World Journal of Zoology 6 (1): 47-51, 2011
ISSN 1817-3098

Toxicity of Neem Based Pesticide Azacel to the Embryo and Fingerlings of Zebrafish *Danio rerio* (Cyprinidae)

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Abstract: It is well known that the natural pesticides are ecofriendly and are safe to the non-target animals. The aim of this work was to evaluate the influence of neem based pesticides Azacel on the embryo and fingerlings of Zebrafish. The embryo and fingerlings of Zebrafish were exposed to different concentrations of Azacel *viz.* 0.02, 0.04, 0.06, 0.08 and 0.10µg/l. The data for embryo and fingerlings toxicity were evaluated by the probit analysis method by using StatPlus® Analyst software. The 72h LC_{50} value of Azacel for Zebrafish embryo was found to be 0.06µg/l. Dose-response in % hatching were recorded. A 24-96h toxicity test was also performed on 5-day old fingerlings of Zebrafish. The number of dead fingerlings significantly increased with increasing concentrations of pesticide. The 24, 48, 72 and 96h LC_{50} values of Azacel for Zebrafish fingerlings were 0.11, 0.08, 0.06 and 0.05µg/l , respectively. It was concluded that although natural pesticides are being considered as less toxic/safe, but it may affect the survival of embryo and fingerlings at nominal concentrations.

Key words: *Danio rerio* · Toxicity · Azacel · LC_{50} · Embryo · Fingerlings

INTRODUCTION

Pesticides are used to increase the agriculture production and in the public health and welfare of mankind. They are carried away by rains and floods to water bodies and alter the physico-chemical properties of water [1]. These chemicals are potentially more toxic to fish and other aquatic organism. Owing to the excessive use of synthetic pesticides, the environment and water resources are being polluted, thus endangering aquatic life directly and human life indirectly [2]. Although, safe to higher animals, both synthetic and natural pesticides are toxic to fish [3,4].

To overcome the hazardous effects of organic chemicals natural pesticides of plant origin are used. Plants are virtually inexhaustible sources of structurally diverse and biologically active substances [5]. Neem (*Azadirachta indica* A. Juss) is a traditional and highly esteemed medicinal tree for the people of Indian sub-continent [6]. Azadirachtin (a tetratriterpenoid) is one of the major components of neem [7,8] which have pesticidal properties [9]. Deshmukh and Pariyal [10] have reported toxic effect of Neemax on *Tilapia mossambica* and *Gambusia sps.* .

It has been reported that neem extract in aquatic environment are lethal to benthic population and drastically decreases the number of organisms in the food web and nutrient cycling process[11,12]. Recently it was observed that neem based pesticide, Achook was toxic to Zebrafish [13]. However, literature on the toxic effect of neem based pesticides on early life stages of Zebrafish is scanty. Hence, a need was felt to evaluate toxicity of Azacel a neem based pesticides on embryo and fingerlings of Zebrafish, *Danio rerio* (Cyprinidae). Animal welfare organizations have increasingly questioned ecotoxicity testing with fish and stimulated efforts to develop various alternatives. A promising alternative approach to classical acute fish toxicity testing with live fish is the fish embryo toxicity test (FET), [14] and fingerlings toxicity test which has been used for the exact evaluation of chemical toxicity to fish [15,16].

The Zebrafish is suitable for this study because it breeds easily under laboratory conditions with a high yield of eggs and viable fry. This fish was selected as the test species for toxicological studies according to the recommendations of the International Organization for Standardization [17] and the Organization for Economic Co-operation and Development [18] for using fish as test organisms for the early life stage toxicity.

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This study was carried out to evaluate the toxic effect of neem based pesticide Azacel to the Zebrafish embryo and fingerlings.

MATERIALS AND METHODS

Zebrafish, *Danio rerio* were reported from Uttar Pradesh [19]. For the present study, Zebrafish were collected, stocked, acclimatized in glass aquaria and bred in the laboratory to obtain the fertilized eggs by the method of Ansari and Kumar [23]. Five concentrations (0.02, 0.04, 0.06, 0.08 and 0.10μg/l) of Azacel was selected for the toxicity test for embryo and fingerlings. The stock solution was prepared by serial dilution of the pesticides in acetone. Acetone alone in the same amount served as control. Water was changed after 24h with fresh treatment of pesticide.

