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Developmental Toxicity of Cyfluthrin in Embryo-larval Stages of Zebrafish

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Abstract

Cyfluthrin is widely used as an agricultural and household pesticide and is both a contact and oral poison for insects, especially chewing and sucking insects. Research work has been carried out on toxicity of cyfluthrin in rats, birds, annelids and arthropods. However so far, there is no data available on the developmental toxicity of cyfluthrin to zebrafish (*Danio rerio*) in its early life stages. In this study, acute toxicity and developmental effects of cyfluthrin were evaluated for embryo-larval zebrafish at 24, 48, 72 and 96 hpf (hours post fertilization). The results showed that the 96 hpf LC_{50} of cyfluthrin to embryos was 3.443 µg/L. Cyfluthrin increased the frequency of spontaneous contractions and hatch rate, while it reduced the body length significantly in a dose and time-dependent manner. Morphological abnormalities including yolk sac edema, tail deformities and curved body axis were induced by cyfluthrin. This study showed that cyfluthrin causes lethality and significant developmental defects in zebrafish in early life stages after short term exposure.

Keywords: *Cyfluthrin, Zebrafish, Acute toxicity, Developmental toxicity.*

1. Introduction

At 157.35 million hectares, India holds the second largest agricultural land in the world and in 2017-18 India's foodgrain production was a record high 277.49 million tonnes [7][14]. This in turn leads to high pest and disease pressure and hence the multitude of agrochemicals used for plant protection in India is diverse. India is the leading manufacturer of basic pesticides in Asia [5]. A major proportion of the pesticides used for agriculture make their way into aquatic systems as run off. Major riverine systems like Ganges and Yamuna also receive huge amounts of untreated or partially treated effluent, which is a source of stress to aquatic community [1,11,19, 21, 26]. Accidental poisoning of farmers due to improper use of highly toxic pesticides is also common in India. Data with the National Crime Records Bureau shows that in 2015, some 7,060 people in India died due to accidental pesticide poisoning [31].

Some of the widely used pesticides are those compounds belonging to pyrethroid family. The synthetic pyrethroids (SPs) are among the most potent and effective insecticides available for agricultural and indoor pest control [28]. Generally, SPs are characterized as low mammalian and avian toxic. However, most aquatic invertebrates and fish are highly susceptible to SPs. [9,10,17,20].

Cyfluthrin is a type II SP that acts on nerve axons by inhibiting neurotransmitter delivery via inhibition of the calcium ion channels coupled with a stimulatory effect on the sodium ion channels, affecting both the peripheral and central nervous systems [27]. Cyfluthrin induces signs of toxicity like increased salivation, uncoordinated movements, increased activity and vocalization, and reduced, labored breathing in human beings and is an eye irritant. It is classified as an endocrine disruptor [34].

The EC50 of cyfluthrin for Daphnia was found to be 0.025 ug/L for 48 hr. The LC50 in male rats was found to be 500-800 mg/kg, >5000 ppm/8 days in Northern bobwhite (*Colinus virginianus*), while in Golden orfe (*Leuciscus idus*) and Mallard duck (*Anas platyrhynchos*), it was 330.9 ng/L/96 hr. [3,33, 35].

Cyfluthrin is widely used in India for agricultural and domestic purposes and is sold under various brand names such as Solomon®(Cyfluthrin + Imidacloprid) for pests like aphids, fruit borer and girdle beetle on brinjal and soyabean crops and Solfac® and Responsar®(Cyfluthrin) for pests like house flies, cockroaches and mosquitoes in houses [2].

In surface water, Cyfluthrin was at a maximum time-weighted mean

concentration of 5 ug/L in an agricultural area in southern Sweden. During 1994, in freshwater sites in England and Wales, cyfluthrin exceeded its environmental quality standards at 1% of the sites tested [13, 24, 38, 39].

Very less ecotoxicological data are available for cyfluthrin [27, 18]. However it is highly toxic to aquatic vertebrates and invertebrates despite being not readily soluble in water with long lasting effects [12, 18, 22, 27]. Hence, it is very important to study the effects of cyfluthrin on living organisms.

