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Developmental toxicity of PAH mixtures in fish early life stages. Part I: adverse effects in rainbow trout

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Abstract:

A new gravel-contact assay using rainbow trout, *Oncorhynchus mykiss*, embryos was developed to assess the toxicity of polycyclic aromatic hydrocarbons (PAHs) and other hydrophobic compounds. Environmentally realistic exposure conditions were mimicked with a direct exposure of eyed rainbow trout embryos incubated onto chemical-spiked gravels until hatching at 10 °C. Several endpoints were recorded including survival, hatching delay, hatching success, biometry, developmental abnormalities, and DNA damage (comet and micronucleus assays). This bioassay was firstly tested with two model PAHs, fluoranthene and benzo[a]pyrene. Then, the method was applied to compare the toxicity of three PAH complex mixtures characterized by different PAH compositions: a pyrolytic extract from a PAH-contaminated sediment (Seine estuary, France) and two petrogenic extracts from *Arabian Light* and *Erika* oils, at two environmental concentrations, 3 and 10 μg g⁻¹ sum of PAHs. The degree and spectrum of toxicity were different according to the extract considered. Acute effects including embryo mortality and decreased hatching success were observed only for *Erika* oil extract. *Arabian Light* and pyrolytic extracts induced mainly sublethal effects including reduced larvae size and hemorrhages. *Arabian Light* and *Erika* extracts both induced repairable DNA damage as revealed by the comet assay versus the micronucleus assay. The concentration and proportion of methylphenanthrenes and methylanthracenes appeared to drive the toxicity of the three PAH fractions tested, featuring a toxic gradient as follows: pyrolytic < *Arabian Light* < *Erika*. The minimal concentration causing developmental defects was as low as 0.7 μg g⁻¹ sum of PAHs, indicating the high sensitivity of the assay and validating its use for toxicity assessment of particle-bound pollutants.

Keywords: PAH mixture; Oil extract; Pyrolytic extract; Embryotoxicity; Developmental defects; Genotoxicity
1. Introduction

Aquatic ecosystems are continuously facing inputs of polycyclic aromatic hydrocarbons (PAHs) of different origins. PAHs sources of contamination are chronic due mainly to human activities such as industry, transport, wood and oil-fired heating or accidental with numerous oil spill accidents, as those involving the Erika, Prestige and Exxon Valdez tankers or more recently the Deep-water Horizon oil spill. Because of their hydrophobicity, PAHs accumulate in sediments over time, which in turn become a continuous source of exposure for benthic organisms.

Fish embryos and eleutheroembryos (yolk fry) are sensitive to a large range of chemicals (Embry et al. 2010; Belanger et al. 2010). Moreover they are also suitable models for chemical testing since they are not covered by the EU regulations on animal experimentation (EC 2010). Among others, salmonids are widely used model species in ecotoxicology (Hawkins et al. 2002; Cook et al. 2003; Carls and Thedinga 2010). They are also suitable species for mesocosm and field studies (Escarti and Porte 1999; Brinkmann et al. 2013). Rainbow trout (Oncorhynchus mykiss) presents several further advantages as being commercially available, easy breeding, having low basal embryo mortality in laboratory settings (below 10 %), a fully documented development and a high sensitivity to pollutants - in particular PAHs - at early life stages (Brinkworth et al. 2003; Hodson et al. 2007).
PAHs occur in complex mixtures in the environment, their composition and toxicity vary markedly depending on their sources: pyrolytic, diagenetic or petrogenic (Wang et al. 2003; Barbee et al. 2008). The additive toxicity of PAH compounds in complex mixtures is a controversial subject. Recent studies disprove the aryl hydrocarbon receptor (AhR) agonist approach to estimate PAH mixture toxicity (Incardona et al. 2005; Billiard et al. 2008). In this study, an embryo-larval assay was developed to compare the toxicity of PAH mixtures of different compositions, to Oncorhynchus mykiss early life stages (ELS). Taking into consideration the natural spawning conditions of salmonids and the poor solubility of PAH in water, the assay was based on direct exposure to PAH-spiked gravel at realistic environmental concentrations. Although aquatic organisms seem to be more sensitive to dissolved PAH (Geffard et al. 2003; Carls and Meador 2009), other studies underlined the evident contribution of the sediment for fish embryo contamination (Kocan et al. 1996). Compared to classical embryo-larval assays using waterborne exposure, this assay mimics the double exposure of fish embryos via the aqueous phase, the pore water and direct contact with contaminated sediment particles.

The experimental design was firstly tested using two model PAHs, fluoranthene and benzo[a]pyrene. Those two compounds were selected for their high capacity to adsorb onto particles, and their ubiquity in aquatic environment (Guasch et al. 2012). The validated experimental design was then applied for toxicity comparison of three different PAH fractions to rainbow trout ELS: 1) a pyrolytic PAH fraction (PY) from a contaminated sediment of the Seine river, 2) an Arabian Light oil PAH fraction (LO) and 3) an Erika oil PAH fraction (HO).

Several endpoints were recorded including survival, hatching delay, hatching success, biometry, developmental defects and DNA damage measured by the comet and the micronucleus assays.

