effective repair mechanisms of DNA lesions. DNA repair has been mainly studied in higher vertebrates and humans (Dianov and Parsons 2007). However, the observation of decreases in the number of induced DNA lesions during depuration suggested the existence of efficient DNA repair systems in fish (Kavouras et al. 2010).

In fish exposed to the petrol elutriate, the amount of lesions still increased during the depuration phase. In this group, the DNA repair mechanisms may be insufficient to cope with the damage induced by the genotoxic PAH metabolites produced in fish.

Interactions between biomarkers

The significant positive correlation observed between concentrations of biliary PAH metabolites and the extent of DNA strand breaks seemed to confirm the contribution of the biotransformation of PAHs to the genotoxic effects measured in turbot. Similar correlations were observed between DNA adducts levels and biliary PAH metabolites in Atlantic cod (Aas et al. 2000) and polar cod (with Pyr- and BaP-type metabolites) (Nahrgang et al. 2010) after chronic oil exposure and in juveniles of sole exposed to food spiked with Fluo, Pyr and BaP (Wessel et al. 2010). A weak but significant positive correlation was also found between the

proportion of micronucleated erythrocytes and the concentration of 3-OHBaP in rainbow trout exposed to resuspended sediments spiked with PAHs (Brinkmann et al. 2010). Because of a rapid bile evacuation, it is noteworthy that the detection of PAH metabolites in fish bile is considered as an early marker of recent PAH exposure. During this study, only a few metabolites were analysed. They were selected because of their representativeness in the assessment of PAH exposure, as well as their easy detection due to their elevated production. When linking genotoxicity and exposure, however, it would have been desirable to focus the chemical analysis on a number of known genotoxic metabolites of PAHs, such as some more reactive phase I metabolites. A large number of research papers (Afshar et al. 1996; Basu et al. 2001; Krahn et al. 1984, 1986; Pangrekar et al. 1995; Varanasi et al. 1986, 1989; Wenzel-Hartung et al. 1990) pointed out the role of diols, diol-epoxides and dione metabolites in the genotoxicity of PAHs. These metabolites can interact either directly with the DNA (diolepoxides) or indirectly via the formation of reactive intermediates (e.g. semi-quinone radicals). However, current analytical methods to quantify these metabolites are limited; efforts are currently underway in the laboratory to develop a method that will allow the detection of these types of metabolites.

The biomarkers proposed in this study were also applied in dab during field campaigns in the Seine estuary (Normandy, France) (Dévier et al. 2012). The accumulation of PAH in dab tissues (white muscle and liver) from the two sites studied was relatively limited and no correlation was found with the level of DNA damage. In contrast, significant correlations were observed between the levels of PAH metabolites and DNA strand breaks in fish erythrocytes, demonstrating the relationship between PAH exposure and biological effects in terms of genotoxicity.

Conclusion

During this study, some differences in tissue PAH concentrations in fish were observed between exposure routes but were mainly related to the PAH concentrations reached in seawater. Bioaccumulation was higher in livers than in muscle tissues but was limited and concentrations returned to background levels at the end of the depuration period demonstrating the capability of fish to eliminate PAHs.

Regardless of the route of exposure, the presence of bile metabolites in fish exposed to PAHs as soon as 24 h after initiation of exposure indicated a rapid uptake and biotransformation of PAHs. Despite the decrease of concentrations of biliary PAH metabolites over the 6-day depuration period, they did not return to basal levels. This is probably explained by the lack of feeding of fish during the exposure experiments

that prevented from normal elimination of the bile into the intestine. When comparing the different exposure routes, although a slower induction of biotransformation enzymes was observed in the sediment exposure, the bioavailability of PAH-bound particles seemed to have played a significant role in the PAH exposure, owing to the metabolite levels observed in fish bile relative to parent PAH levels in the seawater. Regardless of exposure route and levels of individual PAHs reached in water, 1-OHP was the predominant metabolite measured in fish bile, indicating that the low BCF values calculated for this compound were likely to result from a very efficient metabolism rather than from a reduced bioavailability. 1-OHP was confirmed as an early biomarker of PAH exposure even at low level of contamination.

No difference in the genotoxic response was observed between the different routes of PAH exposure. The genotoxic effect was significant after a 4-day delay that can be attributed to the time necessary for the production of a threshold amount of genotoxic metabolites. However, significant positive correlations were observed between the concentrations of biliary PAH metabolites and the extent of DNA strand breaks demonstrating the involvement of PAH biotransformation in the genotoxic response measured by the comet assay.

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