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Preliminary study on adverse effects of phenanthrene and its methyl and phenyl derivatives in larval zebrafish, *Danio rerio*

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ABBREVIATIONS

1M-Ph – 1-methylphenanthrene
4M-Ph – 4-methylphenathrene
1P-Ph – 1-phenylphenanthrene
4P-Ph – 4-phenylphenanthrene
actb – β-actin
AhR – Aryl hydrocarbon Receptor
B[*a*]P – Benzo[*a*]pyrene
BSD – Blue Sac Disease
cDNA – complementary DNA
Ct – Threshold cycle
cyp1a – Cytochrome P450 1A
cyp1b1 – Cytochrome P450 1B1
DC – Dorsal Curvature
DEB – Dynamic Energy Budget
DEPC-H_2O – Diethylpyrocarbonate Treated Water
DMSO – Dimethyl Sulfoxide
ER – Expression Ratio
EW – Egg Water
FW – Fish Water
hpf – hours post fertilization
LC_{50} – Lethal Concentration 50
mRNA – messenger RNA
NEC – No Effect Concentration
OECD – Organisation for Economic Co-operation and Development
PAHs – Polycyclic Aromatic Hydrocarbons
PE – Pericardial Edema
Ph – Phenanthrene
qPCR – quantitative Polymerase Chain Reaction
RNA – Ribonucleic Acid
RT – Reverse Transcriptase
vtg – Vitellogenin

ABSTRACT

Toxic effects of polycyclic aromatic hydrocarbons (PAHs) have been extensively studied in fish, although knowledge concerning biological activities of phenanthrene and its derivatives remains still incomplete. The aim of this work was to evaluate lethal and sublethal effects of benzo(*a*)pyrene, phenanthrene and phenanthrene derivatives (1-methylphenanthrene, 4-methylphenanthrene, 1-phenylphenanthrene and 4-phenylphenanthrene) on zebrafish (*Danio rerio*) larvae. We conducted acute toxicity test, using 96h static renewal exposure to a series of the PAH concentrations (0.05, 0.50, 5.00, 50.00μmol·l^{-1}), to determine the No Effect Concentration (NEC) value for each compound examined. The mean NEC estimates obtained in the study were 5.16±0.45μmol·l^{-1} (B[*a*]P), 4.88±0.13μmol·l^{-1} (Ph), 40.24±12.93μmol·l^{-1} (1P-Ph), 47.92±3.61μmol·l^{-1} (1M-Ph), 24.31±7.33μmol·l^{-1} (4P-Ph) and 3.11±1.01μmol·l^{-1} (4M-Ph) and suggested the following order of PAH toxicities on *Danio rerio* larvae: 4M-Ph>Ph~B[*a*]P>4P-Ph~1P-Ph>1M-Ph. To gain insight into possible molecular mechanisms of apparent toxicity of phenanthrene derivatives on zebrafish larvae, we examined mRNA expression of *cyp1a*, *cyp1b1*, and *vtg* genes in the larvae exposed for 48h to a PAH concentration of 0.50μmol·l^{-1}. Whereas the larvae exposed to

each tested PAH displayed many developmental abnormalities (i.e. pericardial and yolk sac edema, dorsal curvature, or tail malformations), no significant upregulation of *cyp1a* and *cyp1b1* mRNA was observed, except for zebrafish exposed to B[*a*]P. However, significant reduction of *vtg* mRNA was observed in the larvae exposed to phenanthrene and its 4P- derivative. The results may contribute to the development of a new knowledge about effects of structurally diverse phenanthrene derivatives on vertebrate organisms.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are environmentally persistent agents, that are mainly formed in the process of incomplete combustion of organic material. Because of the increasing use of fossil fuels, PAHs composed of two or more fused benzene rings are ubiquitous in industrial and urban areas (Incardona et al. 2006). Among others, phenanthrene and its derivatives are common in crude oil and can be also found in sediments at high concentrations (Leppänen and Oikari 2001; Muri and Wakeham 2009; Rospondek et al. 2009).

Mutagenic activity of some methyl derivatives of phenanthrene has been previously reported (LaVoie et al. 1981), however data concerning biological properties of phenylphenanthrenes is still limited. Carbon skeleton of phenanthrene molecule is one of the best models for studying correlation of PAH structure substitution pattern with its genotoxic activity (Łuczyński et al. 2005).