Although reliable data for extrapolating toxicant effects to humans are obtained through laboratory rodent studies, these are expensive, time consuming, and restricted by law. There are numerous advantages for the use of zebrafish (*Danio rerio*) as a toxicological model species because of short lifecycle, small size, transparent embryos, well established toxicological protocols, genetic and easily accessed genomic tools [29][32]. Very few studies have been done worldwide, with respect to cyfluthrin toxicity in zebrafish (*Danio rerio*).

The objectives of present study were to investigate the acute toxicity and developmental effects of cyfluthrin to embryos and larvae of zebrafish. The results would provide useful information for better understanding the negative effects of cyfluthrin on aquatic organisms.

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^{o}C in darkness. After initial range finding experiments, test solutions of five concentrations – 0.1, 0.4, 1.6, 3.2 and 6.4 µg/L were prepared by diluting the stock in E3 medium [4]. All other chemicals were of analytical grade.

2.2 Zebrafish maintainence and collection

Zebrafish (*Danio rerio*) were obtained from local commercial aquarium. Five breeding pairs were kept in a 10 L glass aquaria, under semi-static conditions in charcoal filtered water. The water was constantly filtered and aerated and 20% of the water was changed daily. They were kept in a light/dark cycle of 14:10 h at 28±1^{o}C. They were fed twice with live blood worms. Embryos were obtained by mass spawning by keeping the fishes in a spawning aquarium with a mesh to prevent cannibalization of the eggs. Spawning was induced in the morning within half an hour of the light being turned on. Eggs were immediately collected and rinsed thrice in E3 medium [4]. Unfertilized eggs were removed after observing them under dissecting microscope.

2.3 Embryo–larvae toxicity assay and microscopic observation

Seven 24-well plates were setup with test solutions with 2ml of solution in each well - one negative control, one solvent control (acetone) and five concentrations of treatment group (0.1, 0.4, 1.6, 3.2 and 6.4 µg/L of cyfluthrin). Fertile eggs were distributed by 3hpf (hours post fertilization), one egg in each well. Each treatment group had twenty embryos in test solutions 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.

The plates were covered and incubated at 28±1^{o}C in light/dark cycle of 14: 10 h for 96 hours. Embryos were observed every 24 hours using a light microscope. Endpoints including mortality, spontaneous contractions (SC), hatch rate, hatchability, yolk sac edema, curved body axis, tail deformities and body length were selected for assessing the effects of cyfluthrin. Every 24 h, embryos with no heartbeat were considered as dead and were removed immediately. For observation of hatching, the embryos were observed from 48 to 60 h at intervals of 2 h. At 24hpf embryos show SC, which were counted for a period of 60 s. Yolk sac edema and tail deformities were observed every 24 h from 48 hpf. Curved body axis and body length was observed from 72 hpf. The body length was measured using an oculometer under 50X magnification from anterior tip of snout to the posterior end of caudal peduncle of the body.

The embryo–larvae toxicity assay was carried out according to some previous studies with some modifications [15, 30] as well as under the guidelines of FET test [23]. All the stages of embryonic development of the zebrafish have been identified using Kimmel [16] as a source of reference.

2.4 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

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ANOVA, followed by Bonferroni and Holm pairwise comparison of means. The differences was 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 LC_{50} and cumulative mortality

The embryos exposed to cyfluthrin were monitored for acute lethality symptoms such as coagualation, lack of somite formation, non-detachment of tail and lack of heart beat every 24 hours for a period of 96 hours. The dead embryos were removed after each observation. The LC_{50} at the end of 96 hpf was calculated to be 3.443 µg/L. The cumulative mortality kept increasing a dose and time dependent manner. The cumulative mortality at concentration of 0.1 µg/L was similar to that of control. However, all the other concentrations showed significant increase in cumulative mortality compared to the control at every stage of observation, i.e. at 24, 48, 72 and 96 hpf. The cumulative mortality rate in the treated groups increased significantly after 48 hours. This could be due to the fact that embryos start hatching out of the chorion asynchronously by 48 hpf. The loss of chorion as a protective barrier from the effects of cyfluthrin must have lead to increase in cumulative mortality rates. (Fig. 1)

