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Cyfluthrin Impairs Cardiac Physiology and Pigmentation in Zebrafish (*Danio Rerio)* Embryos

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Abstract
Widespread and indiscriminate use of insecticides is a major concern for human as well as environmental health. A fraction of the insecticides used in agriculture and domestic use finally enter aquatic ecosystems. While insecticides are used to control insect pests, their mechanisms of action are often not specific enough to prevent undesired effects, such as on non-target aquatic populations. Cyfluthrin is a non-systemic synthetic pyrethroid which affects both the central nervous system and peripheral nervous system and is widely used in agriculture and households as an insecticide. In this study, developmental toxicity of cyfluthrin at sublethal concentrations of 20, 40, 60, 80 and 100 ng/L were studied on zebrafish (Danio rerio) embryos at 24, 48, 72 and 96 hpf (hours post fertilization). The results showed that cyfluthrin caused severe cardiac abnormalities such as failure of embryonic hearts to loop properly, pericardial edema and increased rate of heartbeat. Cyfluthrin also caused reduced skin pigmentation in treated animals, indicating decelerated overall development. This study indicates that sublethal concentrations of cyfluthrin causes developmental defects in zebrafish early life stages after short term exposure. Use of cyfluthrin as an insecticide could potentially affect non-target species like fishes in aquatic ecosystems. Further research is necessary to understand the effect of cyfluthrin on aquatic ecosystems.

Keywords
Cyfluthrin, Developmental toxicity, Heart, Zebrafish.

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1. INTRODUCTION
Today pesticides are widely used all over the world. As a result, pollution of the environment by pesticides is a major concern. In agriculture, aquatic systems function as a source of water as well as drainage. Hence aquatic ecosystems are closely linked to agricultural systems. The current method of pesticide application allows a part of the pesticides used to enter aquatic ecosystems. As a result, non-target species living in water are potentially at risk from the pesticides [1, 2].

Synthetic pyrethroids (SPs) are a major class of widely used insecticides, similar in structure to pyrethrins. They are potent neurotoxicants, having high insecticidal activity and low mammalian toxicity [3]. However, SPs are extremely toxic to fish [4-6] and

beneficial arthropods like lobsters and shrimps [7-10].

Cyfluthrin is a photostable, fluorinated type II SP. It is a non-systemic insecticide widely used in agriculture and household use. Cyfluthrin affects the normal nerve function. It brings about changes in the permeability of sodium ion channels of nerve membranes leading to prolonged depolarization and hyper excitability. Thus it affects both the central nervous system and the peripheral nervous system [11]. It also shows weak anti-estrogenic activity [12-13].

The LC50 values of cyfluthrin toxicity for various fish species are 0.68 mg/L for rainbow trout (*Oncorhynchus mykiss*), 330.9 ng/L/96 hr for golden orfe (*Leuciscus idus*), 28 ng/L/96 hr for bluegill sunfish (*Lepomis macrochirus*) [14]. Cyfluthrin also bioaccumulates in the flesh and the maximum rate of cyfluthrin accumulation in the flesh of Nile tilapia (*Tilapia nilotica*) was 0.009 mg/L after five days, when exposed to a dose of 0.001 mg/L [15].

Cyfluthrin was detected at a concentration of 5 mg/L in freshwaters in southern Sweden. In England and Wales, cyfluthrin exceeded environmental quality standards at 1% of the sites tested [16-19]. Hence the potential movement of cyfluthrin to surface streams and its toxicity on non-target aquatic species is a major emerging concern.

Despite being highly toxic to fish [4, 5], few studies on aquatic toxicity of one of the most widely used SP, cyfluthrin to fish is available in open literature. Hence, it is very important to study effect of cyfluthrin on aquatic organisms.

Zebrafish (*Danio rerio*) is very useful as a toxicological model as it has a short life cycle, small size, transparent embryos and well established toxicological protocols [20,21]. Very few studies have been done on the effect of cyfluthrin on developmental stages of zebrafish.