A number of biological effects in fish of several PAH compounds have been documented, which are particularly significant at early life developmental stages (e.g. Brinkworth et al. 2003). Sublethal effects caused by embryonic exposures include: yolk sac and pericardial edema, hemorrhaging, disruption of cardiac function, craniofacial and spinal deformities, neuronal cell death, or impaired swimming (Barron et al. 2004; Brinkworth et al. 2003; White et al. 1999). Adverse effects of multi-ring carcinogenic PAHs species (i.e. benzo[*a*]pyrene) is believed to be mediated through the AhR pathway, which is activated by dioxins and many dioxin-like compounds. The nature of interactions of 2- and 3-ring PAHs with the AhR is, however, less clear, with some evidence indicating effects mediated by an AhR independent mode of action (Incardona et al. 2005). Recently Scott et al. (2010) identified a possible link between the toxicity of alkylphenanthrenes and their binding affinity to AhR. These findings have implications for understanding the different toxicity mechanisms of structurally diversed PAHs.

The objectives of this paper were to evaluate both the lethal and sublethal effects of phenanthrene and its methyl and phenyl derivatives, as well as benzo[*a*]pyrene on zebrafish (*Danio rerio*) larvae. The embryonic and larval zebrafish has been identified as an ideal organism for *in vivo* bioassays including toxicity tests (Incardona et al. 2006); the replacement of mature stages of fishes with immature ones such as embryos may be a promising alternative based on the hypothesis that these stages may have not evolved pain as that present in mature stages (Huntingford et al. 2006; Lammer et al. 2009). Zebrafish have many advantages over other vertebrate bioassay models with respect to their size, easy reproduction, high reproducibility, or early morphology (Segner 2009). Furthermore, a number of molecular tools for zebrafish are also available to allow integrative studies of the mechanisms of action, underlying observed non-specific biological responses (Vogel 2000).

In the study, we conducted a toxicity test to determine the No Effect Concentration (NEC) value for each PAH examined. Then, based on the obtained NEC data, we performed a low-dose treatment study on larval zebrafish to assess the effect of individual PAH exposures on mRNA transcription of two cytochrome P450 (*cyp1a*, *cyp1b1*) and vitellogenin (*vtg*) genes. The responses of the chosen genes (as expression alterations) are widely accepted indicators of either xenobiotic (cytochromes) or estrogenic (vitellogenin) properties of environmental contaminants (Kausch et al. 2008; Zanette et al. 2009), providing background for more detailed experiments.

MATERIAL AND METHODS

Chemicals

Benzo[*a*]pyrene (B[*a*]P) and phenanthrene (Ph) were purchased from Fluka (Sigma-Aldrich; Schnelldorf, Germany). 1-methylphenanthrene (1M-Ph), 4-methylphenathrene (4M-Ph), 1-phenylphenanthrene (1P-Ph), and 4-phenylphenanthrene (4P-Ph) were synthesized at the Department of Organic Chemistry, Jagiellonian University (Cracow, Poland), according to the published procedures (Rospondek et al. 2009). The nominal concentrations (0.05, 0.50, 5.00, 50.00μmol·l^{-1}) of each tested compound (Figure 1) and one control solution (0.00μmol·l^{-1}) were selected based on available information from studies on other PAH compounds (Incardona et al. 2004; Timme-Laragy et al. 2007). The PAHs examined were dissolved in *fish water* with 0.1% DMSO (FW; Westerfield 2000) and stored in separate bottles. The stock solutions were kept protected from light at 4°C and warmed up for one hour before the fish exposure.

Zebrafish rearing and spawning

Adult male and female zebrafish (Tübingen strain) were reared at the Department of Environmental Biotechnology (University of Warmia and Mazury, Olsztyn, Poland), and maintained at 28°C, 14:10 light-dark photoperiod in 50 litres aquaria with recirculating tap water, conditioned with AquaSafe (Tetra;

