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Influence of sediment composition on PAH toxicity using zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) embryo-larval assays

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Abstract : Due to hydrophobic and persistent properties, polycyclic aromatic hydrocarbons (PAHs) have a high capacity to accumulate in sediment. Sediment quality criteria, for the assessment of habitat quality and risk for aquatic life, include understanding the fate and effects of PAHs. In the context of European regulation (REACH and Water Framework Directive), the first objective was to assess the influence of sediment composition on the toxicity of two model PAHs, benzo[a]pyrene and fluoranthene using 10 day-zebrafish embryo-larval assay. This procedure was undertaken with an artificial sediment in order to limit natural sediment variability. A suitable sediment composition might be then validated for zebrafish and proposed in a new OECD guideline for chemicals testing. Second, a comparative study of toxicity responses from this exposure protocol was then performed using another OECD species, the Japanese medaka. The potential toxicity of both PAHs was assessed through lethal (e.g. survival, hatching success) and sublethal endpoints (e.g. abnormalities, PMR and EROD) measured at different developmental stages, adapted to the embryonic development time of both species. Regarding effects observed for both species, a suitable artificial sediment composition for PAH toxicity testing was set at 92.5 % dw silica of 0.2-0.5 mm grain size, 5 % dw kaolin clay without organic matter for zebrafish and 2.5 % dw blond peat in more only for Japanese medaka. PAH bioavailability and toxicity were highly dependent on the fraction of organic matter in sediment and of the K_{ow} coefficients of the tested compounds. The biological responses observed were also dependent of the species under consideration. Japanese medaka embryos appeared more robust than zebrafish embryos for understanding the toxicity of PAHs due to the longer exposure duration and its lower sensitivity of sediment physical properties.

Keywords: Fish embryo-larval assay, PAHs, artificial sediment, EROD, behavioral performance, teratogenicity.

Abbreviations: AhR: Aryl hydrocarbon Receptor; PAH: Polycyclic Aromatic Hydrocarbon; BaP: Benzo[a]pyrene; Fluo: Fluoranthene; hpf: hours post-fertilization; dpf: days post-fertilization; EROD: Ethoxyresorufin-*O*-deethylase; PMR: PhotoMotor Response.

1. Introduction

The aquatic environment continuously receives anthropogenic pollutants which impact the habitat quality of these ecosystems and can represent an important threat for aquatic life. Natural phenomena – tides, storms and bioturbation - (Hollert et al. 2000) and anthropogenic activities – trawling, dredging and wake waves - (Köthe 2003) can release sediment-linked pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs) into the water column, leading to acute exposure of aquatic organisms. PAHs constitute an important part of pervasive compounds and their emission is increasing with the development of human activities (van der Oost et al. 2003; Shen et al. 2013). The PAH persistence, behavior and distribution in the environment are mediated by their physico-chemical properties (aromaticity and molecular weight). Indeed, the presence of a high number of aromatic rings (=PAH of high molecular weight) dictates their strong hydrophobicity, while their solubility and volatility are reduced (Dabestani and Ivanov 1999). Due to these hydrophobic properties, most PAHs have a high capacity to sorb on particles and favor their accumulation in sediments which can result in high PAH concentrations in aquatic ecosystems. In Europe, PAH concentrations into the sediment can reach up to 50 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight (dw) in area relatively contaminated (Baumard et al. 1999; Benlahcen et al. 1997; Cachot et al. 2006; El Nemr et al. 2007). Sediments are therefore recognized to be a major source of contamination, causing a threat to organisms for which they are habitat and a food source (Ahlf et al. 2002; Wölz et al. 2009). Toxicity assessment of the PAHs is necessary to determine sediment quality values in order to assess their impact on aquatic life.

PAHs are present into the environment as mixture of sole PAH and often associated with others chemicals (metallic and/or organic). The exposure to these PAHs leads to a variety of effects in aquatic organisms. They can bind to the Aryl hydrocarbon Receptor (AhR) and subsequently induce the synthesis of cytochrome P4501A (CYP1A) (specially for the AhR agonists-PAH). The induction of CYP1A is commonly measured through the phase I biotransformation activity, ethoxyresorufin-*O*-deethylase (EROD) activity. The activation of these mechanisms (not necessarily related to the AhR), modulate a battery of genes involved in metabolism impacting negatively exposed-organisms at different biological level (Meador et al. 1995; van der Oost et al. 2003). PAHs are well-described in literature for their genotoxic, mutagenic and carcinogenic mode of action (Larcher et al. 2014; Patel et al. 2006; Wessel et al. 2010). They can also decrease the development, the growth and morphology, which alter the survival and population recruitment of aquatic organisms (Heintz et al. 2000; Incardona et al. 2011; Kerambrun et al. 2012; Scott et al. 2011).

In recent decades, a number of different ecotoxicity tests have been developed for risk assessment of industrial chemicals. However, the extensive use of these tests raises ethical concerns, in terms of the use of live animal and the degree of induced-pain and -suffering. In application of the 3Rs principle (Replace, Reduce and Refine), established by Russell and Burch (1959), and the REACH regulation (Registration, Evaluation, Authorization & Restriction of Chemicals) (Hengstler et al. 2006; Van der Jagt et al. 2004), appropriate methods need to be developed to reduce this number of organisms used (Belanger et al. 2010; Embry et al. 2010; Lammer et al. 2009; Scholz et al. 2013; Strähle et al. 2012). Taking into consideration the regulatory policy of animal welfare, the last thirty years have seen the development of *in vitro* ecotoxicity assays and moves to promote testing of primary cell cultures or fish cell lines. Although these assays are fast, reduce the equipment requirement and limit the use of live animals, they are not completely transposable to the natural environment quality assessment.

These assays can lead to a high variability of responses depending on the physiological status of donor fish and/or the preparation quality of the procedure (Castaño et al. 2003). The use of fish embryo-larval stages - as an experimental model - is one of the robust alternative methods proposed for chemical testing; mixing benefits of both *in vivo* (metabolism and relevance point of view) and *in vitro* models (experimental cost and model sensitivity point of view). These life stages (from the embryonic stage until the onset of exogenous feeding) are not also covered by the EU directive on the protection of animals used for scientific purposes (2010/63/EU).

Five main species have been recommended for chemical risk assessment: bluegill (*Lepomis macrochirus*), fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) (Lammer et al., 2009; OECD 2013a; 1998). In the present study, zebrafish and Japanese medaka were selected as experimental models. The use of zebrafish for toxicity testing offers multiple benefits (Hill et al. 2005; Spitsbergen and Kent 2003). First, they are easily bred in the laboratory and eggs are available in high numbers throughout the year. Second, transparency of both chorion and larvae allow at monitoring the morphological defects and at following all stages of development during exposure, which are well described and illustrated in the literature (Kimmel et al. 1995). Finally, zebrafish have a short embryonic development time (hatching between 48 and 72 hours post-fertilization (hpf) at 28 °C). Japanese medaka offers similar advantages to zebrafish embryos, except that the embryonic development time is longer (10 to 11 days post-fertilization (dpf) at 26 °C). This difference in development time means that medaka have an exposure duration which is longer than zebrafish. Zebrafish and Japanese medaka have shown reliable results in toxicity assessment using aqueous exposure (Fallahtafti et al. 2012; Fang et al. 2013; Hawliczek et al. 2012; Huang et al. 2012; Incardona et al. 2011). In the case of hydrophobic compounds, direct waterborne exposure is not suitable and therefore alternative tests had to be developed. This was achieved by exposing zebrafish and medaka embryos to natural sediment and spiked sediment (Barjhoux et al. 2012; ; Hollert et al. 2003; Höss et al. 2010; Kosmehl et al. 2006; Vicquelin et al. 2011). However, the toxic effects of PAHs are not easily transposable from one study to another due to the natural sediment composition variability (seasonal variability, sampling field, experimental variability), which play a fundamental role in the bioavailability of compounds. To cope with such problems, the use of artificial sediment provides some benefits compared to natural sediment and could be a possible solution to standardize sediment exposure protocols: i) absence of background contamination, ii) well-characterized composition, iii) reproducible composition of sediment over time, and iv) absence of indigenous biota (Burton 2002). In fact, one artificial sediment composition was standardized by the test guideline 218 of OECD (OECD 2004) for the toxicity test using freshwater dipteran *Chironomus* sp.. However, its composition is not suitable for fish embryo-larval assays because it contains a high clay concentration (20 % dw) which may clog chorion pore canals of egg by fine particles, reducing oxygen exchange and optimal embryos development.

