Chapter II
CHAPTER II

DEVELOPMENTAL TOXICITY OF PYRIPROXYFEN ON EMBRYONIC ZEBRAFISH

2.1. Introduction

The increasing usage of pesticides on agricultural practice has resulted in elevated levels in the environments (Adeyemi et al., 2015). Usually, the pesticides used in agriculture often affect the early life stages of aquatic organisms (Pašková et al., 2011). It is well known that the embryonic stages of development are the most susceptible to environmental toxins (Yang et al., 2009). To investigate the toxicity of chemicals, early developmental stage assays have been increasingly used because of its great potential to study the wide range of endpoints (Scholz et al., 2008) including developmental and biochemical parameters (Oliveira et al., 2009). The use of teratogenic parameters as biomarkers in response to environmental contaminants is quite common (Adeyemi et al., 2015).

Zebrafish is a well-established vertebrate model for assessing developmental toxicity of exposure to toxicant during early development in the aquatic environment (Deng et al., 2009). Short life cycle, rapid development (within 72 hours), cost effective maintenance, high fecundity, transparency of embryos, sensitive to toxicants are the advantages of using zebrafish embryos as model system in pharmacology and toxicology (Schmidt et al., 2015). Female zebrafish are able to produce hundreds of eggs and hence large sample sizes are easily achieved which allowing for statistically powerful dose-response studies of a large number of substances in a short period (Truong et al., 2011).

Embryogenesis is highly sensitive to toxicant exposure and zebrafish embryos are the most commonly using model of early life stages in toxicity studies (Miranda-Rodriguez et al., 2017; Sant et al., 2017). Fish embryo toxicity (FET) test was recently validated and adopted as a standard test method in zebrafish embryos by the European Union (Braunbeck et al., 2015). Embryo experiments are useful tools to provide reliable hazard identification and improve the predictive power of developmental toxicity studies of chemicals (Braunbeck et al., 2005; Rial et al., 2013; Kluver et al., 2015).
Zebrafish embryos have been widely used for toxicity assessment of pollutants on growth and development (Liu et al., 2017b). The developmental deformities during the embryonic development, including growth retardation, yolk sac edema, pericardial edema, spinal curvature, hyperemia, pigmentation defects are used as toxicity endpoints in embryo development (Jin et al., 2016; Li et al., 2018). Developmental toxicity of pyrethroid insecticides (Demicco et al., 2010) and the single and joint toxicity effects of chlorpyrifos and beta-cypermethrin (Zhang et al., 2017) have been reported in zebrafish embryos. Embryotoxicity of carbendazim was reported in other fish species prussian carp embryos (Ludwikowska et al., 2013). Recently Li et al. (2018) also reported developmental deformities in zebrafish embryos exposed to pyraclostrobin, trifloxystrobin and picoxystrobin.

Heart is the first functional organ during the early zebrafish embryo development (Shi et al., 2017). Heart rate is often used to assess cardiac function in embryonic tests, as an important toxicological endpoint (Glickman and Yelon, 2002; Li et al., 2018). Hill et al. (2005) have established an excellent correlation with known adult human cardiac toxicity and heart-rate responses of zebrafish. Various studies have shown the effect of pesticides and contaminants in heart development. Yang et al. (2016) have found inhibition of heartbeat in zebrafish embryos exposed to thifluzamide. The cardiovascular toxicity of acetochlor was found in zebrafish embryos pericardial edema, bradycardia, circulation defect, and thrombosis (Liu et al., 2017b). Moreover, heart malformations have been reported in zebrafish embryos exposed to auranofin (Gao et al., 2017) and 2,4-dichlorophenoxyacetic acid have been also been reported (Li et al., 2017).

The toxicity of PPF has been extensively studied in invertebrate species (Ishaaya and Horowitz, 1995; WHO, 2006; Jambulingam et al., 2008; Ginjupalli and Baldwin 2013; Caixeta et al., 2016; Kakaley et al., 2017). However, ecotoxicological effect of PPF in aquatic vertebrates is limited (particularly in fish) to few studies (WHO, 2006; Padilla et al., 2012; Truong et al., 2016; Dzieciolowska et al., 2017). Hence, in this study the adverse effect of PPF was assessed on embryonic developmental stages of zebrafish. Endpoints like developmental abnormalities, heart rate and heart size, were investigated to reveal new insights into the underlying the PPF toxicity.
2.2. Materials and methods

2.2.1. Test compound

Pyriproxyfen with IUPAC name of 4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether was purchased from Sigma Aldrich (≥98.0%) purity (CAS No. 95737-68-1; Cat No. 34174). The compound was dissolved in 100% DMSO at the concentration of 10 mg/mL as stock.

