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Environmental concentrations of benz[a]anthracene induce developmental defects and DNA damage and impair photomotor response in Japanese medaka larvae

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Abstract :

Benz[a]anthracene (BaA) is a ubiquitous polycyclic aromatic hydrocarbon found in numerous aquatic ecosystems. However, ecotoxicological data in aquatic organisms are scarce. To remedy this lack of data, Japanese medaka (Oryzias latipes) embryos were exposed to BaA and toxic effects were investigated at multiple toxicological endpoints. Japanese medaka embryos were incubated onto BaA-spiked artificial sediment for 9 days at low or moderate environmental concentrations ranging from 0.9 to 12 μg g−1 dw. BaA-exposed embryos exhibited significant tachycardia. BaA exposure was also shown to increase CYP1A activity in the hepato-biliary tissue as well as craniofacial deformities and DNA damage in pro-larvae. The photomotor response of BaA-exposed larvae was reduced in comparison to the control group. According to this set of tests, the lowest tested and observed effect concentration (LOEC) for Japanese medaka early life stages was equivalent to 0.92 μg g−1 dw of BaA. This concentration fall into the range of concentrations frequently encountered in sediments of polluted aquatic ecosystems. Taking into consideration these results, BaA represents a threat for fish early life stages in particular those developing onto or into contaminated sediments.

Highlights

► Toxicity of environmental concentrations of benz[*a*]anthracene was assessed in medaka ELS. ► Benz[*a*]anthracene induced teratogenicity, DNA damage and disrupt photomotor response in Japanese medaka larvae. ► Benz[*a*]anthracene lowest tested and observed effect concentration was 0.9 μg g^{-1} dw in sediment.

Keywords : Benz[a]anthracene, Spiked sediment, Teratogenicity, DNA damage, Swimming behavior, In vivo EROD activity

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1. INTRODUCTION

Numerous hydrophobic pollutants enter the aquatic environment as a result of direct discharge in aquatic compartments or via atmospheric particle deposition. These pollutants are mostly transported sorbed onto suspended particulate matter and tend to accumulate over time in sediments. Although not water soluble, this type of compound can represent a threat to aquatic organisms. Generally persistent, these compounds can be bioaccumulated in invertebrates and enter the entire food web. Among those pollutants, polycyclic aromatic hydrocarbons (PAHs) are omnipresent in aquatic ecosystems (Cachot et al., 2006; Gao et al., 2013; Gaspare et al., 2009). Recent work has highlighted that compared to other persistent pollutant such as PCBs and organochlorine pesticides - whose production and use were banned in the 1970s in developed countries - PAH concentrations in the arctic food web have increased 10 to 30 fold over the last 25 years (Laender et al., 2011). This PAH accumulation in tissues was mainly measured in low trophic levels such as invertebrates and fish (Laender et al., 2011). Fish larvae growing in PAH contaminated areas exhibited developmental defects and reduction in species recruitment (Barbee et al., 2008; Norcross et al., 1996). Of the hundreds of environmental PAH congeners, the benz[*a*]anthracene (BaA) is the most hydrophobic (log Kow = 5.6 - 5.9) with high sorption capacity on particle and organic matter and high propensity for accumulation in lipid-rich tissues. Compared to two or three ring PAHs, BaA is considered as a high molecular weight PAH. BaA is predominantly found in pyrolytic contaminated sediment resulting from incomplete combustion of organic matter including biomass and fossil fuels, and to a lesser extent in petrogenic contaminated areas resulting from spillage of petroleum or derivatives (Boll et al., 2008; Yunker et al., 2002). It accumulates in sediments all around the word, accounting for up to 10 % of the total content of PAH (Table 1). BaA is considered a priority substance by the European Union (EC 2000) and the US EPA. As a result of animal testing, the IARC concluded that there was sufficient evidence that this compound was carcinogenic, and classified it in group 2B as possibly carcinogenic to human (IARC, 2010). BaA genotoxicity has been shown *in vitro* using, either SOS Chromotest (Mersch-Sundermann et al., 1992), or *Salmonella tiphimurium* assay after bioactivation with S9 mix (Kido et al., 2013) or the

micronucleus assay in BaA-exposed human lymphocytes (Warshawsky et al., 1995). However, despite all of these considerations relating to the omnipresence of BaA in the environment and potent toxicity, the ecotoxicological data on aquatic organisms available in current scientific literature are sparse.

To remedy this, this study aims to assess BaA toxicity to aquatic organisms, focusing on the fish embryo-larval stage (ELS), because of their high sensitivity to chemical exposure. The medaka embryo larval assay with sediment contact exposure (MELAc) was used in this study because it has been proven to be particularly suitable for chemical testing, notably for hydrophobic pollutants (Le Bihanic et al., 2014a; Barjhoux et al., 2014; Vicquelin et al., 2011). It consists of incubating medaka embryos onto artificially-contaminated sediments in controlled conditions from fertilization all the way up until hatching. To fully qualify the ecotoxic properties of BaA, a large set of toxicity markers able to reveal embryotoxic, teratogenic, genotoxic, cardiotoxic and behavioral effects induced by chemical exposure were recorded at different developmental stages. Induction of CYP1A enzyme activity was investigated through *in vivo* measurement of EROD activity in order to better understand BaA mode of action.

