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Developmental toxicity testing of the fume condensate extracts of bitumen and oxidized asphalt in a series of in vitro alternative assays

Lenny Kamelia [a,1,*], Ivonne.M.C.M. Rietjens [a], Peter J. Boogaard [a,b]

[a] Division of Toxicology, Wageningen University and Research, 6708 WE, Wageningen, The Netherlands
[b] Shell Health, Shell International Bv, PO Box 162, 2501 AN, The Hague, The Netherlands

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ABSTRACT

The potential developmental toxicity and mode-of-action of fume condensate extracts of bitumen and oxidized asphalt were evaluated in the aryl hydrocarbon receptor (AhR) CALUX assay, the zebrafish embryotoxicity test (ZET), and the mouse embryonic stem cell test (mEST). In the AhR CALUX assay, both fume condensate extracts showed a concentration-dependent AhR induction following 6-h of exposure, but this activity was substantially reduced after 24-h, indicating a transient AhR activation. The main effect observed in the ZET was early embryonic lethality that occurred mostly in the 24 h-post-fertilization (hpf). This typically reflects non-specific toxicity rather than in vitro developmental toxicity of the fume condensate extracts tested since this effect was not seen as a result of the whole cumulative exposure period in the ZET (up to 96 hpf). No malformations were seen in any zebrafish embryo exposed to these fume condensate extracts, although some developed pericardial and/or yolk-sac edemas. Furthermore, both fume condensate extracts tested negative in the mEST. In conclusion, the results show that fume condensate extracts of bitumen and oxidized asphalt do not induce any in vitro developmental toxicity, which is in line with the results observed in the in vivo prenatal developmental toxicity studies performed with the same materials.

1. Introduction

Bitumen is a black or dark brown highly viscous material containing a mixture of high-molecular-weight hydrocarbons and is obtained as a residue from crude oil distillation processes. The main application of bitumen is road surfacing (i.e. paving). To increase its hardness bitumen may be blown with hot air, resulting in oxidized asphalt which is mainly applied in waterproofing such as roofing. Two product categories, namely paving grade bitumen (CAS no. 8052-52-4) and oxidized asphalt (CAS no. 64742–93-4) represent >99% of the materials used for paving and roofing applications, respectively (HPV (High Production Volume) Chemical Challenge Program, 2009; Parker et al., 2011). Both bitumen and oxidized asphalt are solid or semi-solid at ambient temperature and need to be heated to lower the viscosity to permit their application. Hence, inhalation of fumes generated from heated products is considered the only relevant route of exposure to these substances for human hazard assessment in the workplace (occupational exposure) as well as for the general population (HPV (High Production Volume) Chemical Challenge Program, 2009).

Both fumes generated from paving grade bitumens and oxidized asphalt are made up of low-molecular-weight substances, including ~70% aliphatic hydrocarbons and ~30% aromatic hydrocarbons, which include predominantly alkylated polycyclic aromatic hydrocarbons (PAHs) and a low concentration of polycyclic aromatic compounds (PACs) containing oxygen, sulfur and nitrogen moieties (Parker et al., 2011). The temperature applied to reduce viscosity to allow handling of the material affects the final relative proportions of individual PAHs in the fume and the amount of fume generated. For instance, fumes generated at high temperature (>220 °C) for experimental purpose are more likely to contain carcinogenic 3- to 7-ring PACs/PAHs than fumes generated at the lower temperatures (<220 °C) usually seen in field samples that match real-world exposures (HPV (High Production Volume) Chemical Challenge Program, 2009; McCarthy et al., 1999; NIOSH, 2000).

Like most petroleum substances, due to their inherent compositional complexity, both bitumen and oxidized asphalt are categorized as substances of Unknown or Variable composition, Complex reaction products or of Biological materials (UVCB) and registered as such under the

* Corresponding author at: PO Box 162, 2501 AN, The Hague, The Netherlands.
E-mail address: lenny.kamelia@shell.com (L. Kamelia).
[1] Present address: Shell Health, Shell International Bv, PO Box 162, 2501 AN, The Hague, The Netherlands

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EU-REACH legislation. Under this regulation, they need to be evaluated for the potential effect on prenatal development (according to the OECD 414 guidelines; OECD, 2018) since they are produced and/or marketed in the EU market at a volume of ≥100 t/year. To date, there are 3 relevant published in vivo studies (Boogaard et al., 2021a, 2021b; Parker et al., 2011) evaluating the effects of fumes of bitumen and oxidized asphalt on prenatal development in mammalian species, where all studies demonstrated that nose-only inhalation to either bitumen fume or oxidized asphalt fume do not cause any effects on reproduction and prenatal development (Boogaard et al., 2021a, 2021b; Parker et al., 2011). For example, in the study done by Boogaard et al. (2021a), rats were exposed to bitumen fumes (at concentrations up to 482 mg/m^3) by nose-only inhalation for 6 h/day from day 1 to 19 post coitum, and the results showed that despite clear maternal toxicity, exposure to these fumes did not induce any significant fetal abnormalities. It is worth mentioning that the exposure levels applied in the study by Boogaard et al. (2021a) are high (i.e. 1–2 orders of magnitudes higher) compared to the current occupational exposure limit (OEL) for bitumen fumes of 5 mg/m^3 (ACGIH, 2020; DECOS, 2008). Nevertheless, the PAH composition of the fumes the experimental rats were exposed to are comparable to the actual composition of fumes that workers may be exposed to during occupational activities (e.g. at the paving site) (Boogaard et al., 2021a). An overview of available in vivo developmental toxicity studies done on fumes of either bitumen or oxidized asphalt is provided in Table 1.

Previous studies have documented that some heavy petroleum UVCB substances, such as those covered in the heavy fuel oil (HFO) category, are able to induce developmental toxicity in pregnant rats exposed dermally to these substances (Feuston et al., 1989; Hoberman et al., 1995; Murray et al., 2013). It was shown that this potency is proportional to the level of 3- to 7-ring PAHs present in these substances (Feuston et al., 1989; Hoberman et al., 1995; Murray et al., 2013). In contrast, when similar developmental toxicity studies (according to the OECD 414) were conducted with gas-to-liquid (GTL) products and highly refined base oil (HRBO), which both contain no aromatics and no PAHs, no prenatal developmental toxicity-related effects were observed (Boogaard et al., 2017; Mobil, 1987). These in vivo data are also in agreement with our recent in vitro findings where a series of PAH-containing DMSO-extracts of petroleum substances were shown to be able to induce concentration-dependent developmental toxicity in vitro, with this potency being proportional to the amount of 3- to 7-ring PAHs present in these substances (Kamelia et al., 2017, 2019b). Moreover, it was found that, among other nuclear receptors, activation of the aryl hydrocarbon receptor (AhR) plays a role in mediating the developmental toxicity of PAH-containing petroleum substances (Kamelia et al., 2018). Altogether, this reflects the usefulness of a modes-of-action-based in vitro assay battery to evaluate the developmental toxicity potency of PAH-containing petroleum UVCB substances.

The present study aims to evaluate the potential in vitro developmental toxicity of the DMSO-extracts of fume condensates of bitumen and oxidized asphalt that were also tested in the in vivo developmental toxicity studies published by Boogaard et al. (2021a, 2021b). It should be noted that petroleum UVCB substances and also the fume condensates generated from bitumen and oxidized asphalt, have a very low water solubility, and consequently, cannot be dosed directly to any of the in vitro assays applied. Hence, an extraction procedure (Roy et al., 1988; McKee et al., 2013) was applied to generate DMSO-extracts from the fume condensates of bitumen and oxidized asphalt, which were then used for in vitro dosing. To this purpose, DMSO-extracts of the fume condensates of a paving grade bitumen and an oxidized asphalt were tested in a series of in vitro alternative assays: the AhR CALUX (Chemical Activated Luciferase Gene Expression) reporter gene assay (Aarts et al., 1995), the zebrafish embryotoxicity test (ZET), and the mouse embryonic stem cell test (mEST). Both ZET and mEST are scientifically

Table 1
Overview of in vivo developmental toxicity data for bitumen fume and oxidized asphalt fume in Wistar rats.