The objective of the present study was to evaluate the sub-lethal effects of cyfluthrin exposure on the early developmental stages of zebrafish. The results would provide better understanding of cyfluthrin toxicity so that the endpoints measured may be used in developing monitoring strategies.

2. MATERIALS AND METHODS

2.1. Chemicals

Cyfluthrin[cyano-(4-fluoro-3-phenoxyphenyl) methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate, 96.4%] was obtained from Sigma (St. Louis, MO, USA). A stock solution was prepared in acetone and stored at 4°C in darkness. Test solutions of concentrations of 20, 40, 60, 80 and 100 ng/L were prepared by diluting the stock solution in E3 medium [22]. These concentrations were selected on the basis of trials done on lethal toxicity of cyfluthrin on zebrafish. All the other chemicals were of analytical grade and were obtained from S D Fine-Chem.

2.2. Maintenance and acclimatization of zebrafish

Five breeding pairs of zebrafish (*Danio rerio*) were obtained from a local aquarium. They were housed under semi-static conditions in a 10 L glass aquaria filled with charcoal filtered water. The water was constantly aerated and filtered. The aquaria was maintained in a light dark cycle of 14:10 h at 28 ± 1°C. The fishes were fed with live bloodworms and readymade fish flakes *ad libitum*. The aquarium was cleaned daily by siphoning the waste and by replacing 10% of the water daily. The fishes were acclimatized for a period of two weeks before breeding.

2.3. Breeding and Egg Collection

Eggs were obtained by mass spawning in a spawning aquarium with a mesh at the bottom to prevent cannibalization of the eggs. Spawning was triggered after half an hour of the light being turned on. The eggs were collected immediately and the debris was removed. The eggs were rinsed thrice in E3 medium. Unfertilized eggs were removed after observation under dissection microscope.

2.4. Experimental Setup and Exposure to Cyfluthrin

Seven 24-well plates were filled with 2 ml test solution in each well – one plate for negative control, one for solvent control and five plates for different concentrations of the cyfluthrin (20, 40, 60, 80 and 100 ng/L). The fertilized eggs at 3 hpf (hours post fertilization) were distributed, one egg in each plate. Each of the treatment groups had twenty embryos in the test solution and four embryos as internal control. The mortality in the internal control wells, negative control plates and solvent controls were kept within the acceptable range of one embryo death for each concentration for internal control and ≥ 90% survival for negative control and solvent control for each concentration [23]. The entire experiment was done in triplicates.

The plates were covered and incubated at 28 ± 1°C under light dark conditions of 14:10 h for a period of 96 h. The test solutions were refreshed every 24 h. The embryos were observed every 24 h. The endpoints selected for assessing the sub-lethal effects of cyfluthrin were survival rate, hatching, body length and heart beat rate. Any malformations like abnormalities in heart formation and skin pigmentation were recorded.

Every 24 h, the dead embryos were discarded. Any embryo without a visible heart beat was considered

as dead. Heart malformations were observed from 48 h onwards. The embryos were mounted in 3% cellulose so as to immobilize and position the embryos in the correct orientation under 100X magnification. The body length was measured using an oculometer under 50X magnification from the anterior tip of the snout to the posterior end of the caudal peduncle of the body at 72 and 96 h. The level of pigmentation was observed at 72 and 96 h. Pigmentation Index (PI) was developed for the present study based on earlier studies [24, 25]. A PI of 1 indicates complete absence of pigmentation, PI of 2 indicates pigmentation only around the eye, PI of 3 indicates pigmentation on the anterior dorsal part of the body, PI of 4 indicates mild pigmentation reaching to the tail end, PI 5 indicates intense pigmentation reaching to the tail, and PI of 6 indicates dorsal and ventral body strips are filling in with pigments. This is from 48 hpf onward according to Kimmel *et al.* [26] (Fig.1).

The toxicity assays were according to the guidelines of FET test [23]. All the stages of embryonic development of the zebrafish have been identified using Kimmel *et al.* [26] as a source of reference. All the protocols have been carried out in accordance with Sophia College Ethical Committee with CPCSEA registration No. 1936/Po/Re/S/17/CPCSEA.

