Effects of Pesticides on the development of Zebrafish Embryo (*Danio rerio*)

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Abstract

The present research article provides some data regarding the effects of pesticides on the development of Zebrafish embryo. Pollution of water is increased due to chemicals present in pesticides. Drainage, rainfall, soil temperature, microbial activity along with half-life of pesticides, solubility and mobility are few factors responsible for pesticides residues' movement to water. Currently Zebrafish is a valid animal model to study treatment with various small molecules, to analyse their biological functions, and to disclose the mechanism of bioactive compounds. Now in toxicological studies, embryos of Zebrafish especially known as Zebra Danio are used as an economic alternative to adult fish. Testing for substance toxicity for living organisms is an important step in the development and adaptation of any chemical for various purposes. Bifenthrin and Deltamethrin are the members of Pyrethroid compound which are group of synthetic pesticides mimicking to the naturally available pesticide Pyrethrum. Pyrethroid insecticides are very commonly used agricultural as well as residential insecticides. Insecticides and pesticides have been found to be inducing several neurological disorders in experimental animals as well as in Humans. However, toxicity assessment of insecticides is complex and some condemning data gap remains. The Zebrafish embryo toxicity test run here is based on exposure of target compounds to newly fertilized eggs for 96 hours in a static system according to OECD (Organisation for Economic Co-operation and Development) guidelines. As toxicological endpoints, coagulation of eggs and embryos, failure to develop somites, lack of heart-beat as well as non-detachment of the tail from the yolk are recorded after 24, 48, 72 and 96 h. Other morphological deformities are also recorded. Overall effects of Deltamethrin and Bifenthrin both are giving teratogenic effects at developing stage of Zebrafish embryo.

Key words: Pyrethroids, Embryo, Toxicity, Morphological deformities

1. Introduction

There have been requirements for low-cost, highly efficient animal models in fields of toxicity assessment, drug screening and other related biomedical research (Lieschke, G. J. & Currie P. D., 2007; Bull, J. & Levin, B., 2000). The Zebrafish embryo has come up as one of the best models for research. The "*Fish Embryo Test (FET)"* (OECD, 2006) has turned out as a substitute test to ascertain the toxicity of chemicals and the Zebrafish has become the best model for evaluating various mechanisms of the substances. The embryos, together with Zebrafish embryos, that are not classified as animals, are not subjected to welfare issues (Nagel, R., 2002). The wide recognition of Zebrafish as a popular model is because of some extraordinary characteristics it harbors. Few are like their tiny size, high reproducibility, rapid development, transparency of the embryo and compliance with genetic and chemical

screenings (Strahle, U. *et al*., 2012; Chakraborty, C. & Agoramoorthy, G., 2010). Presently, over 3.5 billion kilograms of man-made pesticides are being used for agriculture in an over $45 billion industry worldwide (Pretty, J., 2015). The unfavorable effects of pesticides are not observed only in applied areas but also harmful for other regions like grass fields, aquatic environments and even to human colonization as it reaches there because of carried drifts by runoffs. The pesticides' effects on human health typically depend on the chemical toxicity and dimensions and length of exposure (Lorenz, E. S., 2009). A Pyrethroid is an organic compound equivalent to the naturally available Pyrethrins, extracted from the flowers of Pyrethrums (Chrysanthemum cineraria folium and C. coccineum). Pyrethroids are used as household as well as commercial insecticides (Metcalf, R. L., 2000). There are two different types of synthetic pyrethroids on the basis of their chemical structure; type I and II (Rehman, H. *et al*., 2014). Type I includes Allethrin, Bifenthrin, Permethrin, Phenothrin, Resemethrin, Tefluthrin and Teramethrin; whereas Type II includes Cyfluthrin, Cyhalothrin, Cypermethrin, Deltamethrin, Fenvalerate, Fenpropathrin, Flucythrinate, Flumethrin, Fluvalinate and Tralomethrin (Thatheyus, A. J., & Selvam, A. G., 2013). Bifenthrin and Deltamethrin are from Type I and Type II groups respectively. Various research has indicated that animals that are exposed to pesticides have a higher risk of poor reproductive outcomes, along with fetal as well as embryonic death (Hayes, W. J. & Laws, E. R., 1991; Doull, J. *et al*., 1996).