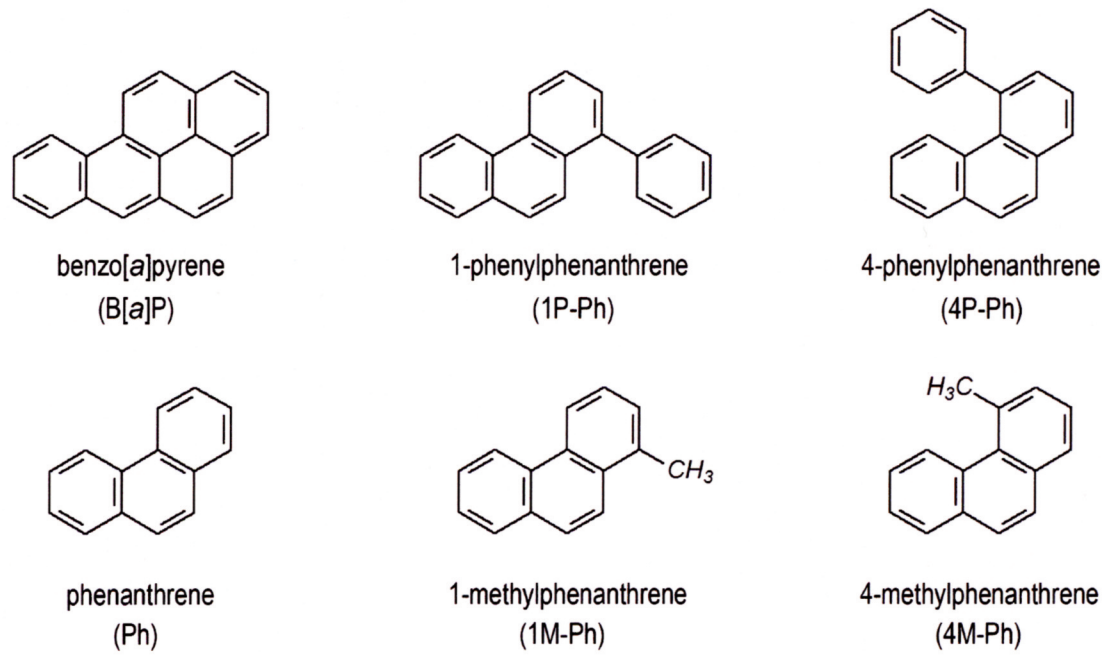

Figure 1. Chemical structure of the PAHs used in this study.

Melle, Germany). The fish were fed with a commercial food two times daily (Tetra, Melle, Germany). During the rearing period no disease symptoms or morphological alterations in the zebrafish individuals were observed.

All experimental individuals were maintained in accordance with the regulations set forth by the Local Ethical Commission No. 33/2010/DTN issued on 24th of February 2010. For treatment, sets of reproductively active fish, each consisting of 2 males and 1 female (above 6 months of age) were transferred to mesh tanks, immersed in the aquarium (3 tanks per aquarium). Eggs were collected after natural spawning, washed, and distributed into Petri dishes; 120 embryos per 50ml of *egg water* (EW; Westerfield 2000), and were allowed to develop at 28.5°C on a 14:10 light-dark photoperiod. EW was exchanged at 20 to 24h intervals and unfertilized eggs and those with visible coagulation were removed each time with the water exchange. After hatching (72 hours post fertilization; hpf) the larvae were transferred to *fish water* (FW), and were left for 24h. After this time, samples of 20 larvae each were transferred to Petri dishes containing respective exposure solutions of the tested compounds. For the experiment, all larvae were staged as described by Kimmel et al. (1995) and selected after visual observations of their swimming activity and mouth movement. Then they were assessed immediately after transfer to the experimental chambers to confirm viability. Fish that did not survive their transfer were not included in the assessment scoring.

Acute toxicity test

Four-day static acute toxicity test was performed to determine the No Effect Concentration (NEC) value for each PAH examined. Both test and control solutions were renewed every 24h. During the acute toxicity test (96 to 192 hpf) fish were not fed. At the 24, 48, 72, 96h of exposure, dead larvae were removed from the Petri dishes and the mortality was recorded. For each compound tested, the acute toxicity tests were prepared in 3 repetitions.

To analyze the mortality data from the conducted toxicity tests, DEBtox 2.0.1 software was used. The basic idea behind the DEB theory is an attempt to model energy management by individuals by inferring a function of time and exposure concentration, in which the parameters used have clear biological meanings (Kooijman and Bedaux 1996). We analyzed with the help of the software the direct impact of studied PAH chemicals on survival of the larvae, i.e. their lethal effects. Choosing the option *survival* in the software, it is possible to estimate several parameters at a time, based on the calculated cumulative mortality data. The DEBtox output parameters may include: an estimate of NEC, blank mortality rate, killing rate and elimination rate. We applied the DEBtox for the estimation of NEC value for each individual PAH compound examined in the toxicity tests. From a practical standpoint, the NEC value offers a facsimile of the threshold that is measurable and that can be used in assessing and managing risk. Finally, Kruskal-Wallis test for several treatments was used (Statistica 9; Statsoft, Tulsa, UK) to test if at least one of the treatments had a different effect on NEC estimate from the others.