2. Materials & methods

2.1 PAH fraction samples

The pyrolytic PAH fraction (PY) was obtained from a superficial sediment (oxic layer) of the Seine River, sampled in March 2010 in Oissel, (Normandy, France). CEDRE (Center of Documentation, Research and Experimentation on Accidental Water Pollution) provided both the Arabian Light oil (LO) and the Erika heavy oil (HO). PY fraction was extracted using pressurized liquid extraction with dichloromethane (Acros Organics, Thermo Fisher Scientific, Geel, Belgium). The two oils were dissolved in pentane to induce asphaltene precipitation. The sediment extract and the two pentane extracts were then reconcentrated using a Vacuum
Evaporation System (Rapidvap, Labconco, Kansas city, USA). All three PAH samples were then purified using alumina columns (preparative liquid chromatography) and eluted with dichloromethane (Acros Organics, Thermo Fisher Scientific, Geel, Belgium). After another re-concentration step, aliphatic fractions obtained after elution on silica columns with pentane were discarded and aromatic fractions were obtained using pentane/dichloromethane (65/35, v/v) as solvents. Atlantic Labo (Bruges, France) provided the pentane solvent. Finally, samples were re-concentrated and the final fractions kept in dichloromethane (stored at -20 °C) for spiking experiments. All the steps were gravimetrically controlled in order to prevent PAH losses and also to be able to express results in terms of quantity of the initial material (i.e. sediment or petroleum).

2.2 Gravel spiking

Silica gravel of 2-4 mm size was provided by Sibelco sandpit (gravel reference BB 2/4, Mios, France). It was washed in HCl (1 %, v/v) and rinsed with reverse osmosis water before use. The clean gravel (50 g dw) was then spiked with 100 mL of dichloromethane (Biosolve, Valkenswaard, the Netherlands) used as a spiking solvent or individual PAHs alone or in mixture in a 1 L glass flask using a rotary evaporator (Rotavapor, IKA, Staufen, Germany). For validation of the experimental design, the gravel was spiked with 3 000 ng g⁻¹ dw of benzo[a]pyrene (BaP) or fluoranthene (Fluo) (Sigma-Aldrich, St Quentin Fallavier, France). For PAH fraction testing, the gravel was spiked with 0.3X and 1X concentrations of each PAH fraction. 1X concentration refers to the total PAH content in sediment from the upper Seine estuary e.g. 10 000 ng g⁻¹ dw (Cachot et al. 2006). Solvent was evaporated at 45 °C, 115 rpm for 2 h. An aliquot of the spiked gravel was stored at -20 °C in anticipation of chemical analysis. Remaining spiked gravel (40 g dw) was kept in the dark and at room temperature prior to the assay.

2.3 Chemical analysis

Internal deuterated standards naphthalene d8 (EGA-CHEMIE), dibenzo[b,d]thiophene d8 (MSD isotopes), phenanthrene d10 (Cambridge Isotope Laboratories), anthracene d10 (Cambridge Isotope Laboratories), fluoranthene d10 (MSD isotopes), chrysene d12 (MSD isotopes), benzo[e]pyrene d12 (Cluzeau – CDN Isotopes), benzo[a]pyrene d12 (Cambridge Isotope Laboratories), and benzo[ghi]perylene d12 (Cambridge Isotope Laboratories) were added prior to extraction, to 1 g of gravel and one blank analysis composed of
PAH compounds were extracted for 10 min at 30 W using microwaves assisted extraction, with dichloromethane as solvent (Budzinski et al. 2000). Samples were then re-concentrated using a Vacuum Evaporation System (Rapidvap, Labconco, Kansas City, USA). Re-concentrated extract samples were purified using alumina micro-columns and eluted with 3 x 5 mL of dichloromethane. After a new re-concentration with gas nitrogen, the aliphatic fraction was eluted on silica micro-columns using pentane/dichloromethane (65/35, v/v) as solvents. Finally, samples were re-concentrated in iso-octane (Scharlau, Barcelona, Spain). Pyrene d10 and benzo[b]fluoranthene d12, both supplied by Cambridge Isotope Laboratories, were added to the samples as syringe standards before injection. PAHs were analysed by gas chromatography coupled to mass spectrometry (GC-MS) as described by Baumard et al. (1998) and included 21 parent compounds: naphthalene, acenaphthylene, acenaphthene, fluorene, dibenzo[bd]thiophene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, triphenylene, chrysene, benzo[b]naphto[2,1-d]thiophene, benzo[b+k+j]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-cd]pyrene, dibenzo[ah]anthracene, dibenzo[ac]anthracene, benzo[ghi]perylene; as well as methyl compounds: 2-methylnaphthalene, 1-methylnaphthalene, 3-methylnaphthalene, 2-methylphenanthrene, 2-methylanthracene, 9-methylnaphthalene+2-methylnaphthalene, 1-methylphenanthrene, 4-methyl dibenzothiophene, 3+2-methyldibenzothiophenes, 1-methyldibenzothiophene, dimethylnaphthalenes, trimethylnaphthalenes, tetramethylnaphthalenes, dimethylphenanthrenes, trimethylphenanthrenes, methylchrysenes. Extraction recoveries calculated with internal and syringe standards were overall very satisfactory, equivalent on average to 87 %. Limits of quantification were evaluated for each PAH compound using the peak to peak method of calculation.

