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# Effects of polycyclic aromatic hydrocarbons (PAHs) on the embryonic hatching rate and development of *Oryzias latipes*

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**ABSTRACT**

Polycyclic aromatic hydrocarbon (PAHs) are hydrocarbons whose structures are made up of aromatic rings. Crude oil spills often expose PAHs to the environment, with negative consequences for the inhabiting organisms. PAHs are known to negatively affect the development of fish. However, it is not known in detail how the embryonic stage of fish is affected by exposure to PAHs. The purpose of this study was to investigate the effects of PAHs on the embryo of the *Oryzias latipes*, by utilizing the partition controlled delivery method to test 6 kinds of PAHs with the polymer polydimethylsiloxane. We investigated the dose-response relationship between the hatching rates of the *Oryzias latipes* and the PAHs at various concentrations. The hatching fish were observed by optical microscopy. The results of the experiment confirmed that exposure to the PAHs caused various deformities.

## 1. Introduction

The use of polycyclic aromatic hydrocarbon (PAHs) in the industry has been on the rise. Consequently, pollution from the industry has exposed the environment to significant doses of PAHs. PAHs contain several different kinds of compounds that can influence the environment. However, environmental risk assessment and management procedures often focus only on individual compounds of the PAHs, rather than evaluating the substance as a whole [1]. Such procedures cannot accurately assess the environmental impact of PAHs, due to the synergetic properties of the compounds within the PAHs [2–4]. Therefore, it is most important to assess the environmental impact of PAHs in its entirety. The most efficient and effective method to do so is to build a model that predicts the effects of the PAHs, rather than direct evaluation [5–7].

Fish exposed to PAHs displayed morphological aberrations and decreased growth [8–10]. Fish affected by the PAHs have developed blue sac disease (BSD). The symptoms of the disease include hemorrhaging, deformities, and edema; the affected fish have released cytochrome P450 (CYP1a) enzymes [11].

Fish affected by BSD at the time of growth showed symptoms similar to fish exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [12]. Alkylated PAHs were found to be the cause of BSD. Study of the effects of the Alaska North Slope oil spill of 2006 found that the concentration of TCDD in the presence of C1 to C4 alkyl-phenanthrenes, alkyl-naphthalenes, and alkyl-chrysenes in the water were found to be responsible for the presence of BSD in nearby salmon [12].

Developing trout and *Oryzias latipes* exposed to 7-isopropyl-1-methylphenanthrene (retene, a C4-phenanthrene)

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displayed similar symptoms [12]. The concentration of this substance in the water at normal levels can be anywhere between 1 and 30 μg/L [13]. While there is a clear general correlation of exposure to PAHs leading to anomalies of fish development, more in-depth studies need to be conducted with regard to the various specific kinds of PAHs, due to its multiplicity in available forms.

Both polydimethylsiloxane (PDMS) silicone rods [14] and silicone cast into glass vials [15,16] were used to partition test compounds within the liquid phase of the bioassays. Partition-controlled delivery (PCD) was used to assess toxicity, using the eggs from *Oryzias latipes* [17]. This partitioning-based method is used to transfer test substances from high concentration PDMS films into the water in test vials. Partitioning works to stabilize the volatility within aqueous solutions, and prevent any dissipation caused by adsorption, allowing the test substance to be maintained at a certain concentration in an aqueous solution for a period of time. This method allows for the hydrophobic materials to be maintained at a certain concentration (5–15 mg) for a period of time. When using the PCD with PDMS polymers, hydrophobic materials in the water can easily be formed and maintained within a small concentration range. This method is economic in making experimental material, and reduces the exposure of the test materials to the experimenter, which is why the method was chosen to evaluate the toxicity of the PAHs within *Oryzias latipes*. *Oryzias latipes* was chosen for the test subjects because of the relatively short time it takes the fish to mature and reproduce, and the fact that the chorion can be clearly observed in the developmental stage, thus making it the ideal candidate. To determine the water concentration of PAHs, all samples were periodically measured by gas chromatography–mass spectrometry (GC-MS).

The PDMS constant ($\log K_{ow}$) and water solubility limits were calculated for every PAHs. The model we built for the study predicts the environmental risk through the analysis of regression using the correlation between the EC50 and $K_{ow}$ values.

The results of this report can be referenced by decision-makers to predict certain risks a crude oil spill may impose on the environment, and to take the proper countermeasures for recovery.