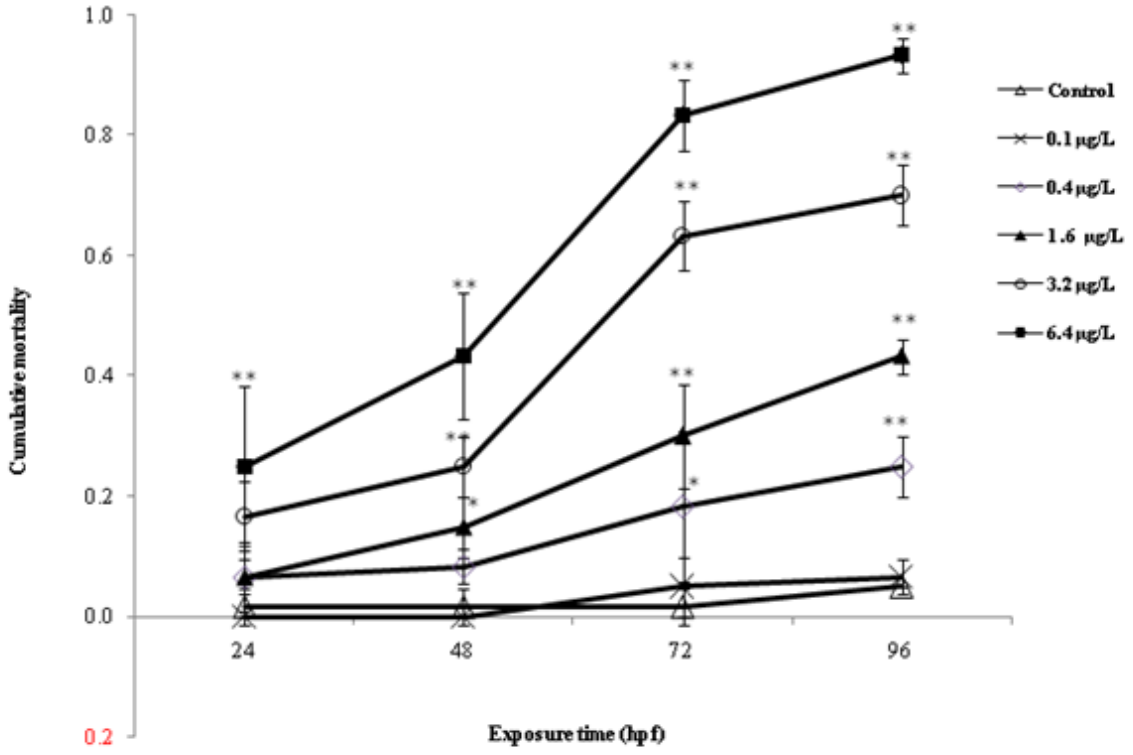

Fig. 1: Cumulative mortality of zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) at 24, 48, 72 and 96 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (*p < 0.5, **p < 0.01)

3.3 Spontaneous contractions (SC)

Spontaneous contractions (SC) in zebrafish embryos start at 17 hpf. This is due to the development of functional neurons adjacent to the somites [8, 15, 25]. The frequency of SC is maximum at 19 hpf and gradually decreases. In this study, we recorded the SC for a period of 60 s at 24 hpf. The values obtained were discrete and normally distributed. The results showed that increasing concentrations of cyfluthrin lead to increase in number of SC, except for concentration of 6.4 µg/L, which showed a drastic decrease with respect to control (Fig. 2). However, observation of the developmental stages of embryos [16] showed no delay in the general development of embryos in treatment as well as control groups.

SC are due to uncontrolled action potential in motorneurons. Disruption of sodium channels can lead to change in frequency of SC[8]. Cyfluthrin, a synthetic pyrethroid acts by interfering with the nerve signals by disruption of the membrane sodium channel systems in target organisms [36]. Cyfluthrin may be increasing the frequency of SC by disrupting the sodium channels, leading to prolonged channel opening which results in repetitive firing of action potentials. However, the embryos exposed to

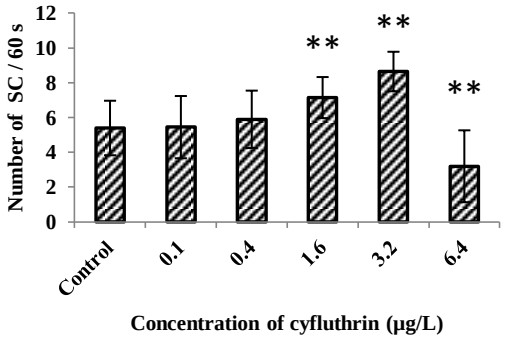

Fig. 2: Number of spontaneous contractions (SC) occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) at 24 hpf for 60 seconds. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

concentration of 6.4 µg/L showed significant decrease in SC compared to control as well as other treatment groups. This could be due to complete inhibition of membrane sodium channels at such high concentrations. Further studies are required to elucidate the correct mechanism involved.