2.5. Statistical analysis

The results were analyzed on the estimation of frequency of abnormalities observed. The data was presented as mean ± standard deviation (SD). Statistical differences between control and treatment groups were evaluated by one-way ANOVA, followed by Bonferroni and Holm pairwise comparison of means. The differences were considered statistically significant when *$p<0.05$ and extremely significant when **$p<0.01$.

3. RESULTS AND DISCUSSION

3.1. Effect of Solvent

Acetone in E3 medium was used as solvent control. Statistical analysis showed that there was no significant difference between the negative control and solvent control for all tests in this study.

3.2. Effect on Survival, Hatching and Growth

The survival rate at all concentrations was similar with respect to control for all observations recorded at 24, 48, 72 and 96 hpf. No mortality was recorded in all the treatment groups during the test period.

Hatching of zebrafish embryos was examined at 72 hpf. The hatching rates were 98.33 ± 2.87%, 98.33 ± 2.87%, 96.67 ± 2.87%, 95.00 ± 5.00%, 95.00 ± 5.00%, and 85.00 ± 5.00% at 0, 20, 40, 60, 80 and 100 ng/L, respectively. The hatching rates at 72 hpf were similar at 0, 20, 40, 60 and 80 ng/L of cyfluthrin with a significant decrease at 100 ng/L with respect to the control (Fig.2).

The body length of zebrafish embryos was measured as an indicator of effect on growth at 72 and 96 hpf. The body lengths at 20, 40, 60 and 80 ng/L of cyfluthrin were similar to control. However, embryos exposed to concentration of 100 ng/L showed significant retardation in growth as compared to control at both 72 and 96 hpf (Fig.3).

3.3. Effect on Cardiac Structure and Function

The cardiac morphology of the embryos was examining at intervals of 24 h, starting from 48 to 96 hpf. In zebrafish, heart is one of the primary organs and is the first one to form and function during embryo development [27,28]. The looping of the heart is a crucial component of heart formation in embryos. Initially, the heart is like a tube which later loops in a curved tube. This will later form the basis of mature heart [29, 30].

In the control group, the heart became S-shaped after looping. The two chambers of fish heart, atrium and ventricle overlapped and could be distinguished separately in lateral view as the ventricle and atrium lay side by side after looping [31].However in the embryos exposed to cyfluthrin, it was observed that the hearts were string-shaped and elongated. The ventricle was positioned in front of the atrium. This suggests that there was a malformation in the looping process. The atrium was thinner and more elongated as compared to control, while the ventricle was smaller and more compact.

The cardiac abnormalities increased in a time and dose dependent manner with the embryos exposed to concentration of 100 ng/L showing maximum abnormalities. The embryos exposed to 20 ng/L of cyfluthrin did not show any adverse effect. However, the embryos showing cardiac abnormalities also showed pericardial edema (Fig.4).

The distance between the junction of heart with inflow tract at sinous venosus (SV) and the junction of the heart with outflow tract at bulbous arteriosus (BA) can be measured as an index of the cardiac looping and is known as SV-BA distance [27, 28]. In this study, the SV-BA distance was measured in zebrafish hearts at 72 and 96 hpf. It was observed that cyfluthrin increases the SV-BA distance as compared to control in a dose-dependent manner. As seen from Fig 5, embryos in control and 20 ng/L of cyfluthrin showed reduction in SV-BA distance from 72 to 96 h, whereas the embryos exposed to 40, 60, 80 and 100 ng/L of cyfluthrin showed an increase in SV-BA distance during the same period. This indicates that normal looping of the heart took place

in control and 20 ng/L, resulting in reduction of SV-BA distance. However, failure of the heart to loop properly resulted in an increase in SV-BA distance in the other treatment groups. This indicates that cyfluthrin induces abnormal cardiac looping in zebrafish hearts (Fig 6).

The rate of heart beat is one of the important cardiac functions [32]. The embryos exposed to increasing dose of cyfluthrin showed increasing heartbeat, thus modifying the cardiac function (Fig.7). The increased heart beat could be due to pericardial edema and abnormal looping of the heart [33]. This study suggests that cyfluthrin impairs both cardiac structure and function of zebrafish embryos.

