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Developmental toxicity, behavioral alterations, and transcriptional responses to selected methylated and oxygenated PACs in zebrafish embryos (Danio rerio)

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Research Article

Keywords: Polycyclic aromatic compounds (PACs), zebrafish embryos, developmental toxicity, teratogenicity, larval behavior, aryl hydrocarbon receptor pathway

Posted Date: November 26th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11701771/v1

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Additional Declarations: No competing interests reported.

Abstract

Background: Substituted polycyclic aromatic compounds (PACs) are frequently detected in the environment but remain insufficiently characterized in environmental risk assessment. This study investigated the developmental toxicity and transcriptional responses of five selected PACs—7,12-dimethylbenz[a]anthracene (DMBaA), 7-methylbenz[a]anthracene (MBaA), 7-methylbenzo[a]pyrene (MBaP), 9-methylacridine (MA), and 9(10H)-acridone (ACR)—using zebrafish embryos. Embryotoxicity was assessed using the Fish Embryo Toxicity (FET) test, complemented by targeted analyses of developmental abnormalities and larval behavior, time-resolved gene expression analysis using qPCR, and chemical characterization of selected exposure solutions by GC-MS.

Results: MA and DMBaA induced acute embryotoxicity, with nominal-concentration-based LC50 values of 2.34 and 9.03 µmol/L at 96 hpf, respectively. DMBaA caused developmental abnormalities, including reduced tail length, lordosis, and pericardial edema, whereas MA reduced larval locomotor activity, with effects differing among light phases. No acute embryotoxicity was observed for MBaA, MBaP, or ACR under the investigated exposure conditions. All five PACs significantly induced cyp1a expression at one or more developmental stages, with differences in the magnitude and timing of AhR-associated transcriptional responses. Measured aqueous concentrations generally declined during exposure, while ACR concentrations remained consistently low relative to nominal concentrations.

Conclusions: The investigated substituted PACs induced distinct developmental, behavioral, and transcriptional responses in zebrafish embryos. However, differences between nominal and measured aqueous concentrations and the absence of internal exposure measurements limit conclusions regarding relative toxic potency. These findings highlight the toxicological relevance of substituted PACs and support their consideration in environmental hazard characterization and risk assessment.

Highlights

• Structural modifications alter the toxicity of polycyclic aromatic compounds.

• DMBaA caused shorter tail length and induced strong AhR-pathway activation.

• MA caused embryotoxicity and reduced larval locomotor activity.

• Measured PAC concentrations differed substantially from nominal concentrations.

• Substituted PACs exhibited distinct developmental and molecular responses.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed environmental contaminants originating from both natural and anthropogenic sources. They are commonly found in air, sediments, soil, surface water, groundwater, and biota [1, 2]. Due to their mutagenic, carcinogenic, teratogenic, and developmental toxic effects, several PAHs are included among the priority pollutants for environmental monitoring and risk assessment. During embryonic development, exposure to PAHs can induce a wide range of adverse effects, some of which are associated with the activation of the aryl hydrocarbon receptor (AhR) pathway and the subsequent induction of cytochrome P450-dependent monooxygenases (CYPs) [3–5]. Developmental exposure to PAHs has been associated with cardiovascular abnormalities, edema formation, skeletal deformities, impaired eye development, altered behavior, and reduced survival in zebrafish and other fish species [3, 4, 6–11].

Despite the broad toxic relevance of PAHs, environmental risk assessment and monitoring frameworks primarily focus on the analysis of the 16 EPA priority PAHs. However, contaminated sites often contain complex mixtures of PAH and other polycyclic aromatic compounds (PACs), including alkylated PAHs, oxygenated PAHs and heterocyclic PACs containing nitrogen, sulfur, or oxygen [13–15]. Among these compounds, alkylated PAHs and heterocyclic analogs are frequently detected in the environment [16, 17]. Previous studies have demonstrated that some substituted PACs exhibit substantial biological activity and toxicity, in some cases comparable to or exceeding that of their parent compounds [18–22]. Therefore, risk assessment based solely on the 16 priority PAHs may not adequately represent the broader toxicological relevance of PAC contamination in environmental samples [23]. Together with recent studies on PAH mixture toxicology using zebrafish [24], these findings emphasize a need to investigate the toxic potential of environmentally relevant substituted PACs.

The zebrafish (*Danio rerio*) embryo has become an established vertebrate model for studying developmental toxicity and is widely used in environmental toxicology because of its rapid embryonic development, optical transparency, and sensitivity to chemically induced developmental disturbances [25]. The standardized OECD Fish Embryo Toxicity (FET) test provides a robust platform for assessing developmental toxicity [26, 27], while the zebrafish model also enables the investigation of additional endpoints, including larval behavior and molecular responses [28, 29]. Despite these advantages, information on the developmental toxicity and associated biological responses of environmentally relevant substituted PACs remains limited, particularly in studies that include analytical verification of exposure concentrations.

In previous studies, we demonstrated the occurrence of substituted PACs in contaminated environmental samples and identified toxicological effects of several methylated and oxygenated PACs using *in vitro* bioreporter assays [30, 18, 13]. However, information on their toxicity in vertebrate *in vivo* models remains limited. Based on these previous findings, five environmentally relevant substituted PACs, including three methylated PAHs and two nitrogen-containing heterocyclic PACs, with different structural characteristics and physicochemical properties were selected to extend their toxicological characterization from previous in vitro studies and to further investigate their toxic potential using zebrafish as a vertebrate developmental model. Developmental toxicity was assessed for all five

compounds using the zebrafish embryo model. Additional analyses included assessment of developmental and behavioral alterations for selected compounds and time-resolved transcriptional analysis. In addition, aqueous exposure concentrations were analytically characterized for selected exposure conditions and time points.

2. Material & Methods

2.1 Chemicals

Toluene (≥ 99.7%), n-hexane (≥ 98%), and anhydrous sodium sulfate (99%) were purchased from VWR (Stockholm, Sweden). 9-methylacridine (99%, MA, $C_{14}H_{11}N$, CAS 611-64-3) was purchased from Chiron AS (Trondheim, Norway). 9(10H)-acridone (99%, ACR, $C_{13}H_9NO$, CAS 578-95-0), 7-methylbenz[a]anthracene (N/A, MBaA, $C_{19}H_{14}$, CAS 2541-69-7), 7,12-dimethylbenz[a]anthracene (99.9%, DMBaA, $C_{20}H_{16}$, CAS 57-97-6), 7-methylbenzo[a]pyrene (98%, MBaP, $C_{21}H_{14}$, CAS 63041-77-0), and dimethyl sulfoxide (99.9%, DMSO) were purchased from Sigma-Aldrich (Stockholm, Sweden). Molecular weight, log Kow, and aqueous solubility were obtained from CompTox Chemicals Dashboard (v2.8.0) (Table 1). The reported aqueous solubility values refer to water without the addition of a carrier solvent and, thus, do not directly represent the experimental exposure conditions, which contained 0.4% DMSO.

Table 1
Molecular weight, log KOW, and water solubility of the investigated PACs obtained from the CompTox Chemicals Dashboard (v2.8.0). Aqueous solubility values refer to water without the addition of a carrier solvent. Values represent experimental or predicted averages as indicated.

Compound	MW (g/mol)	Log K_{OW}	Aqueous sol. (µmol/L)
DMBaA (7,12-dimethylbenz[a]anthracene)	256	5.80[a]	0.163[a]
MBaA (7-methylbenz[a]anthracene)	242	6.31[a]	0.045[a]
ACR (9(10H)-acridone)	195	2.96[b]	41.5[b]
MA (9-methylacridine)	193	3.63[b]	3180[b]
MBaP (7-methylbenzo[a]pyrene)	266	6.22[b]	0.022[b]

[a] Experimental average; [b] predicted average

Stock solutions of DMBaA, MBaA, MA, ACR, and MBaP were prepared in DMSO with a concentration of 2.5 mmol/L, each. Reconstituted water was prepared according to ISO 7346-2 (294 mg/L $CaCl_2$•2 H_2O, 123.3 mg/L $MgSO_4$•7 H_2O, 63 mg/L $NaHCO_3$, 5.5 mg/L KCl in MilliQ water (Millipore®)). Glass beakers used for preparation of exposure solutions were pre-incubated with the respective exposure solutions for 24 hours. For quantification of the PACs in exposure solutions, internal standards (IS) of 9-

methylanthracene-d12, and acridine-d9 purchased from Chiron AS, and a recovery standard (RS) solution of perylene-d12 purchased from Sigma-Aldrich were used.

2.2 Fish maintenance

Adult zebrafish (*Danio rerio*, AB strain; ZFIN ID: ZDB-GENO-960809-7) were obtained from Karolinska Institute (Stockholm, Sweden) and maintained under a 14:10 h light:dark cycle. Standard husbandry conditions were applied with minor modifications. Fish were kept at a water temperature of 26 ± 1°C, pH of 7.2 ± 0.2, and a water hardness of 3–6 °dH. The recirculating system provided a daily water exchange rate of approx. 20%. Conductivity, pH, and temperature were automatically controlled and regulated by a ProfiLux 3.1 controller (GHL Advanced Technology, Kaiserslautern, Germany). Carbonate hardness, nitrates, nitrites (EasyTest strips, JBL, Neuhofen, Germany) and ammonia (Ammonia Test Kit, Tetra GmbH, Melle, Germany) contents were monitored once a week.