3.4 Hatch rate and hatchability

Hatching is an important event in the life cycle of a fish and involves a complex combination of biochemical pathways followed by physical mechanisms which would weaken and destroy the chorion, allowing the embryo to hatch [6]. In this study, we observed the hatching of embryos at intervals of 2 hours from 48 to 60 hpf. The zebrafish embryos started hatching asynchronously at 48 hpf. By 60 hpf, all the embryos had hatched out. The hatch rate was calculated by the following formula: Hatch rate = No of eggs hatched at time 'X'/ No of eggs alive at time 'X'.

It was seen that the hatch rate at concentration of 1.6 and 3.2 µg/L was significantly higher than control in the beginning, but by 54 hpf, the control group had a similar hatch rate to the above treatment groups. The hatch rate of embryos exposed to 6.4 µg/L was significantly lower than the control. This is in agreement with the results obtained for SC, where the frequency of SC was significantly less at 6.4 µg/L. The increased SC at 1.6 and 3.2 µg/L must have lead to quicker destruction of the chorion and faster hatching. Similarly, reduction in SC at 6.4 µg/L lead to a corresponding reduction in the hatch rate. Cyfluthrin induced increase of SC may have resulted in the embryo twisting and tearing up the chorion, thus accelerating the hatch rate. (Fig.4)

The hatchability at 96 hpf was calculated by the formula: Hatchability = No of eggs hatched/Total no of fertile eggs.The hatchability of zebrafish was not affected by the increase in cyfluthrin concentration except for concentrations of 3.2 and 6.4 µg/L, which showed significant decrease with respect to control. (Fig.3)

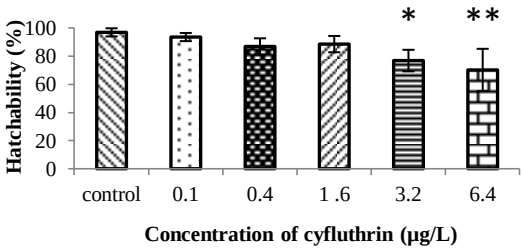

Fig. 3: Hatchability of zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) after 96 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (*p < 0.5, **p < 0.01)

3.5 Non-lethal malformations in zebrafish embryos

Malformations which were not lethal were observed for different concentrations every 24 hours for a period of 96 hours. On observation of the non-lethal endpoints chosen, no effect of cyfluthrin was detected till 48 hours. Cyfluthrin induced non-lethal effects on embryos and larvae in a dose-dependent as well as time-dependent manner, including curved body axis, yolk sac edema, tail deformities and reduced body length (Fig. 5).

Yolk sac edema was observed in larvae exposed to cyfluthrin. Embryos exposed to concentrations of 1.6, 3.2 and 6.4 µg/L were significantly affected by 72 hpf and the toxicity increased with prolonged exposure time (Fig.6).

In freshwater, fishes have to maintain osmotic balance by minimizing entry of water and must excrete excess water that enter within. Cyfluthrin could be impairing the excretion of water allowing the excess water to accumulate as edema fluid. This could be due to kidney malfunction. Cyfluthrin has been shown to depress kidney weight in rats [37].

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Further studies have to be done to confirm the cause of edema formation.