Table 1. Real-Time qPCR primers used in the study.

Primer name (gene abbreviation)		Sequence (5'→3')	Source
β-actin	forward	acatccgtaaggacctg	Timme-Laragy et al. 2007
(actb)	reverse	ggtcgttcgtttgaatctc	
Cytochrome P450 1A	forward	aggacaacatcagacacatcaccg	Timme-Laragy et al. 2007
(cyp1a)	reverse	gatagacaaccgcccaggacagag	
Cytochrome P4501B1	forward	ccacccgaactctgaaactc	Timme-Laragy et al. 2007
(cyp1b1)	reverse	aaacacaccatcagcgacag	
Vitellogenin	forward	ggtgtggttggcaaggtttt	NM_001044897
(vtg)	reverse	ttggcctttggatcctcatt	

Morphological abnormalities were observed and photographed using LEICA MZ 16A stereo microscope (LEICA Microsystems; Wetzlar, Germany) equipped with camera and LEICA QWin Pro (LEICA Microsystems AG; Heerbrugg, Switzerland) soft package.

Gene induction study

Based on the NEC estimates obtained from the above described acute toxicity test we designed a treatment study to analyze possible adverse molecular effects of the compounds on the 96 hpf zebrafish larvae. The nominal PAH concentration of 0.50μmol·l^{-1} was chosen because it was the next lower concentration to the obtained NEC estimates. PAH exposures and controls (FW with 0.1% DMSO) (n=20) were run in triplicate for 48h. After the exposure, 15 zebrafish larvae per replicate were removed from each experimental group, quick-frozen on dry ice, and stored at -20°C in RNA*Later*™ (Sigma-Aldrich) until analysis. Total RNA extractions of pooled zebrafish larvae (n=15) were carried out according to the manufacturer`s protocol (A&A Biotechnology; Gdynia, Poland). Pure RNA was eluted in 50μl of DEPC-H_2O. RNA samples were then incubated at 37°C for 30 minutes with RNase-free DNase I (Roche; Mannheim, Germany). RNA quantity and quality were analyzed using a Bio*Photometer* (Eppendorf; Hamburg, Germany).

Real-Time qPCR primers were either chosen from the literature (*actb*, *cyp1a*, *cyp1b1*; Timme-Laragy et al. 2007), or were designed (*vtg*) with the Primer Express software (Applied Biosystems; Branchburg, USA) based on the sequences available in GenBank for *Danio rerio*. Primer sequences and source sequence accession numbers are provided in Table 1. The assay was performed on ABI 7500 Real-Time PCR system (Applied Biosystems) in one step, singleplex mode, and all samples were analyzed in duplicates. Each PCR reaction tube contained 10μl of Power SYBR Green PCR Master mix (Applied Biosystems), 5pmol of each (forward and reverse) primer, 0.16μl of RT enzyme mix (Applied Biosystems), 100ng of total RNA as a template, and PCR-grade H_2O to a final volume of 20μl. The reaction was performed in standard thermal conditions, i.e. 50°C for 2min, 95°C for 10min, then 40 cycles of 95°C for 15s and 60°C for 1min, followed by a dissociation curve calculation step. Data obtained from the assay was used to estimate ER of each genes relative to β-actin as the endogenous control. The calculations were based on sample Ct values, and for this purpose REST®2008 software was used (Pfaffl et al. 2002).

RESULTS

We examined the effects of six compounds on the survival of zebrafish larvae, between 96 and 192 hpf, using static renewal exposure conditions. The tests fulfilled the validity criteria for control performance required in the OECD guidelines (OECD 1992). Recommended conditions, such as dissolved oxygen concentration, water temperature, concentration of the test substance in solution were not different between the test chambers or between successive days at any time during the test.

