Research Article

Embryotoxicity of Cypermethrin, a Synthetic Pyrethroid Insecticide in Zebrafish

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ABSTRACT

Aim/Background: This study aimed to assess the toxicity of cypermethrin using zebrafish embryos as a model organism. Cypermethrin, a synthetic pyrethroid commonly used in agriculture and pest control, poses significant environmental risks due to its persistence and potential harm to aquatic organisms. **Materials and Methods:** Zebrafish embryos were exposed to varying concentrations of cypermethrin and both lethal and sublethal endpoints were monitored at 24, 48, 72 and 96 hr post-fertilization. Standardized protocols for the Fish Embryo Toxicity Test (FET) were followed to evaluate the effects of cypermethrin on zebrafish embryos. **Results:** The study found that cypermethrin exhibited concentration-dependent toxicity, with a calculated LC_{50} (concentration causing 50% mortality) ranging from 43.75 µg/mL at 24 hr post-fertilization to 8.31 µg/mL at 96 hr post-fertilization. Lethal endpoints such as coagulation, lack of heartbeat, and lack of somite formation were observed, along with sublethal effects such as tail deformation and pigmentation abnormalities. **Conclusion:** The findings indicate that cypermethrin is highly toxic to zebrafish embryos, with mortality increasing over time and with higher concentrations. The study underscores the importance of using alternative models like zebrafish embryos for toxicity assessments, providing valuable insights into the environmental risks associated with synthetic pyrethroids like cypermethrin. Further research is warranted to explore the long-term effects and mechanisms of toxicity and to validate the sensitivity of zebrafish embryos as a model for assessing pesticide toxicity.

Keywords: Cypermethrin, Lethal, Somites, Sublethal.

INTRODUCTION

Understanding the negative and long-term impacts of environmental contaminants is made easier with the help of risk assessment. Through waste and runoff, several pesticides, antibiotics and chemicals used in agriculture, aquaculture and hospitals are regularly released into the aquatic ecosystems nearby.[1] There have been reports of some of these chemicals, such as deltamethrin, cypermethrin and trimethrin, remaining in the environment for many months or years.[2] According to reports, these substances and their residues have long-term negative effects on people[3] and animals[4] especially fish[5] which live in aquatic environments like lakes and rivers and cause ecological degradation. They also negatively affect water taste and odor, have deadly effects on non-target organisms in agroecosystems, and are directly toxic to users.[6,7] Because of its efficiency even at low concentrations, cypermethrin, a synthetic pyrethroid, has replaced naturally occurring pyrethrins as a common insecticide.[8] It has been employed as a chemotherapeutic drug to manage ectoparasitic infestations in fish, in domestic insecticides as mosquito repellent and in agriculture.[9,10] The WHO has categorized this substance as a class II (moderately

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dangerous) chemical. These substances are extremely harmful to fish, bees and aquatic insects, according to the National Pesticides Telecommunication Network (NPTN). Cypermethrin has been discovered to be poisonous to mammals as well as insects.[11] Fish have a slower metabolism and clearance of cypermethrin than other animals.[12] High doses of cypermethrin have been linked to deaths in laboratory animals from respiratory depression, convulsions, ataxia, limb weakness and muscular tremors.[13,14] Skin irritation, reduced food intake, lower body weight and decreased absolute and relative gonadal weight were all observed in rabbits treated with cypermethrin.[15] Consumption of fish and honey from the environment that are polluted with cypermethrin in their meal indirectly impacts humans. Consequently, it is crucial to assess the toxicity of such compounds using appropriate experimental models. The effects of ambient contaminants and their residues are being researched using various experimental models.[16] Several criteria, such as the need for additional space and effort for maintenance, the requirement for ethical clearance, etc., justify using some of the extensively used models for toxicological investigations, such as mice and rat models. Fish embryos serve as a substitute model for determining the environmental risk of substances since they are inexpensive, exempt from current animal welfare laws and simple to operate in a small space. Given their size, husbandry and early morphogenesis, zebrafish are frequently utilized as toxicological models. Zebrafish embryos are regarded as great research models since they are transparent and make assessing toxicity endpoints in toxicity tests simple. Other benefits of employing zebra fish include the availability of mutant strains, c DNA clone collections, physical map, finished genome sequence and well-understood genetic foundation of development.[17-19] In light of this, the study's objective was to assess the toxicity of cypermethrin using Zebrafish Embryos Toxicity Test (ZFET).