Fish were fed twice daily with flakes (TetraRubin®, Tetra) or freshly hatched Artemia (Ocean Nutrition®, Essen, Belgium). The evening before spawning, male and female fish (ratio 3:2, 5 fish total) were placed in spawning tanks (Tecniplast) with a plastic grid. Within 60 minutes after spawning, eggs were collected and rinsed with aerated ISO water. The zebrafish facilities at Örebro University (#5.2.18–20002/2022) hold permission to use (#5.12.18–20142/2022) and breed (#5.2.18–20144/2022) laboratory animals. These permits are affiliated with the Swedish Board of Agriculture, Jönköping, Sweden. Furthermore, the facilities hold ethical permission (#13595 – 2023) affiliated with the same board in Linköping, Sweden.

2.3 Fish Embryo Toxicity (FET) test

Acute and sub-lethal effects of the selected PACs were assessed in accordance with OECD Technical Guideline No. 236 [27], with minor modifications. Fertilized fish eggs at latest in the 64-cell stage without visible deficiencies were selected using a stereomicroscope. Chemicals were tested at five different nominal concentrations in 4–6 independent replicates with 20 embryos per concentration and experiment. The highest exposure concentration was 10 µmol/L for four compounds and 4 µmol/L for one compound (Table 2). Concentration ranges were selected based on previous *in vitro* studies and preliminary range-finding experiments to cover a possible range from low to high toxicity while remaining technically achievable and considering compound solubility [18, 31]. Due to limited aqueous solubility of the investigated PACs, stock solutions were prepared in DMSO, and final exposure solutions contained 0.4% (v/v) DMSO. The same solvent concentration was maintained in all treatments and solvent controls to ensure consistent exposure conditions. Controls included 4 mg/L 3,4-dichloroaniline (positive control), ISO water (negative control), and 0.4% DMSO (solvent control).

Selected eggs were transferred individually into wells of 96-well plates containing 250 µl freshly prepared test solution. Plates were sealed with a gas-permeable and self-adhesive film (VWR International, Sweden) and incubated at 26 (± 1) °C under a 14:10 h light:dark cycle for 96 h. A semi-static exposure design was applied, with 50% of the test solution renewed daily using freshly prepared exposure solution. This approach replenished the exposure medium while minimizing disturbance of the developing embryos, consistent with previous zebrafish embryo studies [32]. Embryo development was

assessed every 24 h using an inverted microscope (Olympus CKX41, Olympus AG, Germany). An experiment was considered valid if mortality in the negative and solvent control was less than 10%, and mortality of the positive control exceeded 30%.

Table 2
Nominal exposure concentrations (C1-C5) of the PACs tested in the FET.

Compound	C1 [µmol/L]	C2 [µmol/L]	C3 [µmol/L]	C4 [µmol/L]	C5 [µmol/L]
DMBaA (7,12-dimethylbenz[a]anthracene)	10	5	2.5	1.25	0.625
MBaA (7-methylbenz[a]anthracene)	10	5	2.5	1.25	0.625
MA (9-methylacridine)	4	2	1.0	0.5	0.25
ACR (9(10H)-acridone)	10	5	2.5	1.25	0.625
MBaP (7-methylbenzo[a]pyrene)	10	5	2.5	1.25	0.625

2.4 Tail length test

During the FET test, exposure to DMBaA induced a visually evident reduction in tail length and tail deformation, whereas this phenotype was not observed for the other investigated PACs. Hence, a targeted follow-up experiment was conducted to quantify effects on tail length. For this purpose, 10 embryos per concentration, not older than 64-cell stage, were exposed under semi-static conditions in a 24-well plate (1 embryo/well) containing 2 ml of the respective DMBaA solution. A 1:2 dilution series of nominal DMBaA concentrations (2.5, 1.25, 0.63, 0.32 and 0.16 µmol/L) was prepared in artificial water before exposure. DMSO concentration was adjusted to 0.4% (v/v) in all exposure solutions. Negative, solvent (0.4% (v/v) DMSO) and positive (DCA) control were prepared as described before.

The experiments were performed in three independent replicates. Alterations of the tail were investigated after 96 hours of exposure using an inverted microscope (Motic AE2000). Tail length was measured from the first anterior somite to the posterior tip of the tail along the spinal column using the Fiji/ImageJ [33]. Prior to imaging, larvae were anesthetized with a saturated benzocaine solution (≥ 1,4 g/L) (Alfa Aeasar®). Dead and coagulated embryos were excluded from the measurement; therefore, the total number of embryos per test condition ranged between 26–30.

2.5 Behavioral analysis

During the FET test, visually apparent alterations in larval activity were observed for MA, whereas comparable behavioral alterations were not observed for the other investigated PACs. Therefore, MA was selected for a targeted behavioral assessment. Based on the FET results, three nominal MA concentrations (1.0, 0.5, and 0.25 µmol/L) associated with no or only minor teratogenic effects were selected for the behavioral experiment. After 96h of exposure, larval locomotor activity was recorded using the DanioVision® automated tracking system (Noldus, The Netherlands) [29].

Behavioral analyses were performed in four independent experimental runs, each comprising 24 larvae per treatment group (negative control, solvent control, and the three MA concentrations). Larvae that died during exposure or for which locomotor activity could not be reliably quantified due to technical tracking errors were excluded from the statistical analyses. For all behavioral experiments, videos were recorded and analyzed using EthoVision XT 17 software (Noldus, The Netherlands). The experiments were carried out at 26°C. The Larval Photomotor Response (LPMR) test was conducted in the early afternoon, as this corresponds to the most stable activity period for larvae at 96 hour-post-fertilization (hpf) [34]. Larval movement was recorded for 15 minutes, consisting of 5 minutes of light (LON1), 5 minutes of darkness (LOFF), and 5 minutes of light (LON2), after a 5-minute acclimatization period in full light conditions.

2.6 qPCR analysis

Based on previous observations of distinct time-dependent transcriptional responses of AhR pathway-related genes following exposure to receptor agonists, the gene expression experiment was designed to characterize temporal rather than concentration-dependent transcriptional responses [35]. Therefore, all five PACs were tested at a common nominal concentration of 1.4 µmol/L, selected based on the results from the FET, and transcriptional responses were assessed at 24, 48, 72, and 96 hpf.

To provide sufficient numbers of embryos for subsequent RNA extraction and gene expression analysis, sets of 30 fertilized eggs at 0–2 hpf were transferred into glass beakers containing 25 mL of ISO water (negative control), 0.4% DMSO (solvent control), or the respective PAC exposure solutions. Each day 20 ml of the different test solutions were renewed. Glass beakers were covered with Parafilm® (Parafilm, Menasha, WI, USA) to prevent evaporation, and incubated at 26 ± 1°C.

For the qPCR analyses, zebrafish embryos were sampled at 24, 48, 72, and 96 hpf. At each sampling timepoint, embryos were euthanized in a saturated ethyl 4-aminobenzoate solution, rinsed in Phosphate Buffer Saline (PBS), and stored in RNAlater® solution (Ambion™) at -20°C. Three independent replicates per treatment and sampling timepoint were collected.

Embryos were homogenized in lysis buffer (Macherey-Nagel NucleoSpin® RNA extraction kit) using a cordless mixer motor and pellet pestles (VWR, Germany). RNA extraction was performed according to the guidelines of the kit. cDNA was synthesized from 1.6 µg of RNA following M-MuLV Reverse Transcriptase (Invitrogen®) associated protocol. All cDNA samples were diluted 10 times and kept at -20°C.

Primers used in qPCR were designed using the online free software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) or taken from a previous study (Appendix A) [36]. Additional genes were selected based on the developmental abnormalities observed during the FET experiments. Specificity, efficiency, and linearity ranges were established for all primer pairs using DNA electrophoresis, melting curve, and standard curve analyses (Appendix B). Each qPCR

reaction was run in technical duplicates and contained 2X Power SyBR green (Applied Biosystems™), 200 nM of each primer, and 2 µL of cDNA dilution topped up to 15 µL with MilliQ water.

2.7 Chemical analysis of exposure solutions

Sample collection for chemical analysis: During the FET tests, 50% of the exposure solution in each well was renewed daily. For chemical analysis, the removed exposure medium from wells belonging to the same treatment was pooled to obtain a total volume of 4 mL. Samples were collected from the two highest nominal exposure concentrations of each test substance (C1 and C2) at selected time points: t_0 (freshly prepared exposure solutions), t_{24} (exposure solution after 24 h of exposure), and t_{96} (exposure solution collected at the end of the 96-h exposure period). Corresponding negative controls were collected at the same time points. Subsequently, 2 mL n-hexane was added to each water sample as a keeper before storage at -20°C to avoid losses of the compounds until extraction.