2.4 Experimental set up validation

To ensure a suitable time period for exposure and stabilized dissolved oxygen concentration, several experimental designs were tested including dynamic water renewal, static daily water renewal, and static water (water not renewed). Since there was no marked difference in trout development according to the experimental design (unpublished data), exposure was set from the eyed stage until the hatching stage in static water conditions in order to limit wash-out of spiked-gravel. To validate this experimental design, a preliminary experiment was performed exposing embryos to two model PAHs, BaP and Fluo.
2.5 Embryo exposure

INRA-PEIMA (Sizun, France) provided rainbow trout embryos at eyed stage, e.g. 180 degree day (DD : number of days x temperature). Each treatment was replicated 3 times. For each single replicate, 25 embryos were laid in a 100 mL glass jar containing 12 g dw of spiked gravel and 50 mL of Saint Martin spring water, dechlorinated with 48 h aeration (St Martin de Gurçon, France, pH = 8.2; 68 mg L\(^{-1}\) Ca\(^{2+}\); 21 mg L\(^{-1}\) Na\(^{2+}\); 11 mg L\(^{-1}\) Mg\(^{2+}\); 2 mg L\(^{-1}\) K\(^{+}\); 216 mg L\(^{-1}\) HCO\(_3\)^{-}; 39 mg L\(^{-1}\) SO\(_4\)^{2-}; 28 mg L\(^{-1}\) Cl\(^{-}\); < 1 mg L\(^{-1}\) NO\(_3\)^{-}). To check if solvent or gravel induced effects on embryos development, a water only control was added. There was no water renewal during the whole exposure and dissolved oxygen was measured daily with a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburg, Germany) compensated with a temperature sensor PT 1000. The oxygen probe was placed at the interface between the water and the gravel. Experiments were carried out in the dark at 10 °C in a climate chamber (Snijdjers Scientific, Tilburg, the Netherlands). At 280 DD, corresponding to 10 days after the start of the experiment, exposure of the embryos was stopped. Embryos were transferred into new glass jars containing 50 mL of spring water without sediment, with 20 mL of water renewed daily. Once hatched, larvae were kept in separated glass jars containing 50 mL of spring water. At 400 DD, corresponding to the end of the 22 days of the experiment, all remaining individuals were euthanized with a lethal dose of 120 mg L\(^{-1}\) ethyl 4-aminobenzoate (benzocaine, Sigma-Aldrich, St Quentin Fallavier, France).

2.6 Phenotypic effects

Embryonic and larval survival was recorded daily. Dead individuals were immediately removed to avoid water alteration. Individuals were considered half-hatched if at least a part of the embryonic body remained inside the chorion. Embryonic, larval or half-hatched mortality refers to the number of dead individuals at the end of the experiment compared to the total number of individuals at the beginning (180 DD). **Hatching time refers to the number of DD from fertilization to hatching.**

Occurrence of developmental abnormalities were microscopically examined on 15 larvae per replicate between 0 and 24 h post hatching. The larvae under examination were laid on a Petri dish containing 1.5 % agarose gel (LB Agar) and 2 mL of spring water. Larvae were photographed at X20 magnification using a stereomicroscope MZ 7.5 Leica coupled with a CCD camera DFP420C Leica (Nanterre, France) and a cold light source source (Intralux® 4100, Volpi AG, Schlieren, Switzerland). Total body length and head length of larvae were measured.
using Leica Microsystems software v3.8 (Nanterre, France). Six types of abnormality were recorded in larvae: edemas (yolk sac, pericardia or skeletal); spinal (scoliosis, lordosis, or cyphosis) and craniofacial (skull and jaw shape deformities) deformities; cardiac anomalies (changes in heart location and ventricle size); hemorrhages (located on the skeleton, yolk sac, skull, or jaw); and yolk sac resorption defects. Abnormal larvae rate referred to the percentage of abnormal larvae presenting at least one type of abnormality. Edema, spinal, craniofacial, cardiac hemorrhage and yolk sac abnormality rates referred to the percentage of larvae presenting one or several abnormalities, counted once. One larva could exhibit several types of abnormalities.

2.7 Genotoxic effects

Genotoxicity assays were performed on 350 DD larvae. Peripheral blood from 5 individuals per replicate treatment was sampled with heparinized tips via cardiac puncture, and mixed immediately with 1 mL of Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, St Quentin Fallavier, France). Cell viability was assessed with Trypan blue exclusion as above 90 % in all samples and cell concentration at around 500 cell µL⁻¹.

Cells used for comet and micronucleus assays were from the same cell suspension sample. For the comet assay, the protocol of Singh et al. (1988) was followed. 50 µL of cell suspension was added to 100 µL of 1 % (w/w) low melting point agarose (LMPA) and two 50 µL gels were laid on slides previously covered with normal melting point agarose (1 %, w/w). Gels were shielded with an 18 x 18 mm glass slip and hardened for 5 min at 4°C. Cover slips were gently slid off and a second LMPA only gel was laid on each of the first gels shields with a new coverslip. After hardening for 5 min again at 4 °C, coverslips were slid off and slides were placed in a freshly prepared lysis solution at 4 °C for 1 h (2.5 M NaCl; 0.1 M EDTA; 0.01 M Tris; pH adjusted to 10 with NaOH). One percent of Triton X-100 was added to the solution extemporaneously. Slides were then placed on a horizontal electrophoresis tray and covered with freshly prepared electrophoresis buffer (0.3 M NaOH; 1 mM EDTA; pH > 13) for 10 min to allow the DNA to unwind. Electrophoresis was carried out at 25 V, 300 mA for 10 min. Subsequently, slides were rinsed three times in a staining tray with neutralization solution (0.4 M Tris; pH = 7.5) for 5 min at 4 °C. Finally slides were dehydrated in absolute ice-cold ethanol for a further 20 min and dried at room temperature. Before reading, slides were stained with 20 µg mL⁻¹ ethidium bromide. Comet analysis was performed at X400 magnification using an Olympus epi-fluorescent microscope (Olympus, Rungis, France) with an excitation filter of 515 – 560 nm, a barrier filter of 590 nm, and a grayscale CCD camera (Zeiss, Germany). DNA damage was measured on 100 cells for each sample with Komet 5.5 software.
(Kinetic Imaging, Liverpool, UK) and expressed as percentage tail DNA. Heavily damaged cells were counted as “hedgehogs”, referring to apoptotic or necrotic cells (Olive and Banáth 1995).