2. MATERIAL AND METHODS

2.1 Chemicals

Sigma-Aldrich supplied benz[*a*]anthracene (BaA), resorufin, 7-ethoxyresorufin, dimethyl sulfoxide, ethyl 4-aminobenzoate (benzocaïne) as well as acridine orange stain (St Quentin Fallavier, France). Acros Organics (Thermo fisher Scientific, Geel, Belgium) supplied the dichloromethane. Cyclophosphamide monohydrate (CP) was purchased from Thermo Fischer Scientific (AC20396, Acros organics, Noisy Le Grand, France) and polychlorobiphenyl 126 from Analytical standard (Dr Ehrenstorfer, Augsburg, Germany).

2.2 Chemical analyses

Sediment samples were stored at -20 °C before chemical analysis. For BaA measurements in sediments, chrysene d12 was added prior to extraction as an internal deuterated standard. Pyrene d10 and

benzo[*b*]fluoranthene d12 were both added as syringe standards before the injection. The extraction method was that described by Budzinski et al. (2000). BaA concentrations were analyzed by gas chromatography combined with mass spectrometry (Baumard et al., 1999). Extraction efficiency was satisfactory, averaging 78 %. The entire analytical procedure was applied several times to certified marine sediment SRM 1944 (NIST, Gaithersburg, MD, USA). A blank of extraction was run for each series of samples.

2.3 Embryo-larval assay with spiked-sediment

The medaka embryo-larval assay in sediment contact (MELAc) was performed as described in Le Bihanic et al. (2014b). An artificial sediment containing 2.5 % dw of blond peat, 5 % dw of kaolin clay and 92.5 % dw of sand was spiked with BaA in order to reach nominal concentrations of 1, 3, 10 and 30 µg g^{-1} dw. Concentrations tested were in the range of environmental sediment concentrations from contaminated areas worldwide (Table 1). Control sediment was spiked with dichloromethane solvent only. Sixty mL of dichloromethane were added to 30 g of artificial sediment in a 250 mL glass balloon. The solvent was evaporated with a rotary evaporator Rotavapor (IKA, Staufen, Germany) at 115 rpm, 45 °C for 50 to 60 min. To ensure complete elimination of residual solvent, spiked sediments were stored overnight at room temperature, in the dark and under a fume hood.

Gis-Amagen (INRA, Jouy-en-Josas, France) provided eggs at early gastrula, stage 14-15 (Iwamatsu, 2004). Exposure consisted of depositing fertilized eggs in a Nitex® thermoformed basket onto the surface of the sediment at 1 day post fertilization (DPF). Exposure units, replicated 3 times per treatment, contained 25 embryos, 3 g dw of sediment and 3 mL of Egg Rearing Solution (ERS: 1 g NaCl, 0.03 g KCl, 4.04 g $CaCl_2$ and 0.163 $MgSO_4$ in 1 L autoclaved ultrapure water) in a 35 mm plastic Petri dish. Experiments were performed at 26 ± 0.3 °C in a temperature-controlled chamber (Snijders Scientific, Netherlands) with a photoperiod light: dark 12:12 and 5000 lx white light. At hatching, each pro-larvae were transferred into glass beaker containing 20 mL of rearing water (dechlorinated tap water mixed with distilled water 1:2 v/v aerated for 24 h). At the end of the exposure (10 DPF), non-hatched embryos were

transferred until hatching into new plastic Petri dishes containing 3 mL ERS without sediment. At the end of the experiment (15 DPF) all remaining individuals were euthanized with a lethal dose of 120 mg L^{-1} Ethyl 4-aminobenzoate. Pro-larvae were not fed during the experiment. Dissolved oxygen was monitored daily and concentrations in average settled above 82 % for all conditions of exposure. Mortality of control embryos and larvae was below 10 %.

Different toxic endpoints were recorded over the embryonic development at different time points on the same individuals. Heart beat measurements were processed in 7 DPF embryos at 23 ± 1 °C under stereomicroscope (MZ75, Leica) using a cold light source (Intralux® 4100, Volpi AG, Schlieren, Switzerland). Heart beat was counted over 3 periods of 20 seconds for a single embryo, with 5 embryos per replicate. Values were summed to obtain beat min^{-1} data for each embryo. At hatching, 15 larvae per replicate were photographed using a stereomicroscope equipped with an image analysis system (CCD camera DFP420C + Leica Application Suite V3.8, Nanterre, France) to record morphological abnormalities, as well as head and body length. The yolk-sac area was measured using a side-on picture of each larva. Morphological abnormalities, e.g. spinal and craniofacial deformities, ocular and cardiac anomalies and edemas, were counted and expressed in percentage regarding the total number of living larvae for each replicate.

2.4 Photomotor response

Photomotor response was assessed by measuring several locomotion variables upon light stimulus. This analysis was carried out on six 2 days post hatching (DPH) larvae per condition replicate, based on published methods (Le Bihanic et al., 2014a). Larvae were randomly selected and placed into a 48-well microplate. Each well of the microplate contained 1 larva in 500 µL of rearing water. Larvae were first acclimatized for 2 h in the dark in a Daniovision chamber (Noldus, Wageningen, Netherlands). Larvae coordinates were then recorded with an IR digital video camera (Ikegami Electronics, Neuss, Germany) and Ethovision 9.0 image analysis system (Noldus, Wageningen, the Netherlands). Measurements were performed at 23 ± 1 °C for 40 min, including 2 cycles of 10 min dark followed by 10 min light. A

dynamic subtraction method was applied with a sampling rate of 25 images per sec, dark contrast 16 - 255, current frame weight 4, subject size 2 - 12500, and no subject contour dilation. To reduce system noise, an input filter of minimal distance moved was set to 10 % of the total body larva equivalent to 0.4 mm. Velocity, distance travelled and mobility were calculated for each larva. Mobility refers to body movement which can be independent of spatial displacement *e.g.* an axial rotation. It is the time period expressed in % for which the larva area is modified. If changes represent less than 20% of time, larvae were considered immobile, between 20 to 60% larvae were considered mobile and over 60% they were considered highly mobile. Each parameter was expressed as mean duration (or percentage) for each 10 min dark or light period. All microplates were analyzed with identical temperature, detection and acquisition settings. Larvae unable to swim because of severe morphological abnormalities were not used for the photomotor assay.