MATERIALS AND METHODS

Test substance

Cypermethrin 25% EC (Cyper 25), a synthetic pyrethroid used in this study was procured from local market.

Brood fish maintenance and collection of eggs

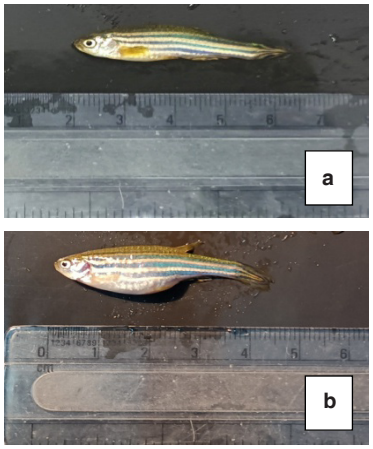


Figure 1: Adult Zebrafish a) male b) female.

Adult zebrafish (offspring of wild-type fish) (Figures 1a and 1b) that were obtained from a nearby fish farm appeared to be in good health and were added to the tank, which has a capacity of roughly 500 L (Figure 2a). The fish were kept in the lab under ideal water quality conditions, which were set at a pH of 7.3-7.6, a temperature of 30 ±1°C, a light-to-dark cycle ratio of 14:10, dissolved O2 of 6.1-6.7 mg/L and a total hardness of 175 mg of calcium carbonate per liter

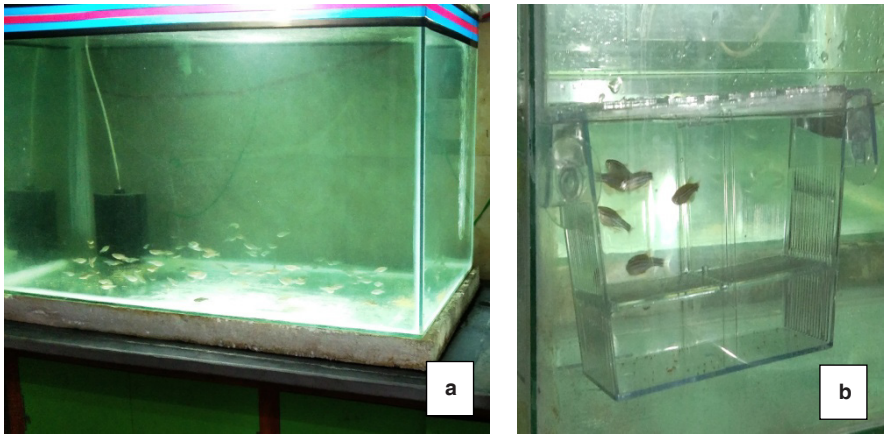


Figure 2: Fish tank a) Reservoir b) Breeding tank.

Table 1: Water quality.

Parameters	Range
Temperature	29-31ºC
pH	7.3-7.6
Dissolved oxygen	6.1-6.7 mg/L (average)
Nitrate	0.18 mg/L (average)
Free carbon dioxide	4.5 mg/L
Alkalinity	118-132 mg/L (as $CaCO_3$)
Total hardness	175 mg/L (as $CaCO_3$)

(Table 1). A fish food (Optimum) readily accessible in stores was used to feed the fish twice daily. The brood fish must mate in pairs to produce zebrafish eggs. A few hours before dusk on the day before the experiment, 4 adult female fish and 3 adult male fish were put together in small glass breeding tanks with a 1 L capacity for breeding (Figure 2b). The following morning, the eggs were retrieved and cleaned to remove waste and trash. Healthy fertilized eggs were microscopically detected, isolated and used in the investigation.

Range finding test

To identify the proper cypermethrin concentration for the Fish Embryo Toxicity Test (FET), a range finding test was performed in zebrafish embryos using a conventional procedure.[20] 100 µg/mL and 6.25 µg/mL were the greatest and lowest concentrations that were evaluated in embryos, respectively.