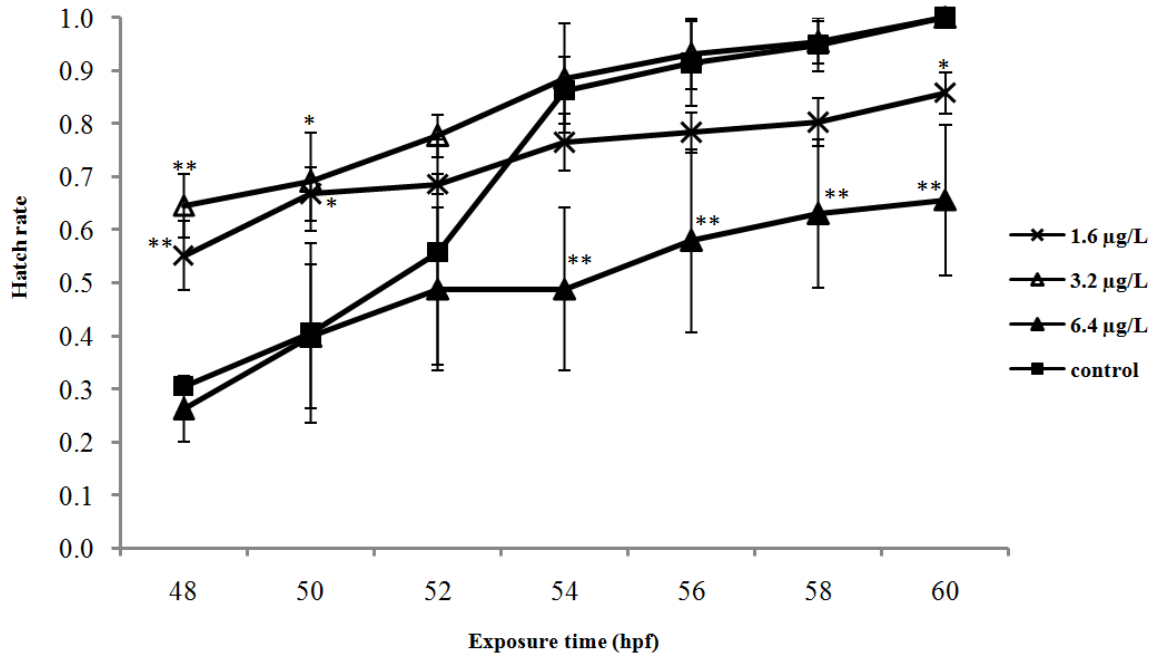

Fig. 4: Hatch rate of zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) from 48 to 60 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (*p < 0.5, **p < 0.01)