![Chemical structure of Pyriproxyfen](image)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC Name</td>
<td>4-Phenoxyphenyl (R/S)-2-(2-pyridyloxy)propyl ether</td>
</tr>
<tr>
<td>Chemical formula</td>
<td>C_{20}H_{19}NO_{3}</td>
</tr>
<tr>
<td>Molar mass</td>
<td>321.38 g mol^{-1}</td>
</tr>
<tr>
<td>Log K_{ow}</td>
<td>5.37</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>0.367 mg/L at 25°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>318 °C</td>
</tr>
</tbody>
</table>

2.2.2. Zebrafish housing and embryo collection

Wild type zebrafish (AB strain) were procured from Zaman aquarist, Chennai, Tamil Nadu and housed in clean well aerated glass tanks. Fishes were fed with commercial feed (Taiyo max) twice daily. Healthy adult male and female fishes (2:1 ratio, respectively) were placed in the spawning tank and light: dark cycle (14:10) was maintained to induce spawning. The fertilized embryos obtained were transferred into a clean petri dish and washed with exposure medium to remove the debris. Then the embryos were staged according to Kimmel et al. (1995) under inverted microscope (EVOS, Life technologies) and removed any unfertilized or dead embryos. Embryos at approximately at 3 hpf (hours post fertilization) were used for the exposure.
2.2.3. Exposure

The exposure medium contains 294 mg/L of CaCl$_2$.2H$_2$O, 63.0 mg/L NaHCO$_3$, 123.3 mg/L MgSO$_4$.7H$_2$O and 5.5 mg/L KCl (ISO, 2007; Ku et al., 2015) and it was prepared freshly before every exposure. According to OECD 236, the guideline on fish embryo toxicity test (FET) was followed to carry out the experiment (OECD, 2013). Three concentrations viz., 0.16, 0.33 and 1.66 µg/mL (0.52, 1.04 and 5.2 µM, respectively) were chosen for the present study, based on the effective concentration 50 (EC 50) value (5.2 µM) determined in zebrafish embryos by Truong et al. (2016). Embryos were gently transferred to the exposure plates containing respective concentrations of the test chemical. The exposure was given in semi static condition in 100 mm diameter sterile glass plates at 50 embryos per 50 mL of exposure medium and the test solutions were renewed daily till 96 hpf. During the exposure period, the dead embryos found were removed immediately to avoid the contamination. Experiments were performed in triplicate with internal replicates and respective control groups were maintained without adding the toxicant.
2.2.4. Quantification of PPF in exposure medium

The nominal exposure concentrations of PPF were determined in the exposure medium by GC-MS analysis. Briefly, 2 ml of exposure medium was collected in triplicate from each PPF treatment group at the time of exposure (T₀) and prior to renewal (T₂₄) of test medium. The samples were diluted with deionized water to the final volume of 500 mL in a separating funnel and 10.0 g of sodium chloride was added to increase the ionic strength. Then the samples were extracted twice with 50 mL of n-hexane and the hexane layer was collected in a conical flask. After, the extracts were added with 2.0 g of anhydrous sodium sulfate for dehydration. The extract were then transferred to condensation flask and condensed to near dryness in the rotary evaporator (Buchi Rotavapor). Finally, the flasks were rinsed well with 2 mL of n-hexane and transferred to auto sampler vial (Agilent Technologies, USA).

