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Embryo and Fingerling Toxicity of Dimethoate and Effect on Fecundity, Viability, Hatchability and Survival of Zebrafish, *Danio rerio* (Cyprinidae)

Shabnam Ansari and Badre Alam Ansari

Department of Zoology, Zebrafish Laboratory,
DDU Gorakhpur University, Gorakhpur-273 009 (UP), India

Abstract: Dimethoate, a broad spectrum organophosphate insecticide is a potential toxic pollutant, adversely affecting the fauna of aquatic ecosystem. In the present study, the adults, embryos and fingerlings of Zebrafish, *Danio rerio* were used as a model to investigate the toxic effects of Dimethoate. The 24 h to 96 h LC_{10}, LC_{50} and LC_{90} for the adults and fingerlings, slope and chi-square values were calculated. The 96 h LC_{50} value of Dimethoate was 60.00 µg/l for adults. The 72 h LC_{50} for the embryo was 24.64 µg/l and for the fingerlings the 96 h LC_{50} value of Dimethoate was 21.64 µg/l. The mortality of embryos at highest concentration *i.e.*, 35 µg/l was increased to 88%. The adult fishes were exposed for one month to four different concentrations of Dimethoate (LC_5 to LC_{20}) and allowed to breed to observe the reproductive ability. The results show significant reductions in fecundity and hatchability in comparison to the control group. The survival of the hatched fingerlings was not affected after one week. The toxicity was concentration as well as time dependent.

Key words: Danio rerio % LC_{50} % Dimethoate % Embryo % Fingerlings % Sub-lethal toxicity

INTRODUCTION

Pesticides of various categories *viz.* organochlorines, organophosphates, carbamates, synthetic pyrethroids and natural products are used against a number of pests, to increase the crop production. These pesticides reach the aquatic environment mainly by runoffs or drainage from treated agricultural lands, inadvertently exposing the non-target organisms especially the fish. They also disrupt the food chain threatening the ecological balance and the biodiversity of the nature. Aquatic contamination of these pesticides cause acute and chronic poisoning of fish and cause severe damage to their vital organs [1 , 2], skeletal deformities [3], reduced reproductive ability [4 , 5] and causes various biochemical alterations [6 , 7]. Recently, deltamethrin and neem-based pesticide achook was found toxic to Zebrafish [8] and it is also reported that pyrethroid lambda-cyhalothrin and neemgold was toxic to the adult and embryo of Zebrafish [9 , 10].

Dimethoate is an organophosphorus pesticide with a contact and systemic action, first described by Hoegberg and Cassaday [11]. It was introduced in 1956 and is produced in many countries for use against a broad range of insects in agriculture and also for the control of the housefly and other household insects. Its mode of action is acetylcholinesterase (AChE) inhibition resulting to nerve exhaustion, nervous system failure and ultimately to death. The signs of the toxicity of Dimethoate in fish, *Channa punctatus* included jumping, erratic movement, imbalance and death [12]. Frequently, organophosphorus contamination has been found in environments, elements of the food chain and humans [13].

Fish as a taxonomic group are the only primarily aquatic vertebrate class and have, thus traditionally regarded as an indispensable component of integrated toxicity testing strategies. 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], which has been used for the exact evaluation of chemical toxicity to fish [15 , 16]. The Zebrafish, *Danio rerio* was selected for the present study because they are model organisms for developmental toxicology research, readily available, produce large number of clear eggs and are sensitive to environmental changes. It is also recommended by International Organization for Standardization [17] and the Organization

Corresponding Author: Badre Alam Ansari, Department of Zoology, Zebrafish Laboratory, DDU Gorakhpur University, Gorakhpur - 273 009 (UP), India, Tel: +91 9452448942, E-mail: ba.ansari@rediffmail.com.

for Economic Co-operation and Development [18] for toxicological studies. The present study deals with the toxic effects of Dimethoate on the adult, embryo, fingerlings and reproductive ability of the Zebrafish.

MATERIALS AND METHODS

Zebrafish (*Danio rerio*) were reported from Uttar Pradesh [19]. They were collected and acclimatized in the laboratory in 35 fingerling for each glass aquaria containing dechlorinated water, aerated continuously through stone diffusers connected to a mechanical air compressor. Water temperature ranged between 25±2ºC and pH was maintained between 6.6-8.5. Fish were fed twice daily alternately with raw chopped goat liver and brine shrimps. *Daphnia* was also given as a live food oftenly as a supplement diet.