It should be noted that abnormalities caused by cyfluthrin did not result from an inability to form a functional heart. Initially at 48 hours, there was no morphological difference between the control and treated hearts. However, with increase in time, the cyfluthrin exposed hearts developed abnormalities and deteriorated in function compared to control hearts.

3.4. Effect on Pigmentation

According to Kimmel *et al.*, pigmentation can be used as an index for determining the stage of development [26]. Since pigmentation increases with embryonic development, decrease in pigmentation indicates retarded overall development. The PI of the cyfluthrin treated embryos at concentration of 20 ng/L showed no significant difference compared to the control. However, the PI of other treated groups was significantly lower than that of the control group. Thus, it can be concluded that cyfluthrin impairs development of the embryo (Fig.8.)

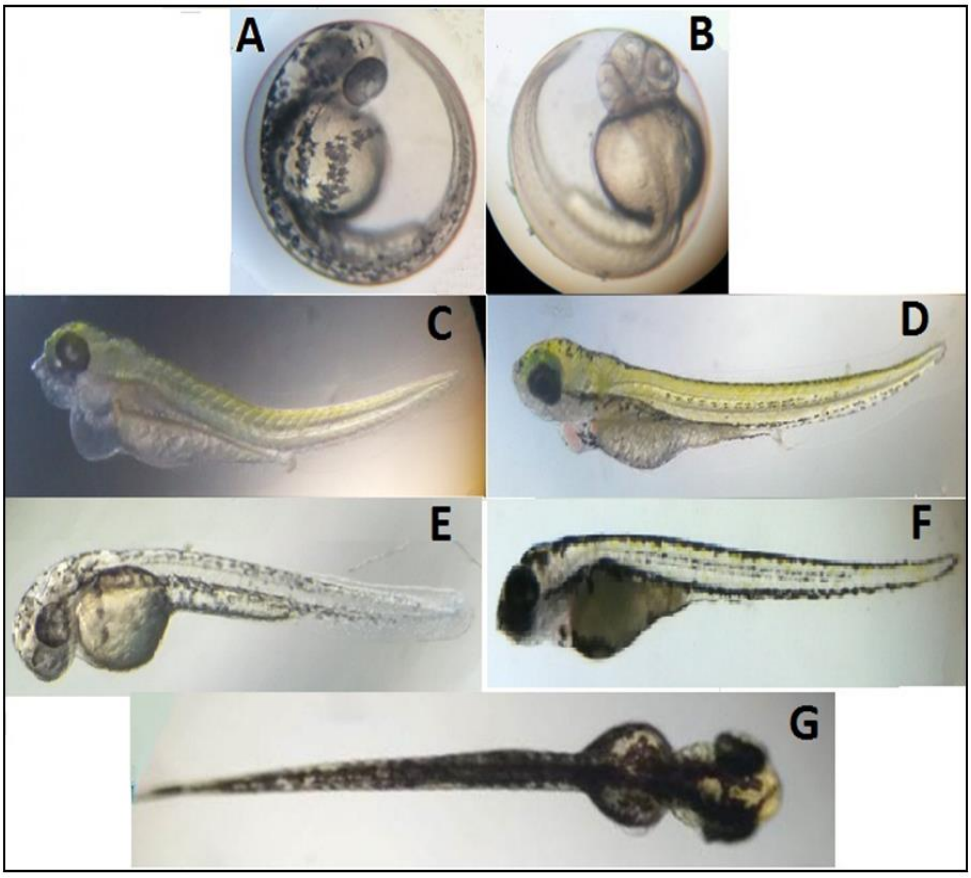

Figure1: Representative photos for the different stages of PI in zebrafish embryos used in the current study. A) Indicates embryo showing normal pigmentation B) Embryo with PI of 1 indicates complete absence of pigmentation C)Embryo with PI of 2 indicates pigmentation only around the eye D) Embryo with PI of 3 indicates pigmentation on the anterior dorsal part of the body E) Embryo with PI of 4 indicates mild pigmentation reaching to the tail end F) Embryo with PI 5 indicates intense pigmentation reaching to the tail, and G) Embryo with PI of 6 indicates dorsal and ventral body strips are filling in with pigments.