Samples were analyzed by using Gas Chromatography - Mass Spectrometer (GC-MS QP 2010) (Shimadzu Corporation, Japan). The analytical separation was carried out with HP-5MS column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness) with the following conditions. Initially, GC oven temperature was held at 150 ºC for 3 min and then the temperature was gradually increased to 280 ºC at the rate of 7 ºC /min. Finally, the column temperature was increased to 320 ºC at the rate of 8 ºC /min and held for 5 min. Sample injection (1 µL) was performed using an autosampler (AOC-20i) in the split-less mode. Helium (99.999 % purity) was used as a carrier gas at a flow rate of 1.50 mL/min. The injector port, interface, and ion source temperatures were set at 280 ºC, 300 ºC, and 230 ºC, respectively. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV and at an emission current of 60 µA. Full scan data was obtained in a mass range of m/z 50-500. Scanning interval and selected ion monitoring (SIM) sampling rate were 0.2 s. The mass selective detector was operated in SIM mode. The PPF in the embryo medium was quantified based on peak areas of external calibration. The calibration curve was generated from 50 to 300 ng/mL using linear regression analysis and the linearity was qualified by linear correlation coefficient, R² (Figure 2.2).
Figure 2.2. Calibration curve of PPF at 50, 100, 200 and 300 ng/ml concentrations with $R^2$ value of 0.998.

2.2.5. Developmental toxicity, heart rate and heart size measurement

During exposure period, the embryos were observed under inverted microscope (EVOS FLc, Life technologies) to study the developmental abnormalities at different time interval viz., 24, 48, 72 and 96 hpf (Figure 2.3). The endpoints used to assess developmental toxicity were pericardial edema, yolk sac edema, hyperemia and spinal deformity. Malformations were described and documented among the embryo from both the control and PPF treated groups. The heart rate was measured in the zebrafish embryos at 48, 72 and 96 hpf by following Ahmad et al. (2015). Embryos (n=30) from each treatment group were anesthetized with 0.016% tricaine (Sigma Aldrich, USA) and heart beat was counted for 30 s under inverted microscope. Further, we also measured the heart size of zebrafish embryos at 48, 72 and 96 hpf using ImageJ software (National Institute of Health, Bethesda, MD).
Figure 2.3. Imaging of zebrafish embryo development

2.2.6. Statistical Analysis

Statistical analyses were performed by using Graph Pad Prism software (version 5.1). The two way ANOVA followed by Bonferroni post-test was employed to determine the significant differences in heart rate and heart size measurement. Data were presented as mean ± SEM. The statistical acceptance level was P<0.05 for heart rate and P<0.01 for heart size measurement.
2.3. Results

2.3.1. Quantification of PPF in exposure medium

The nominal exposure concentrations of PPF (0.16, 0.33 and 1.66 µg/mL) were measured to ensure the concentrations at during exposure time (T₀) and before renewal period (T₂₄). The result shows that at T₀, the measured concentration of PPF in the exposure medium was slightly lower than the nominal concentrations (Table 2.1). The measured values at before renewal (T₂₄) period were decreased than T₀ with the deviation of 25.78, 36.90 and 35.03%, respectively.

Table 2.1. Measured concentrations of PPF (mean ± SD) in the exposure medium

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Measured concentration in the exposure medium (µg/mL) and deviation (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th>Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₀</td>
<td>T₂₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.160</td>
<td>0.141 ± 0.017</td>
<td>0.104 ± 0.014</td>
<td></td>
<td>-25.78</td>
</tr>
<tr>
<td>0.330</td>
<td>0.296 ± 0.022</td>
<td>0.187 ± 0.023</td>
<td></td>
<td>-36.90</td>
</tr>
<tr>
<td>1.660</td>
<td>1.522 ± 0.034</td>
<td>0.989 ± 0.027</td>
<td></td>
<td>-35.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> Deviation = (Measured concentration (T₀- T₂₄))/Measured concentration (T₀) x 100%.

<sup>b</sup>n.d = not detected.

2.3.2. Effect of PPF on zebrafish development

In this study, different concentrations of PPF were evaluated in the zebrafish embryos for the developmental toxicity endpoints like malformation and heart rate. The deformity result shows that in 0.16 µg/mL PPF exposed embryos, the morphological features were quite similar to that of untreated embryos. However, deformities like pericardial edema and scoliosis were observed in the 0.33 and 1.66 µg/mL of PPF treated embryos (Figure 2.4). In addition, elongation of heart, yolk sac edema and hyperemia was found in the embryos exposed with 1.66 µg/mL PPF treatment.
Figure 2.4. Microscopic images showing morphological changes in zebrafish embryos between control and PPF treated with 0.16, 0.33 and 1.66 µg/mL at 24, 48, 72 and 96 hpf time interval. PE - Pericardial edema, YSE - Yolk sac edema, HY - Hyperemia, SC - Scoliosis, H - Heart, DH - Delayed Hatching, ND - Normal Development and NSA - Normal Spine Axis.
2.3.3. Effect of PPF on heart development and heart rate of zebrafish embryo