2.5 Micronucleus assay

MN assay was performed on whole body from a pool of 3 DPH pro-larvae according to the protocol described previously (Le Bihanic et al., 2014a). Seven pro-larvae per replicate were fully minced with razor blades. Minced larvae were then digested with 1.25 mg mL^{-1} dispase *II* from *Bacillus polymyxa* (Roche, Meylan, France) in minimum essential medium eagle (MEM, St Quentin Fallavier, France) at 37 °C, 45 min, under agitation (150 rpm). After centrifugation (1000 rpm, 10 min, room temperature), pellets were rinsed in 80 µL of MEM. Cell concentration and viability were then assessed under microscope after Trypan blue exclusion test. Average cell viability was equivalent to 98 %. After a new centrifugation (1000 rpm, 10 min, room temperature) pelleted cells were fixed in a mix solution of acetic acid and methanol 1:4 v/v during 10 min. Cell suspensions were then smeared on slides (two slides per sample), and slides were dried for 24 h at room temperature. Just before reading, slides were stained with 40 µL acridine orange solution (0.003 % in PBS) and covered with a glass slip. Acridine orange staining was preferred to Wright staining because it guarantees DNA-specific staining and thus minimize artefact MN occurrence (Polard et al., 2011). Reading was processed using an epi-fluorescent microscope at X400

magnification (Olympus, Rungis, France). Micronucleated cells were manually recorded from 1,000 cells per slide using blind review by a single observer. A cell was considered micronucleated if MN was round shaped, distinct from main nucleus, size inferior to a third of the main nucleus, and with a similar green staining to the main nucleus (Hayashi et al., 1998). Non-isolated, stacked, more than bi-nucleated or orange-red stained cells were not taken into account.

As a positive control, 0 DPH Japanese medaka larvae were exposed during 72 h to 5 mg L^{-1} of water-borne Cyclophosphamide (CP). Exposure was performed in 12-well microplates (Greiner, Courtaboeuf, France), 6 larvae per well, three wells per replicate for water control and three replicates for CP treatment. Pro-larvae were not fed during the experiment. No mortality was observed during the exposure.

The MN assay performed on whole body larvae and used in the present study is under the rights of the French patent N°1058505 established the 19/10/2010.

2.6 *In vivo* EROD activity measurement

To investigate EROD activity, a separate exposure experiment was performed on a new batch of individuals. Japanese medaka larvae at 0 DPH were exposed during 24 h to waterborne BaA concentrations ranging from 6.25 to 400 µg L^{-1}. Control larvae were exposed in the same experimental conditions to 0.1 % DMSO used as a carrier solvent. As a positive control, 0 DPH Japanese medaka larvae were exposed during 24 h to 400 ng L^{-1} of water-borne 3,3',4,4',5-pentachlorbiphenyl (PCB126). Exposure was performed in 48-well microplates (Greiner, Courtaboeuf, France) with 1 larva per well and six larvae for control and PCB126 conditions. Pro-larvae were not fed during the experiment. No mortality was observed during the exposure.

EROD activity measurements were performed based on Otte et al. (2010) method. Measurements were conducted on individual medaka pro-larvae. At the end of the 24 h exposure, living larvae were put into individual wells of a 48-well microplate (Greiner, Courtaboeuf, France) and were incubated in 21 µg L^{-1}

7-ethoxyresorufine solution for 5 h in the dark. Larvae were rinsed, anesthetized and then photographed using an epifluorescence microscope at X100 magnification (U-MWIG2 mirror unit, Olympus, Rungis, France) equipped with a monochrome CCD camera (Perceptive instruments, Bury St Edmunds, UK). Wavelengths were 550 nm for excitation and 580 nm for emission. Resorufin-produced fluorescence in the hepato-biliary structure of each individual larva was quantified with ImageJ software (National Institutes of Health, NIH; Bethesda, USA) by selecting a homogenous fluorescence zone. EROD activity was reported as fluorescence unit per pixel (arbitrary unit).

2.7 Statistical analysis

When each condition was identically replicated 3 times, exposure replicates were considered independent samples. When larvae were exposed individually in the case of EROD measurement, each larva was considered as an independent sample. Normality of the data distribution was verified on data residues using the Shapiro-Wilk test ($p < 0.01$). Variance homogeneity was evaluated using the Levene test ($p < 0.05$). For all parameter analyzed, variance was homogenous and data had a normal distribution, thus Anova analysis was performed followed by the Tukey post-hoc test ($p < 0.05$). Spearman's correlation coefficient was calculated to assess the strength of the relation between the different studied endpoints. Statistical analyses were performed using Statistica v7.1 (StatSoft, Maisons-Alfort, France). Data is indicated as mean ± SD.