In the study, the numbers of survivors were recorded for each PAH`s concentration from 24 to 96h during the acute toxicity test. Based on the resulting cumulative mortality data and under default DEBtox software assumptions, NEC values for single individual PAH could be estimated (Table 2). The treatment effects of the PAHs tested were different (p=0.0084; Kruskal-Wallis test), which were revealed in differences in mean NEC values, ranging from 3.11±1.01μmol·l^{-1} for 4M-Ph to 47.92±3.61μmol·l^{-1} for 1M-Ph. With the exception of the 4M-Ph, other phenanthrene derivatives examined in the study showed little effect on zebrafish larvae, for which cumulative mortalities did not exceed 50% at any concentration (data not shown).

Table 2. Estimated No Effect Concentration (NEC) values (μmol·l^{-1}), with their means and standard errors (S.E.) for PAHs examined in 96h semi static tests on larval *Danio rerio*. Results are representative of three independent experiments.

Replicate	PAH					
	B[*a*]P	Ph	1P-Ph	1M-Ph	4P-Ph	4M-Ph
I	4.91	4.97	50.00	50.00	20.20	4.23
II	4.90	4.93	25.58	43.75	19.97	2.83
III	5.68	4.73	45.15	50.00	32.77	2.28
Mean	5.16	4.88	40.24	47.92	24.31	3.11
S.E.	0.45	0.13	12.93	3.61	7.33	1.01

Sublethal adverse effects of the PAH compounds were manifested in the zebrafish larvae by various morphological abnormalities (Figure 2). Whereas no body deformities could be observed in control fish (Figure 2A), the exposed zebrafish showed malformations of the heart, yolk sac, tail and/or notochord (Figure 2B-D). The predominant effect observed after PAHs exposure was pericardial edema (Figure 2B, C). The larvae treated with 1M-Ph and 4M-Ph displayed also dorsal curvature (Figure 2D).