2. Materials & Methods

2.1 Zebrafish Breeding and Embryo collection

Wild species of adult Zebrafish (6-12 months; length 2.9 cm ± 0.05 cm; weight 0.298 ± 0.5 g) were procured from Kamdhenu University, Himmatnagar, Gujarat. A day before, prior to collecting the eggs, males and females were placed in breeding tanks with a 2:1 (male: female) ratio. After collection of embryos, their quality was checked under a microscope to select the fertilized embryos for the experiment.

2.2 Embryo Toxicity Treatment

The Zebrafish embryo toxicity test conducted here is based on a 96h exposure of newly fertilized eggs in a static system according to latest OECD guidelines FET-236; the treatment of commercially available pesticide commenced within 90 minutes of fertilization and the effects of both the substrates were recorded at every 24 hrs till 96 hrs.

Commercially available pesticides were used.

1. Biflex TC (Bifenthrin 2.5%) (Type I)
2. Mosquil (Deltamethrin 2.5%) (Type II)

To evaluate toxic effects of Bifenthrin and Deltamethrin, total 4 concentrations of both the pesticides i.e. 0.5 mg/lit, 1 mg/lit, 2 mg/lit and 3 mg/lit were prepared in distilled water only. The distribution of compounds and eggs was done using 24 well plate according to OECD guidelines along with positive control and negative control. The experiment was designed in 24 well plates for both the substance. The plates were kept at 28.0+- 1^0C temperature.

3. Results

The Zebrafish embryo culture method was first optimised at our lab before going to toxicity treatment. After optimizing culture condition and full development of embryo to adult fish, toxicity experiments were designed. Bifenthrin and Deltamethrin treatment were given to freshly collected embryos for 96 hrs and abnormalities were recorded under microscope every 24hrs. The results according to toxicological endpoints and other than toxicological endpoints like structural abnormalities as scoliosis, yolk edema, and other visual changes like less pigmentation were noted. Abnormality results for Bifenthrin are shown in table 1.1 and for Deltamethrin are shown in table 1.2. The representative photographs of deformities are shown in Fig.1 (Fig 1A-1J). The embryos in the Negative control having water only, grew normally; whereas the embryos in the positive control hardly lived till 24 hours.

Table 1: The Fish embryo toxicity test result

Table 1.1: Effects of Bifenthrin

Bifenthrin Concentration (mg/lit)	0.5				1				2				3			
Observations (% of embryos) according to toxicity endpoints																
Hours	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Coagulation of eggs	8	-	-	-	16	-	-	-	25	-	-	-	25	-	-	-
Failure to develop somites	-	-	-	-	-	-	7	7	-	-	9	9	-	9	9	9
Lack of heart-beat	-	-	8	8	-	-	16	25	-	-	16	35	-	-	25	35
Non-detachment of the tail from the yolk	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Observations (% of embryos) other than toxicity endpoints																
Hatched embryos	0	100	-	-	0	83	100	-	0	50	83	-	0	33	50	-
Yolk edema	7	8	8	8	9	9	9	10	12	12	12	12	20	20	20	20
Pericardial edema	-	-	-	8	-	-	8	8	-	16	16	16	18	18	18	18
Lordosis	-	-	-	10	-	-	-	10	-	-	20	20	-	-	33	33
Kyphosis	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-
Scoliosis	-	-	-	-	-	8	8	8	-	-	11	11	-	-	13	13

Spinal column break	-	-	-	-	-	-	6	6	-	-	12	12	-	-	15	15
Reduced pigmentation	-	-	-	-	-	-	-	-	-	-	9	9	-	10	10	10
Disruption in yolk sac extension	-	-	-	-	-	-	-	-	-	3	3	3	-	5	5	5

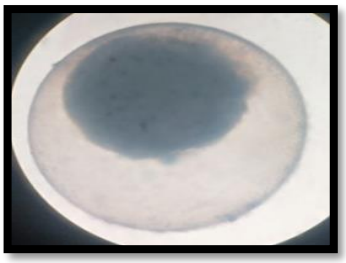
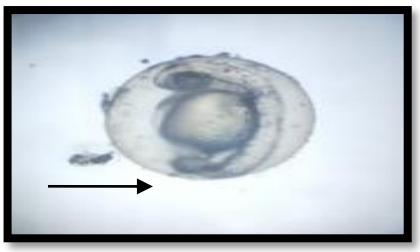
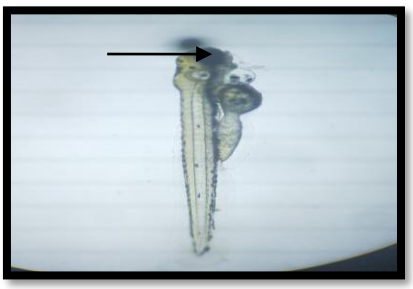
(- indicates the absence of deformity at particular time)