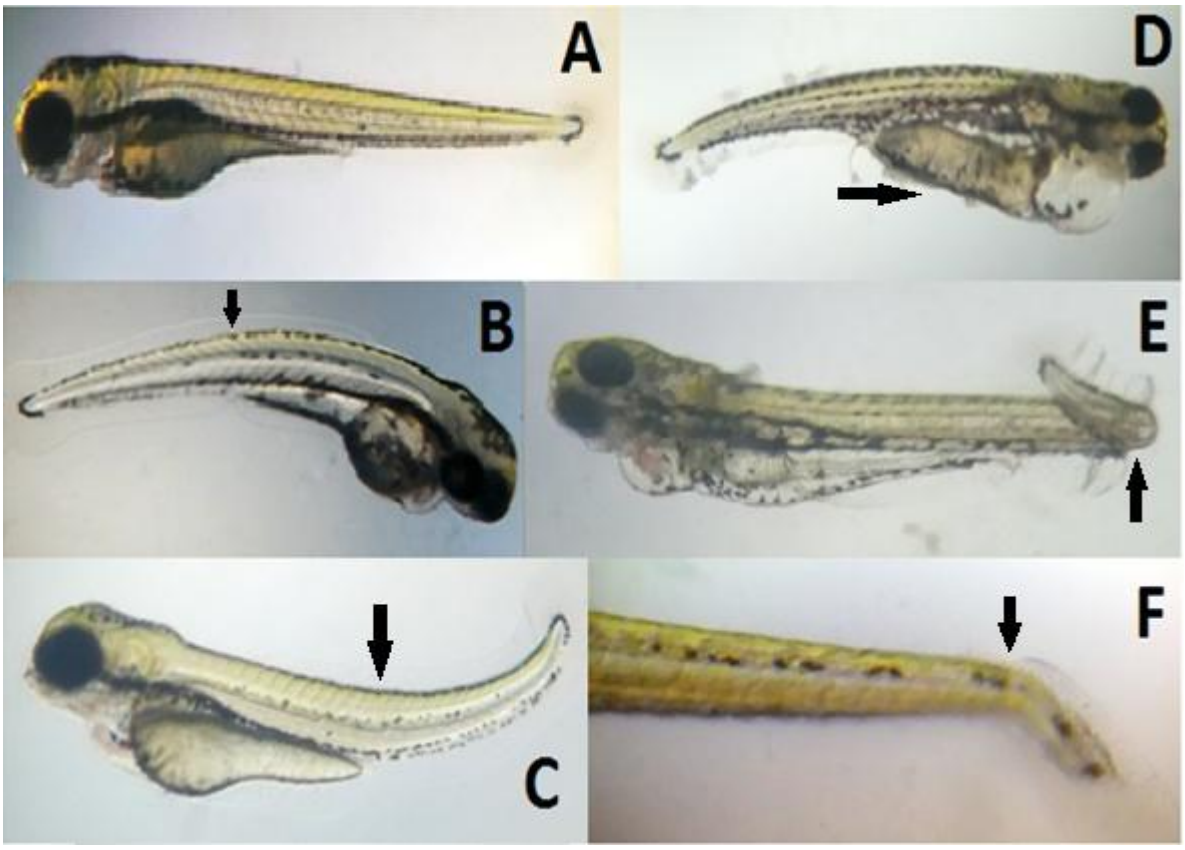

Fig. 5: Morphological effects of cyfluthrin on zebrafish embryos and larva stages. A- Normal embryo at 48 hpf; B and C - embryo with crooked body axis at 48 and 60 hpf respectively; D- embryo with yolk sac edema at 48 hpf; E and F – embryo with tail deformity at 48 hpf

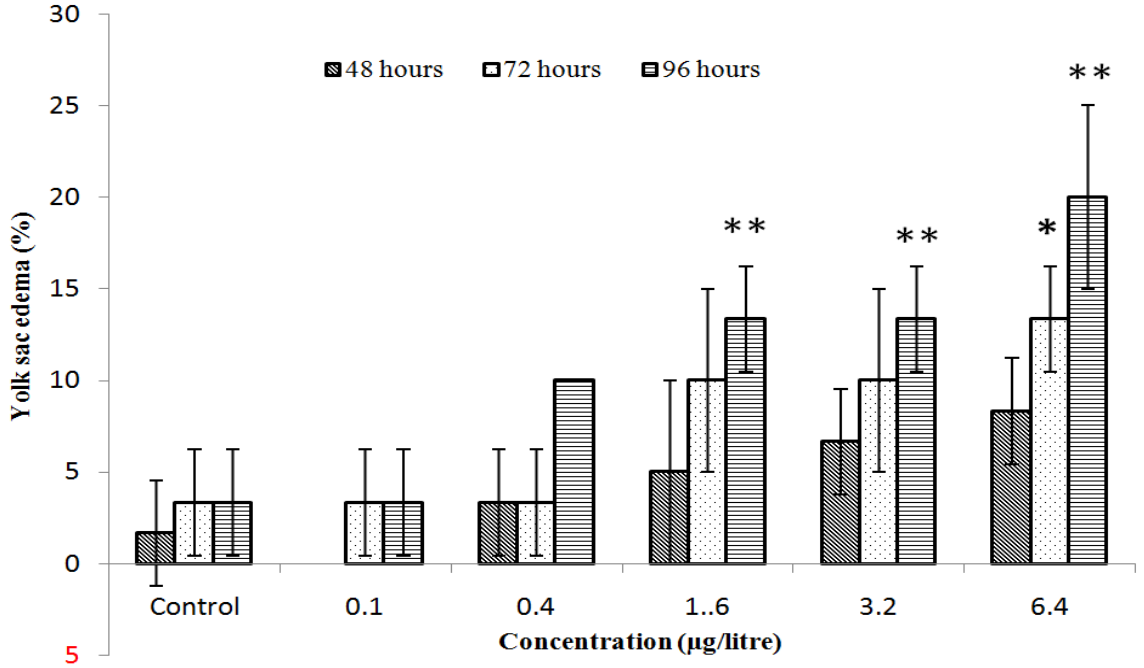

Fig. 6: Yolk sac edema occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) at 48, 72 and 96 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (*p < 0.05, **p < 0.01)