In this context, the present study aimed to evaluate the influence of PAH-contaminated sediment composition on the zebrafish early life stages. First, a screening procedure with artificial sediment reference proposed for the 14-day medaka embryo-larval assays (Le Bihanic et al. 2014b) was used in order to evaluate the influence of different organic matter content on the toxicity of prototypic hydrophobic molecules, the pentacyclic PAH benzo[a]pyrene (BaP) and the 3-benzenic-ring PAH fluoranthene (Fluo) on zebrafish embryos and larvae. This objective will permit also to validate an artificial sediment composition for zebrafish for further research. These two PAHs, listed as priority substances by the European Water Framework Directive 2000/60/EC (EC 2000), were selected for its high adsorption capacities on particles and organic matter, because their effects on fish

development were demonstrated (Wassenberg and Di Giulio 2004) and they are common present in contaminated sites (Cachot et al. 2006). Exposures were performed using a procedure adapted from Le Bihanic et al. (2014) for the 10-day embryo-larval toxicity assay (including 96 hours of exposure, embryos and pro-larvae) with zebrafish (OECD 2013b; 1998). Toxic responses of exposed-embryos and larvae (from the newly fertilized egg to the end of the sac-fry stages) to both of these PAHs were examined through individual, morphological and behavioral endpoints during and post-exposure, for different sediment compositions. In addition, the induction of cytochrome P4501A (CYP1A) of Aryl hydrocarbon Receptor (AhR) agonist PAH was measured by the level of ethoxyresorufin-*O*-deethylase (EROD) activity in fish. The second step consisted in the comparison of the response sensitivity (threshold and intensity) of zebrafish (96-120 hpf) and Japanese medaka (10-14 dpf) embryos and larvae for different sediment compositions, using results from this study and previously published research (Le Bihanic et al. 2014b).

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (CAS No. 50-32-8), fluoranthene (CAS No. 206-44-0) and benzocaine (CAS No. 94-09-7) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Spiking PAH solutions were made in iso-octane (CAS No. 540-84-1, HPLC grade, Scharlau Barcelona, Spain) at $750 \mu\text{g}\cdot\text{L}^{-1}$. PAH spiked-sediment and chemical analysis were performed with dichloromethane (CAS No. 75-09-2) solvent from Biosolve (Valkenswaard, The Netherlands) and Acros Organics (Thermo Fisher Scientific, Geel, Belgium).

2.2. Preparation of spiked artificial sediment

The artificial sediment was prepared according to Le Bihanic et al. (2014). The reconstituted sediment was composed of sand measuring 0.2-0.5 mm (sieved from sand BB 0.2/2 h, Mios, France), 5 % kaolin clay (Merck, Darmstadt, Germany) and *Sphagnum* blond peat (Florentaise, St Mars du Désert, France). Three concentrations of peat (0, 2.5 and 5 %) were also used to evaluate their suitability for the zebrafish embryo-larval assay. Briefly, peat was dried 48 h and sieved (0.5 mm). Milli-Q water was added to the peat (12:1, v/v) in glass bottles and shaken for 48 h at 180 rpm at room temperature. Sand and 5 % kaolin clay were then mixed to humid peat, and shaken for 24 h. pH was adjusted to 6.5 with 10 % CaCO_3 solution. After seven days of stabilization (at room temperature) 1:4 v/v of milli-Q water was added to the sediment. After a 24 h equilibration period, supernatant water was removed and the sediment was dried at 105°C for 14 h. After cooling, the artificial sediment was spiked with two PAHs, BaP ($\text{Log } K_{ow}=6.04$) and Fluo ($\text{Log } K_{ow}=5.16$) at $10 \mu\text{g}\cdot\text{g}^{-1}$ dw sediment each, equivalent to 20-fold the environmental concentration based on the concentration measured in sediments from the upper Seine estuary (France) (Cachot et al. 2006). 60 mL of dichloromethane and PAH solution were mixed to 30 g of sediment in a 250 mL round-bottom glass flask. Then, solvent was evaporated with a rotary evaporator Rotavapor (IKA, Staufen, Germany) for 60 min (115 rpm, 45°C). The spiked sediment was stored overnight in the dark at room temperature under a fume hood to ensure complete residual solvent elimination. A solvent

control sediment (dichloromethane without PAH) was prepared in the same manner. An aliquot (5 g) of each spiked sediment was sampled for PAH chemical analysis.

2.3. PAH chemical analysis of artificial sediment

Sediment samples (a single sample per condition) were stored at -20 °C before chemical analysis. Fluoranthene-d10 and benzo[a]pyrene-d12 were used as internal deuterated standards for the quantification of PAHs. Briefly, organic compounds were extracted from 0.2 g of sediment using a microwave (10 min at 30 W) with dichloromethane (solvent) (Budzinski et al. 2000). Samples were then concentrated into 300 µL of isoctane using a Vacuum Evaporation System (Rapidvap Labconco, Kansas city, USA). Extracts were purified and fractionated on alumina and silica micro-columns, following the procedure described by Behar et al. (1989). Finally, these extracts were concentrated once more in isoctane and analyzed by gas chromatography coupled to mass spectrometry (GC/MS) as described by Baumard et al. (1998). Extraction efficiencies were equivalent on average to 84 %.

2.4. Zebrafish embryo-larval assays

Maintenance and egg production of zebrafish: wild type TU strain (Tübingen, Germany) zebrafish (*Danio rerio*) were maintained in communities in 10 L tanks under standard conditions in water obtained after a mix of reverse osmosis treated water and tap water, both being filtered beforehand with dust and charcoal filters, to obtain a pH of 7.5±0.5 and a conductivity of 300±50 µS.cm⁻¹. Racks aeration allowed an oxygen saturation ≥ 80 %. Water and room temperature were kept between 26 and 28 °C with a 14 h light/10 h dark photoperiod. Ammonia, nitrite and nitrate were monitored daily for 2 months then weekly and remained within recommended ranges (Lawrence 2007). The fish were fed twice a day with commercial granulates (INICIO Plus 0.5, BioMar, France) and an additional distribution of *Artemias* sp. nauplii (INVE, Belgium), occasionally supplemented with red sludge worms (Boschetto-Frozen fish food). Eggs were obtained by random pairwise mating of zebrafish. One adult male and one female were placed together in spawning boxes (AquaSchwarz, Germany) the evening before eggs were required. Spawning and fertilization took place within 30 min after the onset of light in the morning.

Zebrafish exposure (Fig. 1 Supplementary data): Two consecutive experiments were done, the first with BaP and second one with Fluo compound. Fertilized and normally developed eggs were selected at 8-cell stage (1h15 post-fertilization) using a dissecting microscope. The egg fertilization rate had to be ≥ 70 % and a mix of five spawns was used to avoid a potential spawn effect. Artificial water (E3) according to ISO 7346/3 (1996) was used as test medium (0.33 mM CaCl₂, 0.33 mM MgSO₄, 5 mM NaCl and 0.17 mM KCl). Exposure tests were carried out in plastic petri dish (35 mm diameter) with 3 g dw artificial sediment and 3 mL artificial water in triplicates as reported by Daouk (2011) and Vicquelin et al. (2011). 30 embryos were placed randomly on a 1 mm thermoformed Nytex® grid to avoid being buried in the sediment, and exposed for 96 hours in incubator at 28±0.5 °C, with the same photoperiod as the rearing room. During exposure, petri dishes were covered with a lid in order to prevent evaporation. At the end of exposure, larvae were transferred to new petri dishes containing only freshly-prepared E3 medium (30 mL) until 10 dpf for analysis. Larvae were not fed during the experiment.

Survival and hatching success: Embryonic and larvae survival were recorded daily until 10 dpf. Dead individuals were removed. The survival rate (SR) was estimated as: $SR = 100 * (SN_{t_x} / SN_{t_0})$, where SN_{t_x} and SN_{t_0} are the number of live individuals at times t_x (time of the measure, hpf) and t_0 (beginning of the experiment, 0 hpf). Similarly, hatched individuals were counted between 48 and 72 hpf. At the end of exposure (96 hpf), hatching success (HS) was calculated as: $HS = 100 * (HI_{t_{96h}} / SN_{t_{96h}})$, where $HI_{t_{96h}}$ is the number of hatched and living larvae. SR and HS were expressed as percentages.

Biometric measurements and abnormalities monitoring: At the end of exposure (96 hpf), larvae (35-39 per condition) were anesthetized with benzocaine ($16 \mu\text{g.L}^{-1}$). Larvae were immobilized in left lateral view with 3 % methylcellulose gel (dissolved in E3 medium) under dissecting microscope (Olympus SZX9, 10x). Then, larvae were photographed using a DMK 31AU03 camera and IC Capture 2.2 software (both The Imaging Sources, Germany). From these pictures, head length (from terminal point of lower jaw to rear operculum), standard body length (from terminal point of lower jaw to the end of tail without caudal fin) and yolk sac area (including swim bladder area) were measured with ImageJ software (Schneider et al. 2012). Furthermore, morphological abnormalities were recorded individually based on the sublethal endpoints as described in Lammer et al. (2009). Abnormalities scored were: 1- Oedema formation (brain, pericardia, yolk sac); 2- Skeletal deformities (scoliosis, lordosis, tail bud deformities); 3- Craniofacial deformities (jaw, development of eyes, head deformities); 4- Cardiac deformities (anemia, hemorrhage, atrium/ventricle size, blood circulation heart position); 5- Yolk sac malabsorption. Abnormalities were analyzed with a scoring system from 0 to 3 according to their occurrence and severity: (0) healthy larvae, (1) one abnormality or mildly-affected, (2) two abnormalities or moderately affected and (3) three or more abnormalities or severely affected larvae. In addition, general developmental retardation was noted, when the larvae development was delayed (time point of view) compare to normal development. Results were expressed as a percentage of all surviving larvae.