Adult Toxicity: For toxicity test adult Zebrafish of same age were procured from the general culture, for determination of the 24, 48, 72 and 96 h LC_{50} values using five different concentrations of Dimethoate 40, 60, 80, 100 and 120 µg/l (Rogor 30% EC, Rallis India Ltd., Mumbai) purchased from the local market. The pesticide was serially diluted in acetone. The tests were conducted in dechlorinated water glass aquaria containing 10 fingerlings for each. Two replicates of ten fishes for each concentration of pesticide were performed. Randomization of the fish in the test aquaria was done according to the method prescribed by the U.S. Federal Water Pollution Control Administration [20]. Water was changed daily with fresh treatment of pesticide. A fish was considered dead when its gill movements ceased and it did not respond to gentle prodding. Dead fishes was removed carefully from aquaria to avoid deterioration.

Reproductive Ability: For the study of reproductive ability, two months old adult matured fishes (10 females and 20 males) were procured from the general stock and exposed for one month to four different sub-lethal concentrations of Dimethoate (96 h LC_5 24.68, LC_{10} 30.03, LC_{15} 35.05 and LC_{20} 38.09 µg/l). Each set of experiment was accompanied with a control group having no pesticide. After one month continuous stress of the pesticide the adult Zebrafish were brought back to the normal water for breeding. Three matured females along with six males from each test group were placed in 25-1 glass aquaria separately to breed in laboratory by the method of Ansari and Kumar [21]. The eggs were counted and average fecundity was established. Unfertilized eggs were detected by their milky appearance and discarded. The hatched and dead embryos were recorded till 72 h and survival of fingerlings up to one week was observed.

Embryos and Fingerlings Toxicity: For this study Zebrafish were bred in the laboratory to obtain the fertilized eggs. Five concentrations 15, 20, 25, 30 and 35 µg/l of Dimethoate for embryo and fingerling of Zebrafish were selected with three replicates for each concentration. Stock solution was prepared by serial dilution of the pesticides in acetone. Acetone alone in the same amount served as control. Water was changed daily with fresh treatment of pesticide.

For embryos toxicity tests lots of 100 fertilized eggs were separated in 500 ml glass beakers with 250 ml dechlorinated water. Dead eggs were counted and removed daily until the end of the test. The dead embryos became white due to coagulation or precipitation of protein. At the end of the incubation period (72 h) the total hatched eggs were counted.

To determine the toxicity of Dimethoate for fingerlings, 5-days old fingerlings were used. Three replicates of ten fingerlings for each concentration were placed in 500 ml glass beakers having 250 ml of dechlorinated water. Five concentrations *viz*., 15, 20, 25, 30 and 35 µg/l were selected. Mortality of fingerlings was recorded after 24, 48, 72 and 96 h exposure periods. The susceptibility of the adults, embryos and fingerlings of Zebrafish to Dimethoate were established using the probit method of Finney [22] by StatPlus®version 2009 computer software programme to calculate LC_{50} values (with 95% confidence limits), slope and chi-square values.

RESULTS

The exposure of adult fishes to different concentrations of pesticide showed abnormal behavioral changes such as restlessness, aggregations at one corner of the aquarium, erratic and jerky swimming, frequent surfacing, increased mucous secretions and loss of balance. At high concentrations the pectoral and pelvic fins were found to be expanded and they rolled vertically prior to death. It is clear from table 1 that the mortality increases with the increase in concentrations and the LC_{50} decreases with the increase in the exposure period. It shows that toxicity of Dimethoate is concentration as well as time dependent. The 24 h LC_{50} value of Dimethoate was 140.79 µg/l which decreased to 60.00 µg/l after 96 h of exposure. The slope values shown in the table are steep.

Table 1: Toxicity of Dimethoate against Zebrafish. †

Treated Period (h)	Effective Concentrations (µg/l)	95% Confidence limits of LC50 (µg/l) LCL	UCL	Slope	Chi-square Values
24	LC_{10} 53.16	107.59	386.09	2.13	0.22
	LC_{50} 140.79				
	LC_{90} 372.86				
48	LC_{10} 39.44	87	180.49	2.18	0.29
	LC_{50} 107.64				
	LC_{90} 293.73				
72	LC_{10} 37.05	62.99	86.81	1.72	0.28
	LC_{50} 74.36				
	LC_{90} 149.28				
96	LC_{10} 30.03	48.41	69.65	1.71	0.2
	LC_{50} 60.00				
	LC_{90} 119.89				

†Batches of ten fishes were exposed to five different concentrations of Dimethoate (diluted in acetone). Mortality was recorded every 24 h. Each set of experiment was replicated two times. The control groups were treated with acetone simultaneously. The LC50 values of the pesticide showed a significant ($P<0.05$) negative correlation with exposure time. LCL and UCL denote the lower and upper confidence limits respectively for the LC50 values.