3. RESULTS

3.1 BaA induced developmental toxicity from 0.9 µg g^{-1} dw

BaA spiking efficiencies yielded between 41 and 92 % and globally decreased with increasing BaA concentration (Table 2). Embryo-larval survival was above 90 % in all BaA treatments indicating no lethal effects up to 12.2 µg g^{-1} dw sediment. Based on observations, BaA had no noticeable effect on hatching rate or time to hatch. More than 85 % of embryos from each treatment hatched after around 12 days of embryonic development. There was no difference in larva biometry between treatments at hatching. On

average, total larva body length was equivalent to 4.2 ± 0.1 mm. Head length was 0.86 ± 0.02 mm, and yolk sac area was 0.38 ± 0.02 mm^2. In contrast, BaA induced tachycardia in 7 DPF embryos that was significant for the highest tested concentration of 12.2 µg g^{-1} dw. BaA exposure also resulted in the significant enhancement of morphological abnormalities from 0.9 µg g^{-1} BaA dw. Predominant BaA-induced abnormalities were curvature of the spinal axis (11.1 to 27.9 %) and cranio-facial (17.8 to 20.3 %) deformities, including changes in jaw and skull shape. In contrast, cardiac abnormalities, peri-cardiac and yolk-sac edemas were relatively scarce.

3.2 BaA disrupted larval photomotor response from 0.9 µg g^{-1} dw

During the 40 min photomotor assay, a similar response pattern to light stimulation was observed for all examined larvae (Fig. 1A). The first dark period (0 to 10 min) was characterized by larval activity stabilization before light challenge with no statistical difference in larva swimming activity. During the following first light period (10 to 20 min), larva activity increased linearly with time ($R^2 = 0.94$ at 0 µg g^{-1}; $R^2 = 0.79$ at 0.9 µg g^{-1}) but there was no difference between treatments. At 20 min, the light was turned off, and all larvae reacted with a 3 to 4-fold increase in velocity. During the following 10 min dark period (20 to 30 min), larva velocity decreased. The light was turned on again at 30 min and speed dropped down to about 1 mm s^{-1} (equivalent to the initial speed in light at 10 min) and then increased again during the last 10 min light period (30 to 40 min) as observed during previous light periods. However, slopes were on average 1.7 times steeper during the second light period (30 to 40 min) than during the first light period (10 to 20 min). Distance travelled by larvae was markedly reduced during the second light period (30 to 40 min) after exposure to BaA when compared with control larvae (Figure 1B). Indeed, for the second light period, BaA-exposed larvae at all tested concentrations swam a total distance of 0.85 ± 0.07 m - significantly less than control larvae (1.09 ± 0.04 m). Table 3 presents the percentages of immobile, mobile or highly mobile larvae according to BaA concentrations and light conditions. In accordance with results of velocity and distance travelled, larvae exposed to 2.3 and 12.2 µg g^{-1} BaA dw showed a significant decrease in mobility during the last light period (30 to 40 min) compared to control larvae.

3.3 BaA induced micronucleated cells in larvae from 0.9 µg g^{-1} dw

On average, 3 to 5 ‰ micronucleated cells were recorded in control treatments for both BaA and CP exposures (Fig. 2). CP treatment increased MN occurrence up to 12 ‰. The frequency of micronucleated cells was also significantly increased following embryo exposure to 0.9 and 12.2 µg g^{-1} BaA, compared with the control treatment (Fig. 2). Albeit an increase trend was observed, MN frequency was not significantly different from control group for the two intermediate BaA concentrations.

3.4 BaA enhanced *in vivo* EROD activity in larvae

Resorufin fluorescence related to EROD activity was mainly located in hepato-biliary tissue of BaA and PCB126-exposed larvae (Fig. 3A). Similar basal EROD activities were recorded in the control larvae of both experiments (26.0 ± 9.5 integrated density/pixel) (Fig. 3B). Waterborne PCB126 exposure enhanced EROD activity to 2.5 times above the control treatment. After larval exposure to waterborne BaA, EROD activity followed a bell-shape response from 6.25 µg L^{-1}. The intermediate concentration of 25 µg L^{-1} induced the highest activity. At the highest tested concentration of 400 µg L^{-1}, EROD activity decreased to reach values as low as solvent-exposed larvae.

4. DISCUSSION

Embryonic and larval survivals in the control condition were above 90 %. Furthermore dissolved oxygen was on average between 83 and 100 % of saturation throughout the test. Thus these experiments complied with the OECD testing guidelines for fish early life stage toxicity tests (OECD, 1992).

4.1 BaA induces deleterious effects in developing larvae

BaA induced developmental toxicity in Japanese medaka early life stages. Our findings about BaA teratogenicity and cardiovascular toxicity are consistent with developmental abnormalities and heart dysfunction reported by Incardona et al. (2006). Indeed, after zebrafish embryo exposure to BaA, the

aforementioned authors observed defects in cardiac function: pericardial edema, hemorrhage, failure in cardiac looping, degeneration of cardiomyocytes; as well as morphogenesis disruption: spinal curvature, yolk sac edemas and reduced biometry.

BaA also impaired the swimming behavior of fish larvae under challenging light conditions. This result is consistent with recent works reporting lethargy and abnormal swimming in juvenile fish exposed to 2, 3 or 4 aromatic ring PAHs (Gonçalves et al., 2008; Oliveira et al., 2012). Morphological abnormalities and multiple physiological dysfunctions in metabolic, cardio-vascular, or neuro-sensorial systems can stem for this altered swimming activity. Photomotor response disruption was correlated to morphological abnormalities in this study. Even though abnormal larvae were discarded from the photomotor analysis it is possible that subtle lesions not visible under microscope could alter larvae behavior. There are other possible explanations for PAHs changing larva swimming behavior. Indeed, the anti-estrogenic and anti-androgenic activities of BaA have been previously reported *in vitro* (Arcaro et al., 1999; Vinggaard et al., 2000). In addition, concentration of several neurotransmitters such as dopamine, serotonin and noradrenaline can be affected by PAH exposure (Gesto et al., 2009). The alteration of larval behavior and swimming performances can have severe consequences for later developmental stages such as for instance impairment of exogenous feeding or failure to escape predators (Couillard et al., 2011).