Test material	CAS no.	Method and duration of exposure	Dose (mg/m^3)	No. of pregnant dams	No. of resorptions/ no. of implantations	No. of live fetuses/ dam (mean ± SD)	Fetal body weight (mean ± SD)	Main findings and references
Paving grade bitumen	8052-52-4	• Nose-only inhalation • 6 h/day for 20 days post coitum • OECD 414 (prenatal developmental toxicity study)	0, 52, 151, 482	23 21 24 21	20/268 15/277 19/271 23/240	11.6 ± 1.6 10.0 ± 2.7 10.5 ± 2.4 10.2 ± 2.2	5.21 ± 0.25 5.09 ± 0.28 5.13 ± 0.20 4.88 ± 0.21	- Maternal NOAEC: 52 mg/m^3 (based on decreased net body weight gain and reduced food consumption, increased lung weights, histopathological changes in lung and larynx) - Developmental NOAEC: 151 mg/m^3 (based on reduced fetal body weight in the highest-dose group as a consequence of maternal toxicity) - No significant fetal anomalies were observed - Boogaard et al. (2021a)
Oxidized asphalt	64742–93-4	• Nose-only inhalation • 6 h/day for 20 days post coitum • OECD 414 (prenatal developmental toxicity study)	0, 53, 158, 536	22 23 24 21	18/236 18/273 17/264 18/250	9.9 ± 3.0 11.1 ± 1.6 10.3 ± 3.4 11.0 ± 2.1	5.00 ± 0.31 5.11 ± 0.29 5.10 ± 0.30 4.93 ± 0.30	- Maternal NOAEC: 53 mg/m^3 (based on decreased net body weight gain and reduced food consumption, increased lung weight, histological changes in lung, nasal cavity, and larynx) - Developmental NOAEC: 536 mg/m^3 - No significant fetal abnormalities were observed - Boogaard et al. (2021b)
Type III built-up roofing asphalt (BURA)	64742–93-4	• Nose-only inhalation • 6 h/day for 28 days to male rats and 42 days to female rats (including gestation days 0–20) • OECD 422 (repeated dose toxicity study with the reproductive/ developmental toxicity screening test)	0, 30, 100, 300	11 9 11 11	10/118 5/100 13/126 8/98	9.7 ± 1.5 10.3 ± 1.2 10.3 ± 1.5 9.0 ± 2.2	5.8 ± 0.7 5.8 ± 0.5 5.6 ± 0.4 5.7 ± 0.5	- Reproductive and developmental NOAEC: 300 mg/m^3 - No reproductive- and developmental-related effects were observed - Systemic NOAEC: 100 mg/m^3 for males (based on decreased body weight and food consumption, and increased lung weight) and 30 mg/m^3 for females (based on increased lung weight) - Parker et al. (2011)

validated alternative assays widely used to evaluate the in vitro developmental toxicity of chemical substances (de Jong et al., 2009; Genschow et al., 2004; Incardona et al., 2004; Li et al., 2015; Louisse et al., 2011; Selderslaghs et al., 2012; Strikwold et al., 2012), and both assays proved to adequately assess the embryotoxicity of PAH-containing petroleum DMSO-extracts as compared to their in vivo potency (ARCO, 1993; Feuston et al., 1989; Feuston and Mackerer, 1996b; Hoberman et al., 1995; Kamelia et al., 2017, 2019a, 2019b, 2020; Mobil, 1989). Finally, the obtained in vitro results were compared to the in vivo results, to evaluate the applicability of the applied assay battery to assess the developmental toxicity of the fume condensates of bitumen and oxidized asphalt.

2. Materials and methods

2.1. Test materials

DMSO-extracts of the fume condensates of a paving grade bitumen (CAS no. 8052-42-4; EC no. 232–490-9), sample code #016-BFC, and of an oxidized asphalt (CAS no. 64742–93-4; EC no. 265–196-4), sample code #017-OAFC, were tested in the present study. To generate the fume condensates that resembles the fume during occupational exposure, the parent materials were heated under controlled conditions at 180 °C for bitumen and at 215 °C for oxidized asphalt as previously described by Boogaard et al., 2021a, 2021b. The neat bitumen and oxidized asphalt used to generate the fume condensates and subsequently the DMSO-extracts were kindly provided through Concawe (Brussels, Belgium).

2.2. DMSO-extraction procedure and PAH analysis

The DMSO-extraction and PAH analysis were performed at Port Royal Research (PRR) laboratory (Hilton Head, South Carolina, USA) according to the PRR PAC2 Method described in detail by Roy et al. (1988) and McKee et al. (2013). For the DMSO-extraction, 4.0 g of fume condensate samples were dissolved in 10 ml cyclohexane and extracted twice with 10 ml pre-equilibrated DMSO (mixture of 10:1 DMSO/cyclohexane). This mixture was shaken for 1 min and the PAH-enriched extracts were collected and stored in 20 ml capped liquid scintillation vials and stored at 4 °C until further used.

Analysis of the PAC content, i.e. PAHs, was performed to measure the total weight percentage (wt%) of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each test material. To this end, 20 ml of the DMSO-extracts, were transferred to a 125-ml separatory funnel, diluted with twice the volume (40 ml) of 4% aqueous sodium chloride, and back extracted with 20 and 10 ml of cyclohexane. The cyclohexane fractions were combined in a 60-ml separatory funnel, washed twice with 5 ml distilled water, and filtered through anhydrous sodium sulfate into a 50-ml flask. The addition of water is a critical step because the DMSO-PAC (i.e. DMSO-PAH) interaction is very sensitive to the presence of water (enhanced/intensified by the addition of NaCl) (Carrillo et al., 2019; Natusch and Tomkins, 1978). As a result, the addition of water will disrupt the DMSO-PAH interaction and will force the PAHs back to the cyclohexane fractions to assure complete back extraction of all aromatics, including PAHs, for the subsequent analysis by gas chromatography. The cyclohexane fractions were evaporated to near dryness at 40 °C followed by further evaporation at 80 °C (15–20 Torr) for 30 min. Once equilibrated to room temperature, the flask was weighed, and the residue dissolved in cyclohexane (~50 mg/ml). The extracts were then analyzed by gas chromatography with mass spectrometric detection (GC–MS). The gas chromatograph was equipped with a 30 m × 0.25 mm × 0.25 mm Zebron-5HT capillary column. The oven temperature was held at 70 °C for 0.5 min and then programmed to 300 °C at a rate of 5 °C/min and held at the final temperature for 35 min. A split mode of injection (50:1) was used for GC–MS analysis. The GC–MS chromatograms of the extracts were integrated in the slice mode. Naphthalene, phenanthrene, pyrene, benzo[*a*]pyrene, benzo[ghi-]perylene, and coronene were used as standards to define the borderlines (margins) of retention times for respectively 2-, 3-, 4-, 5,- 6,- and 7-ring PAHs. After establishing the retention time markers for 2- to ≥7-ring PAHs, the extracts were subsequently analyzed by GC–MS. Data were presented as the total weight percentage (wt%) of the DMSO-soluble 1- to ≥7-ring aromatic compounds present in each sample, from the starting material of 4.0 g sample. The use of DMSO-extracts, which contain most of the more polar constituents (e.g. 3- to 7-ring PAHs) of the PAH-containing petroleum substances (Carrillo et al., 2019) is a common and standard approach for in vitro testing of these group of highly complex UVCB substances (Concawe, 1994; House et al., 2020). This method is widely applied and routinely used for example for in vitro mutagenicity testing of PAH-containing petroleum substances since the 1980s (Blackburn et al., 1986; Concawe, 1994; Hermann et al., 1980).