The heart rate was studied for the better understanding of the effect of PPF. The images of cardiac region between control and 1.66 µg/mL treated zebrafish embryos was shown in Figure 2.5. The results of heart rate shows that, there was no significant changes in the heart rate in 0.16 and 0.33 µg/mL of PPF treatment groups till 96 hpf (Figure 2.6). Whereas, significant (P<0.05) increase in the heart rate was observed in 1.66 µg/mL PPF exposed embryos at 48 and 72 hpf. Interestingly, at 96 hpf the heart rate was found to be declined significantly (P<0.05) than the control embryos and significant changes in the morphology of cardiac tissue (heart linearization) was also observed.

![Figure 2.5. Effect of PPF on heart development. Images a, b & c were control and d, e, & f were PPF treated with 1.66 µg/mL concentration, respectively. H - Normal Heart, PE - Pericardial edema, A - Auricle, V - Ventricle and * indicates accumulation red blood cells. The yellow marking shows the cardiac region.](image-url)
Figure 2.6. Represents the heart rate of zebrafish embryos at different time points (48, 72 and 96 hpf) exposed to PPF (0.16, 0.33 and 1.66 µg/mL). Two way ANOVA followed by Bonferroni post-test was used. The results are mean ± SEM of triplicate samples and * denotes significant difference at P<0.05.

2.3.4. Effect of PPF on heart size of zebrafish embryo

In addition to heart rate, the heart size was also measured to address the effect of PPF in zebrafish embryo at higher treatment group (1.66 µg/mL) during 48, 72 and 96 hpf. The heart size measurement in the zebrafish embryo is shown in Figure 2.7. The result shows that at 48 and 72 hpf, the heart size was marginally higher than the control embryos however, there was no statistical significance was found in heart size (Figure 2.8). Whereas at 96 hpf, we observed that the heart size was significantly increased (elongated) than the control heart. Moreover, there was no significant changes in the heart size were observed in 0.33 and 1.66 µg/mL exposure groups.
Figure 2.7. Image representing the heart size measurement (yellow line) in the zebrafish embryo at 96 hpf

Figure 2.6. Represents the heart size measurement in zebrafish embryos (n=10) of control and PPF treated with 1.66 µg/mL concentration at 48, 72 and 96 hpf. Two way ANOVA followed by Bonferroni post-test was used. The results are mean ± SEM and * denotes significant difference at P<0.01.
2.4. Discussion

The present study was aimed to explore the adverse effect of PPF in vertebrate model zebrafish embryo using multiple biomarker end points. The results obtained in this study shows that at higher concentration of PPF could cause developmental deformities.

The nominal exposure concentrations of PPF in the exposure medium of all the treatment group were quantified to find the corresponding test concentration. In the present study, we found that measured concentrations of PPF in the medium were slightly lower than the nominal concentrations. The decrease in the measured concentrations could be due to the adsorption (Tu et al., 2014) or by the experimental error (Shi et al., 2017). In similar to our result, Horie et al. (2017) also reported the decrease in the measured concentration of PPF in the exposure medium than the nominal concentration. Further, the measured PPF content in the exposure medium ($T_{24}$) were also decreased than the concentrations of before exposure ($T_0$) in all the treatment groups. Similarly, the decrease in the measured concentrations was also reported in the exposure medium treated with 6:2 chlorinated polyfluorinated ether sulfonate (Shi et al., 2017). The decrease in the PPF concentration in the medium might be due to the uptake by zebrafish embryos (Tu et al., 2014). However, more toxicokinetic data is required to explain the decrease of PPF concentration in the exposure medium and uptake in zebrafish embryos.