Behavioral test by monitoring of PhotoMotor Response: behavioral tests were conducted to monitor photomotor response (PMR), following the procedure described by Péan et al. (2013) with slight modifications. The day before analysis, 4 dpf larvae were acclimated individually in a 24 well-plate (Krystal 24, transparent bottom and opaque walls) with 2 mL of E3 medium in a climate chamber ($28 \pm 0.5 \text{ }^\circ\text{C}$; 14h light/10 h dark photoperiod). Well-plates were covered with a lid to avoid evaporation and were transferred two hours before video tracking in an analysis room ($28 \pm 0.5 \text{ }^\circ\text{C}$ in light) the following day to perform behavioral analysis. Behavioral analyses were performed during a period of stable activity (between 1 and 6 pm) for zebrafish according to MacPhail et al. (2009). Larvae were filmed in a lightproof and temperature-controlled box with a DMK31AU03 camera (The Imaging Sources, Germany) and lens Fujinon (1.4-12.5 mm) and IC Capture 2.2 software (The Imaging Sources, Germany). An infrared floor in the box was used to record in both light and dark conditions. Larvae were placed in the box five minutes before recording their behavior in order to acclimatize them to light. Recording covered three periods: 5 min *light on* (1), 5 min *light off* and 5 min *light on* (2). Video analyses were performed with Ethovision 8.5 software (Noldus, The Netherlands). The behavioral performances of larvae (30 per PAH treatments) were assessed by measuring the distance moved (cm) during the three periods of the light/dark/light

challenge. The distance moved is calculated by the difference between the center point of the larvae from two consecutive X-Y coordinates summed over a 5-min period.

In vivo EROD activity: the CYP1A activation was measured via the EROD activity analysis, according to the *in vivo* assays of Carney et al. (2004) and Otte et al. (2010) with slight modifications. Briefly, CYP1A metabolism converts ethoxyresorufin to resorufin, which was observed and quantified in the gastrointestinal cavity of 96 hpf larvae via fluorescence microscopy. Individual larvae (15 larvae per treatment) from each replicate treatment were incubated in $21 \mu\text{g.L}^{-1}$ 7-ethoxyresorufin (Sigma-Aldrich, St Quentin Fallavier, France) dissolved in 1 mL of E3 for 5 hours. Larvae were then anesthetized with $16 \mu\text{g.L}^{-1}$ benzocaine and mounted in 3 % methylcellulose to immobilize them (left lateral view) for observation of the yolk sac. The entire procedure was performed in light-protected conditions and at 28 ± 0.5 °C. Fluorescence of the resorufin was examined under a fluorescence microscope (Olympus BX41, 100x) equipped with a Rhodamin red filter (excitation/emission: 560 nm/580 nm). The yolk sac of larvae was photographed with a camera (DMK31AU03) and IC Capture 2.2 software (both The Imaging Sources, Germany). Image analysis was done with HeatMap Histogram *plugin* (Péan S., <http://www.samuelpean.com/heatmap-histogram/>) from ImageJ software (Schneider et al. 2012). For EROD analysis, a control treatment composed of 15 non-sediment exposed larvae was analyzed in addition to establish the background. Results were expressed in integrated density of pixels (arbitrary unit).

2.5. *Medaka embryo-larval assay*

Japanese medaka exposure (Fig.1 Supplementary data): The fish embryo-larval toxicity test was performed as described in Le Bihanic et al. (2014). The BaP and Fluo exposures were performed in same time and with the same batch of eggs. 1 dpf medaka embryos (CAB strain providing GIS-Amagen (INRA), Jouy-en-Josas, France) were exposed using the same procedure as for zebrafish embryos with some modifications adapted to the longer embryonic development time. Embryos were exposed throughout embryonic development until the hatching peak (11 dpf) at 26 ± 0.3 °C with a 12h light/12h dark photoperiod. During the exposure, artificial water was renewed daily. Egg Rearing Solution ERS was used as artificial water (85.6 mM NaCl, 0.4 mM KCl, 0.4 mM CaCl_2 and 1.4 mM MgSO_4). After hatching, larvae were observed and transferred to a new petri dish containing a mix of osmose water and tap water. The experiment was halted three days post hatching peak.

Behavioral test by monitoring of PMR: PMRs were measured in similar conditions to zebrafish assay with slight adaptations for Japanese medaka. 14 dpf larvae were acclimated individually for 2 hours in a 48 well-plate with 500 μl of ERS in the dark inside the Daniovision chamber (Noldus, The Netherlands) at 23 °C. 15 larvae per treatment were filmed with an IR digital video camera (Ikegami Electronics, Neuss, Germany) during four periods of 10 min : 10 min *light off* (1), 10 min *light on* (1), 10 min *light off* (2) and 10 min *light on* (2). The analysis time was increased, and a dark period was added at the start of the challenge due to the higher swimming activity in medaka compared to zebrafish. This supplementary phase enabled their swimming to be stabilized so as not to skew the challenge. The video analyses were then performed with Ethovision 9.0 software (Noldus, The Netherlands). As for zebrafish assay, the behavioral performances of larvae were assessed by measuring the distance moved (cm) during the four periods of the dark/light/dark/light challenge.

2.6. Statistical analysis

Statistical analyses were performed using Statistica 9.0 software (StatSoft, USA). A one-way ANOVA was conducted for each variable and treatment to assess differences between experimental replicates. If no difference between replicates was detected, the three replicates were considered as a homogenous group. Normality and homoscedasticity of the data were verified using Shapiro-Wilk and Levene tests respectively. For percent survival, hatching and abnormalities data, Fisher tests were performed. Student's t tests were performed for the biometric analysis and the PMR in order to assess the effects of the different exposure treatment (BaP, Fluo) with their respective solvent control. EROD data were analyzed using a one-way ANOVA supplemented by a *post-hoc* Tukey test. When the parametric assumptions were not respected, the non-parametric Mann-Whitney and Kruskal-Wallis tests were conducted. The behavioral data for Japanese medaka assay were analyzed with an ANOVA. Results were expressed as mean±SEM. The statistical significance threshold was fixed at $p=0.05$.

3. Results

3.1. Chemical analysis of spiked sediments

Spiking efficiencies of PAH on sediment ranged from 17 to 63 % depending on peat concentrations and the compound analyzed (Table 1). For BaP, they were of 17, 18 and 20 % for 0, 2.5 and 5 % peat conditions respectively. Fluo spiking efficiency was slightly higher for no-peat sediment (63 %) than for 2.5 % (59 %) and 5 % peat (44 %).

3.2. Zebrafish embryo-larval assay

Survival and hatching success: the survival and hatching success of zebrafish embryos during BaP and Fluo exposures are shown in Table 1. Whatever the peat proportion, the BaP tested concentration did not induce lethal effects on embryos and larvae, while that was the case for the Fluo exposure. The survival rate for BaP-exposed fish ranged from 89.9 to 95.6 % at the end of the exposure (96 hpf) and from 88.9 to 92.2 % at 10 dpf. In contrast, 0 % and 5 % Fluo-spiked sediments induced a significant decrease in survival compared to the solvent control group with 54.6 % and 83.5 % respectively (Fisher, $p<0.01$). For 2.5 % peat Fluo-spiked sediment, no significant larval mortality was observed with 95.6 % and 96.7 % for the solvent and Fluo survival rate respectively (Fisher, $p>0.05$).

BaP did not induce a hatching delay for all three sediment peat contents (success varied from 95.2 to 100 %). In contrast, hatching success was significantly reduced by Fluo in 0 and 5 % peat sediment as compared to respective control group (decreasing of 10.4 % and 13.5 %, $p=0.005$ and $p=0.004$ respectively).

Biometric measurements: biometric measurements of 96 hpf larvae exposed to BaP or Fluo for each peat content are shown in Tables 2 and 3, respectively. BaP exposure did not induce modifications of larval biometric parameters whatever the sediment peat contents (Table 2). For 0 % peat content (Table 3), standard body length

and ratio head/standard length were significantly reduced in Fluo-exposed larvae (1.1 fold smaller as compared to control larvae, Student's t, $p < 0.001$ each). In contrast the yolk sac area and yolk sac/whole larval area ratio were significantly increased by 1.3 and 1.4 fold respectively (Student's t, $p < 0.001$). For 2.5 % peat content, no biometric modifications were observed between treatments except for the yolk/whole larval area ratio in Fluo-exposed larvae (1.04 fold) that were significantly increased (Student's t, $p = 0.04$). In the case of 5 % peat content, larvae exposed to Fluo had a yolk sac/whole larval area ratio 1.1 fold larger than control larvae (Student's t, $p = 0.04$). These larvae also showed a significant reduction in standard body length (Student's t, $p = 0.004$).