## 2. Materials and methods

### 2.1. Chemical substances

The following PAHs were used for PAHs loading, extraction, and analysis naphthalene (9%, Sigma-Aldrich, Germany), fluorene (99%, Sigma-Aldrich, Germany), phenanthrene (99.5%, Sigma-Aldrich, Germany), pyrene (>99%, Sigma-Aldrich, Germany), dibenzothiophene (98%, Aldrich), 2-methylnaphthalene (97%, Aldrich), methanol (HPLC grade), and methyl chloride (>99.5%, Aldrich) (Merck, Darmstadt, Germany). Milli-Q water (SuperQ-treated, Millipore, MA, USA) was used for rinsing the PDMS polymer (SSP-M823).

### 2.2. Polydimethylsiloxane (PDMS) silicone rods

The PDMS to be used in the experiment was cut into a size of (30 mm × 50 mm × 1 mm) (width × height × depth). The jar was aerated and vented for 48 h at 4°C in a refrigerator, to remove impurities. PDMS polymer was immersed in n-hexane for 1 d, then immersed in methanol for another, to remove any impurities. The PDMS was stored in fresh methanol, before being put into the PAHs jar.

### 2.3. Loading PAHs into the PDMS silicone

The PAHs solution loading method based on partitioning followed the methanol standard solution [16]. The individual PAHs were dissolved in a solution of methanol and tertiary distilled water in a ratio of 7:3 at a concentration of 10 ppm. The PAHs loading solution was mixed with the PDMS for 3 d, for sufficient saturation of the PAHs material. The PAHs material was not dissolved, and the crystal form was removed. After removing the loading solution, the PAHs residue and methanol in the jar were wiped off with a clean cloth. Finally, the solution was rinsed 3 times with a small amount of Milli-Q water (5 mL), to fully remove the methanol in the silicone. The rinsing took approximately 1 h, and the lid on the jar was kept closed, in order to prevent the contents of the jar from being volatilized and lost. The jar was then kept in a dark place.

### 2.4. Collection of the eggs

Fish are kept in a 16:8 light cycle at a water temperature of (26°C ± 1°C). In order to maintain the generation, the fish were fed TetraMin® or TetraBits® three to four times a day. On average, the fish produced (10–30) eggs a day. The eggs formed their chorions after the fertilization. The eggs were then removed from the female within a few hours [18]. Any foreign substances around the eggs were cleaned through washing. Afterwards, the eggs collected in the fish culture were kept, until they were exposed to the experimental material.

### 2.5. Preparation of the toxicity assay

The vials were filled with 50 ml of fish culture medium, and the PDMS loaded with the PAHs was added to the medium, in order to allow for the release of the PAHs into the medium. The control vials were prepared in the same way. The PDMS used for the control vials did not contain PAHs. Five fertilized *Oryzias latipes* eggs were put into each vial, exposing them to the PAHs. The temperature was maintained at (26°C ± 1°C), and the photocycle was maintained at 18:6 h. To maintain adequate oxygen concentration, the vial was opened daily, and also shaken by hand. In order to prevent the water quality from being contaminated by egg decomposition, dead eggs were immediately removed, whenever seen. Six kinds of PAHs were tested six times per kind with a single type of embryo exposure test. The concentration of the test solution was measured daily by GC-MS. All of the samples analyzed by GC-MS were subjected to liquid-liquid extraction, using methyl chloride (DCM) as a solvent. The test solutions extracted by the DCM were concentrated through the use of nitrogen. The DCM was next replaced with hexane. The concentrated solution was then placed in hexane, in order to further purify it. The extracts were analyzed using a GC-MS (Agilent 6890/HP 5973) with

splitless mode injection on a bonded phase fused silica capillary column DB5-MS ((30 m × 0.25 mm) i.d. × 0.25 μm film thickness). The column temperature was 70°C for 4 min, and ramped at 10°C/min to 300°C, which was held for 10 min. The injector and detector temperatures were kept at 260°C and 300°C, respectively. The flow rate of helium was 1 mL/min.