Zebra Fish Embryo Toxicity (ZFET) Test

The cypermethrin bioassay was performed using industry-standard procedures.[20] Before the cleavage of the blastodisc, the chosen zebrafish embryos were subjected to different amounts of cypermethrin (Table 2). In triplicates in petriplates, 20 embryos from each treatment group were subjected to each test concentration. The control group was kept under the same circumstances but not given cypermethrin. The water temperature was kept constant at 30±1vC and a semi-static renewal system that comprises batch-by-batch replacement of the test solution every 24 hr was used. Lethal, sublethal and sublethal continuous endpoints were observed in the embryos in the treatment and control groups at 24, 48, 72 and 96 hr after exposure to determine the lethality of the embryos.[20,21] The observed endpoints and their description are presented in Table 3.

RESULTS

Tables 4-6 give the results of the observations of the lethal categorical (coagulation, lack of heartbeat, lack

Table 2: Concentration of cypermethrin tested in zebrafish embryo toxicity test.

Sl. No.	Group	Concentration (µg/mL)
1	Control	0.00
2	T1	100
3	T2	75
4	T3	50
5	T4	25
6	T5	12.5
7	T6	6.25

Table 3: The observed endpoints and their description.

Endpoints	Description	References
	Lethal endpoints	
Coagulation	Embryos are milky in appearance and are dark when viewed under a microscope.	20
Lack of Heartbeat	In a regularly developing zebrafish embryo (30±1ºC), the heartbeat can be seen at 48 hr. At 48, 72 and 96 hr following hatching, the absence of a heartbeat should be seen with a minimum magnification of 80 X for at least 1 min.	20
Lack of somite formation	Somatization is indicated by spontaneous movements. To note the absence of somites after 24, 48, 72 and 96 hr.	20
Hatching Rate	Hatching rates were monitored and reported for all treatment and control groups starting at 48 hr.	20
	Sub lethal categorical	
Tail deformation	Spinal curvature (scoliosis) or a shorter tail than average.	21
Head Deformity	Head is not normally developed.	21
Lying on the sides	Embryo rests comfortably on its side (not active swimming).	21
Difficulty in hatching	Although the embryo is still alive, it did not hatch when it should have.	21
Pigmentation	Reduced body surface or eye pigmentation compared to normal embryos.	21
	Sublethal continuous	
Movements	Movements were counted throughout the course of one minute and compared to the typical zebra fish embryo.	21
Heart rate	30 heartbeats were time-lapsed, recorded and translated to beats per minute.	21
Time to Hatching	Length of time till the embryo hatches (hpf).	21

of somite formation and hatching rate) and sub-lethal categorical (tail deformation, head deformity, lying on the sides, difficulty in hatching and pigmentation) endpoints in the acute toxicity test for cypermethrin in zebrafish embryo toxicity assay.

In this investigation, the cypermethrin LC_{50} value (i.e., the concentration at which more than 50% mortality occurs) was determined to be 43.75 µg/mL, 20.96 µg/mL, 11.99 µg/mL and 8.31 µg/mL for 24 hpf, 48 hpf, 72 hpf and 96 hpf, respectively. The lowest dose of 6.25 µg/mL had no significant effects. Figure 3 shows the mixture's dose-response curves for 24, 48, 72 and 96 hpf. Four-parameter sigmoidal hill curves were fitted to the curves. At 48hpf, 72 hpf and 96 hpf, the LC_{50} value at 24 hpf was substantially cut in half, one-fourth and one-fifth, respectively. A substantial (p<0.05) inverse connection between the LC_{50} values and the exposure time was observed. Six different cypermethrin concentrations were applied to 20 fish embryos in batches. In three distinct trials, mortality was tracked every 24 hr for a maximum of 96 hr, after which mean% mortality data were calculated. The results of the current investigation showed that zebrafish embryonic development will be impeded even at a low concentration of cypermethrin of 6.25 µg/mL.

Table 4: Observations on lethal categorical endpoints post-exposure of zebrafish embryos to various concentration of cypermethrin (µg/mL).