The early life stage of an organism has been extensively used to assess the developmental abnormalities which is an important toxicological end point (Scholz et al., 2008; Adeyemi et al., 2015). The abnormalities like pericardial edema, altered heart development and spinal and vertebral anomalies are considered as the indicators of toxicity in the zebrafish embryo development (Pamanji et al., 2015). In the present study, we found that the semi static exposure of PPF in zebrafish embryos causes developmental abnormalities like pericardial edema, heart linearization, spinal curvature, hyperemia, and yolk sac edema at higher concentration (1.66 µg/mL or 5.2 µM). Moreover, we have also noticed the abnormal swimming behavior of embryos in the form of circular movements at higher concentration.

In similar to our result, recently Horie et al. (2017) and Dzieciolowska et al. (2017) were also found teratogenic effects in PPF exposed zebrafish embryos at above 0.66 µg/mL and 1 µg/mL concentration, respectively. In addition, Truong et al. (2016) also reported
developmental defects like pericardial edema, yolk sac edema, bent axis and abnormal snout and jaw in PPF exposed zebrafish embryos at above 6.4 µM concentration. Similar developmental deformities has been reported in zebrafish embryos exposed to clomazone (Stevanovic et al., 2017), 6:2 chlorinated polyfluorinated ether sulfonate (Shi et al., 2017) and pyraoxystrobin (Li et al., 2018a). The body curvature observed in this study caused the embryos to swim in a circular pattern (McClain et al., 2012). The RBC accumulation (hyperemia) in the PPF treated embryos is probably due to the weakening of heart muscles, edema formation and reduction in the heart rate.

The heart is the first organ to develop and function in zebrafish (Hill et al., 2005). The developing heart appears to be a sensitive target of developmental toxicity (Kopf and Walker, 2009). Physiologically, the heart rate has been correlated to predict the metabolic rate of fish to some extent (Barrionuevo and Burggren, 1999; Pamanji et al., 2015). The present study shows that, the heart rate of zebrafish embryos was not altered by PPF in the lower concentrations till 96 hpf. But, in case of higher treatment group (1.66 µg/mL), we found significant increase in the heart rate upto 72 hpf and interestingly the count was reduced significantly at 96 hpf. In similar to our findings, recently Horie et al. (2017) also reported that at 3 dpf (days post fertilization) (72 hpf), the heart rate was found to be unchanged in embryos exposed to PPF with the maximum of 0.66 mg/L concentration. However, significant changes in the heart rate were observed in the present study at 1.66 µg/mL PPF concentration which was higher dose when compared to the exposure concentrations of Horie et al. (2017).

Moreover, the present study differs from Horie et al. (2017) in terms of experimental procedure, exposure concentrations and renewal of test solution. Both increase and decrease in heart beat counts has been reported in zebrafish embryos upon other contaminant exposures, for instance Ahmed et al. (2015) shown increased heart rate while, Rana et al. (2010) has reported the decrease in heart rate in cobalt ferrite nanoparticle and caffeine treatment, respectively. Yang et al. (2016) and Qian et al. (2018) have reported inhibition in heart rate exposed to thifluzamide and boscalid respectively. The reduction in heart rate might be probably due to the inhibition of AChE (Lin et al., 2007) and edema formation. Hence, the bibasic response observed in the heart rate at higher concentration of PPF exposed embryos suggests that with increasing exposure time, PPF could affect the cardiac rate in the zebrafish embryos.
Heart size measured in this study reveals important information that at 96 hpf, the heart size was significantly elongated in the embryos of higher PPF exposure (1.66 µg/mL) than the control. Similar results were also reported in zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (Antkiewicz et al., 2005) and monocrotophos (Pamanji et al., 2015). The elongation of heart observed in this study might be due to loss of attachment between the heart and common cardinal vein to migrate dorsally and hence mechanical stretching of heart muscles occurs (Antkiewicz et al., 2005). However, the detailed investigation is warranted to justify the PPF induced cardiotoxicity. Hence, our results suggest that PPF is developmentally toxic and could affect the heart development of zebrafish embryos at higher exposure level.

**Conclusion**

Developmental toxicity of PPF was evaluated in zebrafish development and it resulted in developmental deformities such as pericardial edema, yolk sac edema, hyperemia and scoliosis at higher exposed concentration. PPF also revealed cardiotoxic effects like biphasic effect in heart rate and heart linearization at highest concentration. Hence, PPF exposure in aquatic environment could affect the early life stages of aquatic organisms.