Morphological abnormalities: the percentage of abnormal individuals at 96 hpf, and the different abnormalities and severity are also summarized in Tables 2 and 3 for BaP for Fluo treatments, respectively. Peat concentration in each treatment did not induce significant morphological abnormalities compared to the respective control (Fisher, $p = 0.61$, $p = 0.73$ and $p = 1.0$ for 0 %, 2.5 % and 5 % respectively). No developmental time retardation was observed in BaP exposed larvae whatever the peat concentration. In the absence of peat, 56 % of Fluo-exposed larvae exhibited a delayed development time and 100 % of larvae at the highest peat concentration. 49 % of larvae solvent of the control exposed to sediment with 5 % peat concentration was also retarded time point of view.

In vivo EROD activity: EROD activities in 96 hpf zebrafish larvae exposed to BaP, showed a significant induction over solvent control and control treatment for 0 and 5 % peat (Fig. 1A). EROD activity increased significantly from 2.4 to 1.6-fold compared to solvent control, for 0 and 5 % peat respectively (ANOVA, $p < 0.001$). No increase was observed for 2.5 % peat (ANOVA, $p = 0.12$). For EROD activity measured in larvae exposed to Fluo-spiked sediment with 2.5 % peat (Fig. 1B), a significant 2.4-fold induction was observed over the solvent control (ANOVA, $p = 0.03$) but not over the control treatment (ANOVA, $p = 0.58$). No significant difference in EROD activity was, however, recorded between both controls (ANOVA, $p = 0.33$). Similarly, no significant difference was observed in larvae exposed to Fluo spiked-sediment with 0 % and 5 % peat (ANOVA, $p = 0.22$ and KW, $p = 0.05$ respectively).

3.3. Photomotor behavioral response of larvae

Zebrafish larvae PMR: PMR of zebrafish larvae (at 120 hpf) exposed to BaP in different conditions of peat are shown in Fig. 2. The response patterns of larvae were similar for each peat condition showing an increase in the distance moved during the stress period (*light off*). BaP larvae swimming activity was significantly reduced compared to the control treatment only in *light off* period for 0 % peat condition (Fig. 2A). Fish from all treatments 2.5 % and 5 % showed a similar response pattern to the challenge and no significant differences in swimming activity were observed (Student's t, $p > 0.05$) (Fig. 2B, 2C). PMR of zebrafish larvae (at 120 hpf) exposed to Fluo in different conditions of peat are shown in Fig. 3. Swimming activity was significantly decreased whatever the light/dark periods for the condition without peat (Fig. 3A). No significant differences were observed between Fluo and solvent treatments for 2.5 % and 5 % peat conditions (Student's t, $p > 0.05$) (Fig. 3B, 3C).

Japanese medaka larvae PMR: PMR of Japanese medaka (14 dpf) exposed to BaP and Fluo are shown in Fig. 4. The first dark period is performed to stabilize the swimming activity of larvae. The response patterns of larvae were similar for each peat condition showing an increased swimming activity during *light off* (2) period. The larvae exposed to BaP for 0 % peat condition, swam significantly greater distances than solvent control larvae (Fig. 4A). The swimming activity of larvae exposed to Fluo for 0 % peat condition was not significantly different compared to solvent control (ANOVA, $p > 0.05$). Regarding 2.5 % peat condition, whatever PAH considered, no significant differences in swimming activity were observed (ANOVA, $p > 0.05$). PMR of larvae in 5 % peat condition showed an opposite response pattern to the larvae in condition without peat. The distance moved by BaP-exposed larvae was only significantly lower in the first light period compared to solvent control larvae. Fluo-exposed larvae swam significantly 1.9-fold less for *light on* (1) and 1.8-fold less for *light off* (2) periods than solvent control larvae.

4. Discussion

In recent decades, a number of different ecotoxicity tests have been established demonstrating the robustness of fish embryo-larval assays to reflect the toxicity of organic pollutants. Zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) have shown reliable results in sediment contact tests using natural and spiked sediment (Barjhoux et al. 2012; Hollert et al. 2003; Vicquelin et al. 2011). The responses of zebrafish (this study) and Japanese medaka (Le Bihanic et al. 2014) to two PAH models, BaP and Fluo, were recorded in order to define an artificial sediment while assessing the influence on the sediment composition (mainly organic matter variation) on the toxicity of these both molecules. Using similar PAH-spiked sediment, exposure routes and toxicity endpoints at the molecular (EROD activity), morphological and behavioral levels, it was shown that toxicity thresholds and the intensity of responses of both these species were different.

Our results on zebrafish as well as medaka embryo-larval assays, suggest that PAH bioavailability is reduced by increasing the content of organic matter in the artificial sediment. This finding is consistent with field studies demonstrating that the bioavailability of PAH is highly dependent on the concentration of organic matter and their interaction, which can reduce the freely dissolved PAH and their bioaccumulation in organisms (Fanget et al. 2002; Guasch et al. 2012). Furthermore, the spiking efficiencies of BaP in the prepared sediments also support this postulation, but not for Fluo. Indeed, a reduction of Fluo-spiking efficiency was observed with increasing peat concentration. The high sorption capacity of compounds to the organic matter is linked to their molecular surface area (related to their molecular weight), which manage their high hydrophobicity natures of both BaP ($\text{Log } K_{ow}=6.04$) and Fluo ($\text{Log } K_{ow}=5.16$) and their partitioning and bioavailability. Humic substances act as a trap for hydrophobic molecules which in turn reduce both their dissolved concentrations in the water column and their subsequent bioaccumulation in living organisms (Akkanen and Kukkonen 2003; Akkanen et al. 2005; Haitzer et al. 1999; Laor et al. 1998; Mayer et al. 2007).

BaP exposure did not induce mortality, hatching failure or delayed hatching in zebrafish embryo-larval assays. Moreover, BaP did not lead to teratogenic effects (Table 3). Somewhat different results have been reported for BaP-exposed medaka embryos (Le Bihanic et al. 2014b). A low teratogenic effect was observed in medaka, but contrary to zebrafish, BaP was shown to delay hatching. This compound is not known to directly induce developmental effects. In contrast with BaP-exposed zebrafish larvae, significant biometric developmental

defects were observed in Fluo-exposed embryos. These effects are reflected by an increasing mortality, a lack of embryo hatching and alterations in growth and development (Tables 2 and 3). Although morphological abnormalities are currently used as sensitive endpoints in embryo-larval assay (Lammer et al. 2009; Nagel 2002), neither oedemas, axial skeleton, craniofacial nor cardiovascular abnormalities were significantly recorded during Fluo exposure. Furthermore, the decrease in larval standard length and the increase in their yolk sac area caused by Fluo exposure in the absence of peat attest to a developmental delay.

Zebrafish larval behavioral assay was a robust endpoint in toxicity testing (Ali et al. 2012; Bilotta et al. 2002; MacPhail et al. 2009). The assessment of the developmental neurotoxic effects of various compounds can be analyzed through their swimming capacity, stress-regulating systems and motor responses (perception and reaction) to stimuli (Champagne et al. 2010; Drapeau et al. 2002; de Esch et al. 2012; Linney et al. 2004). Studies regarding behavioral effects in fish have been extensively reported using psychotropic substances (Ali et al. 2012; Cowden et al. 2012; Kokel et al. 2010; Padilla et al. 2011; Rihel et al. 2010) but poorly investigated for other pollutants. In the present study, a significant decrease of PMR in zebrafish was observed for the highest concentration of Fluo in the absence of peat. Moreover for each peat concentration tested, the zebrafish larvae presented a similar behavioral pattern (Fig. 3). The first light period (5 min in duration, *light on (1)*) reflected basal activity which was followed by an excitation phase characterized by an increase in locomotor activity during the sudden darkness period. Then, a return to the basal activity period corresponding to a recovery phase was observed (*light on (2)*). This pattern of response is consistent with previous results reported in the literature (Kokel et al. 2010; MacPhail et al. 2009; Padilla et al. 2011). Concerning Japanese medaka, the potential toxicity of PAH on larval behavior assays has been only recently analyzed and only for PAHs in mixture (Le Bihanic et al. 2014a). BaP led to an increase in PMR in the absence of peat which was suppressed in the highest tested peat concentration. In contrast, Fluo decreased in PMR in this later exposure condition. The Japanese medaka PMR pattern was similar to that for zebrafish. Only a few studies have examined the impact of organic pollutant on the fish PMR. Using a similar approach, Péan et al. (2013) reported a stimulation of this PMR following PCB contamination transferred to offspring zebrafish larvae. Furthermore, changes in zebrafish swimming rates, depending on PBDE congeners, have been shown by Usenko et al. (2011). Additionally, a PAH lethargic effect on hunting behavior and visual acuity was demonstrated by Gonçalves et al. (2008) in juveniles gilthead seabream (*Sparus aurata*). Even more poorly explored, the behavioral effects of organic pollutants clearly merit further research.