Lethal end points	24 hpf	48 hpf	72 hpf	96 hpf
Coagulation				
0.00 (Control)	X	X	X	X
6.25	X	X	X	X
12.5	X	X	X	X
25	X	X	X	✓
50	X	X	X	✓
75	X	X	✓	✓
100	✓	✓	✓	✓
Lack of somite formation				
0.00 (Control)	X	X	X	X
6.25	X	X	X	X
12.5	X	X	X	X
25	X	X	X	X
50	X	X	✓	✓
75	X	✓	✓	✓
100	✓	✓	✓	✓
Lack of heartbeat				
0.00 (Control)	-	X	X	X
6.25	-	X	X	X
12.5	-	X	X	X
25	-	X	X	X
50	-	X	X	✓
75	-	X	X	✓
100	-	✓	✓	✓

** Present x Absent hpf - hours post fertilization.

Table 5: Obsevations on sublethal endpoints post-exposure of zebrafish embryos with various concentrations of cypermethrin.

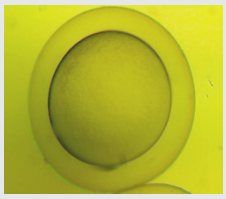
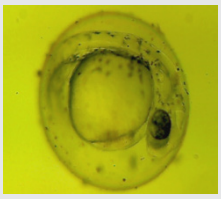
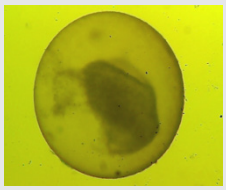
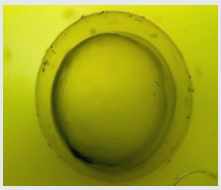
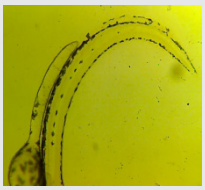
Sub Lethal endpoints	24 hpf	48 hpf	72 hpf	96 hpf
Head and tail deformation				
0.00 (Control)	X	X	X	X
6.25	X	X	X	X
12.5	X	X	X	X
25	X	X	X	X
50	X	X	X	✓
75	X	X	✓	✓
100	✓	✓	✓	✓
Pigmentation				
0.00 (Control)	X	X	X	X
6.25	X	X	X	X
12.5	X	X	X	X
25	X	X	X	X
50	X	X	X	✓
75	X	X	X	✓
100	X	X	X	✓
Lying on side				
0.00 (Control)	X	X	X	X
6.25	X	X	X	X
12.5	X	X	X	X
25	X	X	X	X
50	X	X	✓	✓
75	X	✓	✓	✓
100	X	✓	✓	✓
Difficulty in hatching				
0.00 (Control)	-	X	X	X
6.25	-	X	X	X
12.5	-	X	X	X
25	-	X	X	X
50	-	X	✓	X
75	-	✓	✓	✓
100	-	✓	✓	-

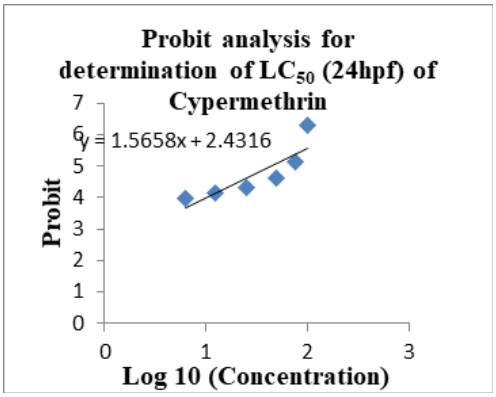
** Present x Absent hpf-hours post fertilization.

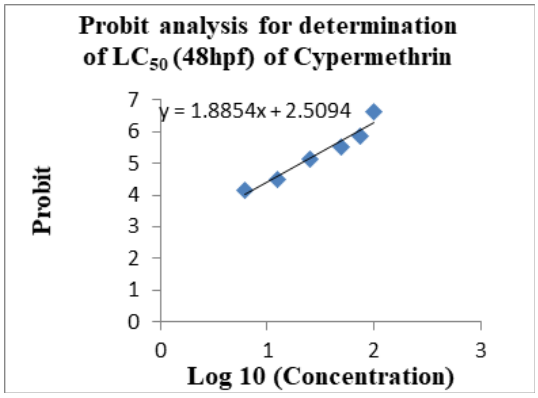
Types of Lethal Endpoints Observed during the Exposure