For embryo toxicity tests lots of 100 fertilized eggs were separated in 500 ml beakers with 250 ml dechlorinated water. Three such replicates were used. The dead eggs were counted and removed after every 24 hours. The dead embryos became white due to coagulations or precipitations of protein. At the end of the incubation period of 72h the total hatched eggs were counted.

To evaluate the fingerling toxicity, 5-day-old fingerlings were placed into 500 ml beaker. Three replicates of ten fingerlings for each concentration were placed in the beakers having 250 ml of dechlorinated water. Mortalities of fingerlings were recorded after 24, 48, 72 and 96h exposure periods. The dead fingerlings were removed from the water.

The susceptibility of the embryos and fingerlings of Zebrafish to Azacel pesticide were determined by the Probit method of Finney [20] using StatPlus® version 2009 computer software programme to calculate the LC_{50} values (with 95% confidence limits) at different exposure periods, slope and chi-square values.

RESULTS

The results of the acute toxicity are illustrated in Tables 1 and 2. It is evident from the Table 1 that the mortality of the treated embryos after 72h at lowest concentration of 0. 02μg/l was 8.67% and at highest concentration (0.10μg/l) it increased to 89.33%. It is clear that at lowest concentration of pesticide the hatching was 274(91.33%) which decrease to only 32 (10.67%) at highest concentration of pesticide as compared to the control group. The 72h LC_{50} value for embryo was calculated to be 0.06μg/l. After the exposure of the pesticides the fingerlings of Zebrafish showed behavioral changes, they aggregated at one corner of the aquarium, swimming at the water surface and erratic swimming throughout the experiment. For fingerlings at lowest concentration of 0.02μg/l the 24h, LC_{50} value of Azacel was 0.11μg/l, while for 48h and 72h it was 0.08μg/l and 0.06μg/l, respectively which decreased to 0.05μg/l after 96h exposure (Table 2).

Table 1: Toxicity of Azacel to Zebrafish embryo and 5-day-old fingerlings.*

	Embryo toxicity		Fingerling toxicity			
			Number of dead fingerlings			
Concentrations (μg/l)	Number of dead embryos	Total hatching in 72h	24h	48h	72h	96h
0.00	05 (1.67)	295 [98.30]	NIL	NIL	NIL	NIL
0.02	26 (8.67)	274 [91.33]	01	02	04	06
0.04	55 (18.34)	245 [81.67]	03	05	07	09
0.06	123 (41.00)	177 [59.00]	06	09	13	16
0.08	211 (70.33)	89 [29.67]	09	14	17	22
0.10	268 (89.33)	32 [10.67]	15	19	24	27

* 300 eggs were used in three batches of 100 each for embryo toxicity. 30 fingerlings were used in three batches of 10 each for fingerling toxicity.
Data in parentheses '( )' shows the % mortality and '[ ]' shows the % hatching of the Zebrafish embryo.

Table 2: Summary of probit analysis of Table 1.

		Effective Concentrations (μg/l)			Confidence limits of LC_{50} (μg/l)			
Test stage	Exposure duration (h)	LC_{10}	LC_{50}	LC_{90}	LCL	UCL	Slope	Chi-square values
Embryo	72	0.03	0.06	0.13	0.04	0.09	1.64	37.71
Fingerling	24	0.04	0.11	0.33	0.09	0.20	1.69	0.75
	48	0.03	0.08	0.25	0.07	0.12	0.86	0.73
	72	0.02	0.06	0.19	0.05	0.08	2.05	1.69
	96	0.02	0.05	0.14	0.04	0.06	2.30	2.35

This shows that the effect of Azacel is concentration as well as time dependent. The slope values shown in the table are steep which indicate that the test animals are very sensitive to even the minor change in concentration of the toxicant. The LC_{50} values of the pesticide showed a significant negative correlation with exposure time.