Table 1.2 Effects of Deltamethrin

Delamethrin Concentration (mg/lit)	0.5				1				2				3			
Observations (% of embryos) according to toxicity endpoints																
Hours	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Coagulation of eggs	16	-	-	-	25	-	-	-	33	-	-	-	33	-	-	-
Failure to develop somites	-	-	-	8	-	-	10	10	-	-	10	10	-	11	11	11
Lack of heart-beat	-	-	8	16	-	-	16	25	-	-	16	25	-	-	25	33
Non-detachment of the tail from the yolk	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Observations (%) other than toxicity endpoints																
Hatched embryos	0	100	-	-	0	66	100	-	0	33	58	100	0	41	66	100
Yolk edema	4	4	4	4	7	7	7	7	15	15	15	15	23	23	23	23
Pericardial edema	-	-	8	9	-	-	6	9	-	13	15	15	18	18	18	18
Lordosis	-	-	-	-	-	-	7	7	-	-	16	16	-	-	20	20
Kyphosis	-	-	-	-	-	-	8	8	-	8	8	8	-	-	11	11
Scoliosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spinal column break	-	8	12	12	-	-	-	-	-	-	12	12	-	-	16	16
Reduced pigmentation	-	-	-	-	-	-	-	7	-	-	-	10	-	-	10	10

Disruption in yolk sac extension	-	-	-	-	-	-	-	-	-	5	5	5	-	7	7	7

(- indicates the absence of deformity at particular time)

1A	Coaggulation of egg	
1B	Failure to develop somites	
1C	Yolk edema	
1D	Pericardial edema	
1E	Lordosis A curving inward of the lower back	

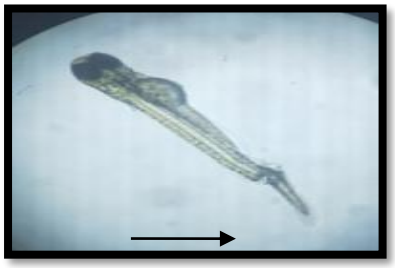
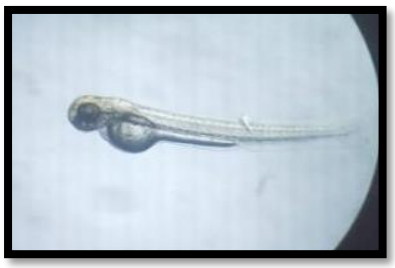
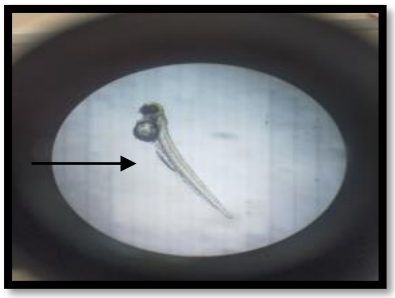
1F	Kyphosis A forward rounding of the back	
1G	Scoliosis A sideway curvature of the spine	
1H	Spinal column break	
1I	Reduced pigmentation	
1J	Disruption in yolk sac extension	

Figure 1 Deformities observed in toxicity experiments

4. Discussion

Earlier research has committed that Zebrafish and mammalian toxicity profiles are much the same and the practice of using of Zebrafish as a model organism for evaluating the toxicity and safety of

chemicals is increasing (Scholz, S. *et al*. 2008).

In the present research, Fish embryo toxicity was conducted in 24 well plates for 4 days. According to toxicological endpoints, developmental problems in Zebrafish embryos exposed to compounds from 1.5 hpf to 96hrs have been recorded. In Bifenthrin treatment, coagulations of eggs (Fig 1A) were observed in 25% of embryos at 24hrs in both 2 and 3 mg/lit. Failure to develop somite (Fig 1B) was observed in 7% of embryo in 1mg/lit and in 9 % of embryos in both 2 & 3 mg/lit. Lack of heartbeat was not visible till 48hrs. The maximum lack of heartbeat observed was in 35% at 96 hrs in 3 mg/ lit concentration. Nondetachment of the tail from the yolk was not observed in any of the embryo.