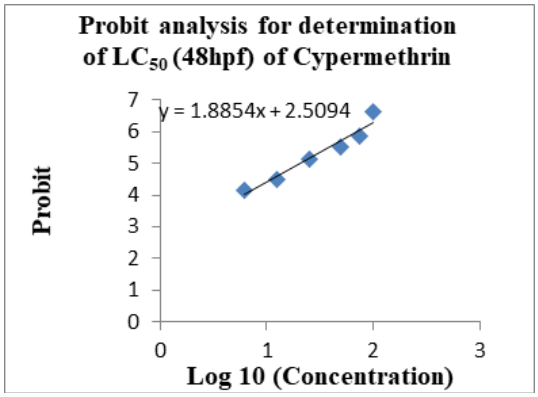
The information on fatal outcomes of 24-96 hr of exposure is compiled in Table 6. The findings show that the most common type of fatal consequence that was

Table 6: Lethal and sublethal endpoints observed in zebrafish embryos exposed to 0.05 µg/L of Cypermethrin.

Control Group	Uncoagulated egg	Well developed somite	Normal tail
			
Treatment group	**Coagulated egg**	**Lack of somite**	**Tail deformity**
			







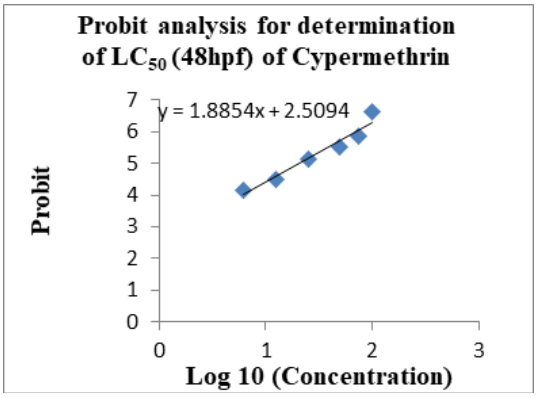


Figure 3: Mortality curve of fish exposed to different cypermethrin concentrations in different times (1 hr-a; 2 hr-

noticed was coagulation (Figure 4). As seen in Figure 4, some embryos also lacked the development of somites and a heartbeat. The number of fish hatching at each interval is shown in Figure 4. Embryos that underwent the test and had no negative effects hatched 48 hr later. Other features of the embryos suggested the fatal outcomes, such as low pigmentation and delayed development. Nevertheless, 96 hr later, the hatched larvae showed no signs of sublethal damage.

DISCUSSION

The LC_{50} values, which indicate over 50% mortality, were 43.75 µg/mL at 24 hr post-fertilization (hpf), 20.96 µg/mL at 48 hpf, 11.99 µg/mL at 72 hpf and 8.31 µg/mL at 96 hpf in the study on the acute toxicity of cypermethrin in zebrafish embryos. This indicates a marked increase in toxicity with prolonged exposure. Coagulation, irregular heartbeat, problems with somite formation