BaA was also shown to induce irreversible DNA damage on medaka larvae. While BaA genotoxicity has already been reported *in vitro* (Kido et al., 2013; Mersch-Sundermann et al., 1992; Warshawsky et al., 1995), to our knowledge this is the first report of BaA-induced DNA damage on fish. For most high molecular weight PAHs, DNA damage is induced by electrophilic metabolites produced through parent compound metabolisation and/or by reactive oxygen species issued from redox cycling of quinone and o-quinone PAH metabolites (Bolton et al., 2000; Burczynski and Penning, 2000). For both pathways, CYP1A induction is required. EROD activity was shown to be significantly increased in medaka larvae following exposure up to 25 µg L^{-1} of BaA. This activity then decreased to baseline levels when BaA exceeded 400 µg L^{-1}. This bell-shaped response is consistent with previous *in vitro* studies (Bosveld et al., 2002) indicating that BaA at high concentrations alters the metabolic function of cells leading to

cytotoxicity. This decrease in EROD activity could also be a consequence of the impairment of cardiac function reported in BaA-exposed embryos, limiting the distribution of 7-ER in the organism.

Interestingly, several toxicity markers were shown to be inter-related. Distance swum by larvae was significantly anti-correlated to the frequency of MN cells ($r = 0.66$, $p = 7.8*10^{-3}$) and to abnormal larvae frequency ($r = -0.71$, $p = 3.2*10^{-3}$). No correlation was observed considering the other endpoints. The three toxicity markers: photomotor response, DNA damage, and developmental defects did not follow a dose-dependent pattern with BaA exposure, and effects were observed from the lowest concentration tested. This means that BaA has no acute effect up to concentrations of about 12 µg g^{-1} dw in sediment. However, it is noteworthy that even at low concentrations BaA can induce sub-lethal effects in different target organs and disrupt various biological functions. From these results it can be concluded that chronic BaA exposure during embryo-larval development could seriously impact health status and biological performances of fish ELS with possible consequences on survival and recruitment.

4.2 BaA accumulated in sediments represents a putative risk for fish early life stage development

In terms of the toxicity threshold for BaA, identical LOEC values were obtained for developmental defects, DNA damage, and locomotion assay equivalent to 0.92 µg g^{-1} dw. From this value it is possible to estimate a predictive no effect concentration (PNEC) for BaA in sediments using a safety factor of 100: PNEC = LOEC / 100 = 9.2 ng g^{-1} dw sediment.

BaA is currently detected at concentrations above this PNEC value in contaminated worldwide sediments (Cachot et al., 2006; Gao et al., 2013; Gaspare et al., 2009) (Table 1) and thus could be a threat for the aquatic environment. The LOEC value of this study was also the lowest concentration tested. We must keep in mind that BaA might induce toxic effect on fish early life stage at concentration below the LOEC value. Moreover, in the field, PAHs generally occur as mixtures and therefore additive and synergistic effects with other compounds can take place (Billiard et al., 2008). This was not taken into consideration in this single compound study. Although BaA is also likely more bioavailable in spiked than in naturally-contaminated sediments (Brinkmann et al., 2013), our data suggest a risk for fish species developing in

direct contact to BaA-contaminated sediments. To ascertain this conclusion, experiments should be performed with wild fish species and naturally-contaminated sediments.

5. CONCLUSION

This study brought new ecotoxicological data about BaA toxicity on fish ELS. BaA was shown to be a potent inducer of CYP1A and embryonic exposure to concentration as low as 0.9 µg g^{-1} dw induced developmental defects, DNA damage and behavioral disturbances in medaka early life stages. BaA could therefore represents a risk to the recruitment of wild fish species developing in direct contact to sediments in the vicinity of urban or industrial areas. To protect aquatic organisms, particularly fish early life stages, environmental quality standards need to be established to cover BaA and other hazardous sediment associated PAHs.

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FIGURES CAPTIONS

Figure 1. Photomotor response of Japanese medaka pro-larvae (2 DPH) following embryonic exposure to BaA-spiked sediment. A) Illustrative graphic of the velocity of control and BaA-exposed larvae, data was averaged over each 1 min interval. B) Distance travelled was recorded during a 40 min video tracked analysis: 10 min light + 10 min dark + 10 min light + 10 min dark. Data was averaged over each 10 min interval. Different letters refer to statistical differences between treatments (Mean ± SD, N = 3, 6 larvae per replicate, Anova, $p < 0.05$).

Figure 2. Micronucleated cell frequency recorded in isolated cells of whole body larvae. Individuals were exposed during all embryonic development to BaA spiked-sediment or during 72h at larval stage to dissolved CP positive control. Different letters refer to statistical differences between treatments and lowercase and uppercase letters refer to two independent statistical analysis (Mean ± SD, N = 3, 7 larvae per sample, Anova or Student t-test, $p < 0.05$).

Figure 3. *In vivo* EROD activity measured in medaka pro-larvae. A) Photos of medaka larvae (ventral view) showing *in vivo* resorufin fluorescence located in hepato-biliary tissue. B) 0 DPH larvae were exposed for 24 h to different doses of dissolved BaA or PCB126 as a positive control. Different letters refer to statistical differences between treatments and lowercase and uppercase letters refer to two independent statistical analysis (Mean ± SD, N = 6, individual measurement, Anova or Student t-test, $p < 0.05$).