In Deltamethrin treatment, coagulation of eggs was observed in 33% of embryo at 2 and 3mg/lit at 24 hrs. Failure to develop somite was observed in 8% of embryos in 0.5 mg/lit, in 10% of embryos in 1& 2 mg/lit and 11% in 3 mg/lit concentration. The onset of appearance of these endpoints was varying in different sets. Lack of heartbeat was highest in 33% of embryos in 3 mg/lit at 96hrs. Whereas nondetachment of the tail from the yolk was not observed in any treatment in any single embryo. Along with two compounds, positive control (PC, 3, 4 dichloroaniline) and negative control (NC, water) plates were also kept where in PC, 100% mortality and in NC, 2% mortality was obtained.

In addition to the four apical lethal endpoints, we also recorded numbers of hatched embryos with other abnormalities at each time point. In the present study, with the increasing concentration of both compounds, the percentage of dead embryos is also increasing, also the hatching time of embryo is affected i.e. late hatching is observed. During adverse conditions delayed hatching permits the protection of embryo and also allow hatching synchronization when environment encourages optimal survival (Nalini, D., 2019). The most commonly found deformities in our study were Yolk edema (Fig 1C), Pericardial edema (Fig 1D), Lordosis (Fig 1E), Kyphosis (Fig 1F), Scoliosis (Fig 1G), Spinal column break (Fig 1H), Reduced pigmentation (Fig 1I) and Disruption in yolk sac extension (Fig 1J) at different stages and at different concentrations. The tables (1.1 and 1.2) indicate the percentage of embryos having any changes in development including total observations with and without toxicological endpoints.

Pericardial edema seems to be the most common abnormality during the development of Zebrafish embryo. In one of the research by Park J. *et al*. (2021), Zebrafish embryo were used to evaluate environmental toxicities of the metabolites of Pyrethroid and Endosulfan. They also found pericardial edema at the concentration of 1000 µg/lit of 3-phenoxybenzoic acid (3-PBA) treated embryos.

Other types of pesticides have also been recorded to induce morphological changes when exposed to Zebrafish embryo. An organophosphate pesticide called Trichlorfon is documented for teratogenicity like anomalies in yolk sac absorption, spine curvature and pericardial edema in Zebrafish embryos (Coelhoa S. *et al*., 2011). Body curvature observed in Zebrafish exposed to Pyrethroid has been described as suggesting neurotoxicity rather than morphological alteration (Lee J. & Freeman J.L., 2014).

A similar study was conducted by Rajini A. and Revathy K. following OECD guidelines. They studied the developmental toxicity and teratogenicity of the pesticides on the embryo-larval stage of Zebrafish (*Danio rerio*). Zebrafish embryos were exposed to different concentrations i.e. 0.0000134, 0.000134, 0.00134, 0.0134, 0.134, 1.34 and 13.4 µg/lit of Chlorpyrifos 50% + Cypermethrin 5% EC. Their results revealed that the lethal as well as sublethal effects on the Zebra fish embryos rose with an increase in the concentration of combination of both the pesticides (Rajini A. and Revathy K., 2015).

Yolk sac edema is ordinarily observed as a deformity in Zebrafish developmental toxicity assays. The large scale of chemical properties exhibited by various chemicals indicates that yolk sac edema is the most sensitive toxicological consequence for embryonic evaluation (Sant, K. E., & Timme-Laragy, A. R. (2018).

In one of the studies, developmental effects and acute toxicity of Cyfluthrin were analyzed for embryo and larval Zebrafish at 24, 48, 72 and 96 hpf (hours post fertilization). The hatch rate and frequency of spontaneous contractions were increased by Cyfluthrin, whereas the body length reduced remarkably in a dose and time-dependent manner. Cyfluthrin also induced morphological deformities like yolk sac edema, tail abnormalities and curved body axis. The study confirmed that short term exposure to Cyfluthrin causes lethality and notable developmental deformations in early life stages of Zebrafish (Kadiru S., 2021).