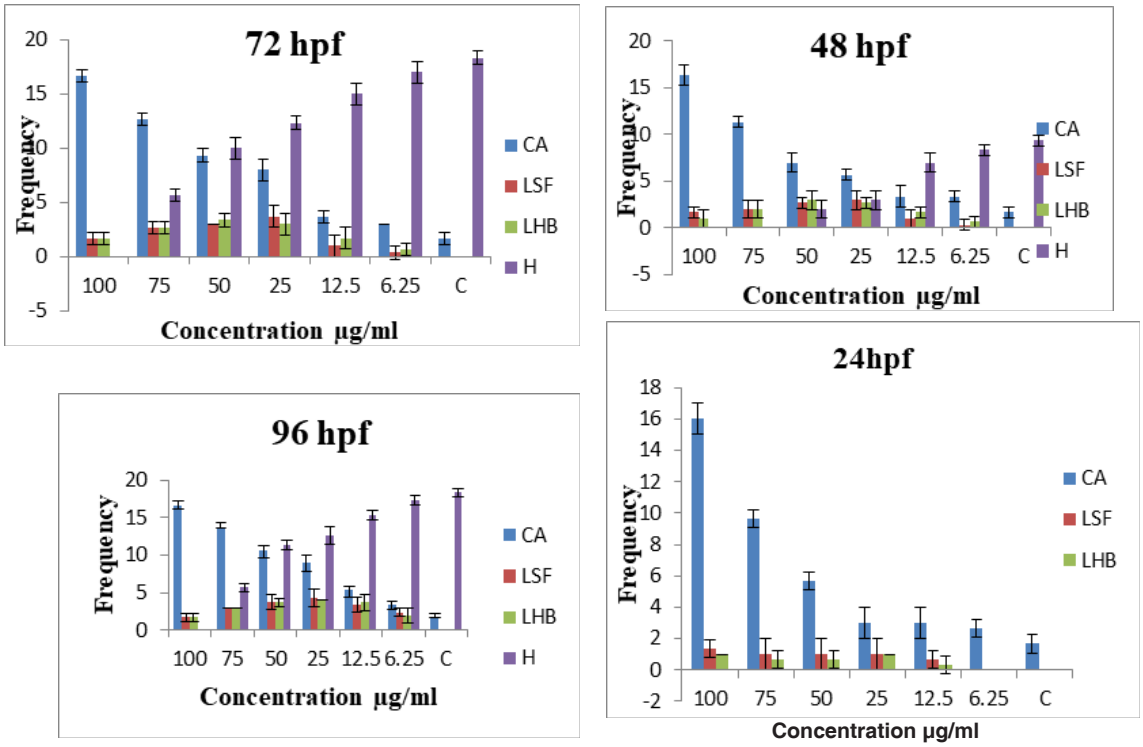


Figure 4: Types and frequencies of lethal effects observed in zebrafish embryos when exposed to cypermethrin at different time intervals: CA: coagulation, LSF: lack of somite formation, LHB: lack of heartbeat, H: number of hatched embryos, C: control.

and decreased hatching rates were among the lethal consequences; tail and head malformations, sideways lying, hatching challenges and pigmentation concerns were among the sub-lethal effects. The compound's significant toxicity to zebrafish embryos was highlighted by the fact that even at the lowest dose of 6.25 µg/mL, it disrupted embryonic development. Across three trials, there was a significant inverse connection ($p<0.05$) between LC_{50} values and exposure time. The highest cypermethrin concentration under investigation (100 µg/mL) was used in this study to quantify categorical lethal and sublethal endpoints starting at 24 hr. The outcomes of this study's observations matched those of the toxicity test on zebra fish embryos that was previously discussed.[20,21] The typical acute toxicity range for cypermethrin in fish laboratory testing has been found to be 1.8-8.2 µg/L by the USDA National Agricultural Pesticide Impact Program.[22] The LC_{50} values and negative effects of cypermethrin and related chemicals have been documented by a number of researchers. *Salmo salar* (96 hr^{-2} µg/L), *S. gairdneri* (96 hr^{-6} µg/L), *Gambusia affinis* (24 hr^{-9} µg/L) and *Cyperinodon macularis* (48 hr^{-6} µg/L) are the fish species for which beta cypermethrin's LC 50 values have been documented.[23] Studies have shown that carp and the freshwater prawn, *Palaemonetes argentines*, have LC_{50} values of cypermethrin of 12.6 µg/L[24] and 0.0031 µg/L,[25] respectively. The beta-cypermethrin study found that the number of dead common carp (*C. carpio*) embryos increased significantly even at extremely low concentrations of the chemical (0.0001, 0.001, 0.01, 0.1, 1, 2, 4 and 8 µg/L).[26]

Fish toxicity shows more promise in environmental toxicological investigations. Most fish toxicity studies have utilized adult or adolescent fish,[27,28] although utilizing live fish has been criticized for being unethical.[29] The most promising substitute technique to the traditional assessment of acute fish toxicity using live fish is the zebrafish embryo toxicity model.[29] Higher fecundity and quick development have allowed testing more samples in less time.[30] In addition, embryos' transparency makes morphological and developmental defects visible during testing.[30] Egg development outside of the mother permits immediate exposure to the toxicants. It allows for dose estimation, in contrast to mammal development, which requires the administration of the toxin to the mother.[31] According to our findings, cypermethrin is extremely harmful to zebrafish embryos. Test embryos were observed after 96 hr of treatment because the toxicity rises with time. It is hypothesised that zebrafish might be more pyrethroid sensitive and that the lethal endpoints of embryotoxicity may not adequately represent delicate neurotoxic consequences.[30] Additionally, it is hypothesised that pyrethroids' stereochemistry may affect their toxicity.