TABLES

Table 1. Concentration range of BaA in various sediments from worldwide areas.

	[BaA] (µg g^{-1} dw)	**[BaA]/[Σ total PAH] (%)**	**Reference**
River			
Pearl river (China/Hong Kong)	0.00 – 0.02	1 – 3	(Luo et al., 2006)
Seine river (France)	0.09 – 1.10	7 – 9	(Cachot et al., 2006)
Mersey river (United Kingdom)	0.04 – 0.30	6 – 10	(Vane et al., 2007)
Little egg estuary (NJ, USA)	0.00 – 0.82	3 – 7	(Vane et al., 2008)
Lenga river (Chili)	0.02 – 0.43	6 – 8	(Pozo et al., 2011)
Scheldt river (France/Belgium)	0.77 – 1.45	-	(Gao et al., 2013)
Coastal area			
Black Sea (Ukraine/Turkey/Russia)	0.00 – 0.05	0 – 9	(Readman et al., 2002)
Dar es Salaam (Tanzania)	0.03 – 1.00	2 – 4	(Gaspare et al., 2009)
Lake			
Lake Superior (Canada/USA)	0.03 – 0.04	3 – 5	(Baker et al., 1991)
European lakes	< 0.001	1 – 2	(Vives et al., 2004)
Pacific Northwest (USA)	2 - 43	4 – 7	(Barbee et al., 2008)

Table 2. Sediment characterization and developmental defects in Japanese medaka following embryonic exposure to BaA-spiked sediment. Different letters refer to significant differences between treatments (mean ± SD, N=3, Anova, $p < 0.05$).

BaA exposure (nominal concentrations μg g^{-1} dw)	**0**	**1**	**3**	**10**	**30**
Measured concentrations (μg g^{-1} dw)	< 0.2	0.917	2.332	5.082	12.154
Spiking efficiencies (%)	-	92	78	51	41
Embryonic survival (%)	94.4±6.3	95.8±3.6	95.7±3.7	95.9±0.1	94.5±4.9
Larval survival (%)	100.0±0.0	98.4±2.8	98.6±2.4	95.6±4.4	98.5±2.6
Hatching rate (%)	88.8±12.0	93.2±2.1	88.8±5.2	94.5±2.0	86.4±8.0
Time to hatch (days)	12.1±0.3	12.0±0.5	12.0±0.3	11.9±0.1	11.8±0.7
Embryonic heartbeat (beat/sec)	**108±7.8^{A}**	**120±13.5^{AB}**	**113±2.1^{A}**	**121±8.6^{AB}**	**130±3.4^{B}**
Larvae total length (mm)	4.28±0.04	4.31±0.03	4.27±0.04	4.18±0.06	4.17±0.09
Larvae head length (mm)	0.88±0.01	0.87±0.01	0.85±0.01	0.85±0.01	0.85±0.01
Yolk sac area (mm²)	0.39±0.01	0.39±0.02	0.38±0.01	0.36±0.02	0.38±0.02
Abnormal larvae (%)	**4.4±2.2^{A}**	**22.2±2.2^{B}**	**28.9±2.2^{C}**	**38.7±6.1^{BC}**	**30.3±1.9^{BC}**
Spinal abnormalities (%)	**2.2±3.9 ^{A}**	**11.1±7.7 ^{A}**	**27.9±7.9 ^{B}**	**15.6±3.9 ^{AB}**	**13.7±6.2 ^{AB}**
Craniofacial abnormalities (%)	**0.0±0.0^{A}**	**17.8±3.9^{B}**	**20.3±6.2^{B}**	**17.8±3.9^{B}**	**18.8±5.0^{B}**
Ocular abnormalities (%)	2.2±3.9	0.0±0.0	0.0±0.0	6.7±6.7	0.0±0.0
Cardiac abnormalities (%)	2.2±3.9	2.2±3.9	11.4±4.2	8.9±7.7	4.4±3.9
Edemas (%)	0.0±0.0	2.2±3.9	2.4±4.1	2.2±3.9	0.0±0.0

Table 3. Japanese medaka larvae mobility after embryonic exposure to BaA-spiked sediments. Values represent averaged mobility percentages over a 10 min time period. Different letters refer to significant differences between treatments (mean ± SD, N=3, Anova, $p < 0.05$).