Thus, results provided evidence that the Azacel pesticide is found to cause mortality of embryo and fingerlings at nominal concentrations. It means that the "safe" neem based products are not so safe to the larval stages of fishes which should be considered when these chemicals are used in agricultural areas near aquatic ecosystems.

DISCUSSION

Toxicity test with embryo and fingerlings are valuable for assessing potential impacts on growth, reproduction and survival of Zebrafish in polluted environment and are important tools for good environmental monitoring [21,22]. The result showed that the neem based pesticide Azacel is toxic to fish embryo and fingerlings. This result is supported by the earlier study of Ansari and Kumar [23]. They examined that malathion did not cause any delay in the hatching of eggs. However, in increasing concentrations the per cent hatching gradually decreased. Also Kaur and Toor [24] reported that at higher concentration of pesticides the eggs of *Cyprinus carpio communis* died before hatching because the pesticide affect the activity of hatching enzymes. Aydin and Koprucu [25] observed that increasing diazinon concentration had significant effects on hatching success. Dave and Xiu [26] found that low concentrations of copper (0.25μg/l); lead (30μg/l), mercury (0.20μg/l) and nickel (80μg/l) can interfere in hatching and survival of the Zebrafish. Azacel, derived from the Neem tree, is supposed to be ecofriendly. The effects of Azadirachtin, the primary active ingredient of Neem on insects include feeding and oviposition deterrence, growth inhibition, fecundity and fitness reduction [27].

In this study, the embryos of Zebrafish during the incubation period show very little voluntary response to changes in their environment and continue to develop by catabolizing their yolk. As soon as the yolk sac is absorbed they become more susceptible to various toxicants. Identical results were observed by Halter and Johnson [28] on Salmon embryos. Hassan *et al.* [29] also reported that the toxicity of quillaja saponin extracted from bark of the tree, *Quillaza saponaria* using Zebrafish embryo and found that at higher concentrations the embryos exhibited shrinkage of the chorion, decreased hatching time and embryonic mortality. In addition, sensitivity to the pesticides may vary with stage and species. Experimental exposure of leopard frogs and green frogs to low concentrations of pyrethroid insecticides indicated that newly hatched tadpoles are considerably more sensitive than embryos [30]. Scheil *et al.* [31] studied the effect of diazinon on early life stages of Zebrafish, *Danio rerio.* It can be concluded that the fish exposed to pesticides caused circulatory failure and death of embryos prior to hatching. The other remarkable finding of the present experiment is that the chorion of the egg provides no protection to the developing embryo. It is evident from the results that the 72h LC_{50} of embryo and fingerling are the same *i.e.* 0.06μg/l.

Recently, Ansari and Sharma [13] reported Achook a neem based pesticide to be toxic to adult Zebrafish. Ansari and Ahmad [4] also studied the comparative toxicity of pyrethroid Lambda-cyhalothrin and Neemgold to the embryo of Zebrafish and reported that the embryos were more sensitive to Lambda-cyhalothrin than to Neemgold. Mondal *et al.* [32] reported the toxicity of two neem based pesticide Nimbecidine and Neemgold on a fresh water loach, *Lepidocephalichthys guntea.* The toxicity of different neem pesticides and extracts on other non-target aquatic organism has been estimated by other investigators [11, 33].

Plant based pesticides contain active ingradients with low half life period and their toxic effects on the environments are not too detrimental [34]. To reduce the contamination in the environment it is suggested that use of plant based pesticides should be encouraged [27] because they disintegrate easily into constituent elements without leaving any indelible impression in different regions of the environment [35]. On the basis of the present investigations it is suggested that concentrations of pesticides in aquatic system should be minimized so that there is no detrimental effects on the developing embryo and fingerlings.

ACKNOWLEDGEMENTS

The authors thankfully acknowledge the UGC New Delhi Project No. F-33-358/2007 (SR) for financial support and to Prof. C. P. M. Tripathi, head of the Department of Zoology, DDU Gorakhpur University, Gorakhpur for providing laboratory facilities.

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