At the molecular level, significant induction of enzymatic EROD activity in zebrafish assays only appeared in the absence of peat and at the highest peat concentration during BaP exposure (Fig. 1A). The EROD activity modulation by BaP is in general agreement with AhR agonist properties of this compound. The ability of BaP to induce EROD activity (indicator of phase I biotransformation activities) through the AhR2 pathway has been established in marine fish (Au et al. 2004; Barron et al. 2004) and freshwater fish (Bols et al. 1999; Costa et al. 2011; Incardona et al. 2011; Noury et al. 2006). Regarding the results of Fluo exposure, no significant induction of EROD activity was observed compared to the control treatment. A mild induction of EROD activity was observed for 2.5 % peat condition compared to 2.5 % peat solvent control. EROD activity in this control is rather low compared to other control conditions; therefore induction of EROD in 2.5 % peat condition may not be meaningful. The absence (or weak) induction of EROD by Fluo is in agreement with the inhibitory activity of

this PAH on cytochrome P4501A enzyme (Van Tiem and Di Giulio 2011; Willett et al. 2001; Willett et al. 1998).

The toxicity of both hydrophobic compounds was consistent with their toxic properties. BaP is known as having a mutagenic mode of action for inducing tumor formation, and is thought to require metabolic activation to become carcinogenic (Carlson et al. 2004; Costa et al. 2011; Wessel et al. 2010). Furthermore, some studies have demonstrated the cardiotoxic action of AhR-mediated compounds such as the BaP (Huang et al. 2012; Incardona et al. 2006; Matson et al. 2008). Our results did not corroborate with previous studies. However, we have to be cautious about these conclusions because we used only crude analysis of cardiotoxicity. Even if relatively few studies focus the toxicity of Fluo, this compound has mutagenic mode of action rather inducing teratogenic defects (Vicquelin 2011; Wessel et al. 2012). When we compare the findings from the experiment on zebrafish and medaka, it appears that the toxicity of PAHs is dependent on their bioavailability, directly related to their adsorption ability on particles of peat. In sediment, the 5-ring BaP appeared less bioavailable than the 3-benzenic rings Fluo for accumulation in fish. These findings are consistent with the studies of Varanasi et al. (1985) and Djomo et al. (1996) which demonstrated that the uptake rate of 4,5-ring PAHs are lower than those of 2,3-ring PAHs. Indeed, in the absence of peat, Fluo may be more labile and consequently more easily uptaken by embryos. Fluo may have passed passively through the chorion, and led to adverse effects on embryos. Indeed, lethal effects were reported in zebrafish and a high proportion of abnormalities were observed in medaka. The bioavailability pattern of Fluo for zebrafish and medaka embryos was somewhat different for 2.5 % and 5 % peat. Particles of blond peat were seen to be fixed to the surface of clay particles and to limit PAHs accessibility to binding sites, decreasing exchanges and PAHs metabolism (Fanget et al. 2002). However, following Fluo exposure, important biometric defects and developmental time retardation were observed in zebrafish at the highest concentration of peat (5 %). Because of the high proportion of peat, PAHs on binding sites could be saturated and would be then slightly less available for embryos than in condition without peat not leading to lethal effects. All these results proved that zebrafish seem to be more sensitive to the physical properties of sediment compared to medaka. This sediment tended to clog egg pores, might limit optimal gas exchange and therefore alter embryonic development.

Taking into consideration the results of both embryo-larval toxicity testing with zebrafish and medaka (Le Bihanic et al. 2014b), an artificial sediment without peat was suitable for zebrafish and with 2.5 % peat for medaka. Similar biological responses of zebrafish and medaka larvae were reported after exposure to BaP for the sediment containing 0 % and 5 % peat (Table 4), leading to any change in survival, hatching kinetic and morphology. At individual level, a modification of the behavioral response were noted for the both species (lethargy or hyperactivity), showing a potential neurotoxic effect of BaP. These effects were also linked with a metabolic activation of detoxification process for the zebrafish. An opposite toxicity response were observed at the intermediate concentration of peat (2.5 %) between the both species. BaP appeared more available for medaka, leading to hatching kinetic and morphometric alterations, whereas no disrupts and metabolic activation were recorded for zebrafish larvae. The biological response of Japanese medaka were in agreement with previous studies (Vicquelin 2011; Wassenberg and Di Giulio 2004). Regarding Fluo exposures, completely opposite toxicity responses were also reported in both species for each artificial sediment tested (Table 5). Acute toxicity effects of Fluo were recorded in zebrafish at 0 % and 5 % peat content compared to Japanese medaka. These effects were expressed by embryo-larval mortality, hatching, morphometric alterations and a significant

lethargic activity of fish. The intensity of effects was more representative of Fluo intrinsic toxicity at 0 % than 5 % peat due to higher bioavailability for zebrafish. In contrast, the embryotoxicity was more pronounced in medaka at 2.5 % peat, which is in total agreement with individual and population-based levels recorded after sediment contact exposures in previous study for similar species (Vicquelin 2011).

The primary purpose of this experiment was to develop an artificial sediment resembling a composition of natural sediment in order to standardize sediment exposure methods. Regarding all results for both species with respect to ethical requirements (3R principles and early developmental stages guidelines), Medaka embryo-larval assay with sediment contact appears to be more robust than zebrafish embryo-larval assay for revealing BaP and Fluo toxic effects and more resilient to the physico-chemical properties of sediment. This higher degree of sensitivity of zebrafish could be due to a higher porosity of chorion and therefore the higher diffusion of compounds (see the section on acute effects). Using a concentration set to 2.5 % peat, the degree of sensitivity of the medaka would be mainly due to its sensitivity to PAHs and to a longer exposure duration during the critical embryonic developmental period, as well as due to the metabolic activity. Indeed, the embryonic phase in medaka at 26 °C ranges from 10 to 11 days compared to 2 days in zebrafish (Kimmel et al. 1995; Padilla et al. 2009). In this context, our findings support the premise that medaka would constitute a better model for testing the toxicity of hydrophobic chemicals (such as PAHs) testing than zebrafish, although zebrafish assay has been demonstrated to be a valuable alternative when short-time exposures are targeted. The use of finer markers representative of the early toxic actions of hydrophobic compounds, in addition to conventional phenotypic endpoints, would appear to be essential in predicting their toxicity (Voelker et al. 2007) as it will increase the sensitivity and relevance of biological responses. In the absence of this information, molecule or treatment safety could be over or underestimated.

5. Conclusion

As a complement to previous research into Japanese medaka by Le Bihanic et al. (2014), our study validated the use of artificial sediment for developmental toxicity testing using fish embryo-larval assay for Japanese medaka. Suitable artificial sediment comprises 92.5 % dw of 0.2-0.5 mm silica, 5 % dw kaolin clay and 2.5 % dw *Sphagnum* blond peat. This composition was not suitable for zebrafish for which responses were more sensitive to sediment without organic matter. Consequently, this suggests that a common protocol cannot be proposed for these two species from an OECD guidelines point of view. Furthermore, this study demonstrated that two physiologically-close fish species exhibited differences in their ability to reflect the relative developmental toxicities of hydrophobic organic pollutants. These differences might be explained by differences in their developmental kinetics (leading to a different exposure duration), 2) in their adsorption/metabolisation capacities and cell damage repair abilities. It also important to take accounts the influence of the sediment composition on the bioavailability and toxicity of chemicals, to better comparison of sediment toxicity tests. We elicit concerns about the real robustness of zebrafish in toxicity testing using sediment contact exposure. Japanese medaka appear better suited to toxicity assessment of hydrophobic compounds such as PAHs. Finally, to avoid any misinterpretation regarding the risk assessment to human or environment health, we recommend that several OECD fish species should be systematically considered.