Table 2: Effect of Dimethoate on the fecundity, viability, hatchability and survival of Zebrafish, Danio rerio. †

Concentrations of Dimethoate of 96 h (µg/l)	Average number of eggs laid/female	Average number of viable eggs	Hatchability after 72 h	Survival of fingerlings after one week
0	320	304	293 (96.38)	287 (980
LC_5 24.68	305	291	267 (91.75)	258 (96.62)
LC_{10} 30.03	293	262	221 (84.35)	203 (91.85)
LC_{15} 35.07	275	237	187 (78.9)	162 (86.63)
LC_{20} 38.09	224	181	97 (53.59)	78 (80.41)

†Fishes were exposed to four different concentrations for one month under pesticidal stress of Dimethoate ranging from 24.68 to 38.09 µg/l. Data in parentheses are percent values. All the data were found significant ($P<0.05$) when Student's t-test was applied between treated and control.

Table 3: Toxicity of Dimethoate to Zebrafish embryo and 5-day-old fingerlings. †

Concentrations (µg/l)	Embryo toxicity Number of dead embryos	Total hatching in 72h	Fingerling toxicity Number of dead fingerlings 24h	48h	72h	96h
0	5 (1.67)	295 [98.30]	NIL	NIL	NIL	NIL
15	34 (11.33)	266 [88.60]	2	3	5	8
20	78(26)	222 [74.00]	4	5	8	12
25	141 (47)	159 [53.10]	8	9	13	17
30	207 (69)	95 [31.50]	13	15	19	21
35	264 (88)	36 [12.00]	18	21	25	28

†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 4: Summary of probit analysis of Table 1.

Test stage	Exposure` Duration (h)	Effective Concentrations (µg/l) LC10	LC50	LC90	Confidence limits of LC50 (µg/l) LCL	UCL	Slope	Chi-square values
Embryo	72	15.52	24.64	39.09	22.62	26.82	1.58	5.61
Fingerling	24	17.84	32.18	58.03	28.79	38.97	1.58	0.37
	48	16.59	29.55	52.66	26.71	34.25	1.57	1.03
	72	14.24	25.16	44.46	22.76	27.98	1.56	1.01
	96	11.92	21.64	39.29	19.07	23.94	1.55	0.97

The LC_{50} values of the pesticide showed a significant ($P<0.05$) negative correlation with the exposure time. The chi-square values were not significant, indicating that the fish populations used in the experiments were homogeneous.

During the experiment a significant ($P<0.05$) reduction in fecundity, viability and hatchability was observed (Table 2). It is revealed from the present experiment that the average number of eggs laid by Zebrafish is 320 under normal conditions whereas this number remarkably reduced to 224 after one month stress of LC_{20} *i.e.* 38.09 µg/l Dimethoate. A significant ($P<0.05$) decrease in hatchability up to 46.41% was observed. Results showed that, the survival of the hatched fingerlings was not affected after one week.

The results of toxicity of Dimethoate to embryos and fingerlings of Zebrafish are illustrated in tables 3 and 4. With increase in concentrations of pesticide the number of dead embryos in treated groups increased to 11.33% at 15 µg/l and at highest concentration *i.e.*, 35 µg/l it was increased to 88% (Table 3). It is clear that at lowest concentration of pesticide the hatching was 266 (88.6%) which decrease to only 36 (12 %) at highest concentration of pesticide as compared to the control group. Mortality of the control as well as treated embryos was observed and the 72 h LC_{50} value for embryo was calculated to be 24.64 µg/l. At 24-96 h, mortality of fingerlings was observed at different concentrations (15, 20, 25, 30 and 35 µg/l). After exposure to the pesticide, the fingerlings of Zebrafish also showed behavioral changes, they aggregated at one corner of the test aquarium, swimming fast at the water surface throughout the experiment. For fingerlings the 24 h LC_{50} value of Dimethoate was 32.18 µg/l, while for 48 h and 72 h it was 29.55 µg/l and 25.16 µg/l respectively which decreased to 21.64 µg/l after 96 h exposure period (Table 4). The number of dead fingerlings increases with increase in concentrations of pesticide. The results showed that, the effect of Dimethoate is concentration as well as time dependent. In this study the slope functions also clearly indicate the acute toxicity of the pesticide. Thus, the results provide evidence that the Dimethoate pesticide is found to cause mortality of adults, embryos and fingerlings of Zebrafish and it is not safe which should be considered when used in agricultural areas near aquatic ecosystems.

