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Preliminary investigations on effects of polycyclic aromatic hydrocarbons on embryos of widow tetra, *Gymnocorymbus ternetzi* Boulenger

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Abstract
Polycyclic aromatic hydrocarbons (PAHs) present in crude oil effluents, when released into aquatic ecosystem pose a major threat to fishes and humans. We investigated the harmful effects of PAHs on early embryonic and larval development of *Gymnocorymbus ternetzi*. Embryos when exposed to samples containing Dibenzothiophene (3 rings), Phenanthrenes (3 rings) and Pyrenes (4 rings) at varying concentrations (15–90 µg/L) exhibited defective embryonic and larval development. As PAHs targets aryl hydrocarbon receptor (AhR) pathway, cardiac deformities were persistent in embryos exposed to PAHs for >48 hrs; concentration specific defects included arrested embryonic development (36 ± 2.6%), cytoplasmic blubbing (24%), pericardial edema and cardiac looping (43 ± 4.2%) and tail bents and body curvature (19%). However, embryonic defects were reversible (>47%) when transferred to clean water immediately after <24hrs of exposure. Suitability of tetras for eco-toxicology studies to investigate effects of aquatic pollutants on embryonic development, advantages and disadvantages are discussed.

Keywords: PAHs, effluents, aquatic toxicology, cardiac looping, body curvature, embryonic development

1. Introduction
Aquatic pollution by organic compounds present in untreated effluents released from oil refineries and oil spills pose a major threat to fisheries and indirectly the aquatic food web. Though, marine ecosystem can reduce their concentrations by upwelling, fresh water lacks such mechanisms [23, 24]. Untreated effluents released into aquatic ecosystem and ground water affects human health; impacts were widely reported from both developed and developing countries therefore, studies on long-term effects of organic pollutants on aquatic life forms were required [32, 28]. Polycyclic Aromatic Hydrocarbons (PAHs) are the most abundantly occurring toxic organic chemicals found in refinery effluents reaching adjacent water bodies [26, 30]. Both marine and fresh water food webs may be altered by PAHs since they can remain persistently in the medium and exposed organisms resulting in bio-magnification. Increasing levels of PAHs were attributed to offshore oil refineries and oil spills for decades, affecting aquatic species and the very livelihood of human population dependant on them [2, 30]; degradation of ecosystem further augmented by urbanization and anthropogenic activities [22]. Consumption of infected fishes induced toxicity in humans, since, physiological and biochemical mechanisms of fish embryonic and larval development are conserved and similar to humans [19]; harmful effects of exposure to organic pollutants upon early embryonic development can be studied using inexpensive ornamental fish models such as Buenos Aires Tetra (BT), *Hemigrammus caudovittatus* [1] and zebrafish [19]; tetras may become a valuable genetic model for assessing pollution in fresh water ecosystem [17, 19]. Tetras are freshwater ornamental fishes with attractive features and amenability to genome manipulation techniques [5-8]. Tetras are excellent vertebrate models for developmental biology, aquatic toxicology and biomedical research owing to genetic homology and visceral organs similarity to humans, optically transparent embryos and larvae that remain amenable to several genome editing technologies, high fecundity (>300 eggs) enabling sibling studies, external and shorter embryonic developmental duration (24 hrs), defined embryonic stages, with fully functional larvae in <3 days post hatching (dph), early (4 months) sexual maturity, and vulnerability to toxic aquatic pollutants attributes to their ability as a humanized vertebrate model [9-11] and

for serving as an *in vivo* screening tool for identifying novel therapeutic targets in human diseases [5, 7-9]. Interestingly, effects of PAHs on zebrafish [18-20] and marine fishes [28, 29, 30] were similar to tetras (present study), and hence may be successfully utilized as biosensors for monitoring aquatic pollution. In the present study, our objectives were to evaluate the impact of 3 major PAHs upon *Gymnocorymbus ternetzi* (Boulenger) [1] embryos (i) early embryonic development defects (ii) Heart formation and function (ii) lethal and sub-lethal effects on larval growth and survival.

2. Materials and methods

2.1 Water sample collection and analyses

Water samples were collected from 3 different locations (Irumapanam (9.96ºN/76.35ºE), Ilanje (9.58 ºN/76.17ºE) and Ambalamukkal (9.96 ºN/76.37ºE) near oil refineries from Kochi, Kerala using glass bottles and brought to lab within 24hrs and preserved using methylene chloride until further experimentation inside a deep freezer (-20 ºC; Haier, China), to prevent degradation of pollutants. Water quality parameters were analysed for quantifying PAHs concentrations using conventional chemical methods [21, 28]. Majority of the PAHs belonged to 2 ringed (Fluorenes and Dibenzothiophenes), 3 ringed (Phenanthrenes) and 4 ringed (Pyrenes) compounds (Figure. 1).