2.3. In vitro alternative assays

DMSO-extracts of the fume condensates of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) were tested in three different alternative assays: the aryl hydrocarbon (AhR) CALUX reporter gene assays, the zebrafish embryotoxicity test (ZET), and the mouse embryonic stem cell test (mEST).

2.3.1. AhR CALUX reporter gene assay (AhR CALUX assay)

The AhR-mediated (i.e. agonistic) activities of DMSO-extracts of the fume condensates of bitumen and oxidized asphalt were evaluated in the AhR CALUX assay. The stably transfected rat hepatoma cell line, H4IIE.luc (Aarts et al., 1995), was used for the AhR CALUX assay. Cells were grown in Minimum Essential Medium alpha (α-MEM) (Gibco, Paisley, UK) supplemented with 10% *v*/v fetal bovine serum (FBS) (Sigma-Aldrich, Zwijndrecht, The Netherlands). Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere and subcultured every 2–3 days, using 0.05% trypsin-EDTA (Gibco) to detach the cells.

For the AhR CALUX assay, H4IIE.luc cells were seeded in a volume of 100 µl into each of the 60-inner wells of 96-well white plates (Greiner Bio-one, Frickenhausen, Germany) at a density of 3×10^5 cells/ml. The 36-outer wells of the same 96-well white plate were filled with 200 µl phosphate buffered saline (PBS) (Gibco) to create an optimal humidity and to limit evaporation from the inner wells. The cells were incubated overnight at 37 °C/5% CO_2 to allow cell adherence and then exposed for either 6 or 24 h to the test materials in triplicate. Two exposure time periods were applied to check whether the AhR induction is transient (6 h) or persistent (24 h). Samples #016-BFC and #017-OAFC were tested in the AhR CALUX at concentrations ranging from 5×10^{-5} to 5 µg raw material/ml. It is worth mentioning that 5 µg raw material/ml was used as the top tested concentration because our previous studies (Kamelia et al., 2018, 2019b) using the AhR CALUX assay with PAH-containing petroleum UVCB extracts showed that the highest AhR induction was already obtained upon exposure to test materials at this concentration. The exposure medium for the AhR CALUX assay was prepared by diluting the test materials (from 400 times concentrated DMSO-stock solutions) with culture medium. A full concentration-response curve of the 5-ring PAH benzo[*a*]pyrene (BaP) (Sigma-Aldrich) (1.3×10^{-5} - 1.3×10^{-1} µg/ml) and of TCDD (Sigma-Aldrich) (8×10^{-8} - 2.4×10^{-5} µg/ml) were included as the reference-standard compounds for the 6 and 24 h exposure regimens, respectively, in the AhR CALUX assay (Vrabie et al., 2009). BaP and TCDD represent commonly used reference-standard compounds for the AhR CALUX assay (Aarts et al., 1995; Vrabie et al., 2009; Pieterse et al., 2013). BaP was selected as a PAH model compound since the test materials are PAH-containing substances, and the maximum AhR induction upon exposure to BaP was obtained following 6 h of exposure (see Supplementary materials). TCDD represents a more persistent AhR inducer and its maximum AhR induction was obtained following 24 h of exposure (see Supplementary materials). The final concentration of solvent, i.e. dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) was kept at 0.25% *v*/v.

Following 6 or 24 h of exposure, medium was removed, cells were washed with 100 µl ½ PBS (1:1 PBS: nanopure water) and then lysed with 30 µl hypotonic low salt buffer (LSB: 10 mM Tris (Sigma-Aldrich), 2 mM dithiothreitol (DTT, Sigma-Aldrich), and 2 mM 1, 2-diaminocyclohexane tetraacetic acid (Sigma-Aldrich), pH 7.8. Afterward, plates were kept on ice for 15 min and subsequently frozen at -80 °C for at least 2 h or until use for the luminescence measurement. For the luminescence measurement, plates were thawed at room temperature for about 30 min to 1 h and then shaken for 3–5 min on a plate shaker. Luciferase activity was determined using a luminometer (Glomax-Multi Detection System, Promega, California, USA) after the addition to each well of 100 µl flashmix solution consisting of an aqueous solution of 20 mM tricine (Sigma-Aldrich), 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2.5H_2O$ (Sigma-Aldrich), 2.67 mM magnesium sulfate ($MgSO_4$) (Merck), 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex III (Merck), 2 mM DTT (Sigma-Aldrich), 0.47 mM D-luciferin (Synchem UG & Co. KG, Felsberg, Germany), and 5 mM adenosine-5-triphosphate (Duchefa Biochemie bv, Haarlem, The Netherlands), pH 7.8. Four independent experiments were done for each test material and results were expressed as fold induction of luciferase activity compared to the solvent control (0.25% *v*/v DMSO).

2.3.2. Zebrafish embryotoxicity test (ZET)

The ZET was performed as described previously by Kamelia et al. (2019a). Fertilized zebrafish embryos (wild-type *Danio rerio* AB line) used for the ZET were obtained from the research facility Carus, Wageningen University and Research (Wageningen, The Netherlands). The ZET was initiated at 4 h post fertilization (hpf) and terminated at 96 hpf. Twenty wells of the 24-well plate (Greiner Bio-One) were used for exposure to one concentration of test material in the ZET and the other 4 wells were used for the internal plate control. The exposure medium was prepared by mixing the 400 times concentrated stock solutions of test materials with egg water. Egg water was prepared by mixing 10 ml 100×-egg water stock solution with 990 ml demi water. 100×-egg water stock solution was prepared by dissolving 3 g sea salt (Tropic Marin, USA) in 500 ml demi water. The exposure medium was then transferred (2 ml/well) into 20 wells of the 24-well plate, and for the internal plate control, 2 ml egg water was added into each of the 4 remaining wells. Afterward, one zebrafish embryo was added to each well of the 24-well plate (1 embryo/well). The plate was then sealed with self-adhesive film cover (Sigma-Aldrich) and was incubated at 26 °C with a photo period of 14 h light:10 h dark. Solvent controls (0.25% v/v DMSO, positive controls (4 µg/ml 3,4-dichloroaniline) (Sigma-Aldrich), and negative controls (egg water only) were included in each independent experiment. Embryos were checked daily for cumulative mortality (decreased survival) and scoring for developmental abnormalities was done at 96 hpf based on the extended general morphology scoring (GMS) system described by Beekhuijzen et al. (2015). The ZET was considered valid if the following was observed: ≤ 1 dead embryo (out of 4) in the internal plate control of the exposed-plate; ≤ 3 dead embryos (out of 24) in the negative control plate (at least 87.5% survival rate); ≤ 2 dead embryos (out of 20) in the solvent control plate (0.25% DMSO); ≤ 14 live embryos (out of 20) in the positive control plate (4 µg/ml 3,4-dichloroaniline). The ZET results of each test material were presented as fraction of total GMS score at 96 hpf (i.e. 17 points, see Supplementary materials). Four independent ZET experiments were done for each test material.

2.3.3. Mouse embryonic stem cell test (mEST)

The third assay applied in the present study was the mEST which was performed essentially as described previously by Kamelia et al. (2017, 2019b). The mEST consists of two assays, namely the ES-D3 cell viability assay (for 1- and 5-days exposure time) and the ES-D3 cell differentiation assay. The viability assay was used to confirm that developmental toxicity was not due to cytotoxicity. In short, for the cell viability assay, ES-D3 cells were seeded (100 µl cell suspension/well) at a density of 20 × 10^4 cells/ml (1 day exposure) or 10^4 cells/ml (5 days exposure) in 96-well plates (Greiner Bio-One). At 24 h after cell seeding, cells were exposed to test materials in triplicate by the addition of 100 µl exposure medium to each well. After 1- or 5-days incubation (37 °C, 5% CO_2), 20 µl of WST-1 reagent (Roche Diagnostics) was added to each well and cells were incubated for 3 h (37 °C, 5% CO_2). The absorbance of the formed formazan, which directly correlates with the number of viable cells, was measured at 440 nm (background at 620 nm) using a SpectraMax M2 (Molecular Devices, UK). Four independent experiments were done for the ES-D3 cell viability assay of each test material.