Lordosis term is often used for all types of back curvatures and deformities, which actually should be subdivided into lordosis i.e. hyperextension of the back, kyphosis i.e. hunchback and scoliosis i.e. sideways curvature. While some reports correctly recognize lordosis as a hyperextension of the back (Matson, C.W. *et al*., 2008; Xu, Z. *et al*., 2011; Labonty, M. *et al*., 2017; Seok, S. H. *et al*., 2006) and other publications use this term to explain what was actually termed as scoliosis (Issa, O. *et al*., 2019).

Recently von Hellfeld, R. *et al.* evaluated the toxicity of the 48 compounds on Zebrafish embryo. They have also recorded scoliosis, kyphosis and lordosis along with other behavioral abnormalities.This research on morphological effects with a wide range of test compounds with a large spectrum of modes of action concludes that any toxic exposure to Zebrafish embryos can lead to many developmental variations over those listed in OECD TG 236 for the FET (von Hellfeld, R. *et al.*, 2020).

Hisada A. *et al*. (2017) explored the possible connection between maternal exposure to Pyrethroid insecticides (PYRs) during pregnancy and infant development. They suggested a positive relation between infant development at 18 months of age and maternal PYR exposure levels during pregnancy, along with maternal fish consumption and home atmosphere quality.

DeMicco, A. *et al*. (2010) investigated similar studies like developmental nurotoxicity of six common Pyrethroids; three compounds from type I (Permethrin, Resmethrin and Bifenthrin) and three compounds from type II (Deltamethrin, Cypermethrin, and l-Cyhalothrin). The study revealed that Pyrethroid exposure to Zebrafish embryos provoked an increase in mortality and pericardial edema being dose

dependent, which showed that type II compounds was the most influential.

Reduced pigmentation has been observed in many Zebrafish embryo toxicity experiments. One of the observations was gained by Zhou S. *et al*. (2009). They explored the potential developmental toxicity of cartap on Zebrafish embryos. They were exposed, from 0.5 to 144 h post-fertilization, to a different concentration between 25–1000 μg/l. They concluded that cartap could weaken melanin pigmentation of embryos of Zebrafish by inhibiting tyrosinase activity.

According to research of Liu, X. *et al.* (2018) Deltamethrin (DM) treatment led to delay in embryo development and a significant rise in mortality of embryo at 24 and 48 h post-fertilization (hpf). DM and Acephate (AP) lowered embryo chorion surface tension at 24 hpf, as well as increase in hatching rate at 72 hours post fertilization. Furthermore, DM induced misexpression of ntl, shh, and krox20 in a dose-dependent manner along with morphological abnormalities at 10 ug/L such as smaller eyes, shorter body length and larger head-body angles.

Jin, M. *et al.* (2009), studied developmental toxicity of Bifenthrin on embryo-larvae of Zebrafish. Developmental deformities were observed for the substance with 96-h EC50 at 256 μg/lit for pericardial edema and 109 μg/ lit for curved body axis. Results from locomotor assays indicated that 96 hpf Zebrafish larvae showed impaired swimming behaviour after exposure to 50, 100 and 200 μg /lit starting from 3hpf to 84 hpf.

Environmentally correlated concentrations of Bifenthrin induced an estrogenic response in juveniles of Zebrafish, but induced an antiestrogenic response in embryos. This indication of life stage–dependent toxicity demonstrates the importance of recognising sensitive stages of thresholds in every risk assessment (Bertotto, L. B. *et al*., 2018).

According to Demoute, J. P. (1989) Pyrethroids are said to be a safer option to other insecticides exhibiting low mammalian toxicity & low environmental perseverance. As Zebrafish share homology with humans, the present research along with other related reports demonstrate the toxic effects of Pyrethroid compounds on the Zebrafish embryo development that may ask attention of organizations and community for the careful usage of Pyrethroids.

5. Conclusion

Present research concludes that, the toxic effect of the Pyrethroid compounds increased with an increase in concentration and exposure time to the Zebrafish embryos. As there is the large-scale use of pesticides, especially in agricultural field and unsupervised access by the general population in residential areas, we might be facing greater risks than presently acknowledged. As a consequence, observations of morphological abnormalities provide a prime source of demonstration for potential toxic mechanisms of Pyrethroid in mammals. So, we suggest that Zebrafish turns out to be an ideal model for the study of toxic compounds in toxicity studies, especially during embryonic development. Additional molecular studies are required to understand the underlying toxicity pathways.

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