Extraction

Test compounds were extracted from the exposure solutions by three consecutive liquid-liquid extractions with n-hexane (2 mL) for 5 min each. Prior to the first extraction, IS (1000 ng) was added to all samples. In addition, a procedure blank consisting of 6 ml of n-hexane was included. Anhydrous sodium sulfate was added to the combined n-hexane extracts to remove water residues, and the n-hexane extracts were then transferred to new amber glass vials. Finally, the extracts were evaporated under a gentle stream of nitrogen gas, solvent exchanged to 0.5 mL toluene and a RS (1000 ng) was added to all vials. Vials were stored at -20°C until GC-MS analysis.

GC-MS analysis

PAC concentrations in the exposure medium were determined using an Agilent 7890A gas chromatograph coupled to a 5975C low-resolution mass spectrometer (GC-LRMS). Separation was achieved on an Agilent Select PAH capillary column (30 m × 0.25 mm, 0.15 µm film thickness). Extracts (1 µL) were injected in splitless mode, and the MS was operated in electron ionization (EI) mode at 70 eV using selected ion monitoring (SIM) mode. The initial oven temperature was 90°C (1 min), 8°C/min to 300°C (3 min), and then 30°C/min to 325°C where it was held for 2 min. Quantification of PAC was performed by use of isotope dilution with a quantification mixture including the five PACs along with IS and RS. Recoveries ranged from 62 to 102% for 9-methylanthracene-d_{12} and 63 to 102% for acridine-d_9.

2.8 Statistical Analyses

FET test. Graphs and statistical analyses were designed and performed using GraphPad Prism® 10. Concentration–response data from the FET test were analyzed by nonlinear regression using a log(concentration) vs. response variable-slope model, with the bottom and top of the curve constrained to 0 and 100%, respectively. LC50 values were calculated according to:

$$Y = \frac{100}{1 + 10^{(logLC50 - X) \times\ HillSlope}}$$

where *X* represents the logarithm of the nominal exposure concentration and HillSlope describes the steepness of the concentration-response curve.

Tail length

Tail length data were analyzed in R (version 4.6.1, R Foundation for Statistical Computing, Vienna, Austria) within the RStudio environment (Posit Software, Boston, MA, USA) using the lme4 package. A linear mixed-effects model was fitted with treatment as a fixed effect and experiment as a random effect to account for variation among the three independent biological replicates. Overall treatment effects were assessed using a Type III analysis of variance with Satterthwaite's approximation for degrees of freedom (lmerTest package). Pairwise comparisons between each treatment and the solvent control were performed using Dunnett-adjusted post hoc tests based on estimated marginal means (emmeans package). Model assumptions were evaluated by visual inspection of residual and normal quantile–quantile plots.

Behavior analyses

Statistical analyses were performed using R within the RStudio environment. Locomotor activity (distance moved) was natural log-transformed using ln(distance + 1) prior to analysis to improve compliance with the assumptions of linear mixed-effects modeling, as verified by graphical assessment of model diagnostics. The effects of treatment and light phase (LON1, LOFF, and LON2) on locomotor activity were analyzed using a linear mixed-effects model implemented in the lmerTest package. The model included treatment, light phase, and their interaction as fixed effects, while experimental run (four independent experiments) was included as a fixed blocking factor to account for potential variation among experiments. Because locomotor activity was measured repeatedly in the same embryos across the three consecutive light phases, embryo identity was included as a random intercept to account for the non-independence of repeated measurements. The significance of fixed effects was assessed using Type III analysis of variance with Satterthwaite's approximation for denominator degrees of freedom. When significant treatment effects or treatment × light interactions were detected, estimated marginal means were calculated using the emmeans package. Pairwise comparisons between each treatment and the solvent control were performed separately for each light phase using Dunnett-adjusted multiple comparisons, while differences among light phases within each treatment were evaluated using Tukey-adjusted pairwise comparisons. Statistical significance was accepted at $p < 0.05$.

qPCR data analysis. Statistical analyses were performed on qPCR data from three independent experiments using R (v4.5.0). Results were normalized using the geometric mean of the expression of *β-actin* and *rpl13a* as they showed the highest stability among four investigated reference genes. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [38]. Quality control assessment of reference gene stability identified systematic instability in biological replicate 2 at 48 hpf across most treatments

(reference Ct differences > 5 between exposed and matched control samples); these samples together with one further sample (MA 24hpf) exceeding the same threshold, were excluded from analysis. Statistical differences in gene expression between treatments and controls were assessed using paired t-tests, with p-values corrected for multiple comparisons within each gene using the Benjamini-Hochberg false discovery rate. A two-way ANOVA (chemical x Time) on log2 fold change values, with Benjamini-Hochberg correction across genes, was used to assess whether chemicals differed in their temporal expression profiles. For both tests, a p-value < 0.05 was considered statistically significant.

3. Results

3.1 Chemical analysis

Measured concentrations in the exposure solutions differed from the corresponding nominal concentrations to varying extents and generally decreased over time, despite daily renewal of 50% of the exposure medium (Table 3). C1 and C2 represent the two highest nominal exposure concentrations tested for each compound and do not represent replicate measurements.

For DMBaA, the measured concentrations at t_0 were 13 and 6 µmol/L for C1 and C2, respectively, compared with nominal concentrations of 10 and 5 µmol/L. Measured concentrations subsequently decreased markedly after exposure. For MBaA, measured concentrations at t_0 were close to the nominal concentrations, with 11 and 4.4 µmol/L measured for C1 and C2, respectively, compared with nominal concentrations of 10 and 5 µmol/L, followed by decreasing concentrations over time. Slightly lower measured concentrations were observed for MA already at t_0 (3.3 and 1.7 µmol/L in C1 and C2, respectively, compared with 4 and 2 µmol/L), followed by a gradual decrease over time.

In contrast, ACR showed substantially lower measured concentrations than the corresponding nominal concentrations. Across all time points, measured ACR concentrations ranged from 0.31 to 0.54 µmol/L, compared with nominal concentrations of 10 and 5.0 µmol/L for C1 and C2, respectively, and no clear temporal trend was observed. For MBaP, measured concentrations at t_0 were below nominal concentrations, with 7.3 compared to 10 µmol/L for C1 and 3.6 compared to 5 µmol/L in C2, followed by decreasing concentrations over time. Overall, all compounds except ACR showed a clear time-dependent decline in measured concentrations, whereas ACR consistently remained at low concentrations and exhibited no temporal trend.

Table 3
Measured concentrations of the tested PACs in the exposure medium at different time points during the FET assay. C1 and C2 represent the highest and second-highest nominal exposure concentrations tested for each compound, respectively. Concentrations are expressed in µmol/L.

PAC	t_0	t_{24}	t_{96}	t_0	t_{24}	t_{96}
	C1 (nominal 10 µmol/L)			**C2 (nominal 5 µmol/L)**		
DMBaA	13	3.7	2.4	6	0.58	0.92
MBaA	11	6.8	4.9	4.4	2.7	2.1
ACR	0.38	0.51	0.38	0.46	0.54	0.31
MBaP	7.3	4.8	3.6	3.6	2.8	1.7
	C1 (nominal 4 µmol/L)			C2 (nominal 2 µmol/L)		
MA	3.3	2.8	2.2	1.7	1.4	1.2

3.2 Embryotoxic and Teratogenic Effects

Among the five tested PACs, MA and DMBaA induced acute embryotoxicity and specific teratogenic effects on zebrafish embryos under the applied exposure conditions. For MA, acute toxicity was already observed at 24 hpf and remained relatively stable throughout the exposure time up to 96 hpf. In contrast, for DMBaA toxicity increased with exposure time with nominal-concentration-based LC_{50} values decreasing from 26.5 µmol/L at 24 hpf to 21.8 µmol/L at 48 hpf, and 15.4 µmol/L at 72 hpf. At 96 hpf, the nominal-concentration-based LC_{50}-values of MA and DMBaA were 2.34 µmol/L and 9.03 µmol/L, respectively (Fig. 1). No acute embryotoxicity was observed for ACR, MBaA, or MBaP within the tested nominal concentration ranges under the applied exposure conditions.

3.3 Developmental abnormalities

DMBaA caused different sub-lethal malformations, including lordosis, shortened tail, deformation of the tail tip, and pericardial edema (Fig. 2). For a few embryos, abnormal heartbeat and blood circulation were also observed visually. Based on the visually evident effects on tail development, tail length was subsequently quantified following exposure to DMBaA.

A statistically significant reduction in tail length was measured for the exposure of zebrafish embryos to 1.25 µmol/L (2566 µm, n = 30, *padj* = 0.0079) and 2.5 µmol/L DMBaA (2413 µm, n = 30, *padj* < 0.0001) when compared with the solvent control (2654 µm, n = 27). At 0.625 µmol/L DMBaA the mean tail length was 2635 µm (n = 30) but did not differ significantly from the solvent control (*padj* = 0.938). Likewise, no significant differences were detected at 0.312 µmol/L (2618 µm, n = 28, *padj* = 0.608) or 0.156 µmol/L DMBaA (2639 µm, n = 29, *padj* = 0.987). The negative control (2698 µm, n = 26) did not differ significantly from the solvent control (*padj* = 0.33; Fig. 3).