2.2. Tetras as Ecotoxicology model:

Tetras [10-16] are attractive models for ecotoxicology research. Fishes were maintained in 15 litre glass tanks with sufficient aeration at 14:10 hr light: dark cycles at room temperature (26 ± 2 ºC); isogenic strains were established [5]. Fishes were fed twice daily with artificial pelleted diet and sexually mature adults with blood worms, twice a day, during breeding. For embryos, gametes were collected from brooders for artificial fertilization following established protocols [5-12].

2.3. Embryo Assay

Developing embryos (3hpf) were sorted into groups of 25 embryos/treatment group (in triplicates) into 12 well plates containing water samples (1millilitre) incubated for about 12, 24, 48 and 72 hrs. Every day, undeveloped and dead embryos were counted before medium renewal until completion of the experiments [27]. Embryos were monitored for lethal and sub-lethal effects i.e. abnormal development and deformities identified were recorded and imaged using stereo-zoom microscope (Lawrence & Mayo, India). For imaging, control and treated embryos and larvae were mounted onto glass slides using 1.5% methyl cellulose and imaged following established protocols [5, 12 -16].

3. Results

3.1. Water quality analyses

Water samples preserved (-20 ºC) using methylene chloride prevented degradation of organic constituents. Analyses of Polycyclic Aromatic Hydrocarbons (PAHs) in the water showed concentrations ranging from 16 – 90 µg/L with a pH ranging from 6.2- 7.1pH. Samples were found to contain predominantly more Dibenzothiophenes (43±3.4%) Phenanthrenes (27±4.1%), Fluoranthenes (17±1.4%) and Pyrenes (13±1.7%). Alkalinity of the water samples ranged from 59 – 196 mg/L (CaCo3). Lead (Pb) levels were 4 – 16 µg/L and Cadmium (Cd) levels were 1.2 – 4.37 µg/L (Table 1).

3.2. Effects of PAHs on tetras embryos and larvae

Concentration specific defects were noted in embryos (73 ± 3.2%) exposed to water samples for >48 hr; of the defective embryos; of which, 36 ± 2.6% underwent arrested embryonic development before hatching or exhibited larval deformities (64 ± 3.7%) after hatching. However, upon prolonged exposure (72hr), 27 ± 4.7% of the hatchlings had irreversible morphological deformities (Figure. 1), exhibited pericardial edema, cardiac looping, tail bent and lordosis (Table 1). When embryos were exposed to PAHs for overnight (24hr) alone, 63 ± 4.7% were able to complete embryonic development and interestingly, some of the defects such as yolk sac edema and block in blood vessels (37 ± 3.2%) exhibited mild abnormalities that were partially reversed after rearing the larvae in fresh autoclaved tap water. Representative samples of treated embryos, arrested embryonic development and control embryos and deformed hatchlings along with control larvae are shown in Figure 2. In embryos exposed to extended durations >72hr, effects were more pronounced with >76 ± 2.6 embryos failed to survive, while remaining hatchlings succumbed after 5 days of exposure, even after incubation in autoclaved tap water.

Table I: PAHs and heavy metal concentrations in water samples and their effects on embryos and larvae

S.no	Sample/ Location	PAHs (µM/L) and metal (µg/L) concentrations	Effects on embryos/larvae
1	Irumpanam / Kochi	pH: 6.17; Dibenzothiophene- 47 Phenanthrene- 34; Fluoranthenes- 11 Pyrene- 14; Lead – 4.16 ± 1.64; Cadmium- 2.43 ± 0.56 ; CaCo3 – 64 ± 7.46	Dorsal curvature, Pericardial edema, Yolk sac edema; cytoplasmic blubbing; Tail bents
2	Ambalamukal / Kochi	pH: 6.86; Dibenzothiophene- 103; Phenanthrenes- 81 ;Fluoranthenes -37 ;Pyrenes -31; Lead – 13.43 ± 2.31; Cadmium- 3.76 ± 1.23; CaCo3 – 168 ± 12.61	Arrested embryonic development; Pericardial edema and Cardiac looping; heart beat conduction defect- Bradycardia; Tail and body curvature
3	Ilanje / Kochi	pH: 7.19; Dibenzothiophene- 69; Phenanthrenes- 54; Fluoranthenes - 28; Pyrenes - 27; Lead – 4.16 ± 1.64; Cadmium- 2.43 ± 0.56; CaCo3 – 193 ± 10.31	Cytoplasmic blubbing; arrested embryonic development; pericardial and yolk sac edema; Tail bents