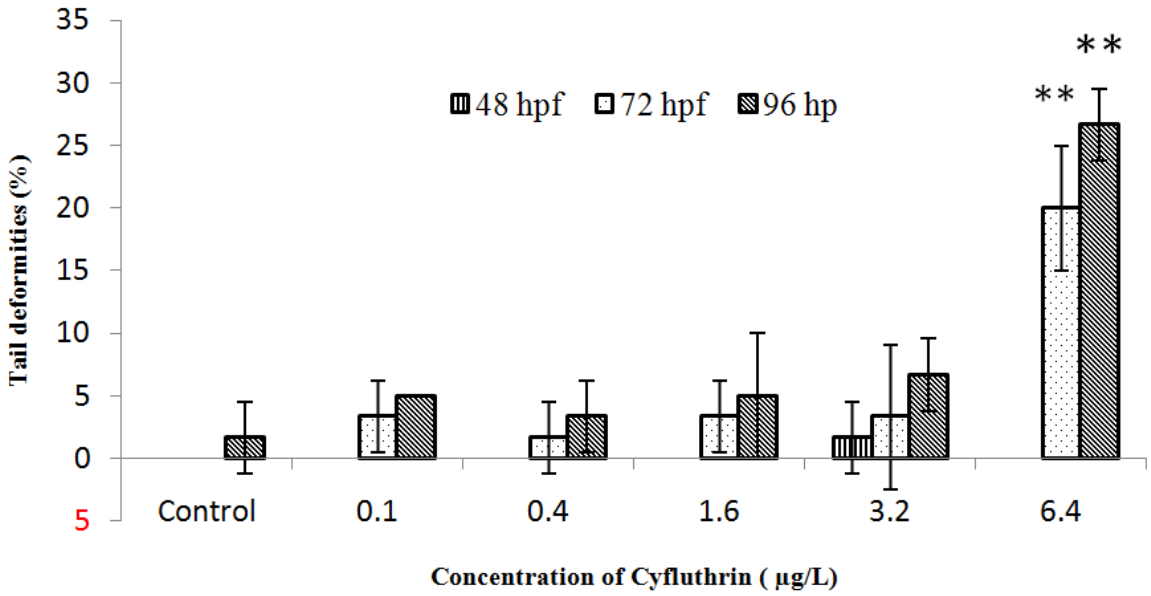

Fig. 7: Tail deformities occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) at 48, 72 and 96 hpf. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

The occurrence of tail deformities in the form of bent tail, damaged caudal fin, broken tail was significantly higher in larvae exposed to concentration of 6.4 µg/L compared to control. The frequency of tail deformities also increased with exposure time. At 72 hpf, 20% of the embryos exposed to 6.4 µg/L showed tail deformities which increased to 26.66% by 96 hpf (Fig.7)

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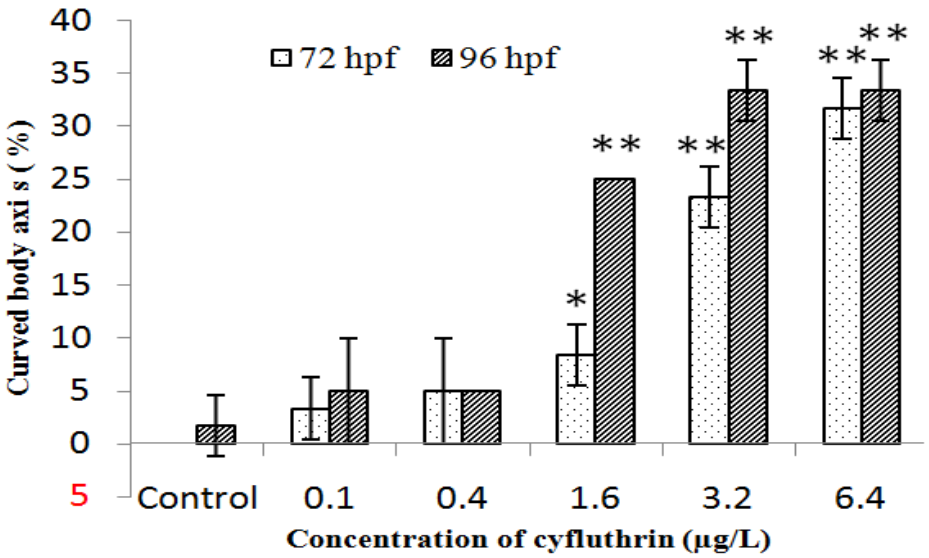