### 2.6. Embryo observation and EC50 determination

The exposure time of the *Oryzias latipes* to the PAHs in the vials was 17 d. Both the control and test embryos were observed once a day by optical microscopy. The yolk sac edema confirmed that fluid accumulation occurred on the yolk sheet. Pericardial edema is caused by the accumulation of liquids in the pericardial sac and occurs along with general swelling. Hemorrhages were observed in many areas of the fish. Spinal deformities were identified by the curvature of the spinal column. Yolk sac absorption delayed swim bladder inflation, and abnormal swimming motions were all observed after hatching. Both the hatching and mortality rate was recorded at the end of each experiment. For each test substance, the values of the toxic effects of the PAHs were expressed as a percentage. Median effective concentration (EC50) values were obtained using both the ToxCalc 5.0 program and the sigmoidal dose-response graph.

### 2.7. Optical microscopy

Images were captured with Clemex Captiva (v. 6.0.023, Clemex Technologies Inc., Longueuil, QC, Canada) software, and exported as (720 × 480) BMP file format images. Compound optical microscope (Olympus SZ61, Olympus America Inc., Center Valley, PA, USA) was used to observe the physical anomalies of the *Oryzias latipes*. When compared with the fish of the control group, the fish exposed to the PAHs-polluted vials all displayed abnormal hatching rates and high malformation rates.

## 3. Results and discussion

### 3.1. Characterization of the assay

The test solution that the *Oryzias latipes* were exposed to was prepared by releasing PAHs into the water using a specially prepared PDMS polymer. The PDMS polymer was immersed in the water for 24 h, to allow the PAHs to equilibrate well in [15,17]. While the amount of PAHs contained in the PDMS was the same regardless of the type, the amount of PAHs that was released into the water depended on the substance.

Fig. 1 (the molecular structure of PAHs) and Table 1 (the $K_{ow}$ value of the molecular weight of the PAHs) show the molecular structure and basic information of the six types of PAHs to be studied in this study. The six different kinds of PAHs were each tested six times. The embryos were exposed to the PAHs for 17 d, but were not exposed to the PDMS polymer. The test solution was analyzed by GC-MS to confirm that the PAHs were successfully being released from the PDMS into the water. Fig. 2. (GC-MS) shows the results. Table 2 shows the concentrations of the PAHs used in the experiment. The process of releasing the

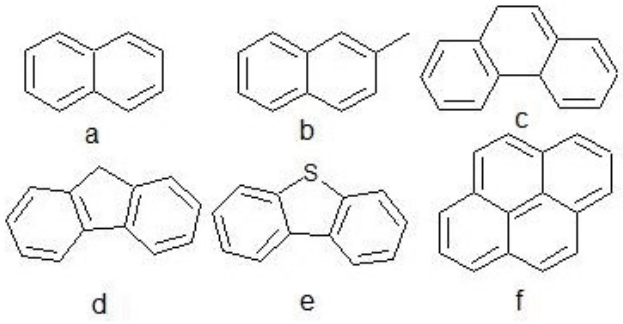


Fig. 1. Molecular structure of PAHs in this study (a) naphthalene, (b) 2-methylnaphthalene, (c) phenanthrene, (d) fluorine, (e) dibenzothiophene, and (f) pyrene.

Table 1
Basic information of PAHs studied in this study

| PAH | MW | Water solubility | log$K_{ow}$ |
|---|---|---|---|
| Naphthalene | 128.17 | 31 mg/L (25°C) | 3.34 |
| 2-methylnaphthalene | 142.20 | 24.6 mg/L (25°C) | 3.86 |
| Fluorene | 166.22 | 1.69 mg/L (25°C) | 4.22 |
| Dibenzothiophene | 184.26 | 1.47 mg/L (25°C) | 4.44 |
| Phenanthrene | 178.23 | 1.15 mg/L (25°C) | 4.53 |
| Pyrene | 202.25 | 0.135 mg/L (25°C) | 5.07 |

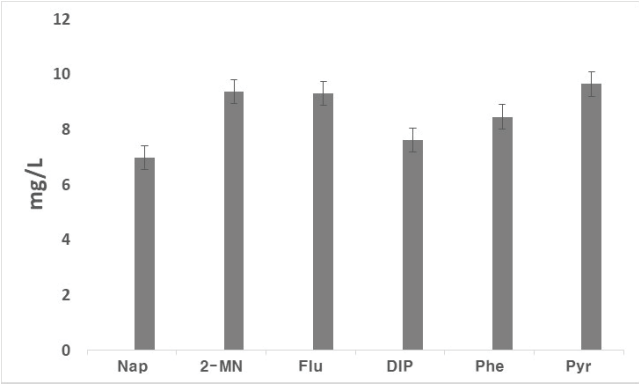


Fig. 2. Analysis of PAHs by the concentration of initial concentration test solution using GC-MS (naphthalene, 2-methylnaphthalene, fluorene, dibenzothiophene, phenanthrene, pyrene).