For the micronucleus (MN) assay, samples were centrifuged (at 1000 rpm, for 10 min at room temperature) and **pelleted cells** were fixed in 200 µL of a mixed solution of acetic acid: methanol (1:4, v/v). Cell suspensions were then smeared onto slides and then dried for 24h at room temperature. Just before reading, slides were stained with 40 µL of acridine orange solution (0.003 % in PBS) and covered with a glass slip. Reading was processed using an epi-fluorescent microscope at X400 magnification (Olympus, Rungis, France). The frequency of MN cells was manually recorded on 1000 cells per slide using blind review analysis by a single observer. A cell was considered micronucleated if the MN was round-shaped, distinct from main nucleus, its size was less than a third of the main nucleus, and with a similar green staining as the main nucleus (Hayashi et al. 1998). Non-isolated, stacked, more than bi-nucleated or orange-red stained cells were not considered.

### 2.8 Statistical analysis

Each exposure condition was replicated 3 times and each replicate was considered as an independent sample. Data is indicated as mean ± standard deviation (SD). Normality of the data distribution was tested on data residues using the Shapiro-Wilk test (p < 0.01). Variance homogeneity was evaluated using the Levene test (p < 0.05). In cases of homogenous variance and normalized data, one-way ANOVA was performed, followed by the Tukey post-hoc test (p < 0.05). In opposite cases, data was analyzed using the Kruskal-Wallis non parametric test (p < 0.05). Statistical analyses were performed with Statistica software v7.1 (StatSoft, Maisons-Alfort, France). A principal component analysis (PCA) was performed with R software (http://cran.r-project.org/).

### 3. Results

#### 3.1 Experimental set up validation

Oxygen saturation ranged between 85.3 and 103.9 % over embryonic exposure. Fluo spiking efficiency was 2.4 fold higher than BaP efficiency and nominal concentrations reached 2.7 and 1.1 µg g⁻¹ dw for Fluo and BaP respectively (Table 1). No significant mortality was observed in any exposure condition. Mortality rate of solvent control embryos, half-hatched individuals and larvae were very low (below 2.2 %). Only 10.0 % of
control larvae presented developmental abnormalities. Exposure to BaP and Fluo induced significantly more abnormal larvae than control solvent. The majority of BaP and Fluo-induced developmental defects were spinal curvatures and edemas. BaP and Fluo did not affect larval time to hatch or hatching rate. Given the low mortality and abnormality levels in the solvent control, combined with the significant adverse effects induced by BaP or Fluo exposure, the preliminary experimental set up appeared to be suitable for sensitive toxicity testing of PAH with rainbow trout ELS.

3.2 Gravel characterization

Composition of PAH fractions differed in molecule proportions in relation to the number of cycles and the methylation level of PAHs (Table 2). The proportions of individual PAHs in spiked sediments were comparable for the 0.3X and 1X concentrations, apart from the 0.3X PY condition. Indeed, PY spiked gravels were mainly composed of four aromatic ring compounds, representing 36 % and 33 % of 0.3X and 1X total PAHs, respectively. LO spiked gravel was mainly composed of two aromatic ring compounds, representing 44 % and 60 % of 0.3X and 1X total PAHs, respectively. HO spiked gravel was mainly composed of three ring compounds representing 54 % and 50 % of 0.3X and 1X total PAHs, respectively. Spiking efficiencies ranged between 40 % and 50 %, apart from 0.3X LO gravel which yielded 24 %.

3.3 Developmental toxicity of PAH fractions

Dissolved oxygen concentrations varied between 80 % and 103 % over embryonic exposure. No significant embryonic or larval mortality was observed during exposure to PAH fractions (Table 3). No statistical difference was detected between control groups incubated in water only or to solvent-spiked gravel. Water and solvent control mortalities were below 3.9 %. Embryonic or larval mortality and time to hatch were not affected by PAH exposure. In contrast, half-hatched embryos frequency was significantly increased after exposure to 0.3X LO (10.7 %) and 1X HO (14.7 %) and 1X HO reduced significantly hatching success by 28 %. In addition, LO and HO treatments but not PY had significant effects on larvae biometry. Larvae exposed to LO and HO were 0.8 mm and 1.4 mm shorter than solvent-exposed larvae, respectively (Fig. 1A). LO and HO-exposed larvae had a smaller head with head length reduction by 6.5 %, 7.2 % and 8.8 %, for 1X LO, 0.3X and 1X HO treatments, respectively (Fig. 1B). Finally, all PAH fractions significantly impaired normal embryo-larval development. For
1X HO, 100% of larvae presented at least one type of abnormality compared with 82.2% for 1X LO and 68.9% for 1X PY fraction (Table 3). There was no treatment effect on yolk sac resorption with an average 9.3% of individuals exhibiting yolk sac anomalies (Fig. 2A). About 72.4% of 1X HO larvae had a spinal curvature, 66.1% craniofacial abnormalities, 29.3% cardiac anomalies and 56.6% edema (Fig. 2B, 2C, 2D & 2E). Furthermore, all three PAH fractions significantly induced hemorrhages from 44.4% for PY-exposed larvae to 91.1% for HO-exposed larvae (Fig. 2F).