The ES-D3 cell differentiation assay of the mEST lasted for 10 days. On day 0, droplets of 20 µl of ES-D3 cell suspension (3.75 × 10^4 cells/ml) in exposure medium (1 plate for 1 concentration of test material), were placed between the well borders on the inner side of the lid of a 96-well plate, and incubated as follows to allow formation of embryoid bodies (EBs) or spheroids. The exposure medium was prepared by mixing 400 times concentrated DMSO stock solutions of the test materials with the growth medium HyClone AdvanceSTEM™ Low Osmo Dulbecco's Modified Eagle Medium (Fischer Scientific, The Netherlands), supplemented with 15% (*v*/v) FBS (ATCC, USA) and 1% (v/v) Penicillin-Streptomycin-Glutamine 100× (Gibco, UK). All wells of the 96-well plate were filled with 250 µl PBS to create an optimal humidity and to prevent evaporation of the hanging drops. Sterile caps of Eppendorf tubes were placed in the corner of the plates in order to prevent direct contact of the drops with the plate and the plate was then sealed with Micropore tape (3M, Germany). The hanging drop cultures were incubated for three days at 37 °C and 5% CO_2. On day 3, the resulting EBs were collected and transferred to 60 × 15 mm bacteriological petri dishes (Greiner Bio-One) containing 5 ml exposure medium. Petri dishes were incubated (37 °C/5% CO_2) for another two days. On day 5, the EBs were transferred to a 24-well plate (Greiner Bio-One) (1 EB/well and 1 plate/concentration), containing 1 ml exposure medium with or without test material. The EBs in 24-well plates were then incubated for 5 days. On day 10, the number of wells containing beating cardiomyocytes was determined by visual inspection using an inverted microscope. The ES-D3 cell differentiation assay was considered valid if the solvent control in each experiment (0.25% *v*/v DMSO) had at least 21 out of 24 wells that contained beating cardiomyocytes. The inhibition of differentiation by the test material was presented as the fraction of total EBs plated in the 24-well plate. In addition to solvent control, 0.065 µg/ml (0.5 µM) 5-fluorouracil (5-FU, Sigma-Aldrich) was included in each experiment as a positive control of the assay. Four independent experiments were performed for each of the test materials.

2.4. Data analysis

Figures of concentration-response curves upon exposure to the DMSO-extracts of the fume condensates of bitumen and oxidized asphalt in the AhR CALUX assay, ZET, and mEST were made using GraphPad Prism 5.0 (California, USA). For this purpose, data obtained from all assays applied were analyzed using non-linear regression analysis of GraphPad and fitted to a sigmoid dose-response curve with 3 parameters.

For the AhR CALUX assay, the AhR-mediated activity was presented as an induction factor (IF) relative to the response induced by solvent control 0.25% v/v DMSO (IF set to 1). The EC50 value (50% effective concentration) representing the concentration for 50% induction of luciferase activity upon exposure to the respective test material, was determined using GraphPad. For the ZET, results were expressed as a fraction of the total GMS score at 96 hpf (the maximum score being 17 points; see Supplementary materials) and are presented as mean ± standard error of the mean (SEM). For the mEST, data from the ES-D3 cell viability (1- and 5- days exposure) were expressed as % cell viability compared to the solvent control 0.25% v/v DMSO, whilst data from the ES-D3 cell differentiation assay were expressed as a fraction of differentiated embryoid bodies (EBs) into beating cardiomyocytes, out of the total EBs plated in the 24-well plate. Data obtained in the mEST

are all presented as mean ± SEM.

To determine the lower bound of the 95% confidence interval for the benchmark concentrations (i.e. BMCL), concentration-response curves obtained in the ZET and ES-D3 cell differentiation assay of the mEST were fitted to all quantal concentration-response models available in the European Food Safety Authority (EFSA) benchmark dose (BMD) modeling web-tool (https://shiny-efsa.openanalytics.eu/) based on the R-package PROAST version 66.40 developed by the Dutch National Institute for Public Health and the Environment (RIVM). The analysis was performed according to the manual provided on the EFSA BMD web-tool site. The quantal models of the ESFA web-tool include two-stage, log logistic, weibull, log probit, gamma, logistic, probit, exponential, and hill models. For BMCL50 determination, the benchmark response (BMR) was set at 50%, representing the concentration that induces either a 50% inhibition of EBs differentiated into contracting cardiomyocytes in the ES-D3 cell differentiation assay of the mEST, or a 50% reduction of total GMS at 96 hpf in the ZET. All fitted and accepted models from the BMD analysis were further used to perform model averaging, in which a weighted-average BMCL50 value was estimated using parametric bootstrap sampling. The bootstrap was set at 200 (default). The performance of each fitted model was evaluated based on the goodness-of-fit, the scaled residuals, and the visual inspection of model fitting. Weighing in model averaging is based on the accepted model Akaike's Information Criterion (AIC) values where the accepted model with a lower AIC value counts with larger weight when calculating the final BMCL50.

3. Results

3.1. PAH content analysis for the DMSO-extracts of the fume condensates of bitumen and oxidized asphalt tested

DMSO-extracts of the fume condensates of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) were analyzed for their PAH content to determine their 1- to ≥7-ring PAH profile. As illustrated in Fig. 1, samples #016-BFC and #017-OAFC contain 13% and 16% weight percentage (wt%) of total PAHs, respectively. Moreover, this PAH analysis also revealed that the majority of PAH present in samples #016-BFC and #017-OAFC belong to the groups of 2- and 3-ring PAHs, amounting to 75 and 81 wt% of the total amount of all PAH present, or 10 and 13 wt% of the total extracts, respectively.

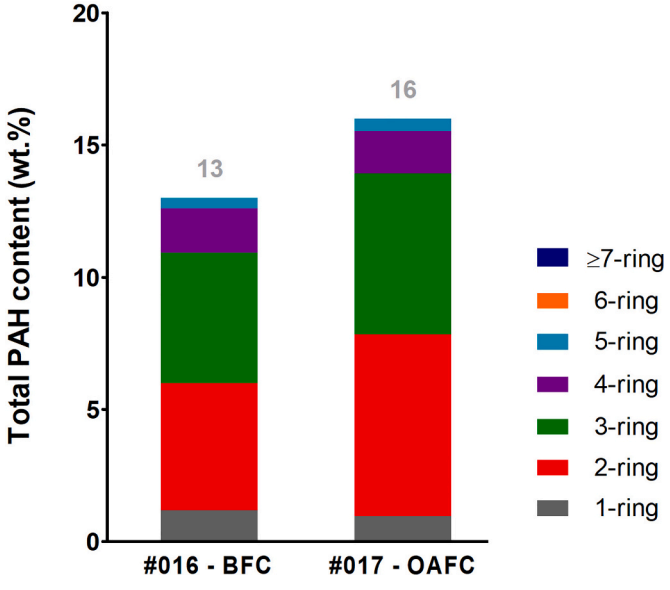

Fig. 1. PAH profiles of the DMSO-extracts of the fume condensates of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) tested in the present study. The presented PAH profile represents the weight percent of the DMSO-soluble 1- to ≥7 PAHs present in each test material, determined by the PRR PAC2 method described previously by Roy et al. (1988) and McKee et al. (2013). *Abbreviations. #016-BFC: DMSO-extract of bitumen fume condensates, #017-OAFC: DMSO-extract of oxidized asphalt fume condensates.*

3.2. AhR-mediated activity of fume condensate extracts of bitumen and oxidized asphalt

AhR-mediated activities of the DMSO-extracts of the fume condensates of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) were evaluated in the AhR CALUX assay, as this endpoint was assumed to play a role in a battery of in vitro assays able to detect the developmental toxicity potency of PAH-containing petroleum substances (Kamelia et al., 2017, 2018, 2019a). Results show that both samples are able to induce concentration-dependent AhR-mediated activities in the AhR CALUX assay. The maximum AhR induction was obtained following 6 h of exposure and this activity was substantially reduced after 24 h of exposure to these substances (Fig. 2). These concentration-response curves were then used to derive the EC50 values, defined as the concentrations that induce half of the maximum AhR-mediated activity induced by samples #016-BFC and #017-OAFC relative to the solvent control (induction factor of the solvent control was set at 1) (Fig. 2). The derived EC50 values for sample #016-BFC following 6 or 24 h of exposure in the AhR CALUX assay were 3.3×10^{-2} and 2.1 μg raw material/ml, respectively (Fig. 2). For sample #017-OAFC, EC50s of 5.0×10^{-2} and 3.0 μg raw material/ml were obtained upon exposure for 6 or 24 h, respectively (Fig. 2).