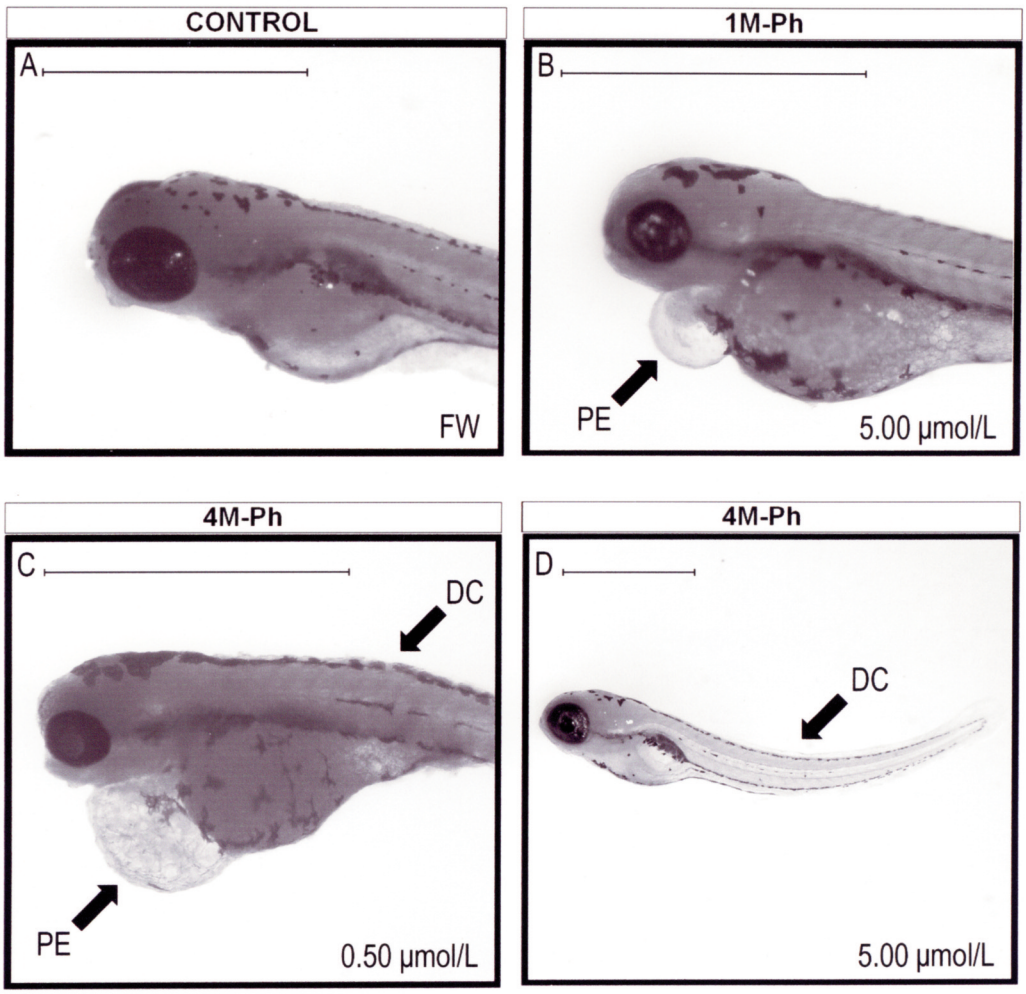

Figure 2. Control (A) and exposed (B, C, D) zebrafish larvae (fixed in 4% paraformaldehyde) with abnormal developmental morphological changes. 96 hpf zebrafish larvae were exposed to 1M-Ph for 24h (B) and 4M-Ph for 24h (C) and for 72h (D) dissolved with DMSO (0.1%) in *fish water* (FW; negative control). Representative sublethal toxic effects, as observed under stereo microscope. Pericardial edema (PE) and dorsal curvature (DC) are indicated by arrows. Scale bar represents 1mm.

To gain a more detailed understanding on the adverse molecular effects caused by the PAH compounds, the mRNA levels of three genes, *cyp1a*, *cyp1b1*, and *vtg* were examined, after 48h of treatment with a concentration of 0.50μmol·l^{-1} (Figure 3). For the two CYP genes involved in PAH biotransformation pathways, transcript quantification performed on the pools of zebrafish larvae revealed significant mRNA accumulation [ER_{cyp1a}=52.22 ($p<0.05$) and ER_{cyp1b1}=26.11 ($p<0.001$)] only in the fish exposed to B[*a*]P. On the other hand, vtg gene transcription in zebrafish larvae after the exposure to two PAH compounds, Ph and 4P-Ph was significantly reduced [ER_{vtg}=0.41 ($p<0.001$) and ER_{vtg}=0.43 ($p<0.05$), respectively].

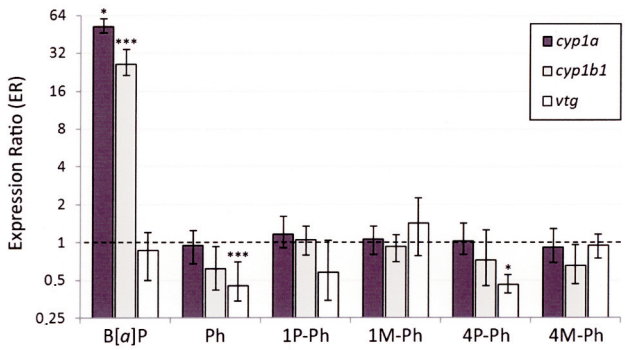

Figure 3. mRNA expression of *cyp1a, cyp1b1,* vtg genes in zebrafish larvae exposed for 48h to 0.50μmol·l^{-1} of B[*a*]P, Ph, 1P-Ph, 1M-Ph, 4P-Ph and 4M-Ph. Bars represent mean values of expression ratios (ER) with their respective standard errors of the mean (S.E.; n=3), normalized by β-actin as an endogenous reference, and relative to control group (ER=1.00; dashed line). The ER values calculated by REST© indicate significant difference between the control and treated group of fish (*, $p<0.05$; ***, $p<0.001$).

DISCUSSION

It is known that phenanthrene and its alkyl derivatives, such as retene, may cause a variety of effects in early life-stages of fish and chronic exposures to the PAHs often lead to deformities, edemas and embryo mortality (Incardona et al. 2006, Scott et al. 2010). However, knowledge concerning biological effects of phenylphentanthrenes is limited.

This study reports on the adverse effects of two methyl- and two phenyl- derivatives of phenanthrene on zebrafish larvae, which are compared to those of negative control sample and two positive controls (benzo[*a*]pyrene or phenanthrene). The six examined PAH compounds had clearly different effects on the larval survival, yielding different individual NEC estimates (Table 2). Based on the estimated NEC values, the results from the acute toxicity test may suggest the following order of PAH toxicity on *Danio rerio* larvae:

4M-Ph>Ph~B[*a*]P>4P-Ph~1P-Ph>1M-Ph.