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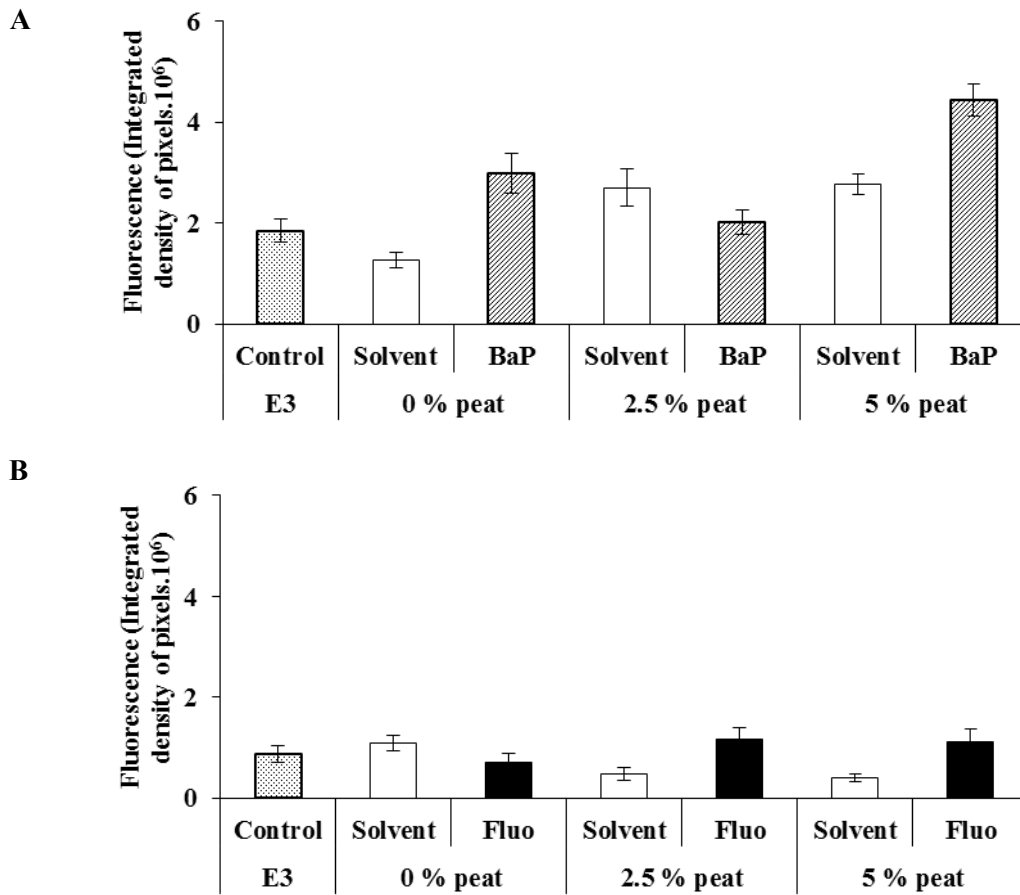


Figure 1. *In vivo* measurement of EROD activity in 96 hpf zebrafish larvae exposed to BaP (A) and Fluo (B) for different peat sediment contents. All data are presented as mean of integrated density of pixels \pm SEM, n=15 per treatment. Asterisks indicate significant differences with control treatment and Dark traits between others treatments (ANOVA, * $p < 0.05$ and *** $p < 0.001$)

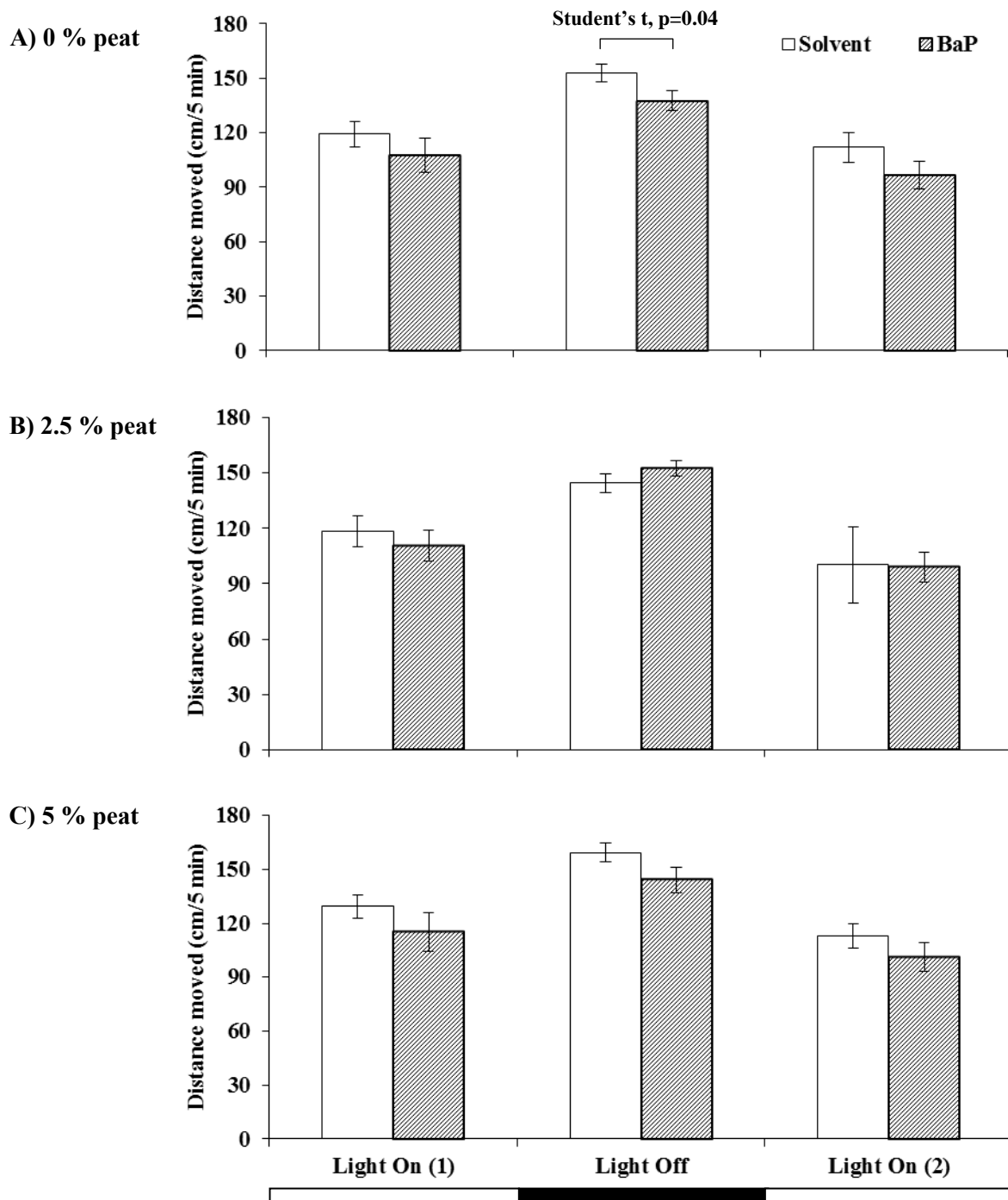


Figure 2. PMR measured in 120 hpf zebrafish larvae exposed to BaP. The locomotor activity of larvae in each 5-min periods of light/dark challenge was measured for different peat sediment contents: 0 % peat (A), 2.5 % peat (B), 5 % peat (C). All data are presented as Mean±SEM, n=30 per treatment. Dark traits indicate significant differences with respective solvent control (Homogeneous triplicates, Student's t, $p < 0.05$)