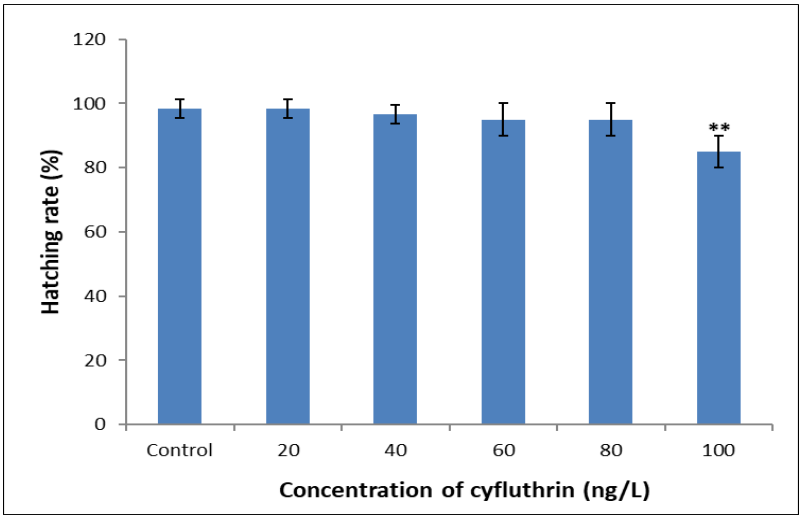

Figure 2: Hatch rate of zebrafish embryos exposed to different concentrations of cyfluthrin (ng/L) at 72 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

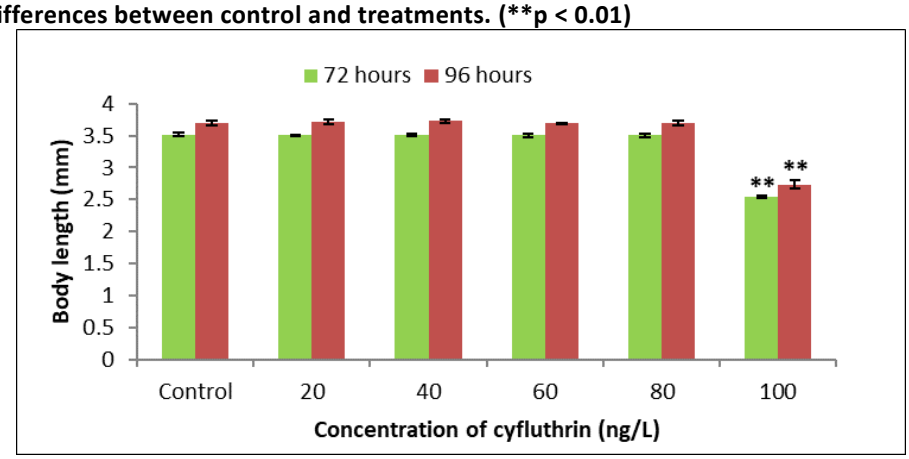

Figure 3: Body length of zebrafish embryos exposed to different concentrations of cyfluthrin (ng/L) after 72 / 96 hours exposure. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