3.4 Behavioral Analysis

Locomotor activity in the larval photo-motor response (LPMR) assay was analyzed using a linear mixed-effects model with treatment, light phase (LON1, LOFF, and LON2), and their interaction as fixed effects, experimental run as a fixed blocking factor, and embryo identity as a random effect. Distance moved was ln-transformed prior to analysis to improve compliance with model assumptions. The analysis revealed significant effects of treatment ($F_{4,428.7} = 7.68$, $p < 0.001$), light phase ($F_{2,852.0} = 384.70$, $p < 0.001$), and the treatment × light phase interaction ($F_{8,851.9} = 7.37$, $p < 0.001$), whereas no significant effect of experimental run was detected ($F_{3,428.8} = 2.01$, $p = 0.113$). The significant treatment × light phase interaction indicates that the effect of MA exposure on locomotor activity differed among the three light phases.

No significant differences in locomotor activity were observed between the negative control (NC) and the solvent control (SC) during any of the three light phases (Dunnett-adjusted $p > 0.05$), demonstrating that the solvent did not affect larval behavior. All treatment groups exhibited the characteristic visual motor response, with significantly increased locomotor activity during the dark phase (LOFF) compared with both light phases (LON1 and LON2) (Tukey-adjusted $p < 0.0001$ for all treatments), whereas no significant differences were detected between the two light phases within any treatment group (Fig. 4). Thus, the characteristic increase in locomotor activity following the light-to-dark transition was retained across all treatment groups, although the magnitude of locomotor activity differed among treatments.

Dunnett-adjusted post hoc comparisons using the solvent control as the reference demonstrated phase-specific effects of MA exposure on locomotor activity (Fig. 4). During the first light phase (LON1), all three MA concentrations exhibited significantly lower locomotor activity than the SC (0.25 µmol/L, *padj* < 0.0001; 0.5 µmol/L, *padj* = 0.0018; 1.0 µmol/L, *padj* = 0.0363). During the dark phase (LOFF), significant reductions were observed at 0.25 µmol/L (*padj* = 0.0157) and 1.0 µmol/L (*padj* = 0.0003), whereas the 0.5 µmol/L treatment did not differ significantly from the SC (*padj* = 0.765). During the second light phase (LON2), locomotor activity was significantly reduced at all three MA concentrations compared with the SC (0.25 µmol/L, *padj* < 0.0001; 0.5 µmol/L, *padj* = 0.0004; 1.0 µmol/L, *padj* = 0.0036).

3.5 Gene Expression Analysis

Exposure to the investigated PACs resulted in time-dependent changes in the expressions of genes associated with AhR pathway (Fig. 5). In contrast, no significant changes in the expression of *wnt5b, otx2, mitfa*, *spt6, dnmt1, dnmt3ba* and *tp53* were detected under the investigated exposure conditions (Appendices C and D).

All tested PACs significantly induced *cyp1a* expression, although the temporal patterns and magnitude of the responses differed among compounds (Fig. 5a, b). DMBaA significantly increased *cyp1a* expression at all developmental stages investigated (Fig. 5b). Exposure to MBaA and MBaP also significantly increased *cyp1a* expression across the investigated developmental stages, while MBaA showed

significant induction at the investigated time points except 48 hpf, for which only two replicates were available (Fig. 5a). In contrast, effects of ACR and MA were restricted to later developmental stages, with significant *cyp1a* induction after ACR exposure at 72 and 96 hpf, and exposure to MA at 96 hpf only (Fig. 5b).

Significant changes were also observed for other AhR-associated genes. Expression of the AhR repressor (*ahrrb*) was significantly upregulated after DMBaA and MBaP exposure at 72 hpf (Fig. 5b). Expression of *cyp1b1* was significantly induced after exposure to DMBaA at 24, 48, and 72 hpf and MBaP at 24 and 72 hpf (Fig. 5b).

4. Discussion

4.1 Chemical analyses

In the present study, the exposure concentrations of the investigated PACs were analytically verified at selected timepoints during the exposure period for the two highest concentrations used in the FET test. These measurements showed that the aqueous concentrations of DMBaA, MBaA, MBaP, and MA generally declined over time despite daily renewal of 50% of the exposure medium, whereas ACR remained consistently low relative to its nominal concentrations. Similar losses of hydrophobic PACs have previously been reported in FET and *in vitro* assays and may result from several processes, including sorption to experimental material, limited aqueous solubility, and potential degradation or transformation processes [13, 28, 29]. In addition, for DMBaA, MBaA and MBaP the highest exposure concentrations also exceeded their reported aqueous solubilities. However, these solubility values refer to water without a carrier solvent and, thus, do not directly represent the present exposure conditions containing 0.4% DMSO. Low concentrations of DMSO have been shown to increase the aqueous solubility of hydrophobic PAHs, with the magnitude of the cosolvent effect increasing with compound hydrophobicity [39]. Nevertheless, the extent to which 0.4% DMSO increased the solubility of the specific substituted PACs investigated here is unknown, and heterogeneous exposure systems, including metastable dispersions or colloidal material, cannot be excluded at the highest nominal concentrations [40].

Hydrophobic PACs are known to exhibit strong affinity for experimental surfaces, including plastic well plates, and thereby reduce their concentrations in the aqueous phase. Additionally, partitioning to embryos and chorion structures may also contribute to the removal from the exposure medium [9, 29]. In early life stages of fish, uptake and accumulation of PACs can be pronounced due to high lipid content and limited metabolic capacity, although internal accumulation is strongly dependent on molecular size, planarity, and hydrophobicity [41]. In the present study, the biological responses observed for several of the investigated PACs are consistent with exposure of the embryos to the compounds. However, as internal concentrations were not measured, the extent of uptake remains unknown, and the absence of biological effects cannot be distinguished from insufficient internal exposure. Likewise, the relative

contributions of sorption, partitioning into embryos or chorion structures, transformation, and other loss processes to the decline in aqueous concentrations cannot be determined.

Among the compounds investigated in the present study, DMBaA showed a pronounced decline in measured aqueous concentrations. Relative to the initial measured concentrations, C1 decreased by approximately 72% at 24 h and 82% at 96 h, whereas C2 decreased by approximately 90% at 24 h and 85% at 96 h. MBaA and MBaP also showed substantial decreases, with concentrations at 96 h approximately 50% lower than their respective initial measured concentrations for both C1 and C2. These compounds have relatively high hydrophobicity (log K_{OW} 5.80–6.31), consistent with a high potential for partitioning from the aqueous phase, although the present study does not allow the processes responsible for the observed losses to be quantified.

MA showed a less pronounced decline in measured aqueous concentrations, with reductions relative to the initial measured concentrations of approximately 15% and 33% for C1 and 18% and 29% for C2 at 24 and 96 h, respectively. MA has a lower hydrophobicity and higher aqueous solubility than the investigated methylated PAHs, which may contribute to reduced partitioning to plastic surfaces and improved stability in the exposure medium. In contrast, measured ACR concentrations remained consistently low (3–11% of nominal concentrations) without a clear temporal trend. The reason for the low apparent recovery of ACR cannot be determined from the present data. However, the physicochemical properties of ACR in combination with the analytical procedure, particularly liquid-liquid extraction with n-hexane, may have contributed to a reduced extraction efficiency and consequent underestimation of ACR concentrations. Further experimental investigations would be required to identify the factors contributing to the low apparent recovery.

Together, the analytical measurements demonstrate that nominal concentrations did not consistently reflect measured aqueous concentrations during exposure. The measured aqueous concentrations are influenced by a combination of analytical recovery, partitioning, and redistribution processes within the system and cannot be assumed to correspond to freely dissolved or internal embryo concentrations. Phase separation processes, including the formation of metastable dispersions, may further influence compound distribution by facilitating transfer from the freely dissolved phase to aggregated, surface-associated, and biological compartments over time [40, 42]. Furthermore, because chemical analysis was restricted to the two highest nominal FET concentrations and selected time points, the available measured concentrations do not directly represent the biologically relevant exposure, which is governed by the freely dissolved concentration of the compounds. Therefore, the LC50 values reported in this study are based on nominal concentrations and should be interpreted within the applied exposure conditions rather than as estimates of intrinsic compound potency.

4.2 Embryotoxicity, teratogenicity, and larval behavior

Investigations of the embryotoxic and teratogenic effects of the selected PACs revealed that MA (LC_{50} 2.34 µmol/L) and DMBaA (LC_{50} 9.03 µmol/L) caused acute embryotoxic effects at 96 hpf, based on nominal exposure concentrations. Exposure to DMBaA caused distinct developmental and teratogenic

malformations in zebrafish embryos. In contrast, MBaA, MBaP, and ACR did not induce acute embryotoxicity within the tested nominal concentration ranges.