3.4 PAH fractions genotoxicity

For the comet assay, cells from the control larvae had only 3.5% of tail DNA (Fig. 3A). 1X LO significantly increased tail DNA to 14.1% in larvae erythrocytes and 0.3X and 1X HO to 17.0% and 27.0%, respectively. There was no evidence of significant DNA damage induction in erythrocytes from larvae exposed to the PY fraction. Albeit the prevalence of hedgehog cells ranged from 2.6 ± 1.8% (mean ± SD) in controls to 17.7 ± 2.0% for 1X HO treatment, there was no significant difference between treatments. The MN assay did not reveal significant increase of DNA damage with any treatment (Fig. 3B). MN erythrocytes ranged from 0.3‰ for the 0.3X PY treatment to 2.0‰ for the 0.3X LO treatment.

3.5 Principal Component Analysis of PAH fractions toxicity

The selected PCA featured 85.1% of nine variable variances: five toxicity endpoints and four PAH gravel concentrations (Fig. 4A). Along axis 1 (70.0%), comet assay, developmental abnormalities and half-hatched embryos variables, with high normalized coefficients, were negatively correlated to hatching rate and total larvae length variables. Moreover, the toxicity of PAH fractions - featured by these five toxicity variables - were positively correlated (comet assay, percentage of deformities and half-hatched embryos) or negatively correlated (hatching rate and total larvae length) to the methylphenantrene concentration especially. In contrast, the toxicity variables were not correlated to the methyl dibenzothiophene concentration. The plot of scores for each replicate revealed no overlapping treatment group and a toxicity gradient along axis 1 as following: solvent control < 0.3X PY < 1X PY < 0.3X LO = 0.3X HO < 1X LO < 1X HO (Fig. 4B). Low toxic fractions are located on the left negative side of axis 1 while high toxic fractions are located on the opposite side.
4. Discussion

In this paper a new gravel-contact exposure protocol was specifically designed for toxicity assessment of hydrophobic pollutants on rainbow-trout embryos. **Dissolved oxygen remained high in the different assays although no water renewal was made during the experiment.** Furthermore, low mortality and abnormality rates were observed in control conditions, while exposed larvae exhibited significant developmental abnormalities. Incubation of trout embryos in direct contact to non-spiked or solvent-spiked gravels did not affect embryonic development. Therefore, the experimental design with direct exposure to spiked gravels and no water renewal appeared suitable for toxicity testing of chemicals with rainbow trout ELS.

Despite a lower Log $K_{ow}$, Fluo was shown to better adsorb onto gravels than BaP. Liu et al. (2011) have shown that PAHs adsorption on inorganic particles including quartz sand probably follows the Langmuir equation and is related to the hydrophobicity of the compound. Our unexpected result might be explained by the singular nature of the gravel which is exclusively composed of silica grains of relatively large size (2-4 mm). The low specific surface area of the studied gravel might favor sorption of smaller size molecules such as Fluo (4 rings, MW 202.25) instead of larger ones such as BaP (5 rings, MW 252.31).

The PAH composition of the three fractions was well reproduced in the different spiked gravels except for the 0.3X PY-spiked gravel for which four ring compounds adsorbed much better than for the 1X PY-spiked gravel. This may be due to the loss of low molecular weight compounds during the spiking process. PAH fractions mainly induced sublethal effects in embryos and larvae including cardio-vascular defects and reduced growth for PY and LO conditions. Our observations support recent studies reporting embryotoxicity in rainbow trout ELS after exposure to PAH-contaminated sediment extracts (Sundberg et al. 2005; Karlsson et al. 2008); and juvenile mullet after exposure to LO (Milinkovitch et al. 2011b). Milinkovitch et al. (2011a; 2011b; 2013) also reported bioaccumulation of PAHs in muscle and increased levels of PAH metabolites as well as cardiac toxicity after fish exposure to LO crude oil and its water soluble fraction. Numerous studies about the toxicity of the Alaska North Slope Crude Oil (ANSCO) on pink salmon, herring, mummichog and zebrafish, reported the induction of blue sac disease syndrome, including pericardial edemas, haemorrhages, spinal deformities as well as reduced growth (Heintz et al. 1999; Couillard 2002; Barron et al. 2003; Hicken et al. 2011). The HO displayed a comparable PAH composition and featured a similar spectrum of developmental defects to the ANSCO. Nevertheless, no developmental delay was observed after rainbow trout ELS exposure to HO or LO extracts.
Petrogenic fractions but not pyrolytic one also resulted in a significant increase of tail DNA in peripheral blood cells from exposed larvae. Comet assay detects repairable DNA damages such as single/double strand breaks and also alkali labile sites. Comet assay in fish blood cells have been shown to be a sensitive and convenient assay to measure DNA damage induced by genotoxic pollutants in aquatic environment (De Andrade et al 2004; Frenzilli et al, 2009). Conversely, no significant increase of micronucleated blood cells was observed. The MN assay measures irreversible DNA damage, resulting from clastogenic (chromosomal breakage) or aneugenic events (mitotic spindle dysfunction) occurring later in the time course of the mutagenesis process (Udroiu 2006). We can assume that when MN assay was performed (350 DD), DNA damage may not have been definitively fixed in the larva blood cells. These results can be explained by the kinetics of induction and repair of DNA strand breaks (Carls et al. 1999; Nahrgang et al. 2010) but also by the low mitotic index of peripheral blood cells (Fujiwara et al. 2001; Wang et al. 2010).