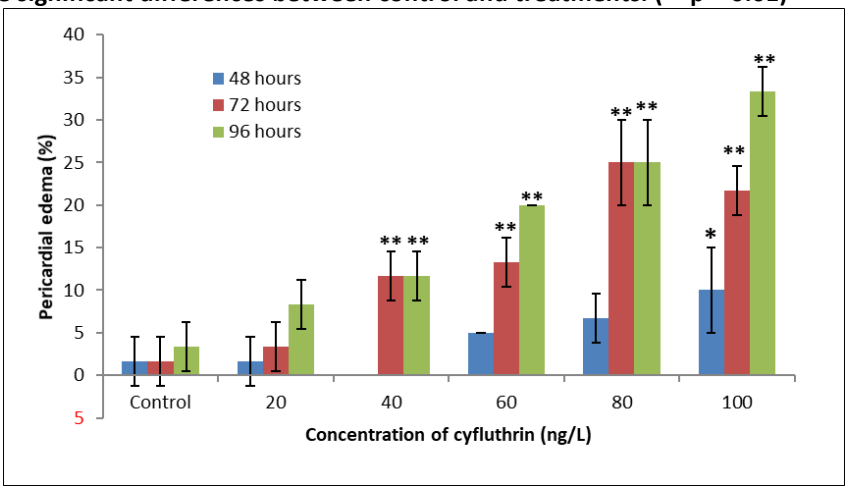

Figure 4: Pericardial edema occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (ng/L) at 48, 72 and 96 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (*p < 0.05, **p < 0.01).

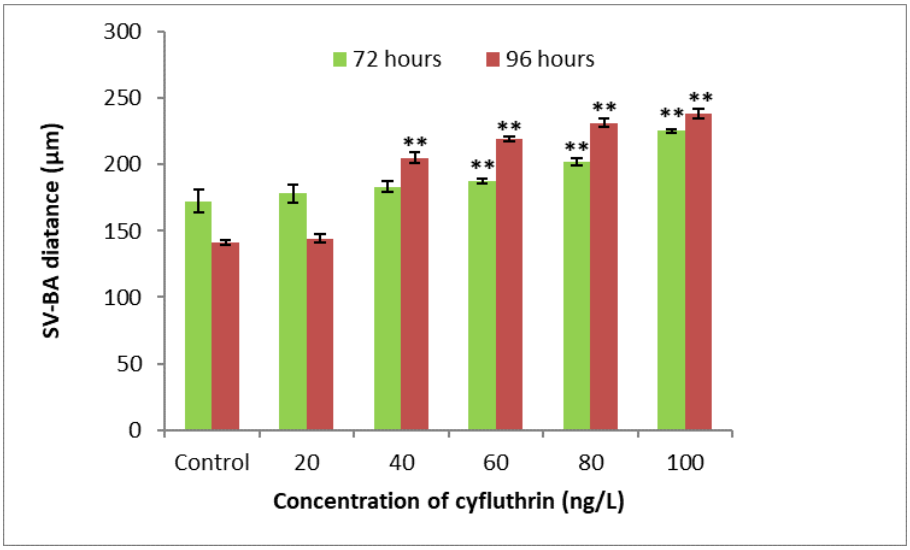

Figure 5: SV-BA distance in zebrafish embryos exposed to different concentrations of cyfluthrin (ng/L) after 72 / 96 hours exposure. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

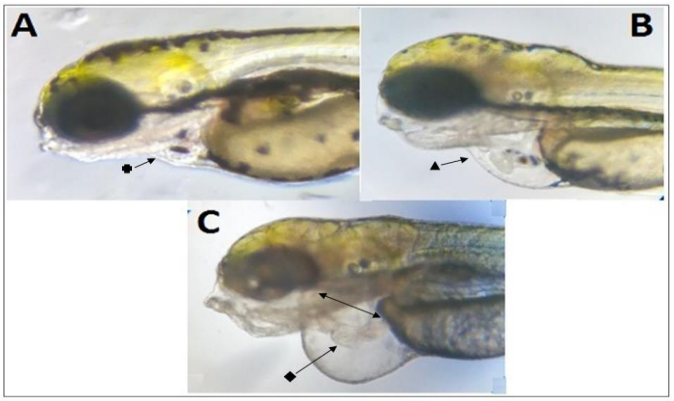

Figure 6: A) indicates embryo at 72 hpf with normal heart B) ▯ indicates embryo at 72 hpf with mild pericardial edema C) indicates embryo at 96 hpf with severe pericardial edema and heart which has failed to loop correctly, indicates the SV-BA distance.