Previous studies reported acute zebrafish embryotoxicity for acridine, the parent compound of MA, with LC_{50} values between 4 and 7 µmol/L at 96 hpf [21, 43]. Legler et al. [44] demonstrated that exposure to 5 µmol/L MA resulted in developmental effects such as lack of swim bladder inflation at 144 hpf. However, this was not observed in the present study after a shorter exposure time of 96 hpf at a slightly lower exposure concentration (4 µmol/L). The nominal LC_{50} determined for MA in the present study was lower than the previously reported values for acridine, although differences in experimental conditions and chemical exposure preclude a direct comparison of their intrinsic toxicities. Similarly, benzo[a]pyrene (BaP), the parent compound to MBaP, has been shown to cause acute embryotoxicity in zebrafish, with an LC_{50} of 5.1 µmol/L and 4.1 µmol/L at 72 hpf and 96 hpf, respectively [45, 46]. In the present study, MBaP did not induce acute embryotoxicity, indicating that the parent compound is more embryotoxic to zebrafish than its methylated variant.

Apart from acute embryotoxicity, DMBaA caused several developmental abnormalities, including pericardial edemas, lordosis, and a concentration-dependent reduction in tail length, which became statistically significant at the two highest exposure concentrations. Dietary exposure to DMBaA has been shown to cause an adverse impact on growth and hematological alterations in Korean Rockfish (*Sebastes schlegeli*) [47]. Similarly, Vicquelin et al. [48] reported significant reduction in body length, skeletal deformities (body axis curvature), and pericardial edemas in larvae of Japanese medaka (*Oryzias latipes*) after exposure to sediments spiked with DMBaA. For the parent compound BaA, Incardona et al. [4] demonstrated that exposure to 22 µmol/L resulted in cardiovascular defects by 48 hpf in zebrafish. In another study by Fang et al. [49], BaA induced similar effects on the cardiovascular system, including pericardial edema, but also deformations of the tail and body shape, as well as yolk sac edema, at concentrations between 0.5 and 15 µmol/L. Lethality was only observed at concentration of 50 µmol/L. In the present study, DMBaA exposure at 1.25 µmol/L also induced pericardial edema, while abnormal heartbeat and blood circulation were observed only occasionally. In contrast, cardiac abnormalities and pericardiac edemas were relatively rare in Japanese medaka exposed to sediments spiked with BaA [50]. DMBaA is known as a highly carcinogenic PAC and has been associated with the formation of DNA adducts in fish [51, 52]. Together, the present results and previous studies demonstrate that DMBaA can induce developmental toxicity in fish, including skeletal abnormalities and cardiovascular defects. Additionally, DMBaA appears to be more acutely embryotoxic and teratogenic to zebrafish than the parent compound benz[a]anthracene. Surprisingly, in the present study, the mono-alkylated MBaA was neither acutely toxic nor teratogenic to zebrafish embryos, which confirms results presented by Fang et al. [49].

Investigations with embryos of Japanese medaka reported that alkylation generally increases toxicity, suggesting a structure-activity relationship for alkyl-PAHs [53]. Peddinghaus et al. [21] reported that mono- and di-alkylated derivates of benzofuran were more embryotoxic to zebrafish than the parent compound, while the di-alkylated variant being more toxic than the mono-alkylated. However, in our

study, the mono alkylated variants MBaP and MBaA did not induce acute toxic effect within the investigated concentration ranges. In contrast, mono-alkylated acridine was more toxic than its parent compound. Therefore, a direct structure-activity relationship, as suggested by Lin et al. [53], cannot be entirely confirmed for the alkylated PAHs of BaA and BaP in zebrafish embryos. However, the embryotoxicity of an alkylated chemical depends on the position of the alkylation [53, 54]. Therefore, further studies under comparable exposure conditions are needed to investigate how the number and position of alkyl substituents influence PAC induced embryotoxic and teratogenic effects in zebrafish.

The LPMR assay is widely recognized as a sensitive endpoint for detecting developmental neurotoxicity in zebrafish larvae because it reflects the integrated function of sensory processing, central nervous system activity, and motor performance [55]. In the present study, MA exposure reduced zebrafish larval locomotor activity across the investigated light phases. Nevertheless, larvae in all treatment groups exhibited increased locomotor activity during the dark phase compared with the light phases, indicating that the characteristic behavioral response to changes in illumination was retained. Such responses depend on coordinated sensory and neural processing and motor function [56, 57]. Therefore, the observed phenotype is likely consistent with a reduction in general behavioral activity or neuronal function, potentially involving altered neurotransmitter signaling or neuronal excitability [58]. In previous studies, such alterations in locomotor activity have been linked to disturbances in multiple neurotransmitter systems, including dopaminergic, glutamatergic, and GABAergic pathways, which are essential in regulating spontaneous and stressor-induced behavioral changes [58, 59]. However, the present study demonstrates only MA-associated alterations in larval locomotor behavior, while the underlying biological mechanisms remain unresolved and would require further investigations. Importantly, no significant differences were detected between the negative and solvent controls, indicating that the observed behavioral effects were attributable to MA rather than the solvent.

The OECD TG236 guideline recommends keeping solvent concentrations as low as possible and preferably below 0.01% (v/v) DMSO [27]. In the present study, a higher solvent concentration (0.4%) was required because of the extremely low aqueous solubility of several investigated PACs. Although this exceeds the OECD recommendation, no statistically significant differences between the negative and solvent controls were observed for tail length or larval behavior, indicating that the solvent concentration did not measurably influence these endpoints under the present experimental conditions. Previous studies have reported differing conclusions regarding the effects of DMSO on zebrafish embryos. Hallare et al. [60] observed no developmental abnormalities or mortality at concentrations up to 0.1% DMSO, whereas morphological effects became evident at higher concentrations, although molecular stress responses were detectable at lower concentrations. More recently, Hoyberghs et al. [61] demonstrated that DMSO concentrations up to 1% did not increase lethality or developmental malformations in a standardized zebrafish embryo developmental toxicity assay. Together, these studies suggest that while elevated DMSO concentrations may influence certain molecular or physiological processes, apparent developmental toxicity is not necessarily expected at concentrations around 0.4%. Therefore, the use of 0.4% DMSO should be regarded as a limitation of the present study that may influence absolute exposure conditions. However, because all treatments and the solvent control contained identical DMSO

concentrations, and no statistically significant differences between the negative and solvent controls were detected for the investigated morphological and behavioral endpoints, the observed differences among treatments are most likely attributable to the investigated PACs rather than to the solvent itself.

4.3 Gene expression analyses

The selection of genes investigated in this study was based on known toxicological effects of the selected PACs and their parent compounds, as well as observed developmental abnormalities. The DNA methyltransferases DNMT1 and DNMT3ba are epigenetic regulators of DNA methylation and play important roles in gene expression regulation [62]. Neither *dnmt1* nor *dnmt3ba* expression was significantly altered by the investigated PACs. However, DMBaA has been associated with alterations in methylation patterns in hamsters [63]. Similarly, TCDD exposure has been shown to alter promoter methylation of different AhR genes in zebrafish embryos, including hypermethylation of *ahrra* and hypomethylation of *c-fos* [64]. However, since DNA methylation can be regulated independently of changes in DNMT transcription, the absence of significant expression changes does not exclude epigenetic effects. Direct methylation analyses would therefore be required to determine whether DMBaA alters methylation at specific loci of the AhR pathway.

The genes *wnt5b* and *spt6* are known to be essentially involved in zebrafish development, including body axis formation and cardiac function [65, 66]. Previous studies demonstrated that a decreased expression of *wnt5b* affects posterior development and somite formation [65, 67], while the injection of wild type *spt6* mRNA rescued several developmental abnormalities, including tail length, in zebrafish *pandora* mutants. Despite the observed tail deformities after DMBaA exposure, the expression of *wnt5b* and *spt6* was not significantly altered at the investigated time points. Previous other studies have also suggested a crosstalk between the AhR and of Wnt/β-catenin signaling pathway [68–70]. Such interactions may interfere with signaling processes involved in embryonic patterning and organ development, providing a potential link between AhR-associated responses and the developmental abnormalities observed following DMBaA exposure. However, the present results did not directly assess Wnt/β-catenin signaling activity, and the unchanged *wnt5b* expression does not exclude interactions between these pathways. Similarly, *tp53*, *mitfa*, and *otx2* expression remained unchanged. However, unchanged *tp53* does not exclude DNA damage or cellular stress, as *p53* in zebrafish is regulated predominantly at the post-translational level [71]; particularly, since several of the investigated PACs are metabolically activated to diol-epoxides [72].

Among the investigated genes involved in the AhR pathway, *cyp1a* was most significantly altered after exposure to all five PACs at one or more developmental stages. DMBaA showed the highest increase in *cyp1a* expression, accompanied by significant induction *cyp1b1* and *ahrrb* at selected time points. However, differences in transcriptional response magnitude cannot establish relative AhR-activating potency because only one nominal concentration was investigated and equivalent aqueous or internal exposure was not demonstrated. DMBaA caused similar effects as previously shown for BaP, e.g. lordosis, reduced tail length, and pericardial edemas. Specifically, cardiovascular failures in zebrafish larvae caused by BaP are related to the induction of *cyp1a* gene expression [5, 73, 74]. These similarities

suggest potentially overlapping biological responses in zebrafish embryos, although a causal relationship between AhR-pathway associated transcription and DMBaA-induced developmental abnormalities remains to be established.