In general, fish are more sensitive to cis isomer of pyrethroids than the trans-isomer.[32,33]

The challenge in sustaining concentrations and the fact that exposure concentrations frequently fall well below the test concentration are two of the largest hurdles in zebrafish embryo toxicity assays.[34] Therefore, the discrepancy between the test and exposure levels could cause the zebrafish embryo's decreased sensitivity. Therefore, additional research is necessary to verify the assay's exposure concentration. Furthermore, a comparable study involving young fish can be used to confirm the susceptibility of zebrafish embryos to pyrethroids.

At similar identical quantities, pyrethroids are reportedly up to thousand times more hazardous to fish species than to mammals.[32] Compared to other vertebrates, the sensitivity of fish species to pyrethroids is mainly explained with the sluggish rate of biotransformation.[32] The pyrethroid hydrolyzing enzymes are lacking in fish.[35] Additionally, fish are particularly susceptible to pyrethroid toxicity due to their nervous systems' sensitivity to these chemicals and their bloodstreams' ease of absorption through the gills.[32,36]

The most frequent fatal outcome seen at all pyrethroid exposure levels and exposure times was egg coagulation, which was followed by heartbeat and somite formation defects. Embryonic coagulation was listed as a common deadly effect in a previous experiment on malathion,[37] which is compatible with our observation. Even though the focus of the current investigation was on fatal outcomes, identifying sublethal and teratogenic deformities is crucial for a thorough understanding of the toxicity of these pyrethroids. Therefore, additional research is encouraged.

Due to their extreme toxicity to fish species and other aquatic animals, the use of pyrethroids is limited.[38] They are instead used as very low volume applications, also referred to as thermal or cold fogs.[39] The anti-mosquito pesticide K-OTHRIN 1 ULV, which contains the active component deltamethrin, was determined to be the cause of 2 severe eel (*Anguilla anguilla*) destructions that occurred in Lake Balaton, Hungary, in the years 1991 and 1995. Other afflicted animal species were Bream (*Abramis brama*), Pike perch (*Stizostedion lucioperca*) and Common Gull (*Larus canus*).[40]

CONCLUSION

The zebrafish embryos were found to be more sensitive to even low concentrations of cypermethrin (6.25 g/mL) and the toxic effect of cypermethrin on zebrafish embryos increased with increase in concentration and exposure time. This was determined by analysing the lethal and sub lethal categorical endpoints. It is simple to carry out the suggested toxicity testing using the zebra fish embryo model. The information gleaned from this study will be valuable in determining the potential damage that cypermethrin poses to aquatic life.

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We acknowledge Dr. Apurba Bandhyopadhyay, Principal of Mrinalini Datta Mahavidyapith, for providing us the infrastructural and moral support for the work.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The work has been done with indigenous fish, ensuring fish well-being with proper care, reducing harm and stress during research, treating fish with dignity and gentleness, recognizing cultural and community significance, sharing findings and methods openly, and adhering to all regulations and guidelines.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The study aimed to assess cypermethrin toxicity using zebrafish embryos as a model. Cypermethrin, a synthetic pyrethroid, is commonly used in agriculture, aquaculture, and public health. The research was motivated by the persistent presence of environmental contaminants like cypermethrin, which are detrimental to aquatic ecosystems and organisms.

Zebrafish embryos were chosen for their suitability as an experimental model for toxicity testing. The study conducted a range-finding test to determine appropriate cypermethrin concentrations for the Fish Embryo Toxicity Test (FET). Subsequently, the Zebra Fish Embryo Toxicity (ZFET) Test was performed, subjecting embryos to various cypermethrin concentrations and observing lethal and sublethal endpoints over 24 to 96 hr.

Results revealed significant cypermethrin toxicity to zebrafish embryos, with increasing mortality observed with higher concentrations and longer exposure times. Lethal endpoints included coagulation, lack of heartbeat and lack of somite formation. The study also highlighted the simplicity and efficiency of using zebrafish embryos for toxicity testing, providing valuable insights into the potential harm cypermethrin poses to aquatic life.

In conclusion, the study emphasizes the sensitivity of zebrafish embryos to cypermethrin, even at low concentrations and underscores the importance of monitoring and regulating the use of such chemicals to safeguard aquatic ecosystems.

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