It is likely that PAH metabolites and reactive oxygen species may be responsible for the DNA damage recorded in this study (Regoli et al. 2002; Fallahtafti et al. 2012). We could expect MN, mutations and tumors induction at later developmental stages as reported after fish exposure to Erika oil, Staffjord oil, or organic extract from the Seine river sediment (Amat et al. 2006; Baršienė et al. 2006; Cachot et al. 2007). However, aromatic extracts may also contain traces of other chemicals which can also be toxic to fish in particular saturated hydrocarbons or mono-aromatic compounds (González-Doncel et al. 2008). In fact, Arabian light crude oil contains 75 % of saturated hydrocarbons, 15 % of aromatic hydrocarbons and 10 % of resins and asphaltenes (Wang et al. 2003). Erika oil contains 22 % of saturated hydrocarbons, 42 % of aromatic hydrocarbons and 35 % of resins and asphaltenes (Baars 2002). Although most of them were removed during the extraction process, some traces may remain in the aromatic fraction.

Toxicity did not increase linearly with increasing proportions of high molecular weight PAHs. Indeed, the PY fraction which contains a high proportion of high molecular weight PAHs was the least toxic mixture to rainbow trout ELS. The LO fraction, mainly composed of two and three ring PAHs, was moderately toxic. Finally, the HO fraction, mainly composed of two to four ring PAHs, was the most toxic fraction. The toxicity gradient illustrated in figure 4B, highlights differences in toxicity according to the proportion and concentration in methylated phenanthrenes and methylated anthracenes. Genotoxicity and developmental toxicity of the PAH mixtures are mainly driven by the concentration and proportion of three ring methylated-PAHs that predominate in the HO fraction. It is now well documented that different PAH compounds have different modes of action and toxicity that are assumed not to be additive (Billiard et al. 2008). Low molecular weight PAHs act preferentially...
through the narcosis pathway while high molecular weight PAHs involve binding to aryl hydrocarbon receptor AhR (Di Toro et al. 1991; Wassenberg and Di Giulio 2004). It has been recently demonstrated that three ring compounds can also act through an unknown AhR-independent pathway (Hawkins et al. 2002; Incardona et al. 2005). Previous works reported the highest toxicity to fish ELS of three ring compounds either alone (Incardona et al. 2004) or in mixture (Sundberg et al. 2005; Incardona et al. 2009). Based on their embryo-larval toxicity, an alkyl-phenanthrene model was recently developed on methylated-phenanthrene from two to four carbons to better evaluate the complex toxicity of PAH mixtures (Barron et al. 2004). Alkyl-phenanthrenes toxicity can be explained 1) by indirect toxicity due to reactive oxygen species production and 2) by direct toxicity of the compound itself or its metabolites (Billiard et al. 2008). Indeed, biotransformation of alkyl-phenanthrenes generates highly toxic metabolites by hydroxylation on double bonds of rings or on the alkyl side chains. These molecules are able to disrupt the cellular homeostasis resulting in cytotoxicity directly related to the genotoxic potency of the compound (Burczynski and Penning 2000). Para substituted quinones appear to be one of the most toxic hydroxylated metabolites of alkyl-phenanthrenes to fish ELS (Fallahtafti et al. 2012).

In an ecological point of view, the exposure conditions in our experiments are consistent with the natural habitat conditions of salmonid embryos and environmental concentrations of PAHs in contaminated sediments (Cachot et al. 2006; Karlsson et al. 2008). Indeed, salmonid embryos are usually buried into the gravel of the river bed throughout their embryonic development. This exposure design did not consider the phototoxicity of PAHs since embryos were incubated in complete darkness. This could have led to underestimating PAH toxicity to rainbow trout ELS (Barron et al. 2003; Hatlen et al. 2010).

It was shown here that direct exposure of rainbow trout ELS to spiked gravels is a suitable approach to assess toxicity of PAHs. Further experiments will be carried out to validate this approach with other hydrophobic pollutants.

**Conclusion**

To summarize, this study succeeded in designing a suitable and environmentally realistic protocol for hydrophobic compound testing with *Oncorhynchus mykiss* ELS. **Teratogenic and genotoxic effects of three** PAH mixtures were reported. **Toxicity of PAH mixtures was positively related to the concentrations of**
methylphenanthrenes and methylanthracenes. Our data support PAH mixtures containing high amount of alkylphenanthrenes are likely a threat for fish early life stages.

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**FIGURE CAPTIONS**

**Fig. 1**: Exposure to PAH fractions affected rainbow trout larvae biometry at hatching. A) *Larvae total length*; B) *Larvae head length*. Individuals were exposed during embryonic development to chemical-spiked gravels. Different letters refer to significant differences within treatments (Mean ± SD, N = 3, Anova, p < 0.05).
**Fig. 2:** Exposure to PAH fractions induced developmental abnormalities in newly hatched rainbow trout larvae. A) Yolk sac resorption anomalies; B) Spinal abnormalities including scoliosis, lordosis, cyphosis; C) Craniofacial deformities including changes in skull and jaw shape; D) Cardiac anomalies including changes in heart location and ventricle size; E) Edema occurrence; F) Haemorrhage occurrence. Individuals were exposed during embryonic development to chemical-spiked gravels. Different letters refer to significant differences between treatments (Mean ± SD, N = 3, Anova, p < 0.05).

**Fig. 3:** Exposure to PAH fractions induced genotoxicity in rainbow trout larvae. A) Comet assay. DNA damage is expressed in percentage of DNA in the comet tail. B) Micronucleus assay. DNA damage is expressed in micronucleated cell frequency. Individuals were exposed during embryonic development to chemical-spiked gravels. Different letters refer to significant differences between treatments (Mean ± SD, N = 3, Anova, p < 0.05).