The pronounced induction of *cyp1a* is consistent with its established metabolization of PACs in zebrafish embryos [28, 75, 76]. Early induction was observed at 24 hpf following DMBaA, MBaA, and MBaP exposure, whereas significant responses to ACR and MA were first detected at 72 and 96 hpf, respectively. Previous studies already indicated that the time-dependent transcription of *cyp1a* is compound specific, as also observed for other genes involved in the AhR pathway [28, 35, 36, 77, 78]. However, although the observed profiles differed descriptively, the statistical analysis did not identify a significant chemical × time interaction for the genes presented in Fig. 5B. In comparison to *cyp1a*, significant *cyp1b1* induction was restricted to DMBaA at 24–72 hpf and MBaP at 24 and 72 hpf. Both cytochrome P450 systems play an important role in the metabolic activation of PACs to carcinogens [72]. In mammalian systems, DMBaA can undergo a three-step bioactivation pathway involving CYP-mediated epoxidation, epoxide hydrolysis, and a second CYP-mediated oxidation, producing the DNA-reactive metabolite DMBaA-3,4-diol-1,2-epoxide [72, 79, 80]. However, the observed transcriptional induction does not demonstrate increased CYP activity or formation of these metabolites in zebrafish embryos.

For MBaA, Myers and Flesher [81] showed that the methyl group of MBaA underwent enzymatic hydroxylation in rat lung cytosol preparations. Other studies showed that MBaA exhibited only limited AhR-mediated activity in rat hepatoma cells [18, 82]. In contrast, MA and ACR exhibited only minimal AhR-mediated activity after 24 h of exposure *in vitro*. The present findings extend these observations by demonstrating AhR-associated transcriptional responses to MBaA, MA, and ACR in zebrafish embryos, including responses that became significant only at later developmental stages.

No expression changes for *ahr2* were observed in our study, for any of the compounds tested. Previous studies on compounds activating the AhR pathway showed a similar low and rather stable *ahr2* expression in zebrafish embryos, suggesting a recurring pattern of AHR2 regulation as already shown in rats [28, 35, 83]. The stable expression of *ahr2*, despite pronounced induction of downstream AhR-responsive genes, is consistent with the known mechanism of ligand-dependent AhR signaling, in which transcriptional activation of target genes does not necessarily require increased receptor expression [84].

We observed a progressive and time-dependent increase of *ahrrb* expression after DMBaA exposure from 24 to 72 hpf (reaching significance at 72hpf), followed by a decrease at 96 hpf. Significant induction of *ahrrb* at 72 hpf was likewise observed after MBaP exposure, although of lower magnitude. Despite its role as an AhR pathway regulator, the temporal pattern of *ahrrb* expression did not consistently correlate with a reduction in downstream gene expression across all compounds and time points. The significant induction of *ahrrb* following DMBaA and MBaP exposure is consistent with transcriptional regulation of the AhR-associated repressor, although its functional relationship with

downstream target genes remains unclear. Since AhRR is thought to act at the protein level, its functional impact cannot be inferred from transcript abundance alone. Several hypotheses have been proposed regarding the exact feedback mechanism by which the repressor inhibits the activity of the other components of the AhR pathway [85], including competition for binding factors like ARNT [86] or for DNA binding sites [87]. Resolving which of these mechanisms operates in zebrafish embryos exposed to PACs will require protein-level analyses of AhRR and ARNT together with extended sampling beyond 96 hpf, at which point *ahrrb* expression had already begun to decline.

5. Conclusion

This study demonstrates distinct developmental, behavioral, and transcriptional responses to selected methylated and oxygenated PACs in zebrafish embryos. Among the five investigated compounds, MA and DMBaA induced acute embryotoxicity, whereas no acute embryotoxicity was observed for MBaA, MBaP, or ACR under the investigated exposure conditions. DMBaA additionally induced developmental abnormalities, including reduced tail length, lordosis, and pericardial edema, while MA altered larval locomotor activity, with effects differing among light phases.

All five PACs induced cyp1a expression at one or more developmental stages, with compound-specific differences in the magnitude and timing of transcriptional responses. DMBaA produced particularly pronounced AhR-associated transcriptional responses, although the relationship between these responses and the observed developmental abnormalities remains unresolved. Differences between nominal and measured aqueous concentrations, together with the absence of internal exposure measurements, limit conclusions regarding relative toxic potency and the interpretation of compounds showing no acute embryotoxicity.

Overall, the findings demonstrate that the investigated substituted PACs can induce distinct biological responses during early zebrafish development and highlight the importance of considering these compounds in chemical hazard characterization and environmental risk assessment.

Abbreviations

ACR	9(10H)-Acridone
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BaA	Benz[a]anthracene
BaP	Benzo[a]pyrene
CYP	Cytochrome P450
DCA	3,4-Dichloroaniline
DMBaA	7,12-Dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
EI	Electron ionization
FET	Fish embryo toxicity
GC-LRMS	Gas chromatography–low-resolution mass spectrometry
GC-MS	Gas chromatography–mass spectrometry
hpf	Hours post-fertilization
LC50	Median lethal concentration
LON1	First light phase
LOFF	Dark phase
LON2	Second light phase
LPMR	Larval photomotor response
MA	9-Methylacridine
MBaA	7-Methylbenz[a]anthracene
MBaP	7-Methylbenzo[a]pyrene
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

SIM	Selected-ion monitoring
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Declarations

Ethics approval and consent to participate

The study was conducted using zebrafish (*Danio rerio*) maintained at Örebro University, Sweden. The animal facility was authorized under permit No. 5.2.18-20002/2022, with permissions to use (No. 5.12.18-20142/2022) and breed (No. 5.2.18-20144/2022) laboratory animals issued by the Swedish Board of Agriculture. Ethical approval was obtained under permit No. 13595-2023 by the Linköping Animal Ethics Committee (Linköpings djurförsöksetiska nämnd) (Linköping, Sweden).

The study did not involve human participants.

Consent for publication

Not applicable.

Availability of data and materials

The data generated and analyzed during this study are included in this article or are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was conducted as part of the EnForce platform, financially supported by the Swedish Knowledge Foundation (grant agreement No. 201660019).

Authorship contribution statement

- Steffen H. Keiter: Writing – original draft, Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Supervision, Resources, and Funding acquisition.
- Manon Fallet: Writing – original draft, Investigation, Formal analysis, Data curation, Visualization.
- Melanie Blanc: Conceptualization, Methodology, Investigation, and Formal analysis.
- Flora Borchert: Investigation, Formal analysis, and Visualization.
- Kai Horvath: Investigation, Formal analysis, and Visualization.
- Nikolai Scherbak: Investigation, Formal analysis, Data curation, and Visualization.

- Maria Larsson: Writing – original draft, Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Supervision, Resources, and Funding acquisition.

Acknowledgments

The authors thank Lina Lundin and Marta Szot for their assistance with the husbandry, feeding, and rearing of zebrafish, as well as with the fish embryotoxicity tests.

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Figures

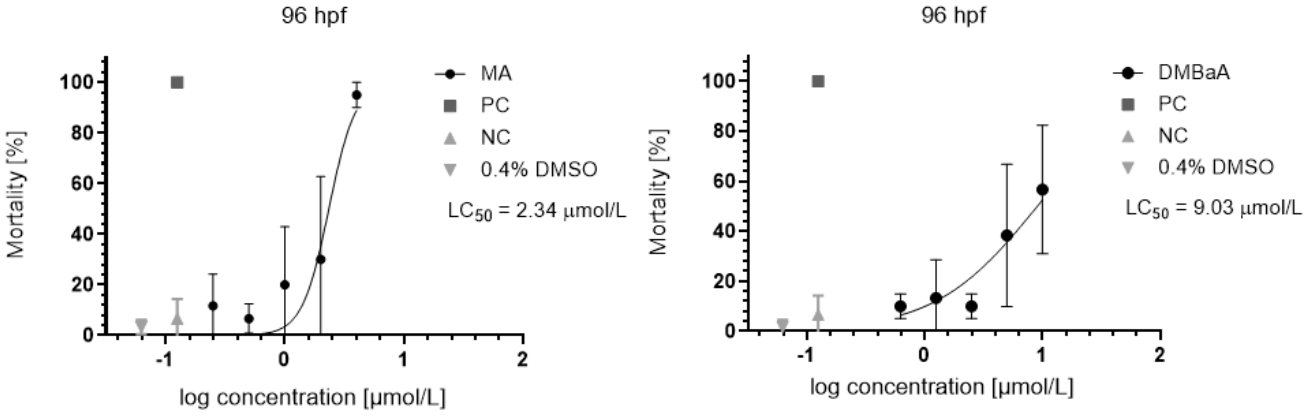

Figure 1

Concentration-response relationships for acute embryotoxicity of 9-methylacridine (MA), and 7,12-dimethylbenz[a]anthracene (DMBaA) in zebrafish embryos. PC, NC, and SC represent the positive, negative, and solvent controls (0.4% DMSO), respectively. Experiments were conducted as 4-6 independent replicates.