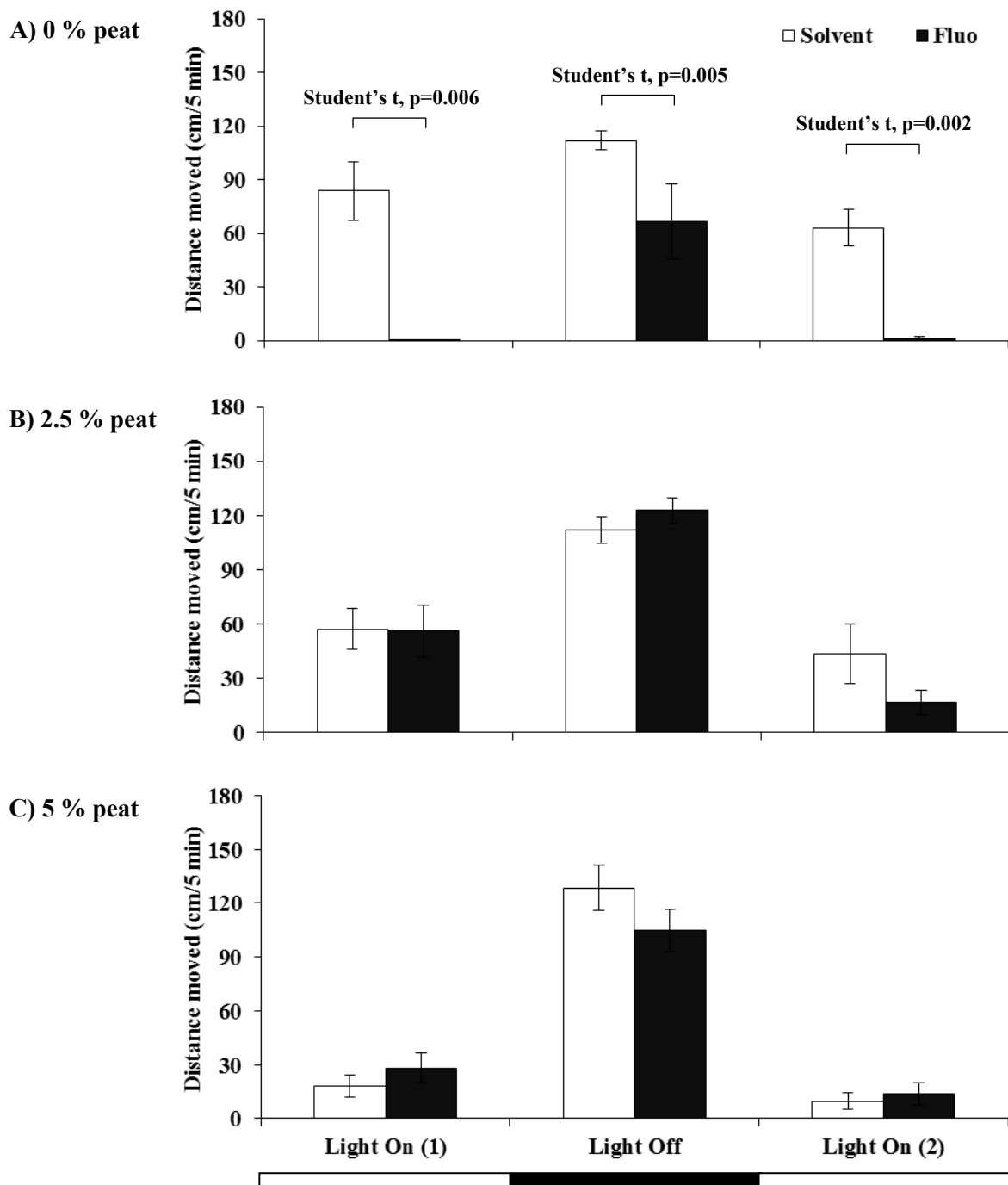


Figure 3. PMR measured in 120 hpf zebrafish larvae exposed to Fluo. The locomotor activity of larvae in each 5-min periods of light/dark challenge was measured for different peat sediment contents: 0 % peat (A), 2.5 % peat (B) and 5 % peat (C). All data are presented as Mean±SEM, n=30 per treatment. Dark traits indicate significant differences with respect to solvent control treatment (Homogeneous triplicates, Student's t, $p < 0.05$)

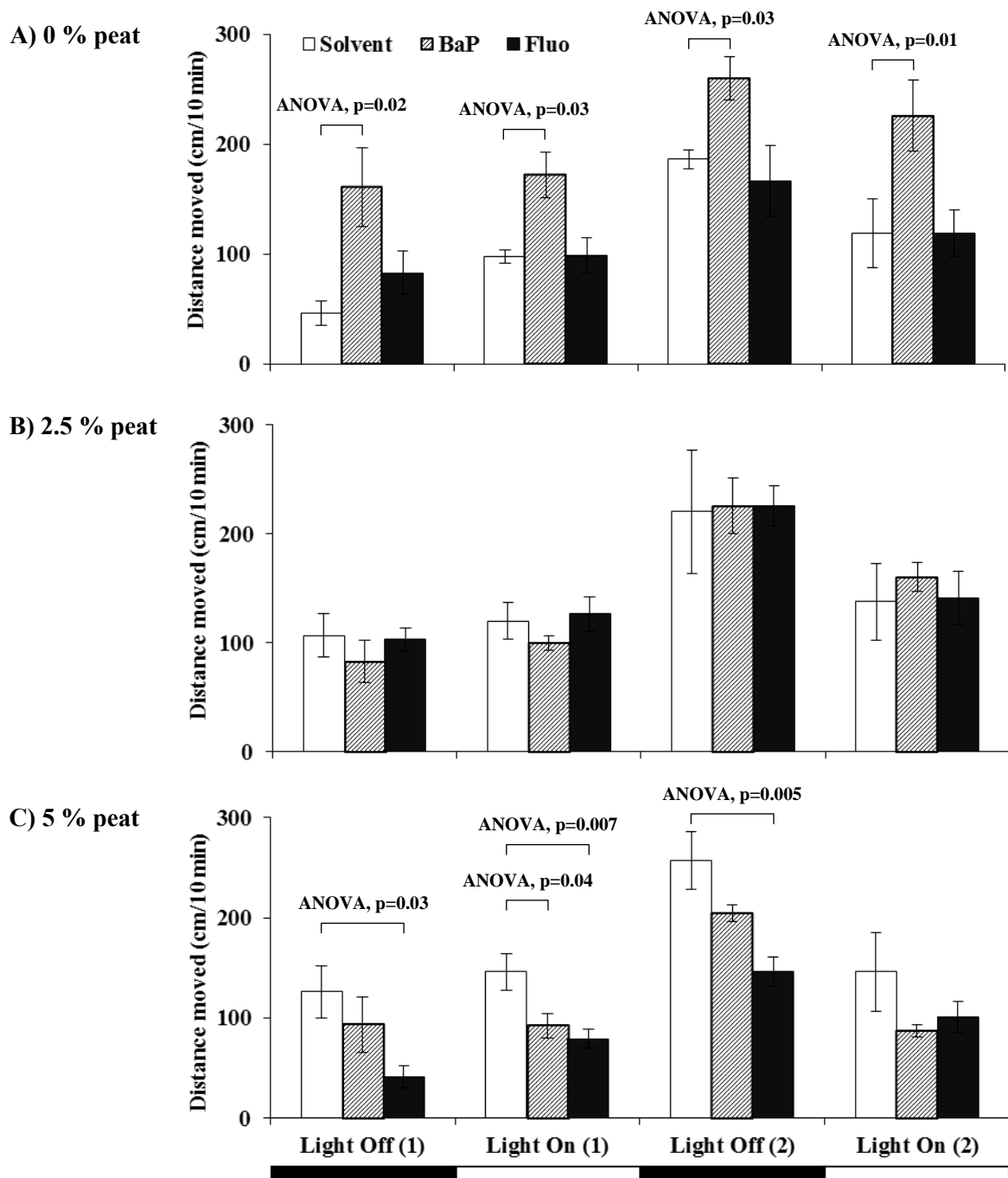


Figure 4. PMR measured in 14 dpf Japanese medaka larvae exposed to BaP and Fluo. The locomotor activity of larvae in each 10-min periods of light/dark challenge was measured for different peat sediment contents: 0 % peat (A), 2.5 % peat (B) and 5 % peat (C). All data are presented as Mean±SEM, n=15 per treatment. Dark traits indicate significant differences with solvent control treatment (ANOVA, $p < 0.05$)