**Fig. 4:** Methylphenanthrene concentrations drove PAH fractions toxicity in rainbow trout early life stages. A) Variable factor map; B) Individual factor map. PCA representing normalized coefficients on the first two axes (axis 1: 70.0 %; axis 2: 15.1 %) for nine variables: five toxicity variables and four PAH gravel concentrations. Toxicity endpoints referred to HR: Hatching rate; LengthT: Larvae total length; D: Developmental abnormalities; Comet: percentage tail DNA; MortHH: half-hatched mortality. PAH gravels concentrations refer to MP: methylphenanthrene, DMP: dimethylphenanthrene, TMP: trimethylphenanthrenes; MDBT: methyl dibenzothiophene.
Figure 1

**A**

![Graph showing Larvae total length (mm) for different treatments](image)

**B**

![Graph showing Larvae head length (mm) for different treatments](image)
Figure 3

A

Tail DNA (%)

0 (water) 0 (solvent) 0.3X PY 1X PY 0.3X LO 1X LO 0.3X HO 1X HO

B

Micronuclei (‰)

0 (water) 0 (solvent) 0.3X PY 1X PY 0.3X LO 1X LO 0.3X HO 1X HO
Table 1. Experimental set up validation. Rainbow trout embryos were exposed to FLUO- or BaP-spiked gravel. Different letters refer to significant differences with solvent control (Mean ± SD, N = 3, Anova, p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>0 (Solvent)</th>
<th>BaP 3 µg g⁻¹ dw</th>
<th>FLUO 3 µg g⁻¹ dw</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH concentration (ng g⁻¹ dw) (spiking efficiency %)</td>
<td>ND¹</td>
<td>1110 (37)</td>
<td>2657 (88)</td>
</tr>
<tr>
<td>Embryonic mortality (%)</td>
<td>2.0±2.8</td>
<td>2.7±2.3</td>
<td>6.7±2.3</td>
</tr>
<tr>
<td>Larval mortality (%)</td>
<td>2.2±3.1</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Half-hatched embryos mortality (%)</td>
<td>0.0±0.0</td>
<td>2.7±4.6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Time to hatch (DD)</td>
<td>359.2±9.4</td>
<td>362.7±4.2</td>
<td>360.4±4.7</td>
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<tr>
<td>Hatching rate (%)</td>
<td>94.0±2.8</td>
<td>86.7±2.3</td>
<td>92.0±10.6</td>
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<tr>
<td>Larvae head length (mm)</td>
<td>12.6±0.6</td>
<td>12.4±0.3</td>
<td>12.2±0.1</td>
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<tr>
<td>Larvae total length (mm)</td>
<td>2.7±0.13</td>
<td>2.6±0.06</td>
<td>2.6±0.06</td>
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<tr>
<td>Larval abnormalities (%)</td>
<td>10.0±4.7a</td>
<td>64.4±13.9b</td>
<td>56.7±8.8b</td>
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<tr>
<td>Yolk-sac</td>
<td>0.0±0.0</td>
<td>8.9±10.2</td>
<td>5.0±4.4</td>
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<tr>
<td>Spinal</td>
<td>0.0±0.0a</td>
<td>17.8±3.8b</td>
<td>16.7±3.3b</td>
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<tr>
<td>Craniofacial</td>
<td>0.0±0.0</td>
<td>4.4±3.8</td>
<td>16.1±9.5</td>
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<tr>
<td>Cardiac</td>
<td>0.0±0.0</td>
<td>2.2±3.8</td>
<td>2.2±3.8</td>
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<tr>
<td>Edema</td>
<td>10.0±4.7a</td>
<td>35.6±7.7b</td>
<td>38.3±10.4b</td>
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<tr>
<td>Hemorrhages</td>
<td>0.0±0.0</td>
<td>20.0±11.5</td>
<td>25.0±14.4</td>
</tr>
</tbody>
</table>

¹Non Detected
Table 2. Measured PAH concentrations in gravels spiked with three different PAH fractions LO, HO or PY (ng g⁻¹ dw).