PAHs into the water from the PAH-loaded PDMS resulted in concentrations that were close to the aqueous solubility. The aqueous solubility of the PAHs depended on the type of PAH. Creating PAH solutions using the PDMS polymer produced solutions with the required concentration range, without any loss of the PAHs. Through this method, the PAHs were able to be safely and practically kept at a constant concentration in the water. It was imperative to keep the concentration of the PAHs constant, as the fish was only affected at a certain concentration [15].

The number of hatching fish from the embryos exposed to PAHs was routinely checked, and Fig. 3 shows the dose-response graph for the effects of the PAHs on the *Oryzias*

Table 2
Measurement of PAHs concentration (mg/L) in test solution made with PDMS polymer using GC-MS

| | Naphthalene | 2-methylnaphthalene | Fluorene | Dibenzothiophene | Phenanthrene | Pyrene |
|---|---|---|---|---|---|---|
| 100% | 6.99 ± 0.18 | 9.38 ± 0.14 | 9.32 ± 0.47 | 7.62 ± 0.30 | 8.46 ± 0.35 | 9.65 ± 0.45 |
| 50% | 3.49 ± 0.09 | 4.69 ± 0.07 | 5.66 ± 0.23 | 3.81 ± 0.15 | 4.23 ± 0.18 | 5.82 ± 0.22 |
| 25% | 1.75 ± 0.05 | 2.35 ± 0.03 | 2.83 ± 0.12 | 1.91 ± 0.07 | 2.11 ± 0.09 | 2.912 ± 0.11 |
| 12.5% | 0.87 ± 0.02 | 1.17 ± 0.02 | 1.42 ± 0.06 | 0.95 ± 0.04 | 1.06 ± 0.04 | 1.46 ± 0.06 |
| 6.25% | 0.44 ± 0.01 | 0.58 ± 0.01 | 0.71 ± 0.03 | 0.48 ± 0.02 | 0.53 ± 0.02 | 0.73 ± 0.03 |
| 3.125% | 0.22 ± 0.01 | 0.29 ± 0.01 | 0.35 ± 0.01 | 0.24 ± 0.01 | 0.26 ± 0.01 | 0.36 ± 0.01 |