Figure Supplementary data

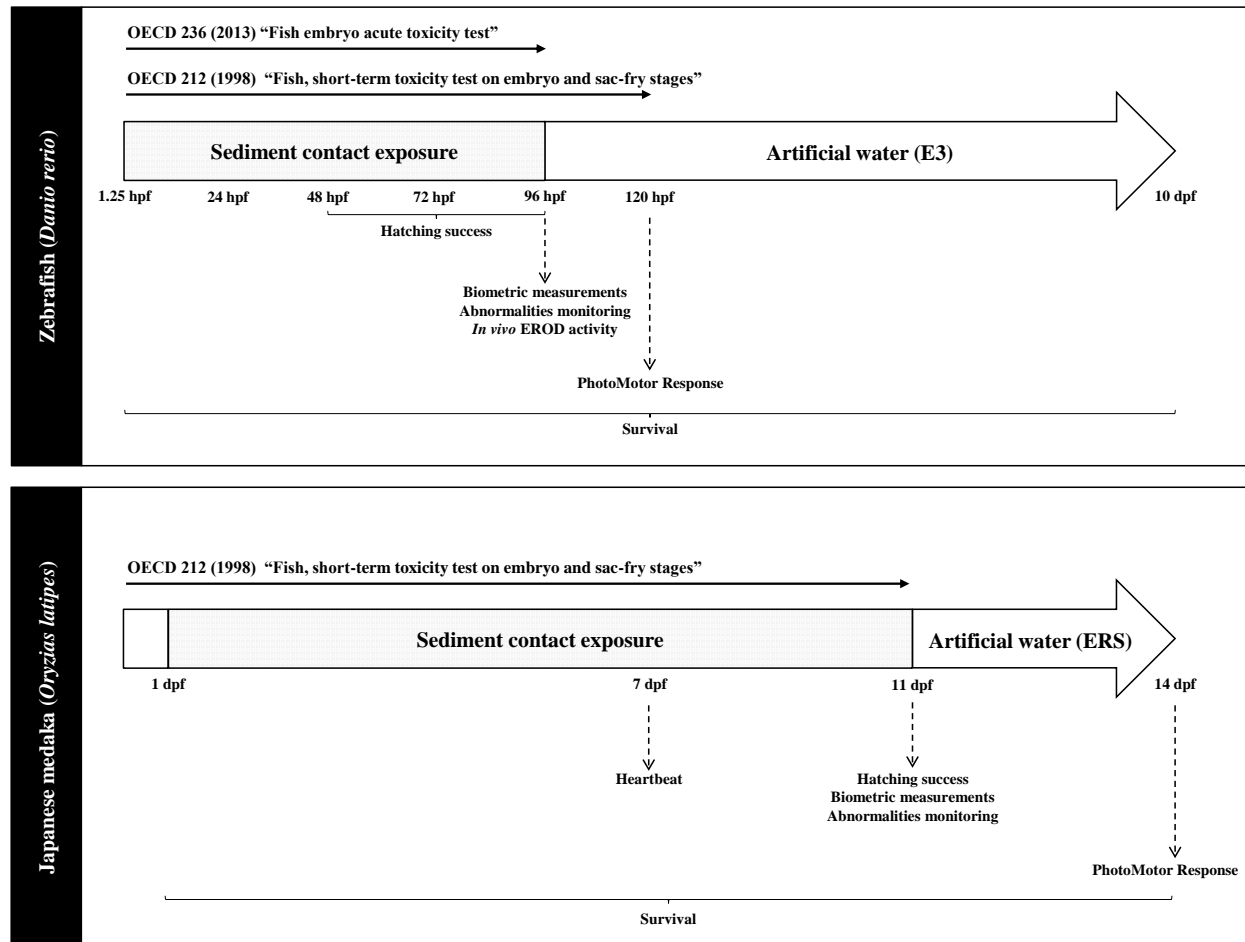


Fig. 1 Supplementary data Experimental design of zebrafish and medaka embryo-larval assays

Table 1 Viability and hatching success of zebrafish assessing BaP and Fluo toxicity regarding different peat sediment contents

		n=	Measured concentration ng.g ⁻¹ dw (Spiking efficiency %)	Survival rate (%)		Hatching success (%)
				96 hpf	240 hpf	96 hpf
0 % Peat	Solvent	89		89.9	89.9	100
	BaP	90	1655 (17)	95.6	92.2	100
2,5 % Peat	Solvent	90		93.3	87.8	95.2
	BaP	90	1736 (18)	94.4	92.2	100
5 % Peat	Solvent	90		93.3	88.9	100
	BaP	90	1969 (20)	92.2	90.0	100
0 % Peat	Solvent	90		97.8	96.7	100
	Fluo	90	6321 (63)	54.6*	46.6*	89.6*
2,5 % Peat	Solvent	90		95.6	93.3	100
	Fluo	90	5912 (59)	96.7	96.7	96.5
5 % Peat	Solvent	90		96.7	94.4	97.7
	Fluo	90	4396 (44)	83.5*	82.4*	84.2*

Asterisks indicate significant differences with respective Solvent control (Homogenous triplicates, Fisher test, p<0.05)

Table 2 Teratogenic effects on larval zebrafish exposed to BaP with different peat sediment contents

	0 % peat		2.5 % peat		5 % peat	
	Solvent	BaP	Solvent	BaP	Solvent	BaP
	n=3	n=3	n=3	n=3	n=3	n=3
Standard length (mm)	3.57±0.03	3.57±0.03	3.58±0.03	3.62±0.02	3.60±0.03	3.60±0.02
Ratio Head/Standard length (%)	18.3±0.11	18.6±0.14	18.6±0.15	18.2±0.18	18.6±0.14	18.5±0.11
Yolk sac area (mm ²)	0.33±0.01	0.32±0.01	0.34±0.01	0.33±0.01	0.35±0.01	0.34±0.01
Ratio Yolk sac/Whole larval area (%)	26.4±0.41	26.4±0.53	26.6±0.42	25.5±0.37	26.6±0.68	26.4±0.48
Abnormal individuals (%)	2.86	7.89	14.29	10.00	7.69	5.26
Severity of abnormality (scoring/3) (%):						
No affected (score 0)	97.14	92.11	85.71	90.00	92.31	94.74
Mild (score 1)	0.00	2.63	2.86	7.50	0.00	0.00
Moderate (score 2)	0.00	2.63	2.86	0.00	0.00	0.00
Severe (score 3 or more)	2.86	2.63	8.57	2.50	7.69	5.26
Abnormalities among abnormal individuals (%):						
Oedemas	1.43	2.63	5.10	3.33	3.21	1.50
Axial skeleton	0.00	1.32	4.08	5.00	1.92	1.50
Craniofacial	0.71	1.32	4.08	1.67	2.56	1.50
Cardiovascular	0.00	0.00	0.00	0.00	0.00	0.00
Yolk sac malabsorption	0.71	2.63	1.02	0.00	0.00	0.75
Developmental retardation (%)	0.00	0.00	0.00	0.00	0.00	0.00

No significant differences were observed (homogenous triplicates, t-test, $p>0.05$)

Table 3 Teratogenic effects on larval zebrafish exposed to Fluo with different peat sediment contents

	0 % peat		2.5 % peat		5 % peat	
	Solvent	Fluo	Solvent	Fluo	Solvent	Fluo
	n=3	n=3	n=3	n=3	n=3	n=3
Standard length (mm)	3.45±0.02	3.11±0.09*	3.41±0.02	3.40±0.02	3.30±0.02	3.17±0.02*
Ratio Head/Standard length (%)	18.3±0.10	16.4±0.42*	17.7±0.16	17.2±0.20	17.0±0.16	17.3±0.20
Yolk sac area (mm ²)	0.31±0.01	0.40±0.02*	0.31±0.01	0.32±0.01	0.33±0.01	0.35±0.01
Ratio Yolk sac/Whole larval area (%)	27.0±0.40	39.0±2.50*	27.0±0.40	28.0±0.50*	30.0±0.80	32.0±0.90*
Abnormal individuals (%)	4.65	22.22	7.32	2.56	2.44	10.53
Severity of abnormality (scoring/3) (%):						
No affected (score 0)	95.35	77.78	92.68	97.44	95.12	78.95
Mild (score 1)	2.33	11.11	7.32	2.56	2.44	15.79
Moderate (score 2)	0.00	11.11	0.00	0.00	2.44	5.26
Severe (score 3 or more)	2.33	0.00	0.00	0.00	0.00	0.00
Abnormalities among abnormal individuals (%):						
Oedemas	1.16	11.11	7.32	2.56	0.00	3.51
Axial skeleton	1.16	7.41	0.00	0.00	2.44	3.51
Craniofacial	1.16	0.00	0.00	0.00	0.00	3.51
Cardiovascular	0.00	0.00	0.00	0.00	0.00	0.00
Yolk sac malabsorption	1.16	3.70	0.00	0.00	0.00	0.00
Developmental retardation (%)	2.0	56.0*	0.0	10.0	49.0	100.0*

Asterisks indicate significant differences with respective solvent control (homogenous triplicates, t-test, $p < 0.05$)

Table 4 Comparison of BaP toxicity to zebrafish and Japanese medaka (Le Bihanic et al., 2014) early life stages for different peat concentrations in artificial sediment

		zebrafish			Japanese medaka		
Exposure time		96 hpf			12 dpf		
Peat concentration		0 %	2.5 %	5 %	0 %	2.5 %	5 %
Survival/Hatching	Embryo-larval mortality	No	No	No	No	No	No
	Hatching success	No	No	No	No	No	No
	Hatching time	No	No	No	No	Yes (+)	No
Morphology	Larval body length	No	No	No	No	No	No
	Yolk sac area	No	No	No	No	Yes (-)	No
	Abnormal individuals	No	No	No	No	No	No
Behavior	Photomotor response	Yes (-)	No	No	Yes (+)	No	No

Signs in brackets indicate significant inductor/increase (+) or inhibitor/decrease (-) effect compared to the solvent control respective

Table 5 Comparison of Fluo toxicity to zebrafish and Japanese medaka (Le Bihanic et al., 2014) early life stages for different peat concentrations in artificial sediment

		zebrafish			Japanese medaka		
		96 hpf			12 dpf		
Peat concentration		0 %	2.5 %	5 %	0 %	2.5 %	5 %
Survival/Hatching	Embryo-larval mortality	Yes (+)	No	Yes (+)	No	No	No
	Hatching success	Yes (-)	No	Yes (-)	No	No	No
	Hatching time	No	No	No	No	Yes (+)	No
Morphology	Larval body length	Yes (-)	No	Yes (-)	No	No	No
	Yolk sac area	Yes (+)	No	No	No	Yes (-)	No
	Abnormal individuals	No	No	No	Yes (+)	Yes (+)	Yes (+)
Behavior	Photomotor response	Yes (-)	No	No	No	No	Yes (-)

Signs in brackets indicate significant inductor/increase (+) or inhibitor/decrease (-) effect compared to the solvent control respective