Thus, in the present study we did not only confirm previous findings that B[*a*]P, Ph or its alkyl derivatives may elicit lethal effects to the fish larvae (e.g. Hawkins et al. 2002; White 1999), but we also showed moderate toxicity of phenyl phenanthrene derivatives to zebrafish. Being cautious in making comparisons of different toxicity parameters, the mean NEC estimates for Ph (NEC=4.88±0.13μmol·l^{-1}) obtained in this work may suggest that zebrafish larvae are more tolerant to this PAH compound than turbot larvae for which acute toxicity was found at much lower concentration (0.29μmol·l^{-1}; Mhadhbi et al. 2010). The results also suggest that fish may be more tolerant to 1M-Ph than invertebrates. For example, 1M-Ph LC_{50} for polychaete worm (*Nereis arenaceodentata*) was ~1.56μmol·l^{-1} (PAN Pesticide Database 2010), which was much lower concentration than the mean NEC estimate obtained in this study for zebrafish larvae (NEC=47.92±3.61μmol·l^{-1}).

Furthermore, the observations on PAH toxicity made in the study, may suggest that there is a compound structure-activity relationship with regard to: i) the alkylation position of the phenanthrene molecule, and ii) the presence of phenyl substituents in the molecule. These structural features may play a role in determining bioavailability of PAH compounds. It is known that unsubstituted phenanthrene could be toxic to fish, but only at high concentrations and long-term exposure. Depending on the position which substituents occupy on the parent compound, uptake and metabolism can be affected. For example 7-isopropyl-1-methyl-phenanthrene (retene) is more readily taken up by fish and its potency of CYP1A enzymes` induction is stronger than 1-methyl or unsubstituted phenanthrene (Basu et al. 2001; Hawkins et al. 2002). In the present study, the transposition of a methyl group from a position 1 to 4 in the parental, phenanthrene molecule (Figure 1), clearly led to the increased mortality of zebrafish larvae. Interestingly, the two methyl derivatives, when compared one to another, showed over 15-fold difference in the NEC estimate. On the other hand, the unsubstituted phenathrene structure was more toxic than its phenyl derivatives. Further research should focus on uncovering the structure-activity relationship and bioavailability of these and other phenanthrene derivatives, considering equally kinetic and biological dynamic effects of these compounds.

Structure activity relationship studies of alkyl-PAHs, identified a possible link between the toxicity of alkylphenanthrenes and their binding affinity to the aryl hydrocarbon receptor, AhR (Scott et al. 2010). AhR-binding alkylphenanthrenes produced dioxin-like toxicity in Japanese medaka (*Oryzias latipes*), which generally follows a rank order of potency in accordance with an increase of lipophilicity (Turcotte et al. 2011). In the present study, similar dioxin-like symptoms in larval zebrafish were observed upon treatment with either methyl- or phenyl- derivatives of phenanthrene. The abundance of various deformities in the zebrafish larvae prompted us to assess other biological effects of the PAH, by measuring mRNA expression of three genes, *cyp1a*,

cyp1b1, and *vtg* in the treated larvae. However, no significant modulation of gene expression was observed in the larvae for either genes, except for those exposed to B[*a*]P. This result is in general agreement with a recent report on molecular toxicity of a retene, for which an AhR-dependent, CYP1A-independent mechanism of toxicity was proposed (Scott et al. 2010). Further research should consider the use of histological and immunohistochemical analyses, directly investigating the involvement of CYP1A and AhR in the intoxication process.

Whereas the mRNA levels of genes involved in biotransformation pathways remained unaffected by either phenanthrene or its derivatives, mRNA for zebrafish vitellogenin was significantly reduced in two cases (Ph and 4P-Ph). *Vtg* levels in male and sexually immature fish are normally very low (Jin et al. 2008), and the biological meaning of the apparent reduction of *vtg* expression is unclear. Further research, analyzing more mRNA profiles and integrating cellular biology techniques may help identify mechanisms associated with toxicity of phenanthrene derivatives.

From environmental health perspective, the data obtained in the present study may complement environmental monitoring systems that utilize the embryonic zebrafish model as a rapid throughput bio-analytical tool for determining the toxicity of environmentally relevant complex mixtures (e.g. Hillwalker et al. 2010). As more and more structural and toxicological data for the various derivatives of phenanthrenes are described, the sensitivity of the bio-analytical tool for detecting spatially distinct toxicity in aquatic systems will increase.

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