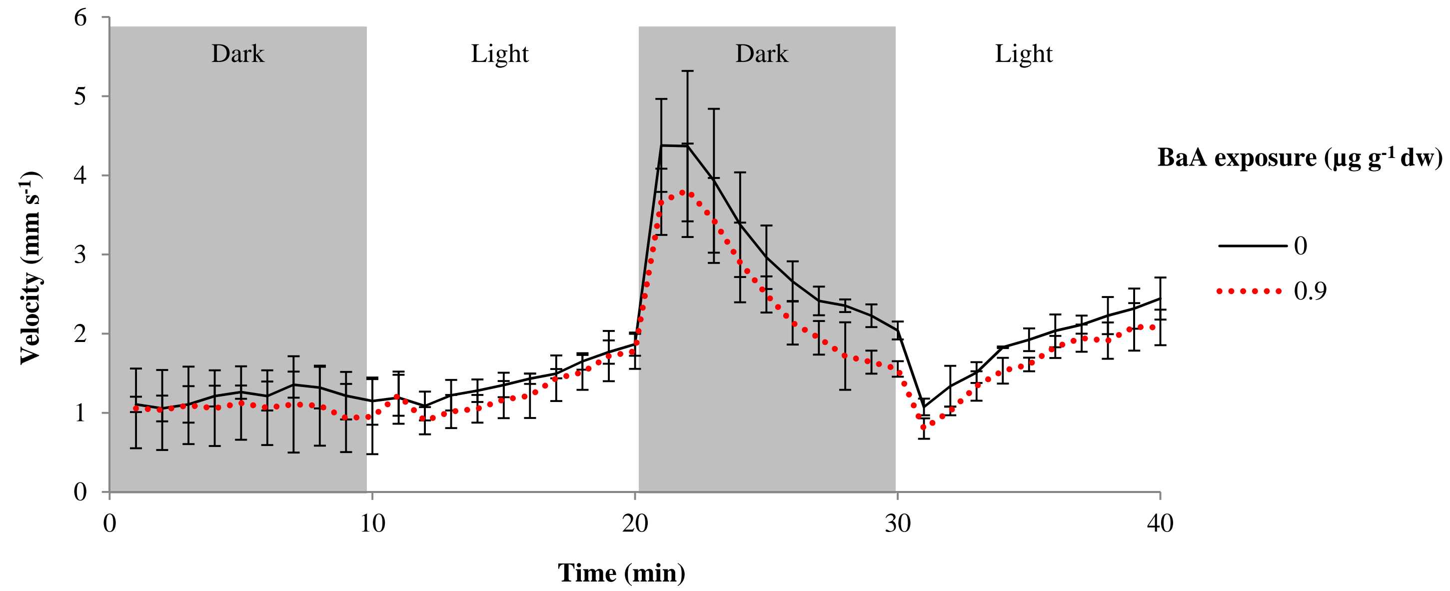
			BaA concentration (µg g^{-1} dw)				
	Time (min)		**0**	**0.9**	**2.3**	**5.1**	**12.2**
Immobile (%)	0 – 10	Dark	91.9±1.2	92.9±3.0	94.6±1.1	93.5±0.7	92.0±2.1
	10 – 20	Light	90.3±0.5	91.3±0.9	91.4±0.4	90.9±0.9	89.8±0.7
	20 – 30	Dark	79.5±1.8	82.1±2.2	79.5±0.5	78.7±0.8	79.8±2.4
	30 – 40	Light	87.3±0.2	89.0±0.3	89.7±1.1	89.2±0.4	89.2±1.6
Mobile (%)	0 – 10	Dark	3.1±0.4	2.9±0.8	2.3±0.3	2.5±0.1	3.2±0.7
	10 – 20	Light	4.8±0.2	4.4±0.7	4.3±0.1	4.6±0.5	4.9±0.5
	20 – 30	Dark	7.0±0.6	6.3±0.4	7.1±0.4	6.8±0.5	6.9±0.5
	30 – 40	Light	5.6±0.3	5.2±0.2	4.8±0.3	4.8±0.5	5.1±0.7
Highly mobile (%)	0 – 10	Dark	5.0±0.8	4.2±2.3	3.0±0.9	4.0±0.5	4.9±1.4
	10 – 20	Light	4.9±0.6	4.3±0.4	4.2±0.3	4.5±0.5	5.3±0.7
	20 – 30	Dark	13.5±1.4	11.6±2.3	13.4±0.4	14.6±1.2	13.3±1.9
	30 – 40	Light	**7.1±0.3^{A}**	**5.9±0.5^{AB}**	**5.5±0.8^{B}**	**5.9±0.1^{AB}**	**5.7±1.3^{B}**

Table 4. Lowest Observed Effect Concentration (LOEC) of BaA on Japanese medaka embryos and larvae.

	LOEC (µg g^{-1} dw)
Embryonic heartbeat	12.154
% of abnormal larvae	0.917
MN assay	0.917
Photomotor assay	0.917

Figure 1

A



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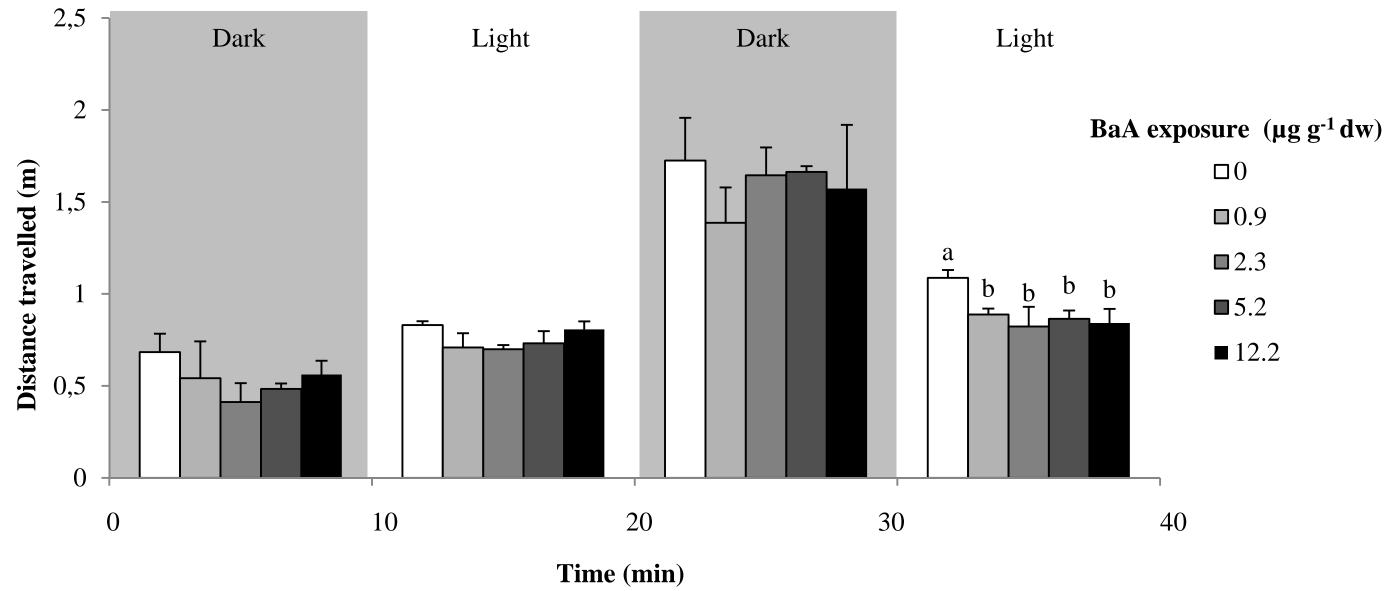