3.3. Effects of fume condensate extracts of bitumen and oxidized asphalt in the ZET

To evaluate the developmental toxicity potential of the fume condensate extracts of bitumen and oxidized asphalt, the effects of samples #016-BFC and #017-OAFC on the developing zebrafish embryos were determined. As shown in Fig. 3, exposure to both #016-BFC and #017-OAFC induced a concentration-dependent decrease in the

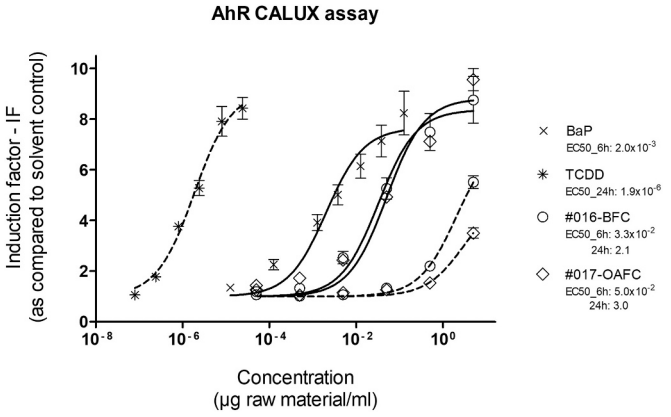

Fig. 2. Effects of the DMSO-extracts of the fume condensates of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) in the AhR CALUX assay following 6 (continuous line) or 24 h (dashed line) of exposure to these substances. Benzo[*a*]pyrene (BaP) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was included in each independent experiment as the reference-standard compound for 6 or 24 h exposure time, respectively. Results are expressed as fold induction factor (IF) of luciferase activity relative to the solvent control (0.25% *v*/v DMSO) and are presented as mean ± SEM from four independent experiments. *Abbreviations. AhR: aryl hydrocarbon receptor, CALUX: chemical activated luciferase gene expression, BaP: benzo[a]pyrene, TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin, IF: induction factor, #016-BFC: DMSO-extract of bitumen fume condensates, #017-OAFC: DMSO-extract of oxidized asphalt fume condensates.*

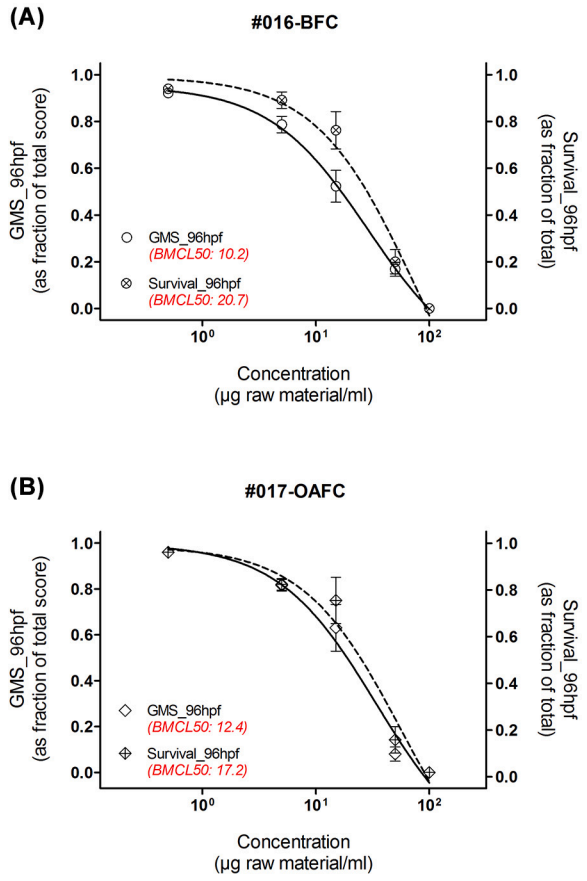

Fig. 3. Concentration-dependent effects of the DMSO-extracts of the fume condensates of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) in the ZET on GMS score (continuous line) and embryo survival (dashed line). Results represent data from four independent experiments ($n = 4$) and are presented as mean ± standard error of the mean (SEM). *Abbreviations. GMS: general morphology scoring, #016-BFC: DMSO-extract of bitumen fume condensates, #017-OAFC: DMSO-extract of oxidized asphalt fume condensates, BMCL50: the lower bound of the 95% confidence interval for the benchmark concentrations* (i.e. *BMCL), representing the concentration that induces a 50% reduction of GMS score or embryo survival at 96 hpf in the ZET.*

GMS score that coincided with concentrations causing a decrease in embryo survival at 96 hpf in the ZET. In other words, the decrease of the GMS score (at 96 hpf) was mainly due to the early embryo lethality that mainly occurred from 24 to 48 hpf (or when scored at 24/48 hpf time-points; see Supplementary materials). Using the obtained concentration-response curves in the ZET, the BMCL50 value for both GMS and survival (lethality) were determined and the resulting BMCL50s are presented in Fig. 3 as well. For sample #016-BFC, a BMD analysis of the data for GMS and survival (lethality) resulted in BMCL50 values of 10.2 and 20.7 µg raw material/ml, respectively (Fig. 3A). For sample #017-OAFC, the analysis resulted in BMCL50s of 12.4 and 17.2 µg raw material/ml for GMS and survival (lethality), respectively (Fig. 3B). All results from the BMD analysis of the ZET data for samples #016-BFC and #017-OAFC are presented in the Supplementary materials. In addition to the GMS endpoints, other developmental effects induced by samples #016-BFC and #017-OAFC in the ZET (at 96 hpf) included pericardial edema and/or yolk deformation, which occurred starting at a tested concentration of 5 µg raw material/ml for sample #016-BFC and 15 µg raw material/ml for sample #017-OAFC and occurred at concentrations where also embryo lethality was observed (Fig. 4).

3.4. Effects of fume condensate extracts of bitumen and oxidized asphalt in the mEST

Finally, the mEST was performed to further evaluate the developmental toxicity potency of the fume condensate extracts of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC). In the mEST, both samples did not affect either the cell viability or the differentiation of ES-D3 cells into contracting cardiomyocytes up to the highest concentration tested (100 µg raw material/ml) (Fig. 5). In other words, both samples tested negative in the mEST, and consequently, no BMCL50 value could be derived for either sample #016-BFC or sample #017-OAFC from the results obtained.