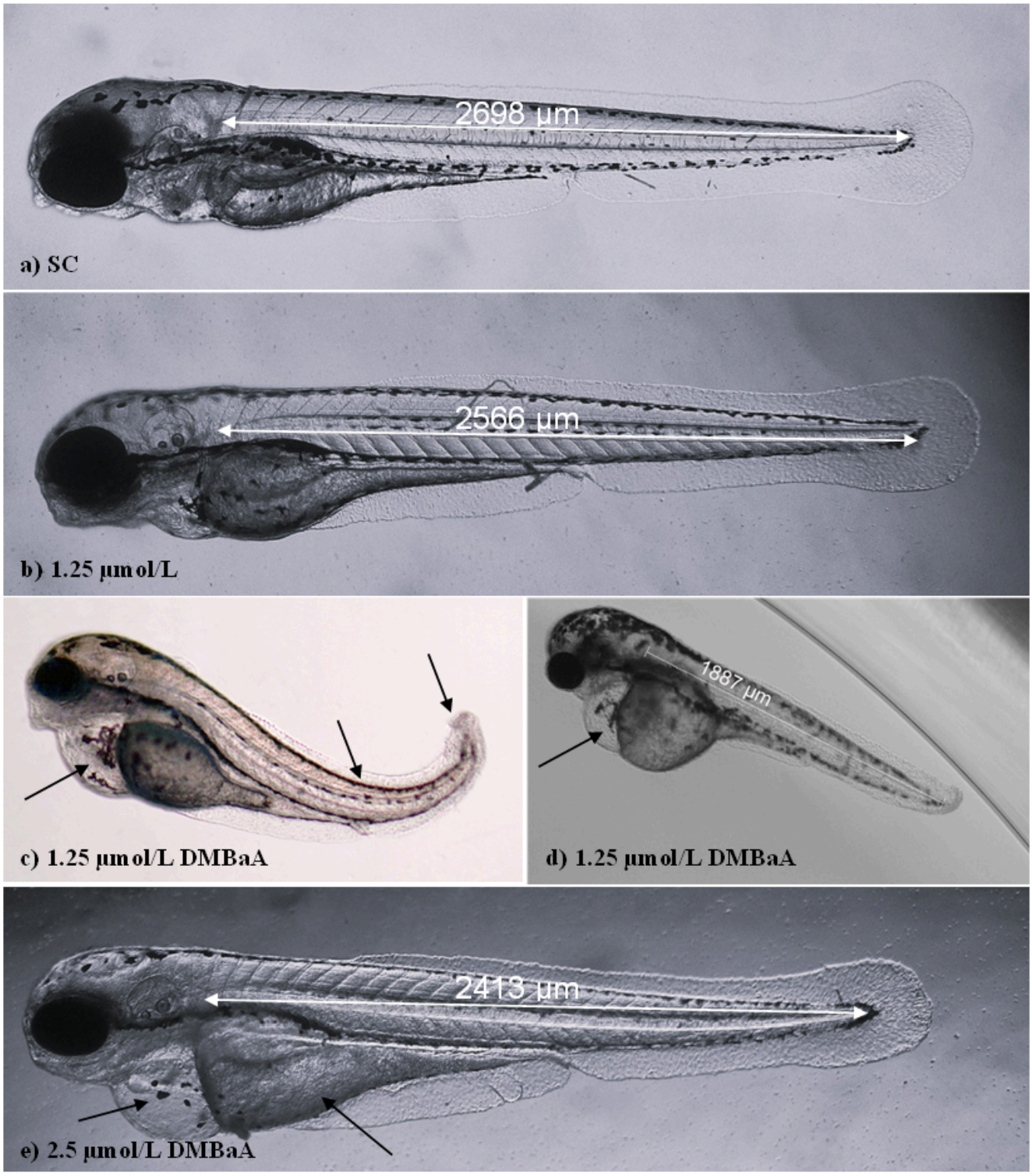

Figure 2

Representative images of developmental abnormalities in zebrafish embryos after 96 hpf of exposure to 7,12-dimethylbenz[a]anthracene (DMBaA): a) solvent control (SC); b) shortened tail; c) pericardial edema, lordosis, and deformation of the tail tip; d) pericardial edema and shortened tail (1887 µm); and e) pericardial edema, yolk deformation, and shortened tail (2413 µm). Panels (b–d) show embryos exposed to 1.25 µmol/L DMBaA, and panel (e) shows an embryo exposed to 2.5 µmol/L DMBaA.

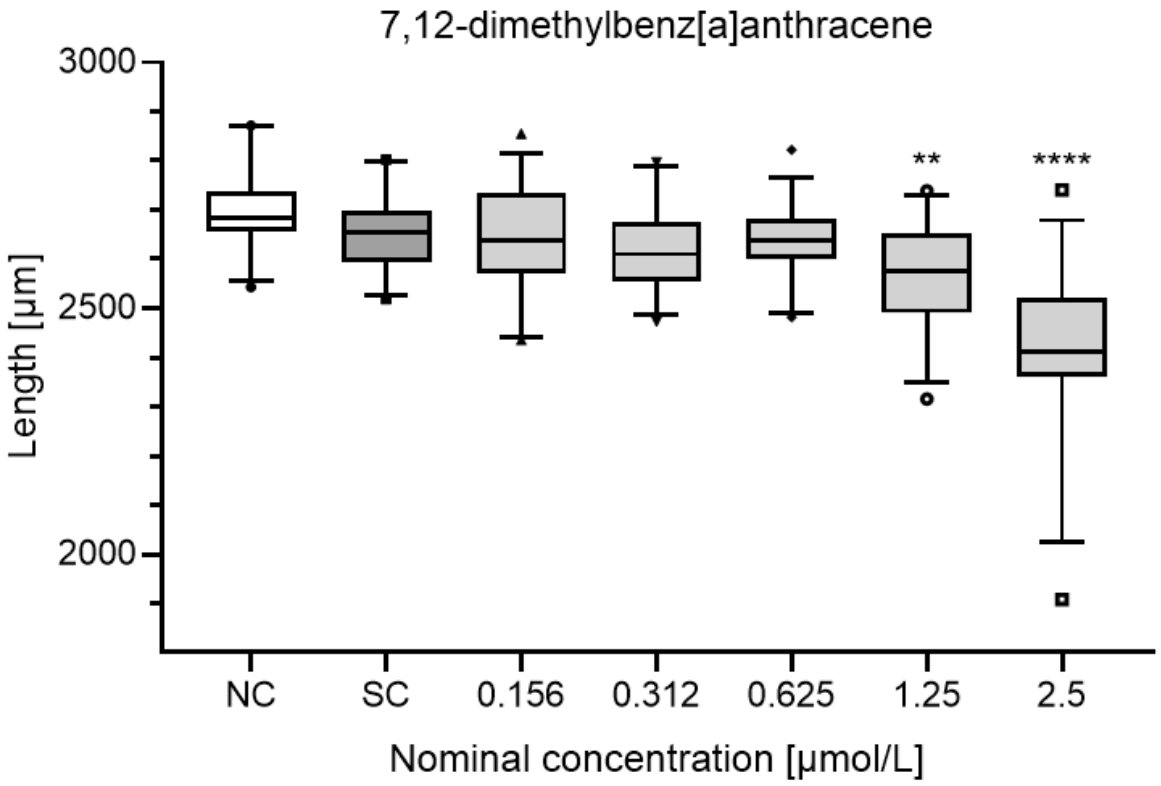

Figure 3

Tail lengths of zebrafish embryos exposed to a 1:2 dilution series of 7,12- dimethylbenz[a]anthracene (DMBaA), together with the negative control (NC) and solvent control (SC, 0.4% DMSO) (n=26-30 per treatment). The data are represented as box-and-whisker plots, including 25 and 75% percentiles (box) and standard error (whiskers). Central solid lines represent the median. ** $p < 0.01$; **** $p < 0.0001$.