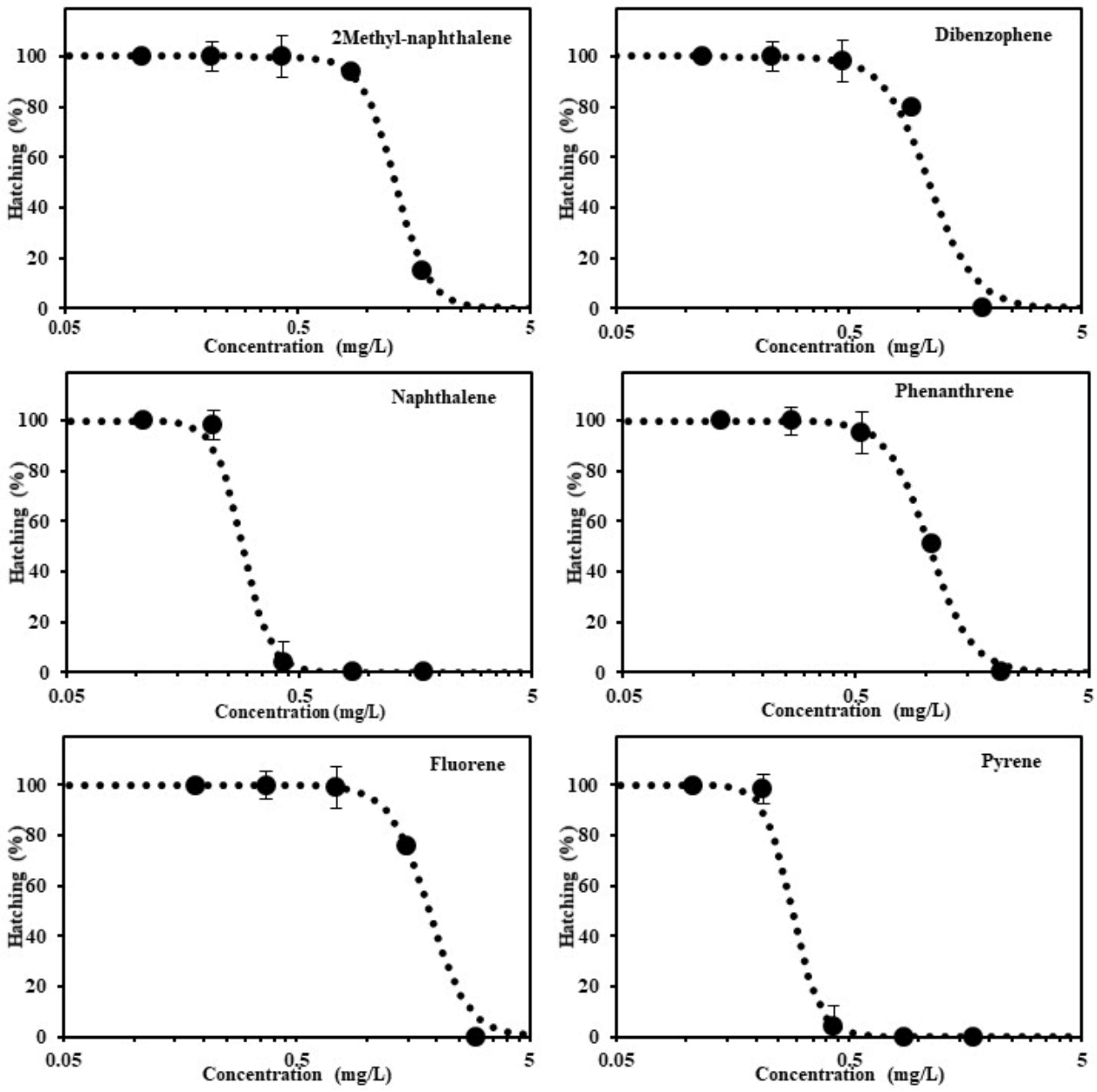


Fig. 3. Hatching rate (%) from the embryo of *Oryzias latipes* according to PAHs concentration.

*latipes* embryos by observation of the *Oryzias latipes* hatchlings. The median effective concentration (EC50), a concentration that could affect 50 % embryo hatchlings of the *Oryzias latipes*, varied depending on the type of PAHs.

The most notable difference among the effects of the PAHs was the difference between naphthalene and 2-methylnaphthalene. Although the difference between the two molecules is only a single methyl group substitution, the EC50 value that affects the embryo birth rate was vastly different. Table 3 shows the EC50 values of each PAHs. The highest required EC50 value was for 2-methylnaphthalene, while the lowest required value was for naphthalene. The EC50 values decreased as the log$K_{ow}$ values increased across all PAHs, with the exception of naphthalene. The hatching fish from the embryos affected by PAHs were observed by optical microscopy, and Fig. 4 shows photographs of the fish that were observed.

### 3.2. Effects of the PAHs

Embryos that were affected by a concentration of PAHs above a certain level failed to hatch. While the hatching