4. Discussion

The present study evaluated the usefulness of in vitro alternative assays to assess the developmental toxicity and underlying mode-of-action of fume condensate extracts of bitumen and oxidized asphalt, as compared to their activities observed in vivo. The in vitro assays applied were the AhR CALUX assay, the ZET, and the mEST. This assay battery has previously been shown to be able to predict the developmental toxicity potency and possible underlying modes-of-action of a range of petroleum UVCB substances, within and across product categories, as the results obtained with their DMSO-extracts in these in vitro assays were fully in line with the corresponding in vivo developmental toxicity potency (ARCO, 1993; Feuston et al., 1989; Feuston and Mackerer, 1996a, 1996b; Kamelia et al., 2017, 2019a, 2019b; Mobil, 1989). Results obtained in the present study show that, despite a transient concentration-dependent AhR-mediated activity induced by the fume condensate extracts of both bitumen and oxidized asphalt, these two materials were not able to induce any developmental toxicity in the ZET or mEST. Results obtained in the ZET and mEST are in line with in vivo findings where the fumes of both bitumen and oxidized asphalt tested negative for effects on prenatal development in studies according to the OECD 414 testing guideline (Boogaard et al., 2021a, 2021b). In brief, Boogaard et al. (2021a, 2021b) reported that nose-only exposure to fumes from either bitumen or oxidized asphalt did not cause any significant fetal abnormalities, and all pregnant dams had viable fetuses without any dead fetuses and without pre- or post-implantation loss. The only developmental-related effect observed was reduced fetal body weight at the highest dose tested, and this only occurred in the study with bitumen fumes while it was not observed in the study with oxidized asphalt fumes. This decrease in fetal weight is most likely a secondary effect to maternal toxicity, as reflected by the reduction of maternal body weight gain (26% lower as compared to the control group) as a consequence of decreased maternal food consumption (Boogaard et al., 2021a). Similarly, Parker et al. (2011) also reported the results of a combined repeated dose toxicity study with a reproduction/developmental toxicity screening study according to the OECD 422 protocol, in which exposure (via nose-only inhalation) of roofing asphalt fume to pregnant rats did not induce any adverse effects on either reproductive or developmental parameters.

The present study makes use of well-validated alternative assays, ZET and mEST, for evaluating the in vitro developmental toxicity potency of the fume condensate extracts of bitumen and of oxidized asphalt. In the ZET, the main observed effect following exposure to these materials was early embryo lethality, while almost no effect was seen for other developmental endpoints scored. As a result, the decrease in the total GMS score at 96 hpf was largely due to this early embryo lethality (Fig. 3). Most of embryo lethality was spotted at 24 hpf (see Supplementary materials), although this event may already occur at earlier time points because the most vulnerable time-window for effects on the survival rate of zebrafish embryos is at the early stages of their development, i.e. around gastrulation (6–10 hpf) and early segmentation periods (10–22 hpf) (Kimmel et al., 1995; Uchida et al., 2018). Available literature showed that exposure of zebrafish embryos to especially

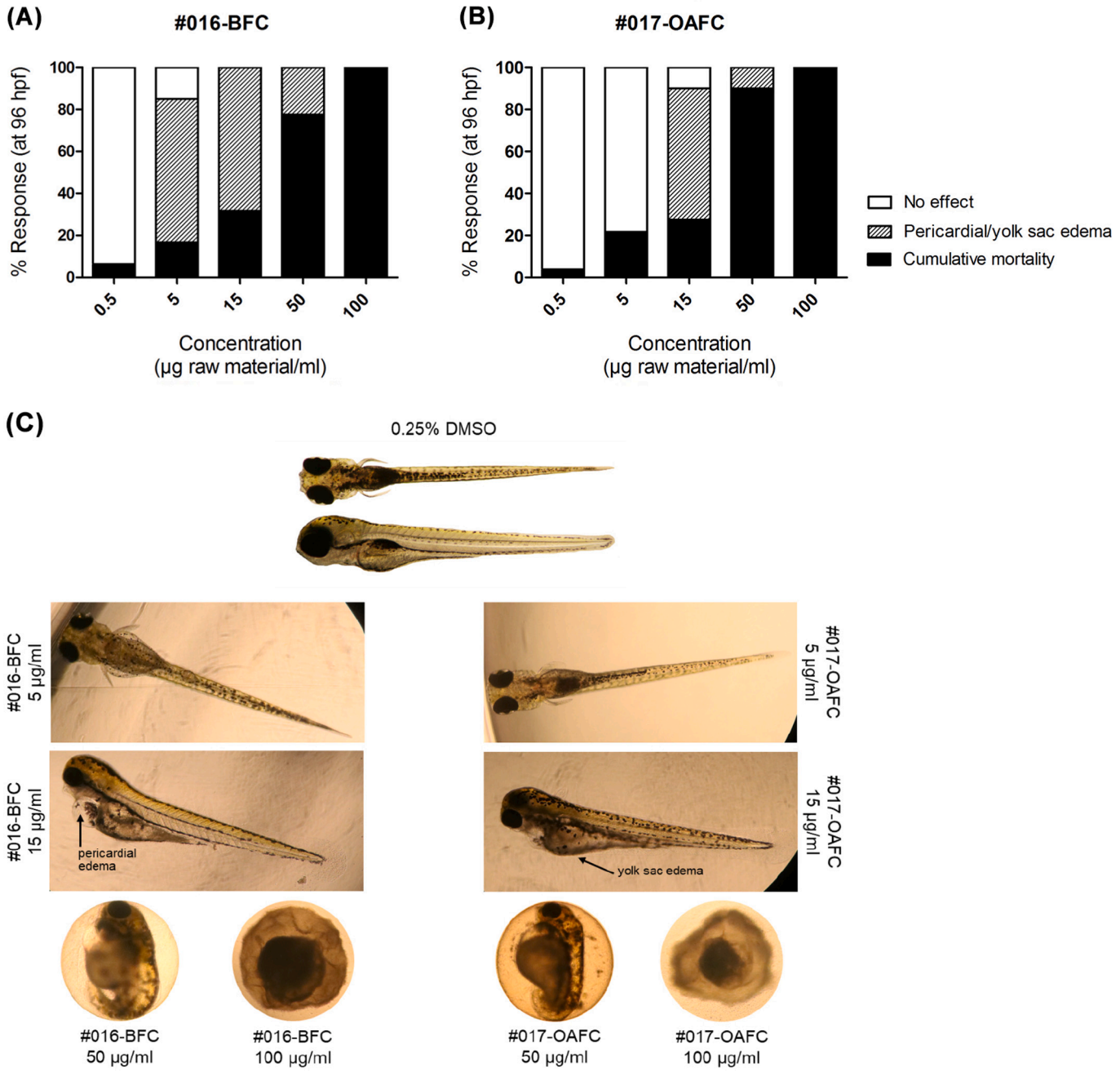

Fig. 4. (A-B) Effects on embryo lethality and pericardial and/or yolk sac edema following exposure to the samples (A) #016-BFC and (B) #017-OAFC at 96 hpf in the ZET. (C) Representative images of morphological effects of zebrafish embryos exposed to different concentrations of the samples #016-BFC and #017-OAFC at 96 hpf. *Abbreviations. #016-BFC: DMSO-extract of bitumen fume condensates, #017-OAFC: DMSO-extract of oxidized asphalt fume condensates.*

volatile compounds, such as ethanol, at ±4–22 hpf resulted in high mortality, while exposure at later stages (±24–30 hpf) showed more developmental-related effects (Ali et al., 2011a, 2011b). Hence, considering that both tested materials contain substantial amounts of volatiles, one could adjust the starting time point of exposure from 4 to 24 hpf to avoid high embryo mortality at the beginning of the ZET. In the present study, the observed embryo lethality was exposure time-related, however, embryonic lethality at an early stage alone is not sufficient to conclude that test materials induce developmental toxicity, in particular when it is not observed as an outcome over the whole exposure period in the ZET. It is more likely that the observed early embryonic lethality reflects non-specific toxicity rather than developmental toxicity of the fume condensate extracts. This is supported by the results obtained in the mEST where both fume condensate extracts tested negative. Together these results show that the fume condensate extracts of both bitumen and oxidized asphalt do not cause any in vitro developmental toxicity, in agreement with their test results observed in vivo. Moreover, exposed zebrafish embryos also developed pericardial and/or yolk sac edema, and such effects are common manifestations/profiles of zebrafish embryos exposed to individual PAHs or PAH-containing substances in the ZET, which are believed to be mediated via the AhR (Goodale et al., 2013; Gu et al., 2010; Huang et al., 2012; Incardona et al., 2004; Wincent et al., 2015; Geier et al., 2018). Hence, it is not surprising to see those edema effects as both test materials showed transient AhR induction in the AhR CALUX assay applied. More importantly, no malformations (e.g. curved tail or body shape) were observed on any zebrafish embryo after exposure to the DMSO-extracts of the fume condensates of bitumen or oxidized asphalt in the ZET.