<table>
<thead>
<tr>
<th>Compound</th>
<th>N cycles</th>
<th>Solvent</th>
<th>PY</th>
<th>LO</th>
<th>HO</th>
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<tr>
<td>Naphthalene*</td>
<td>2</td>
<td>1</td>
<td>&lt; LQ</td>
<td>&lt; LQ</td>
<td>9</td>
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<tr>
<td>Acenaphthylene*</td>
<td>3</td>
<td>1</td>
<td>&lt; LQ</td>
<td>7</td>
<td>27</td>
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<tr>
<td>Acenaphthene*</td>
<td>3</td>
<td>1</td>
<td>&lt; LQ</td>
<td>2</td>
<td>12</td>
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<tr>
<td>Fluorene*</td>
<td>3</td>
<td>5</td>
<td>&lt; LQ</td>
<td>7</td>
<td>38</td>
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<tr>
<td>Dibenzo[bd]thiophene</td>
<td>3</td>
<td>5</td>
<td>&lt; LQ</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Phenanthrene*</td>
<td>3</td>
<td>10</td>
<td>&lt; LQ</td>
<td>58</td>
<td>256</td>
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<tr>
<td>Anthracene*</td>
<td>3</td>
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<td>&lt; LQ</td>
<td>21</td>
<td>118</td>
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<tr>
<td>Fluoranthene*</td>
<td>4</td>
<td>5</td>
<td>&lt; LQ</td>
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<td>490</td>
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<tr>
<td>Pyrene*</td>
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<tr>
<td>Benzo[a]anthracene*</td>
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<td>&lt; LQ</td>
<td>79</td>
<td>234</td>
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<td>Triphenylene + Chrysene*</td>
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<td>&lt; LQ</td>
<td>101</td>
<td>315</td>
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<td>Benzo[b]naphto[2,1-d]thiophene</td>
<td>4</td>
<td>10</td>
<td>&lt; LQ</td>
<td>65</td>
<td>626</td>
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<tr>
<td>Benzo[b+k+j]fluoranthene*</td>
<td>5</td>
<td>10</td>
<td>&lt; LQ</td>
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<td>626</td>
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<tr>
<td>Benzo[e]pyrene</td>
<td>5</td>
<td>5</td>
<td>&lt; LQ</td>
<td>76</td>
<td>235</td>
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<td>Benzo[a]pyrene*</td>
<td>5</td>
<td>10</td>
<td>&lt; LQ</td>
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<td>Perylene</td>
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<td>Indeno[1,2,3-cd]pyrene*</td>
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<td>&lt; LQ</td>
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<td>Dibenzo[ah]anthracene*</td>
<td>5</td>
<td>5</td>
<td>&lt; LQ</td>
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<td>281</td>
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<td>2-Methylnaphthalene</td>
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<td>1</td>
<td>&lt; LQ</td>
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<td>29</td>
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<td>2</td>
<td>1</td>
<td>&lt; LQ</td>
<td>4</td>
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<td>3-Methylphenanthrene</td>
<td>3</td>
<td>10</td>
<td>&lt; LQ</td>
<td>14</td>
<td>66</td>
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<tr>
<td>2-Methylphenanthrene</td>
<td>3</td>
<td>10</td>
<td>&lt; LQ</td>
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<td>108</td>
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<tr>
<td>2-Methylnaphthalene + 9-Methylphenanthrene + 2-Methylanthracene</td>
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<td>10</td>
<td>&lt; LQ</td>
<td>23</td>
<td>&lt; LQ</td>
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<tr>
<td>1-Methylphenanthrene</td>
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<td>4-Methyl dibenzothiophene</td>
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<td>40</td>
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<tr>
<td>3+2-Methyl dibenzothiophene</td>
<td>3</td>
<td>2</td>
<td>&lt; LQ</td>
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<td>13</td>
</tr>
<tr>
<td>1-Methyl dibenzothiophene</td>
<td>3</td>
<td>2</td>
<td>&lt; LQ</td>
<td>14</td>
<td>81</td>
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<td>Dimethylnaphthalenes</td>
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<td>5</td>
<td>&lt; LQ</td>
<td>10</td>
<td>100</td>
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<td>Trimethylnaphthalene</td>
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<td>5</td>
<td>&lt; LQ</td>
<td>17</td>
<td>76</td>
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<td>Tetramethylnaphthalenes</td>
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<td>&lt; LQ</td>
<td>14</td>
<td>81</td>
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<td>Dimethylphenanthrenes</td>
<td>3</td>
<td>10</td>
<td>&lt; LQ</td>
<td>72</td>
<td>345</td>
</tr>
<tr>
<td>Trimethylphenanthrenes</td>
<td>3</td>
<td>10</td>
<td>&lt; LQ</td>
<td>57</td>
<td>281</td>
</tr>
<tr>
<td>Methylchrysesnes</td>
<td>4</td>
<td>10</td>
<td>&lt; LQ</td>
<td>93</td>
<td>335</td>
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</table>

Σ US EPA priority PAHs*  < LQ  1038  3503  108  1082  535  2040

Spiking efficiency (%)  40  42  24  42  42  50

* PAHs labelled as priority substances by the US Environmental Protection Agency

1 Limit of quantification
Table 3. Developmental defects in rainbow trout following embryonic exposure to the three PAH fractions PY, LO and HO. Different letters refer to significant differences with solvent control (Mean ± SD, N = 3, Anova / KW, p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>0 (water)</th>
<th>0 (solvent)</th>
<th>0.3X PY</th>
<th>1X PY</th>
<th>0.3X LO</th>
<th>1X LO</th>
<th>0.3X HO</th>
<th>1X HO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic mortality (%)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±2.3</td>
<td>1.3±2.3</td>
<td>1.3±2.3</td>
<td>1.3±2.3</td>
<td>0.0±0.0</td>
<td>13.3±8.3</td>
</tr>
<tr>
<td>Larval mortality (%)</td>
<td>3.9±6.8</td>
<td>1.7±2.9</td>
<td>3.5±6.1</td>
<td>8.7±5.5</td>
<td>8.0±8.9</td>
<td>6.3±10.8</td>
<td>2.6±3.7</td>
<td>25.1±31.2</td>
</tr>
<tr>
<td>Half-hatched embryos (%)</td>
<td>1.3±2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7±2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.7±4.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.7±2.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.0±4.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0±0.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.7±2.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hatching rate (%)</td>
<td>98.7±2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>100.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.3±2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>94.7±6.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>86.7±2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>94.7±4.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>96.0±2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>72.0±6.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to hatch (DD)</td>
<td>315.6±5.9</td>
<td>318.9±6.5</td>
<td>316.5±3.1</td>
<td>311.1±5.5</td>
<td>310.5±12.0</td>
<td>316.7±6.2</td>
<td>308.5±8.0</td>
<td>308.0±6.8</td>
</tr>
<tr>
<td>Abnormal larvae (%)</td>
<td>26.7±6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.6±9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.9±7.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>68.9±10.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>81.7±10.4&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>82.2±10.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>95.6±3.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100.0±0.0&lt;sup&gt;d&lt;/sup&gt;</td>
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