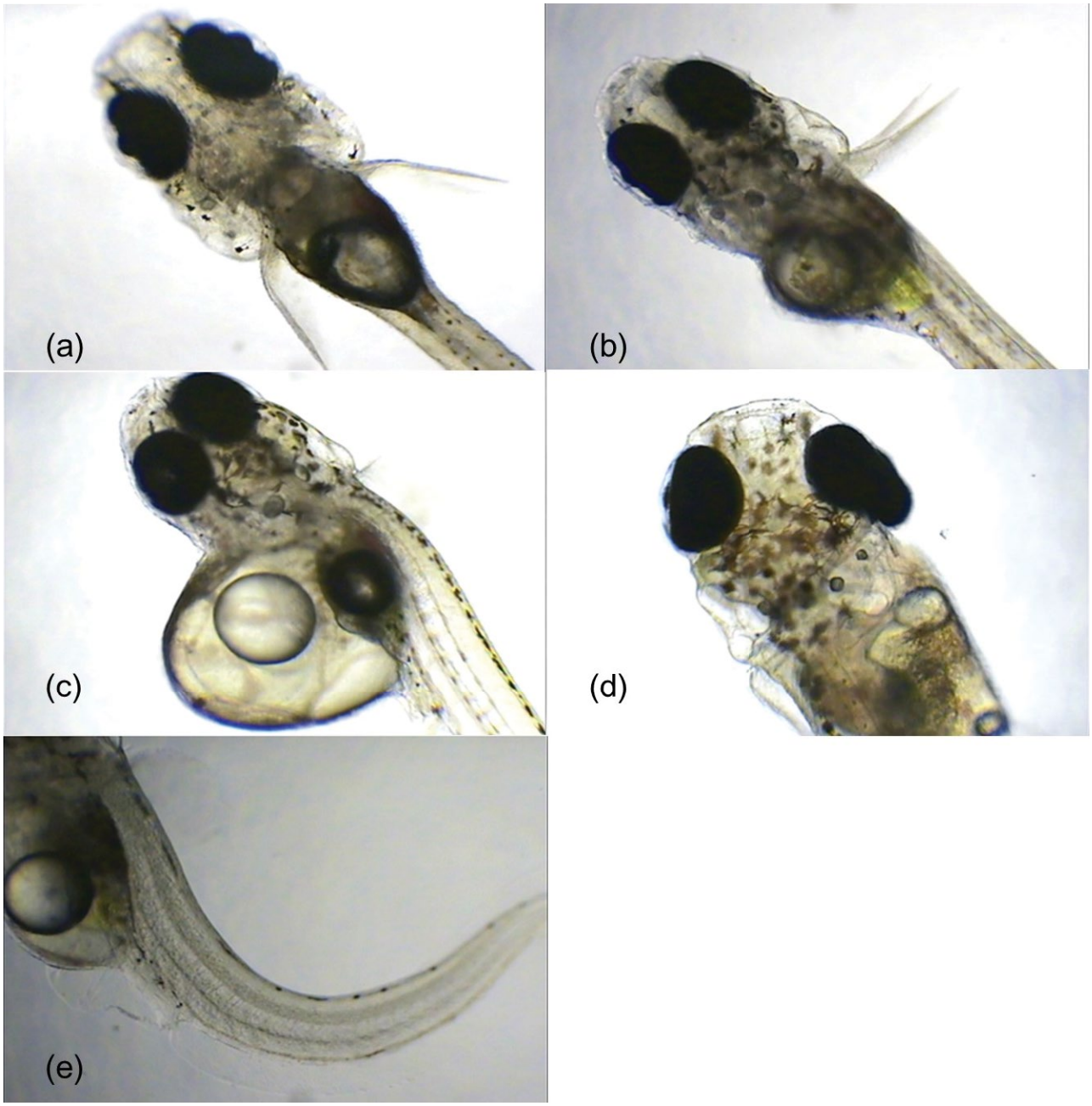


Fig. 4. Influence of PAHs on *Oryzias latipes* born from the embryo by the substance. (a) Control, (b) pyrene, (c) phenanthrene, (d) naphthalene, and (e) dibenzothiophene.

rate of the embryos was different depending on the type of PAHs the fish were exposed to, all of the PAHs above a certain concentration affected the rate. PAHs not only affected the embryo hatching rate. General anomalies observed across all PAHs included anomalies of facial structure, fusiform, and swelling of the abdomen. Table 3 shows the EC50 values affecting the birth of the embryos. One of the most striking findings was the different end results when the embryos were exposed to naphthalene, as opposed to 2-methylnaphthalene. The fish exposed to naphthalene showed the lowest hatching rate of the embryos. However, in the case of exposure to 2-methylnaphthalene, the hatching rate was relatively very high.

The embryos affected by the PAHs developed various mutations that led to morphological aberrations, as the PAHs acted as a chronic toxicity. The aberrations included BSD, hemorrhaging, and vertebral and facial structural deformities. The enzyme cytochrome P450 (CYP1A) was induced, in order to counter some of these aberrations.

Optical microscopy was used to observe the hatching *Oryzias latipes*, and Fig. 4 shows photographs comparing the control group *Oryzias latipes* and the exposed *Oryzias latipes*. The fish hatched from embryos exposed to dibenzothiophene presented spinal deformities, while the hatchlings of the embryos exposed to phenanthrene presented abnormal swelling of the abdomen. The fish exposed to pyrene had differing fin sizes, while the ones exposed to naphthalene displayed facial anomalies. Both BSD and abnormal swimming patterns were observed on the majority of the hatchlings, although the severity of both depended on the type of PAHs.