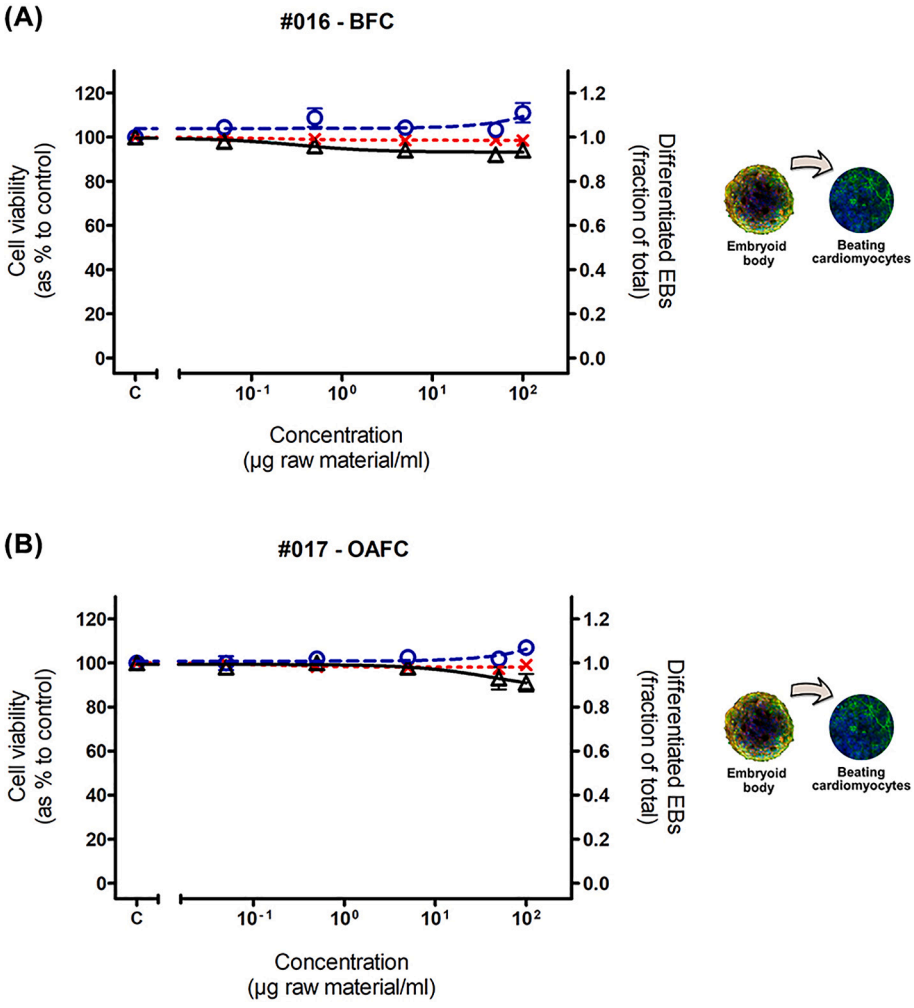

Fig. 5. Concentration-dependent effects of the fume condensate extracts of bitumen (#016-BFC) and oxidized asphalt (#017-OAFC) on ES-D3 cell viability upon 1-day (x and dotted line) and 5-days (o and dashed line) exposure and on inhibition of ES-D3 cell differentiation into contracting cardiomyocytes (Δ and continuous line). Results represent data from four independent experiments and are presented as mean ± SEM. *Abbreviations. #016-BFC: DMSO-extract of bitumen fume condensates, #017-OAFC: DMSO-extract of oxidized asphalt fume condensates, EB: embryoid body.*

Bitumen and oxidized asphalt are typically made up of high-molecular-weight compounds and may contain significant amounts of 4- to 7-ring naked and alkylated PAHs, whereas fumes generated from these materials contain mainly lighter PAHs (Fuhst et al., 2007; Parker et al., 2011). At ambient temperatures bitumen and oxidized asphalt are solid materials and to reduce viscosity to allow handling of the materials they are heated which may release fumes that contain the lighter, more volatile compounds of the neat material including 2- to 4-ring PAHs (such as naphthalene, acenaphthylene, fluorene, phenanthrene, and triphenylene). For testing purposes, these fumes can be collected by condensation and analysis of the condensates confirmed the predominance of the lighter polycyclic aromatic constituents in these fume condensates (Boogaard et al., 2021a; HPV (High Production Volume) Chemical Challenge Program, 2009; Kriech et al., 2002). The analytical data obtained in the present study showed that the DMSO-extracts of the fume condensates of bitumen and oxidized asphalt contained primarily 2- to 3-ring PAHs (75–81% of the total PAHs), and a low amount of 4-ring PAHs (10–13% of the total PAHs) and 5-ring PAHs (<5% of the total PAHs) (see Supplementary materials). The group of 3- to 7-ring PAHs are regarded as the main constituents in heavier petroleum UVCB substances (e.g. HFO) responsible for the developmental toxicity observed following exposure to these substances (Feuston et al., 1989; Feuston and Mackerer, 1996a; Murray et al., 2013). The more 3- to 7-ring PAHs these substances contain, the more likely for them to induce developmental toxicity both in vivo and in vitro, as shown in previous studies where the developmental toxicity potency of petroleum UVCB extracts, within and across product categories, was highly correlated with the level of 3- to 7-PAHs present in these substances (Feuston et al., 1989; Feuston and Mackerer, 1996a; Hoberman et al., 1995; Kamelia et al., 2017, 2019b; Murray et al., 2013). The five product categories tested in these previous studies included straight-run gas oils (GO), distillate aromatic extracts (DAE), vacuum and hydrocracked gas oils (VHGO), residual aromatic extracts (RAE), and heavy fuel oils (HFO). Based on the results obtained in the present study, the analytical characteristics (i.e. PAH profiles) of the extracts tested, at first glance, do not seem to match the relationship previously observed between PAH content and potency, reporting that petroleum UVCB extracts with substantial 3–5 or 3–7 ring PAH content did test positive for in vitro developmental toxicity in the ZET or mEST (Kamelia et al., 2017, 2019a, 2019b). Based on results obtained in the present study, fume condensate extracts of bitumen and oxidized asphalt tested negative in the ZET as well as the mEST, in full accordance with their in vivo results. It should be noted that 3- to 5-ring PAHs comprise approximately 50% of the total amount of PAH in the extracts of the fumes of both bitumen and oxidized asphalt (see Supplementary materials) while the amount of 5-ring PAHs is only 3% and no detectable amounts of 6- or 7-ring PAHs are present. The difference in PAH profiles of the fume condensates compared to those of the petroleum UVCB substances tested to establish the correlation model (i.e. between PAH content and potency) is the most likely explanation for the apparent discrepancy. Furthermore, this is the first time that fume condensates instead of the neat petroleum UVCB substances were extracted to be tested in the current assay battery designed for developmental toxicity testing in vitro. Apparently, as above explained, the correlation between PAH content and in vitro