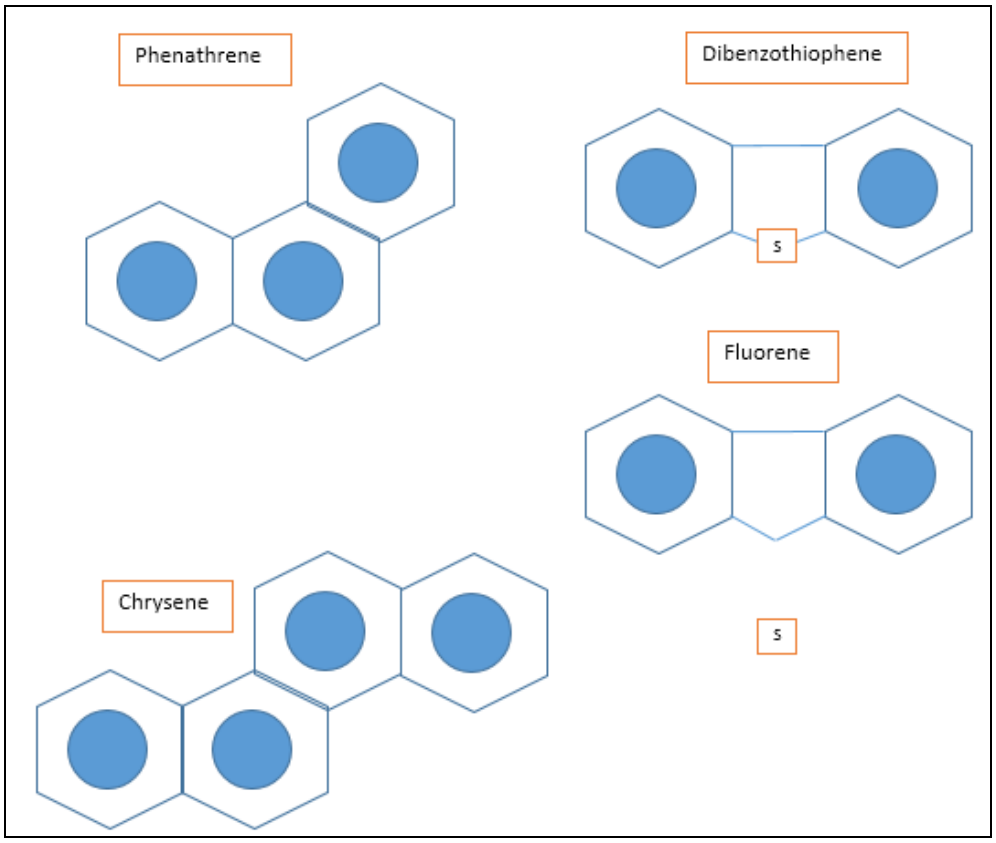

Fig 1

Fig 2: Effects of PAHs on embryonic development of widow tetra. 1, Control embryos at 80% epiboly stage 2. Arrested embryonic development ~1000 cell stages. 3. Cytoplasmic blubbing 4. Control larvae 5. Control larvae with tubular heart 6. Pericardial edema with cardiac looping. 7. Tail and body curvature. 8. Yolk sac edema with tail bents. 9. Hatched but deformed larvae exhibiting pericardial and yolk sac edema