### 3.3. Characterization of the assay

This study was conducted to investigate the effects of exposure to embryos of *Oryzias latipes* on 6 PAHs. The embryo exposure period was 17 d, and the endpoint of the experiment was determined by consideration of the hatching rates. The PAHs-loaded PDMS released the PAHs into the water of the test vials by using GC-MS (Fig. 2). Despite the volatility and hydrophobic property of the PAHs, the PDMS successfully created the PAHs solution with little trouble. Based on the experimental data, we found that PDMS itself does not affect the embryo birth rate of *Oryzias latipes*.

The PAHs pass through the aryl hydrocarbon receptor (AHR) pathway, and induces the expression of the enzyme cytochrome P450 1A (CYP1A). PAHs with high molecular weight (>5 rings) have been reported to have carcinogenic and immunotoxic effects on certain organisms.

Incardona et al. [19] investigated exposure to PAHs using Zebrafish embryo, and found that the forms of anomalies that were induced depended on the number of benzene rings that made up the PAHs [19]. For substances that affected the AHR pathway, edema and cardiovascular diseases were frequently observed.

Dibenzothiophene and phenanthrene, despite not being as complex in molecular structure as PAHs, also induced similar defects as did PAHs [11,20–22]. However, it is still unclear as to what mechanisms these substances follow, to cause such defects. It is necessary to analyze how the molecular structure, specifically the number of benzene rings, affects the embryo. Intensive research should be conducted into exactly what mechanisms are involved when PAHs affect fish embryos.

Although the effects of PAHs on the embryo differed depending on the type of PAHs used, they commonly affected the fin structure of the fish, regardless of the type and structure of the PAHs, as observed by optical microscopy.

The degree to which the embryo of the *Oryzias latipes* was affected depended on the molecular structure of the PAHs. Naphthalene, which has the smallest number of benzene rings, was relatively less prone to causing malformations in the fish after hatching. However, the hatching rate was found to have been much lower, as compared to the other PAHs. In the case of phenanthrene and naphthalene, the numbers of benzene rings were 3 and 2, respectively, so the two were thus expected to have similar results. However, this was not the case.

This result is in line with the findings of [23], who studied how lipophilic substances affect early-stage fish [23]. In a study of the bioaccumulation and depuration of radiolabeled PAHs in zebrafish embryos and larvae, naphthalene and phenanthrene were found to bioaccumulate to similar levels after 24–30 h of exposure 7–8 mmol/kg of dry weight for embryos, 4–5 mmol/kg for larvae). Phenanthrene was found to cause cardiovascular diseases in fish, but naphthalene did not. Naphthalene, which only has two benzene rings, did not affect the fish as hazardously as did the three-ringed phenanthrene. The effects of naphthalene and phenanthrene on the hatching fish were similar to those reported by [19].

Fluorene and naphthalene have different molecular structures, despite having the same number of benzene rings. Therefore, the effects on the *Oryzias latipes* embryos and larvae were different. Using data from zebrafish to determine the correlation between PAHs $\log K_{ow}$ and bioconcentration [23], fluorene could be estimated to accumulate 96% of the accumulation of dibenzothiophene in organisms. The effects on the cardiac system of the fish hatched from the embryos were slightly different. Fluorene did not affect the embryo birth rate of *Oryzias latipes*, but it did induce BSD in the hatchlings, and affected the formation of the dorsal fins.

While 2-methylnaphthalene is a type of alkyl-PAHs of naphthalene that is similar to naphthalene in basic structure, it has a slightly different molecular composition. The alkyl groups vary in size, and can be located at different positions in the benzene ring, so there are more molecules of different alkyl-PAHs per PAHs. The hatchlings exposed to 2-methylnaphthalene had a higher hatch rate, as compared to those

Table 3
EC50 values affecting the embryos of *Oryzias latipes*

| PAHs | Median effective concentration (EC50) (mg/L) |
|---|---|
| Naphthalene | 0.33 ± 0.01 |
| 2-methylnaphthalene | 3.23 ± 0.15 |
| Fluorene | 1.98 ± 0.07 |
| Dibenzothiophene | 1.29 ± 0.02 |
| Phenanthrene | 1.10 ± 0.12 |
| Pyrene | 0.55 ± 0.02 |

exposed to naphthalene. However, as mentioned before, BSD and fin anomalies appeared in most of the hatching fish, regardless of the PAHs. This is because naphthalene and 2-methylnaphthalene affect the embryo and larvae through different mechanisms [24–26].