developmental toxicity potency derived for petroleum UVCB substances cannot be applied to the extracts of the fume condensates. A possible reason may be that the type of PAH constituents, for example not only the level of 3- to 5-ring and 6- and 7-ring PAHs but also the degree of alkylation of the PAHs, in these fume condensates is different from those present in the petroleum UVCB extracts tested previously (Kamelia et al., 2017, 2018, 2019a, 2019b). It has been reported that fumes derived from bitumen and oxidized asphalt contain more alkylated than unsubstituted 2- to 3-ring PAHs (Kriech et al., 2002; Osborn et al., 2001). Recent studies demonstrated that the alkylation of PAHs shifts metabolism from aromatic ring oxidation to alkyl side chain oxidation (Wang et al., 2020). In addition, metabolism of alkylated 2- to 3-ring PAHs becomes less efficient with elongation of the alkyl chain, starting from the alkyl side chain having 3 or more carbon atoms, and the overall metabolism was greatly reduced with n-hexyl and completely absent with a n-dodecyl substitution (Wang et al., 2020, 2021 *in preparation*). Also, the more alkylation of a PAH, the more they behave like an aliphatic than an aromatic compound, which affects their bioavailability and consequently their toxicological profiles. Therefore, current research investigates to what extent substitution (i.e. the degree and position of alkyl substitution) affects the developmental toxicity potency of PAHs. The currently available PRR PAC2 methodology does not allow characterization of the PAH profile with regard to the substitution patterns. Hence, advanced analytical methods (e.g. NMR) should be considered for future research to further unravel specific types of PAH constituents present in fume condensate extracts, since this could explain the differences in their developmental toxicity as compared to that of other PAH-containing petroleum substances.

Published studies have noted the importance of the (in)activation of nuclear receptors, including the AhR, in mediating the developmental toxicity of PAH-containing substances (Billiard et al., 2006; Goodale et al., 2013; Puga et al., 2005). The AhR CALUX assay was included in the present study as part of the battery of in vitro assays for screening the developmental toxicity potency and mode-of-action of PAH-containing petroleum substances. However, it should be kept in mind that having tested positive in the AhR CALUX assay does not directly imply that the substance tested would induce developmental toxicity in vitro or in vivo. Basically, the AhR CALUX assay is merely a screening tool to detect activation of the AhR, among other nuclear receptors (e.g. retinoic acid receptor, estrogen receptor alpha), which may play a role in the observed developmental toxicity by some PAH-containing petroleum substances (Kamelia et al., 2019b). Hence, results obtained in the AhR CALUX assay do not necessarily reflect what happens in vivo since physiological feedback mechanisms and metabolism are lacking in these in vitro systems. In the present study, DMSO-extracts of fume condensate of bitumen and oxidized asphalt induced only a transient (not persistent) AhR activity, as quantified in the AhR CALUX assay. The fume condensate extracts tested not only contain 2-ring PAHs, which are unable to bind and activate the AhR, but also a group of 3- to 5-ring PAHs of which several are known ligands of the AhR (Machala et al., 2001; Pieterse et al., 2013; Vondráček et al., 2017). Thus, it was not surprising to see concentration-dependent AhR-mediated activities induced by samples #016-BFC and the #017-OAFC in the AhR CALUX assay. Two exposure time periods, 6 and 24 h, are applied in the current AhR CALUX assay to investigate whether any observed AhR induction is transient or persistent (sustained). The results from the 6 h of exposure showed clear AhR induction by both fume condensate extracts, but this effect was significantly reduced following 24 h of exposure, implying a transient or short-lived AhR activation. It is known that AhR plays an important role in regulating a protective adaptive response to xenobiotics, in addition to its involvement in maintaining cellular functions (Hankinson, 1995; Larigot et al., 2018; Puga et al., 2002). Transient AhR activation by (endogenous) ligands is important in the maintenance of cell homeostasis (Bock, 2013; Mitchell et al., 2006). In contrast, persistent (or sustained) AhR induction may culminate in adverse toxic outcomes (e.g. developmental toxicity), such as those observed following TCDD exposure (Mitchell et al., 2006; Mitchell and Elferink, 2009). Altogether, this absence of sustained AhR induction by the fume condensate extracts of bitumen and oxidized asphalt tested compliments the current finding that both test materials tested negative for in vitro and in vivo developmental toxicity.

Fume condensates of bitumen and oxidized asphalt, like most petroleum UVCB substances, are highly lipophilic and it is impossible to directly dose them into the vast majority of in vitro test systems, as their bioavailability would be very limited or absent. To overcome this challenge, the DMSO-extraction procedure (Roy et al., 1988; McKee et al., 2013) was applied to selectively extract most of the biologically active compounds (i.e. 3–7 ring PACs / PAHs) present in the petroleum substances (Carrillo et al., 2019; Natusch and Tomkins, 1978) that are relevant for evaluating the developmental toxicity potency of these substances in vitro (Kamelia et al., 2017, 2019a, 2019b) and ensure that the test substances are formulated in such a way that they are compatible with the aqueous surroundings of the in vitro assays applied. In other words, the use of these DMSO-extracts for in vitro dosing of PAH-containing petroleum substances improves the biological interaction of potentially toxic constituents (i.e. potential developmental toxicants) with aqueous medium in in vitro test systems. A recent study showed that the chemical profiles, in particular PAH/PAC profiles, of these highly complex substances remain consistent following DMSO-extraction (Luo et al., 2020). Also, this approach has been widely used and was extensively validated for in vitro carcinogenicity and mutagenicity testing of petroleum UVCB substances (Concawe, 1994; Blackburn et al., 1984, 1986). In addition to 3- to 7-ring PAHs, DMSO also extracts other polar constituents present in petroleum substances but it is known that these groups of hydrocarbon constituents do not induce any reprotoxic or developmental effects (Feuston and Mackerer, 1996a, 1996b; Tsitou et al., 2015). Altogether, the current results confirm the suitability of applying DMSO-extracts of petroleum UVCB substances for hazard assessment as they adequately capture the expected absence (present study) or presence (Kamelia et al., 2019a) of developmental toxicity of these substances, in line with their potencies observed in vivo.

5. Conclusions

In summary, the present study extends the usefulness of a battery of in vitro alternative assays to assess the developmental toxicity of PAH-containing petroleum substances. The DMSO-extracts of fume condensates of bitumen and oxidized asphalt showed a concentration-dependent AhR induction following 6-h of exposure, but this activity was substantially reduced after 24-h, indicating a transient AhR activation. The results obtained in the mEST and ZET show that the fume condensate extracts of bitumen and oxidized asphalt do not induce any in vitro developmental toxicity, which is in line with the results observed in the in vivo prenatal developmental toxicity studies performed with the same materials (Boogaard et al., 2021a, 2021b). Complex materials like petroleum substances may possess multiple modes-of-action in causing their adverse effects, including developmental toxicity, due to diverse and variable groups of constituents present in these substances. The ability of ZET and mEST to capture these underlying mechanisms is reflected by a good correlation (R^2: 0.91-ZET and R^2: 0.97-mEST) when comparing the obtained in vitro developmental toxicity potency of PAH-containing petroleum substances extracts with their potencies observed in vivo (Kamelia et al., 2017, 2019a). Furthermore, our findings (Kamelia et al., 2017, 2019a) also indicate that the ZET does not outperform the mEST as a stand-alone assay for assessing the developmental toxicity of PAH-containing petroleum substances extracts, but the ZET is a useful addition to the battery of in vitro tests able to predict the in vivo developmental toxicity of these substances. Finally, more fume condensates, ideally from different product categories than those included in the present study, like HFO fume condensates, should be tested in the current assay battery to broaden the applicability domain of the proposed testing strategy to further support regulatory hazard

assessment for the developmental toxicity endpoint of these highly complex substances.

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Declaration of Competing Interest

LK and PJB are employed by Shell International Bv, a member company of Concawe, and PJB is the chairman of the health management group of Concawe. Both authors are totally free (by contract) to freely design and conduct research and express their own scientific opinion without any obligation towards either Shell or Concawe. The current findings are not intended to constitute any product endorsement.

The other author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2021.105195.

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