Fig. 8: Curved body axis occurring in zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) after 72 / 96 hours exposure. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (*p < 0.5, **p < 0.01)

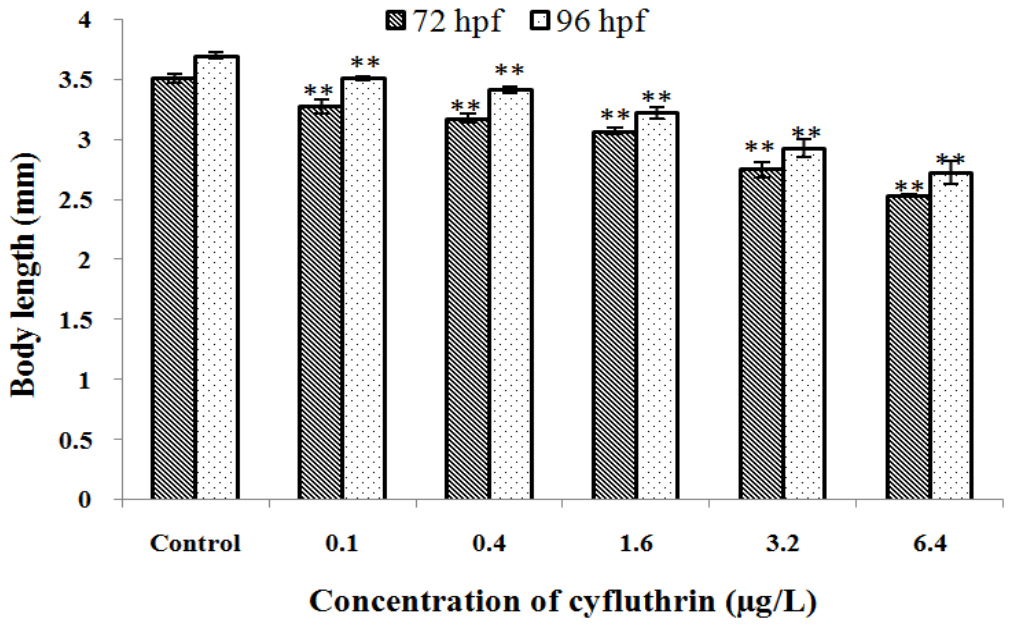

Fig. 9: Body length in zebrafish embryos exposed to different concentrations of cyfluthrin (µg/L) after 72 / 96 hours exposure. Data are expressed as mean ± SD (n=3 replicates of 20 embryos in each concentration). Asterisks denote significant differences between control and treatments. (p < 0.01)**

Curved body axis was also detected in zebrafish exposed to cyfluthrin at concentrations of 1.6, 3.2 and 6.4 µg/L. There was a significant increase at the above concentrations compared to control group. The occurrence of curved body axis increased with increase in exposure time (Fig.8).

Cyfluthrin induced growth retardation was observed at 72 and 96 hpf. All the treatment groups showed growth retardation in a dose and time-dependent manner. At the maximum concentration of 6.4 µg/L, the body length was 2.53 mm and 2.73 mm at 72 and 96 hpf respectively (Fig. 9). This was significantly higher compared to control groups.

4. Conclusion

Developmental abnormalities are crucial endpoints for determining teratogenecity of chemicals [22]. The results of this study show that cyfluthrin

causes acute developmental toxicity in zebrafish embryos and larvae in a dose and time-dependent manner. This also correlates with earlier work done on effect of cyfluthrin on other animals. The increasing use of cyfluthrin may have adverse effects on aquatic ecosystems. Therefore cyfluthrin should be used in a scientific and sparing manner in domestic as well as agricultural use. It is very important that further studies are done to study the mechanisms for acute developmental toxicity of cyfluthrin.

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