DISCUSSION

During the present study, the erratic and abrupt swimming in fishes after pesticide exposure may be due to obstruction in AChE activity as suggested by other workers [23, 24]. A significant decrease of AChE activity in *Danio rerio* adult specimens has been occurred in chronic tests using 0.27 µg/l of parathion solution within a Non Observed Effect Level (NOEL) of 0.12 µg/l [25]. It is also reported that AChE inhibition decrease the feeding rate due to impairment of impulse transmission [26].

Exemplifying [27] calculated the 96 h LC_{50} values to be 4.57 µg/l for *Saccobrachus fossilis* exposed to Dimethoate. The 96 h LC_{50} values of Dimethoate for catfish, *Clarias batrachus* was 65 mg/l [28]. In *Heteropneustes* it was 2.98 mg/l [29]. It was reported earlier that Zebrafish exposed to long term concentrations of malathion, failed to spawn and showed skeletal deformities [3], inhibited acetylcholinesterase (AChE) activity in the nervous tissue (Brain) of Zebrafish [30]. During the present study 96 h LC_{50} values of Dimethoate for adult fishes was 60.00 µg/l. Steep slope functions of the toxicity curves of 96 h mortality concentration data for Dimethoate indicate a large increase in the mortality associated with the relatively small increase in the concentration of this pesticide. This may be due to the rapid absorption of the pesticide and rapid onset of effects [31].

The embryos and fingerlings toxicity tests are valuable for assessing potential impacts on growth, reproduction and survival of Zebrafish in polluted environment and are important tools for good environmental monitoring [32, 33]. It has been observed that increasing Dimethoate concentration had significant effects on hatchability. Supporting this observation [34] who reported that early embryonic exposure in Medaka was the greatest effect on hatching success. For example, when embryos were exposed to 26 mg/l diazinon from days 1-5, only 16% hatched whereas 100% hatched when exposed to the same concentration from days 5-9. At higher concentrations of pesticides the eggs of *Cyprinus carpio communis* died before hatching because the pesticides affects the activity of hatching enzymes [35]. Sub-lethal effect of diazinon resulting AChE inhibition could drastically affect growth, survival, feeding and reproductive behavior of fishes [36]. Moore and Wairing [37] and Wall [38] reported significant reduction in the levels of the reproductive steroids in Zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) after sub-lethal doses of diazinon. Hatchability was observed to be 30 and 50% in eggs obtained from the mother fish exposed chronically to 2.6 and 1.3 mg/l for 30 days respectively [39]. In the present study, it can be attributed that the hatching was affected due to the inhibition of some hatching enzymes.

During the development sensitivity may change with some compounds showing higher sensitivity in embryos whereas others are more toxic to larvae [40 , 41]. Also, [15] found that, early life stages of *Oryzias latipes* were the most sensitive to toxic effect. Annune and Ajike [42] reported a very low LC_{50} of 0.018 mg/l for *Oreochromis niloticus* juveniles exposed to Dimethoate (Rogor). Oh *et al*., [43] present three factors causing the selective toxicity of diazinon for various fish species; different inhibition of AChE, different detoxification and absorption.

During the present study there was a remarkable observation that the 72 h LC_{50} for embryos and free swimming fingerlings were almost the same. This indicates that the chorion of the egg could not act as a barrier for Dimethoate and incapable to protect the embryo. This is in agreement with the finding of Scheil, *et al.,* [44] who observed that the organophosphate, diazinon affected the embryo of Zebrafish and caused death due to circulatory failure. Several investigators have shown that organophosphates can penetrate the chorion and cause various teratogenic effects and death of fish embryos [45-47]. Very recently we also reported that the chorion of Zebrafish provides no protection to the developing embryo exposed to neem pesticides Neemgold and Azacel [9, 48]. It is concluded from the present study that Zebrafish and its early life stages are sensitive to low levels of Dimethoate in aquatic environment and significantly affect its populations. Therefore, these pesticides should be used with great caution and in a sustainable way so that it may not be hazardous to aquatic environment and human beings. Moreover, extensive investigations should be done for their safe use in aquaculture.

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