In a study of bioaccumulation using zebrafish, it was estimated that the radiolabeled pyrene accumulated to about 3 mmol/kg dry weight in the zebrafish larvae [23]. In the case of toxic effects caused by pyrene, the fish were mostly affected during their early stages of growth, rather than it being chronically toxic. These actions include anemia, edema, loss of peripheral circulation, and cell death. Pyrene-induced toxic mechanisms have mechanisms similar to TCDD, and have pathways that act strongly on AH receptors (AHRs). There have been many studies that have exposed zebrafish to TCDD. The results of a series of studies show that zebrafish exposed to TCDD display erythropoiesis, apoptotic cell death, and pericardial [25,27–30]. The results induced by TCDD are shown by inducing CYP1A, and may be affected by the use of an AH receptor antagonist or CYP1A inhibitor [27,28,31]. In a study conducted by [32], gene silencing of AHR or CYP1A antisense morpholinos revealed that the AHR pathway was required for pericardial edema induced by TCDD in zebrafish [32]. Many studies on pyrene and TCDD have yielded a number of results that suggest that the AHR pathway may be affected by both pyrene and TCDD. In our study, when *Oryzias latipes* embryos were exposed to pyrene, when the concentration of pyrene was high, the fish did not hatch in the embryo. When the concentration decreased, the fish eventually hatched from their embryos, but displayed various anomalies, such as malformed dorsal fins and facial structures.

### 3.4. Observation of the hatching fish

The hatching *Oryzias latipes* were observed by optical microscopy. When compared with the control group, the *Oryzias latipes* exposed to the PAHs displayed various abnormal physical features and behaviors. Each of the PAHs affected the *Oryzias latipes* at different concentrations. However, even at low concentrations, certain traits, like BSD, were observed across all subjects exposed to PAHs.

One of the most significant results of this study was the difference in birth rates between fish exposed to naphthalene vs. 2-methylnaphthalene. Fig. 4 shows a photograph of a fish hatched from an embryo exposed to 2-methylnaphthalene. Among embryos exposed to naphthalene, the rate of hatching was low. However, the amount of observed physical deformities was low as well. On the other hand, fish born from embryos exposed to 2-methylnaphthalene bore opposite results: a relatively high hatching rate, but also a high rate of visible deformities.

2-methylnaphthalene is a naphthalene with an alkyl group attached, and is thus a different molecular structure. Alkyl-PAHs have different mechanisms to induce toxicity than do PAHs. The respective toxicities of C1 and C2 naphthalene were not expressed in the fish embryos. These results suggest that the mechanisms by which PAHs and alkyl-PAHs may affect the fish embryo differ from each other. Incardona et al. [19] observed the fish hatching from zebrafish embryos affected by PAHs. In this study, the fish hatching from embryos affected by fluorene, dibenzothiophene, and phenanthrene showed bends in the spine of the tail, and significantly decreased growth, especially at the head. In addition, in embryos affected by dibenzothiophene and phenanthrene, we observed pericardial and yolk-sac edema. On the other hand, in embryos affected by fluorene, mild pericardial edema appeared. In the case of anomalous anomalies that are affected by PAHs, genetic testing, rather than just microscopy, can be used to determine genetic anomalies from PAHs.

## 4. Conclusion

This study was an experiment to evaluate the toxicity of PAHs using *Oryzias latipes* embryo. Based on the toxicity experiment, the correlation between the $\log K_{ow}$ value and the EC50 value of the PAHs used in the experiment was derived. Although the number of PAHs used in the experiment is small, it may be sufficient to elucidate the correlation between the chemical structure and toxicity of PAHs. However, this experimental data may help to evaluate the toxicity of PAHs in crude oil. The $\log K_{ow}$ value of selected PAHs in the experiment was chosen between (3 and 5). However, similar results were obtained when the *Oryzias latipes* embryo affected by selected PAHs was affected by crude oil. More in-depth studies have shown that selecting PAHs in the wider $\log K_{ow}$ range, except for the PAHs used in the experiment, can more accurately assess the toxicity of PAHs, and can better judge what the level of contamination will be when contaminated with crude oil.

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