Figure 2

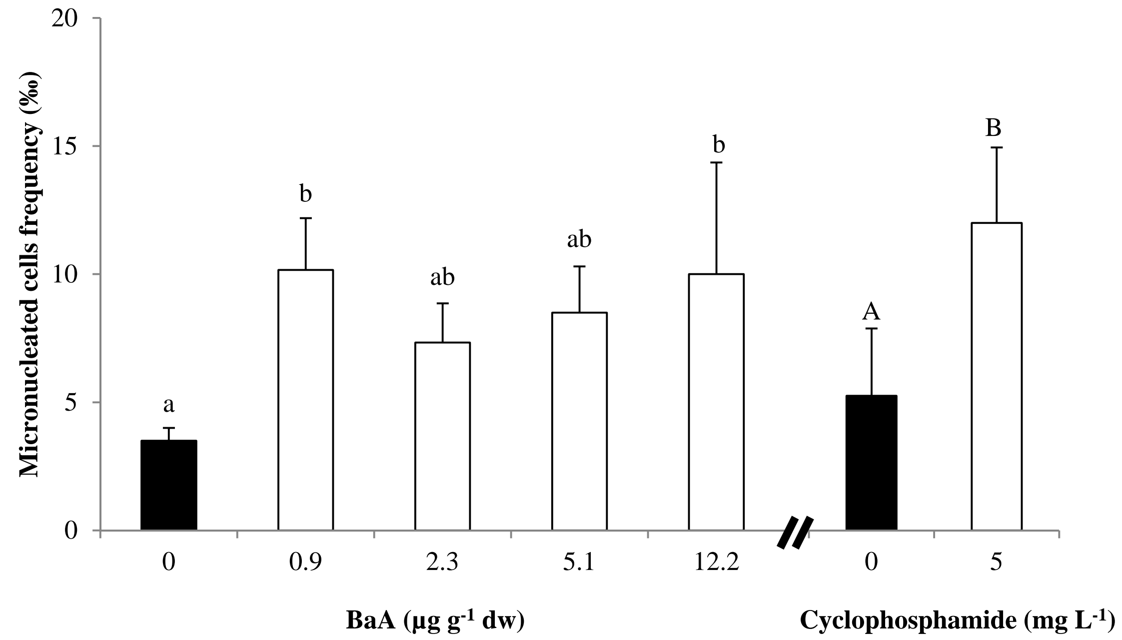

Figure 3

A

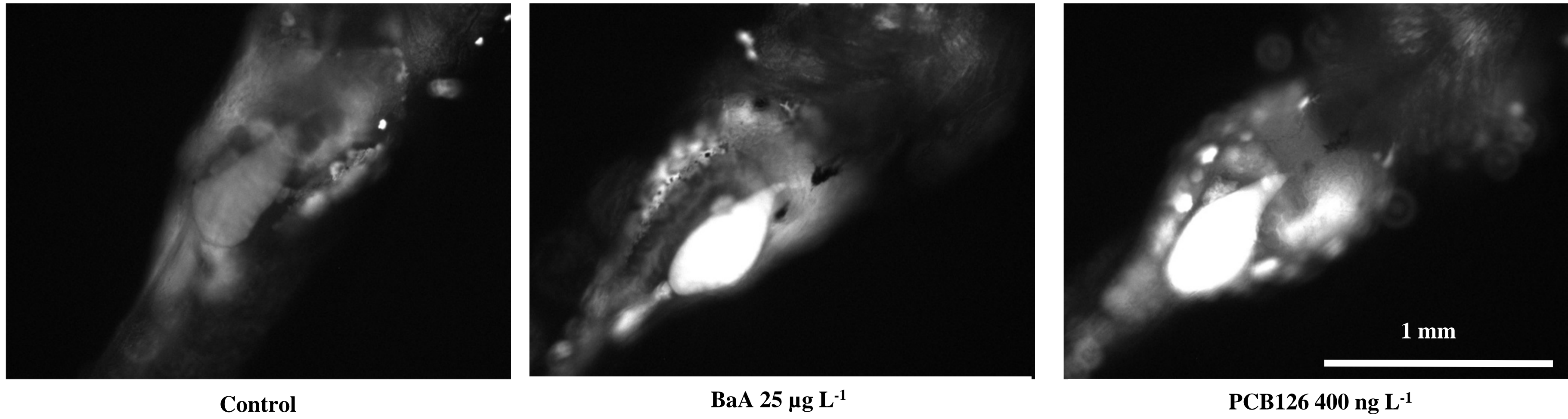

Figure 3