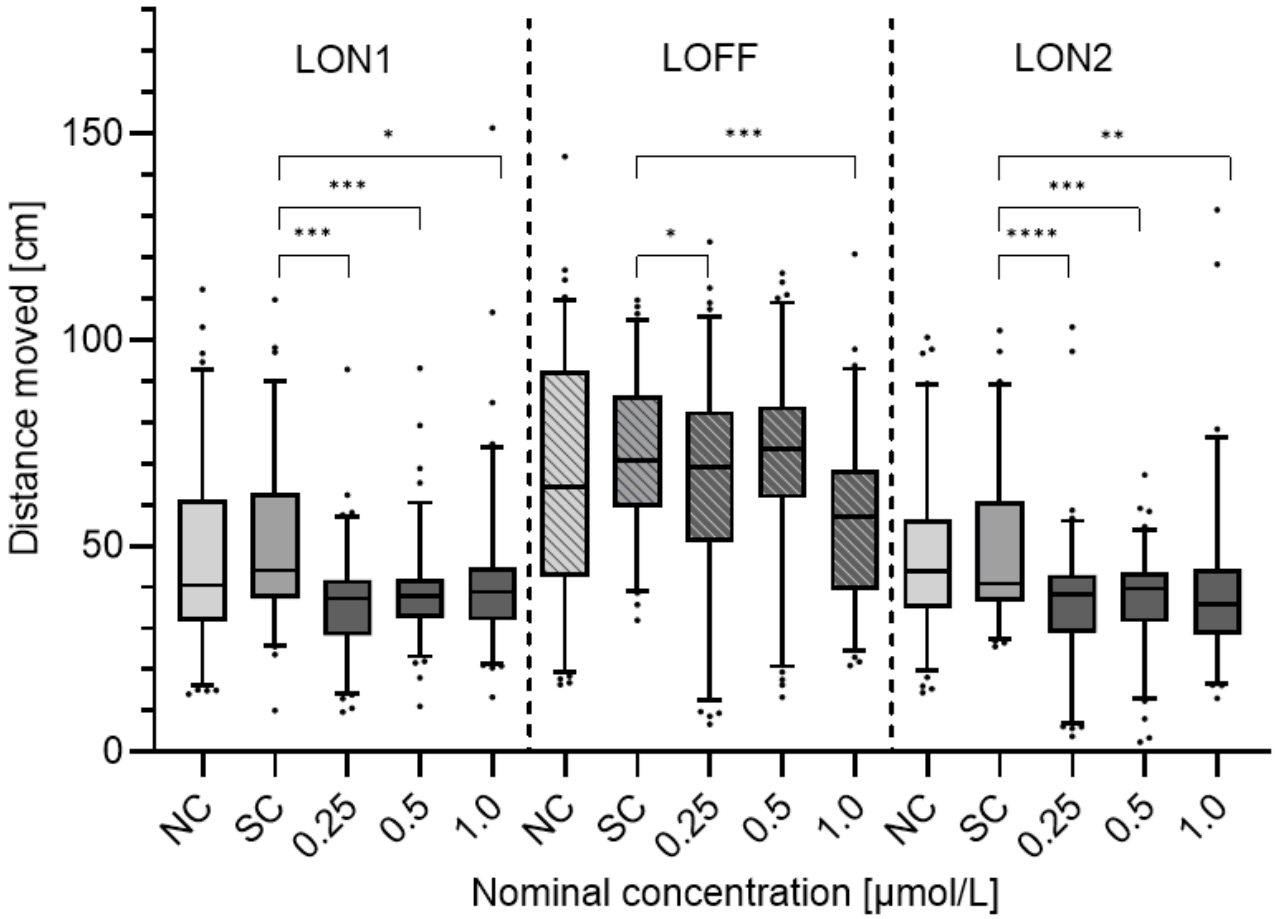

Figure 4

Locomotor activity of zebrafish larvae exposed to 9-methylacridine (MA), assessed using the larval photo-motor response (LPMR) assay at 96 hpf. Distance moved (cm) is shown during the first light phase (LON1), dark phase (LOFF), and second light phase (LON2) for the negative control (NC), solvent control (SC; 0.4% DMSO), and nominal MA concentrations of 0.25, 0.5, and 1.0 µmol/L. Data are presented as box-and-whisker plots. Statistical differences between treatment groups and the solvent control (SC) were determined using Dunnett-adjusted post hoc comparisons following a linear mixed-effects model. Asterisks indicate significant differences (*$padj$ < 0.05, **$padj$ < 0.01, ***$padj$< 0.001, ****$padj$ < 0.0001).

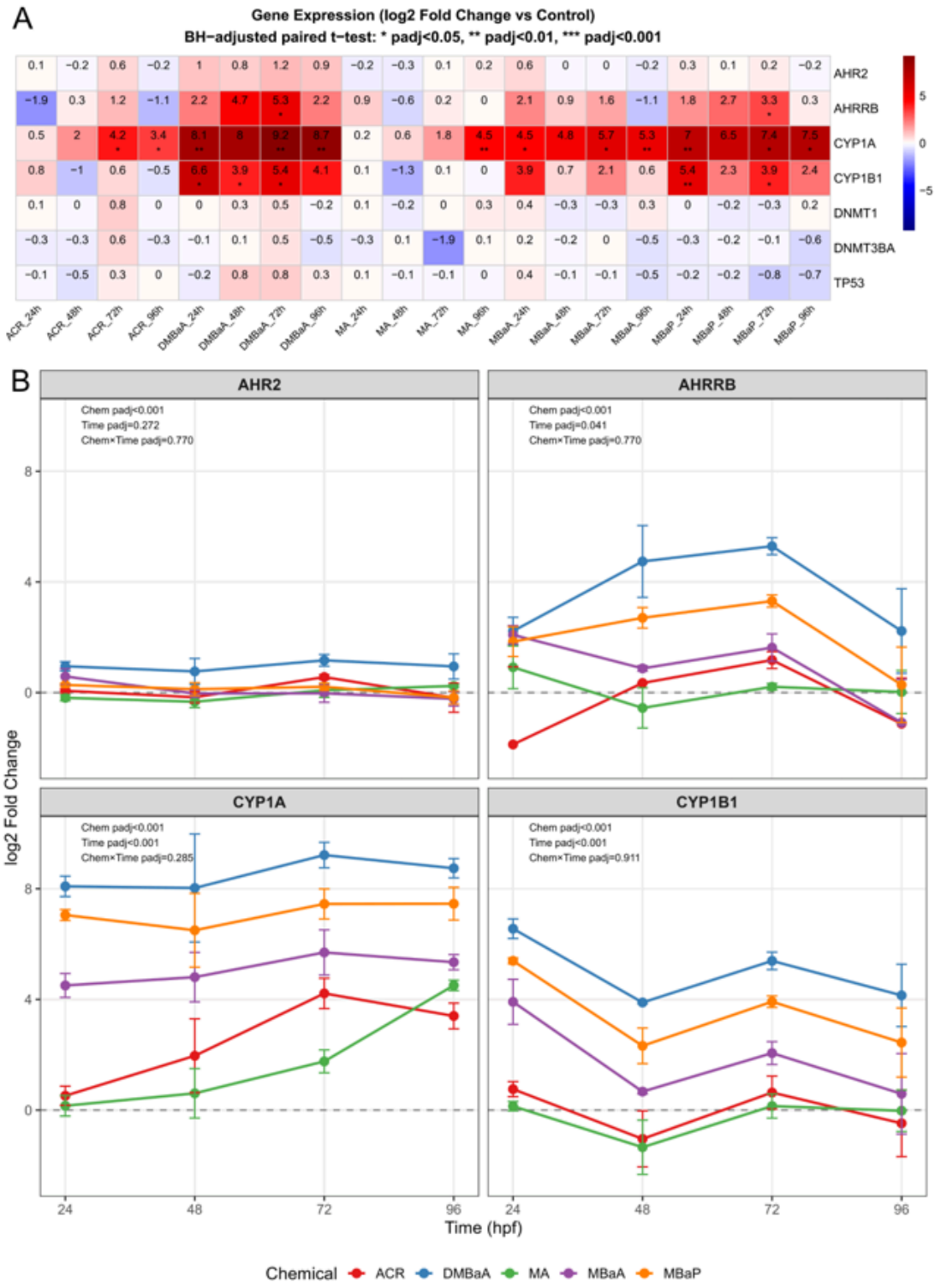

Figure 5

Gene expression changes in zebrafish embryos following exposure to the investigated PACs. (A) Heatmap showing mean log2 fold changes in target-gene expression relative to the corresponding controls across developmental time points (24-96 hpf) for all treatments. Asterisks indicate statistically significant differences (paired t-test, Benjamini-Hochberg adjusted). Significant induction of *cyp1a* was observed across all time points after exposure to DMBaA and MBaP, as well as for MBaA except 48 hpf,

for which only two replicates were available. ACR significantly induced *cyp1a* at 72 and 96 hpf, whereas MA induced cyp1a expression at 96 hpf. Significant induction of *ahrrb* was observed at 72 hpf after exposure to DMBaA and MBaP. Expression of *cyp1b1* was significantly induced from 24 to 72 hpf after exposure to DMBaA, and at 24 and 72 hpf for MBaP. Data are expressed as mean ± SD (n=3), except for MBaA at 48hpf and MA at 24hpf (n=2). ****padj* < 0.001, **: *padj*< 0.01, *: *padj* < 0.05. (B) Time-course profiles of log2 fold change for genes showing a significant overall chemical effect (two-way ANOVA, Benjamini-Hochberg adjusted across genes). Lines represent mean ± SE of each treatment group across developmental time points. ANOVA p-values for Chemical, Time and Chemical x Time interaction effects are shown within each panel. These plots illustrate differences in temporal expression profiles between chemicals.

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