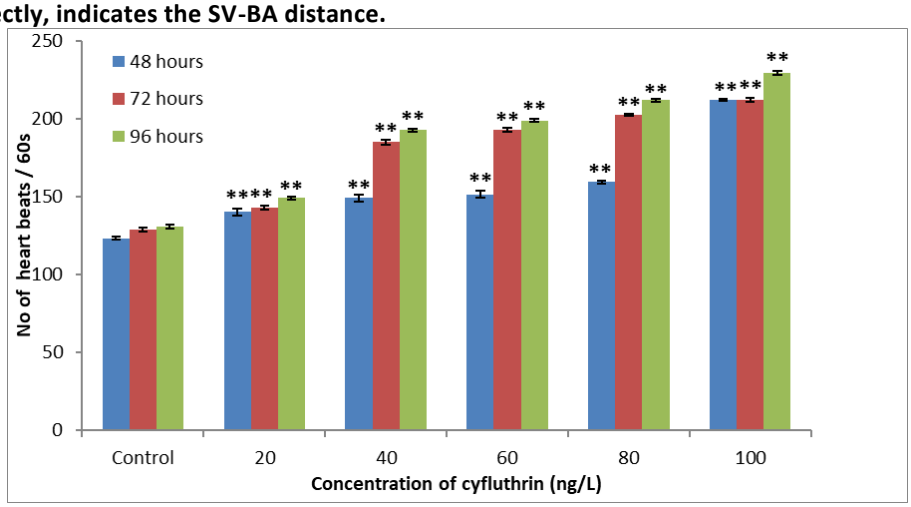

Figure 7: Number of heart beats occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (ng/L) at 48/72/96 hpf for 60 s. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

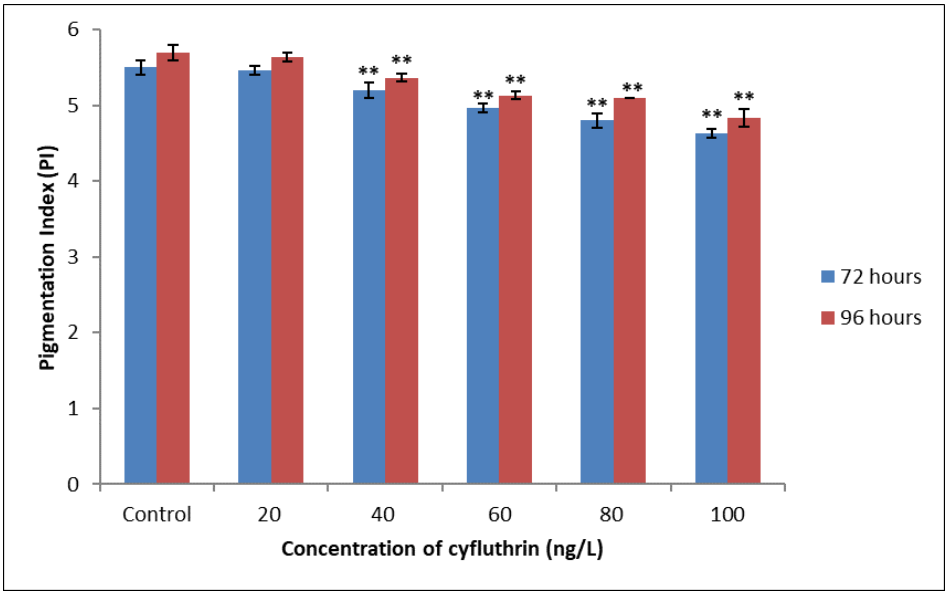

Figure 8: Pigmentation index (PI) occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (ng/L) at 72/96 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

4. CONCLUSION

The results of this study show that cyfluthrin causes sub-lethal developmental toxicity in zebrafish embryos and larvae in a dose and time-dependent manner. Cyfluthrin induced developmental toxicity and resulted in abnormal cardiac structure and function as well as reduced pigmentation. This study provides the foundation for further investigation into cardiac developmental toxicity of cyfluthrin. Since cyfluthrin is a widely used insecticide, the potential risk to non-target species is very high. Therefore application of cyfluthrin should be done in a manner which reduces the risk of cyfluthrin reaching aquatic ecosystems. Further work needs to be done to elucidate the underlying mechanisms of developmental toxicity of cyfluthrin in zebrafish (*Danio rerio*).

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