4. Discussion

Polycyclic Aromatic Hydrocarbons in effluents contain Phenanthrenes (3 ringed) and Dibenzothiopenes and Fluranthenes (2 ringed) and Pyrenes (4 ringed) as major constituents that induced cardiac deformities i.e. edema and looping, defects in neural tube and cranio-focal, skeleton deformation in vertebrates [32]. PAHs act through nonpolar necrosis by activating aryl hydrocarbon receptor (*AhR*) pathway inducing Cytochrome P4501A1 (CYP1A1) protein expression in most vertebrates. Activated *AhR* dimerizes with *AhR* nuclear translocator to bind with response elements such as Xenobiotic Response Elements (XREs) and DREs in the CYP1A1 expression pathway [34]. Recently, *in vitro* studies conducted in zebrafish liver cell lines with PAHs detected toxicity in aryl hydrocarbon receptor binding, confirming the harmful effects of PAHs on fishes [19, 20]. Earlier studies have confirmed the carcinogenic potential of PAHs to embryos and larvae in fishes upon exposure during development [28, 29]. Tetra embryos also exhibited similar deformities such as cardiac looping and pericardial edema after prolonged exposure (>24 hr) to PAHs thus confirming the conserved mechanism of action through *Ahr* pathway [34]. Heintz *et al.* (1999) [14] have reported gross malformations in fish larvae exposed to chemical pollutants; most common being the pericardial and yolk sac edema, lordosis (tail bent) or scoliosis (dorsal curvature), bradycardia (reduction in heartbeat) and arrhythmia leading to death of the larvae. Tetras also displayed similar phenotypic defects especially pericardial and yolk sac edema which were more pronounced with increasing duration of exposure while lordosis and scoliosis appeared only after >72 hr exposure (Figure. 1) hence, may be beneficial for eco-toxicological studies. Earlier studies on pacific herring embryos exposed to petroleum –derived PAHs also confirm above reported developmental deformities [33]. However, in tetras, three major deformities were more pronounced firstly, arrested embryonic development by cytoplasmic blubbing before reaching 1000 cell stage. Secondly, hatchlings (>48 hr) exhibited cardiac defects, pericardial and yolk sac edema and thirdly, dorsal tail and trunk curvature disrupting blood circulation resulting in premature death of hatchlings in accordance with earlier reports [27].

From the present study, we infer that though both three ringed and two ringed PAH compounds differ in structure, it is evident that they target *Ahr* pathway alone following a common biochemical pathway resulting in cardiac dysfunction by disrupting membrane integrity; incorporation of hydrophobic organic compounds induced bradycardia by repolarizing of potassium or calcium channel in zebrafish heart [18-20]. Based on gene expression studies in tetras, these compounds altered (down regulated) the expression of functional genes in cardiomyocytes but not those involved in cardiac development and morphogenesis (data not shown). We also confirm that dibenzothiophene and phenanthrenes induced mostly cardiac defects but curvature of the trunk or tail and stunted growth resulted only upon prolonged treatment while Pyrenes induced less pronounced body curvature as reported in other fish species [4]. There are other class of PAHs such as dioxins which may act on distinct biochemical pathways detrimental to early embryonic development and larval growth [15], which were not included in the present study. The observed effects upon exposure to PAHs on developmental abnormalities in tetra embryos and larvae resembled earlier studies conducted in zebrafish larvae [16] and inland silverside, *Menidia beryllina* [25].

Exposure of zebrafish embryos to the non-alkylated PAH compounds at higher concentrations lead to cardiac looping and pericardial edema and dorsal body curvature correspondingly with a marked reduction in jaw size and facial deformities [13]. We also confirm that in tetras, exposure to phenanthrenes affects formation of heart chambers and initial stages of heart looping and also inhibited later stages of morphogenesis resulting in cardiac defects such as dilated and extended loop like heart structure indicating the failure to complete cardiac looping (Figure. 1). Earlier studies have recorded loss of circulation, comparable to the silent heart *sih* zebrafish mutants that lacked heartbeat conduction due to loss of Troponin T, an essential component of sarcomere assembly [31]. Our present study also confirms mode of action and similar targets of these PAHs especially on heart beat conduction culminating in bradycardia and dysregulated auricular-ventricular (AV) conduction; effects were highly dose dependant and increased with increasing exposure duration and concentration of the compounds [35]. But, these effects were reversed when tetra embryos were re-incubated in clean water for extended duration (~5 days) after <24 hr exposure. Thus from the present study, we suggest that tetras can serve as potential model for studying harmful impacts of organic PAHs on embryonic development, organ formation and sexual maturity in adult fishes. Studies on molecular pathways and gene expression disrupted by PAHs in economically important food fishes may help better understand long-term detrimental effects of PAHs toxicity to fishes and humans.

5. Conclusion

PAHs present in untreated effluents from oil refineries are toxic and pose severe threat to aquatic ecosystem and humans. We successfully utilized tetra embryos and larvae for testing harmful effects of the aquatic pollutant and confirmed their ability to serve as inexpensive models for eco-toxicology studies since, genetic, molecular and physiological mechanisms are similar to humans. Elucidation of molecular pathways dysregulated by PAHs in tetras may help better understand long-term effects of hazardous environmental pollutants on aquatic species and ecosystem.

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