

EFFECT OF NEEM-BASED FORMULATIONS (NIMBECIDINE AND ULTINEEM) ON THE SPAWNING SUCCESS OF ZEBRAFISH *DANIO RERIO* (CYPRINIDAE)

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ABSTRACT

Present research work was based on the evaluation of the toxic effects of two Neem-based pesticides viz. Nimbecidine and Ultineem on the reproductive ability of zebrafish. Four month old zebrafish (both male and female) were exposed to 96-h LC₅ values (0.27µg/L for Nimbecidine and 0.08µg/L for Ultineem) for one month. Fishes were brought back to normal water and allowed to breed to observe the fecundity and hatchability. Results show significant reduction in fecundity and hatchability as compared to that of control. It was observed that the fecundity was reduced by 21% in Nimbecidine and 37.77% in Ultineem treated fishes. A significant decrease in hatchability up to 21.69% in Nimbecidine and 26.99% in Ultineem treated fishes were observed respectively. The number of unhatched/dead eggs in treated fishes increased significantly ($p < 0.05$ for each case). The results suggest that low concentrations (96-h LC₅ values) of both the pesticides may have a significant effect on the reproduction of zebrafish.

INTRODUCTION

In developing countries anthropogenic activities have caused profound alteration in physical and chemical characteristics of the aquatic environment. Industrial, municipal as well as agricultural water containing hazardous chemicals like hydrocarbons, and halogenated hydrocarbons accumulate in aquatic food webs causing chronic or acute effects in fish population (Herric, 1991). The reduced fitness and growth of fish occurs at sub-lethal levels depending on exposure time, toxicity and concentration of the chemical substances involved (Lanno and Dixon, 1994). The reproductive ability and early life stages of fish, like eggs and larvae are particularly sensitive to contaminants (Fiuman, 1993).

All kinds of pesticides (synthetic as well as natural) are being used extensively in the control of crop pests, mosquitoes and vector born disease. With rapid industrialization and increase in human population, the pollution of water bodies has become a universal phenomenon in the present day world. Synthetic insecticides are used widely for the control of various insect pests because they can be applied whenever and wherever needed, economical and most important thing is the reliability of control method. Hence, the production and consumption of pesticides has greatly increased in recent years. The contribution of pesticides to increase agricultural production cannot be denied, but synthetic pesticides have also caused unprecedented ecological damage, also induced

serious health hazard among workers during manufacture, formulation and field applications (Dikshit and Dutta, 1973; Anonymous, 1975; Kashyap, 1984; Ansari and Kumar, 1988).

To overcome the problems of synthetic chemical hazards, one of the best control measures is the use of plant origin products. Their biodegradability, least persistence and least toxic to non-target organisms, economic and easy availability made them popular now-a-days. Today about 200 plants containing insecticidal activities are known (Tripathi *et al.*, 1999; Tripathi *et al.*, 2000 and Singh *et al.*, 2001). Among all these natural products, one of the most promising natural compound is *Azadirachtin*, an active compound extracted from the Neem tree (*Azadirachta indica*) whose antiviral, antifungal, antibacterial and insecticidal properties have been known for several years (Isman *et al.*, 1990; Harikrishnan *et al.*, 2003). Since, these kinds of pesticides are supposed to be non-toxic to non-target organisms; hence the present study was conducted to justify it. Literature review reveals that most of the studies with reference to neem based formulations as well as raw plant extracts are limited up to testing its toxicity on insect pests. Intensive research projects on the biological evaluation of neem products are being conducted in several countries (Jilani and Su, 1983; Islam, 1984; Saxena *et al.*, 1984; Ansari and Sharma, 2009). Hazard assessment of chemicals to aquatic organisms involves the use of many toxicity tests. Acute toxicity tests, embryo-larval toxicity tests and chronic toxicity tests that measures survival, growth and

reproductive effects now provide the most relative utility for evaluation of potential chemical hazards to aquatic life. Physiological, biochemical and histological measurement have a low relative utility as diagnostic tests in aquatic toxicology because it is not possible to relate changes in these sub-lethal responses to adverse environmental impacts.

However, little work has done on the study of toxic effects of neem based formulations on the reproductive ability of fish. Hence, a need was felt to investigate the effect of two neem based pesticides Nimbecidine and Ultineem on the reproductive ability of zebrafish, *Danio rerio* (Cyprinidae). This fish was selected as test species according to the recommendation of the International Organization for Standardization (1976) and the Organization for Economic Co-operation and Development (OECD 210, 1992).

MATERIALS AND METHODS

Zebrafish recorded for the first time from Uttar Pradesh (Ansari and Kumar, 1982) were collected from local pond and acclimatized. They were bred in the laboratory in 25-L glass aquaria containing 10-L of dechlorinated water, aerated continuously through stone diffuser connected to the mechanical air compressor. Water temperature ranged between 25-28°C and pH was maintained between 6.6-8.5. The fishes were fed twice daily; in the morning they were given finely chopped raw goat liver and eggs albumin and in the evening, powdered brine shrimps. To evaluate the toxic effect of Nimbecidine and Ultineem on the reproductive ability and spawning success of zebrafish, 10 females and 20 males were exposed for one month to sub-lethal concentrations of both the pesticides (LC₅₀ value of 96hr i.e. 0.27 µg/L and 0.08 µg/L of Nimbecidine and Ultineem respectively). In our earlier experiment the 96-h LC₅₀ of Nimbecidine and Ultineem was calculated to be 2.37 µg/L and 0.83 µg/L respectively, for adult zebrafish. The water of aquaria was replaced alternate day along with the fresh treatment of Nimbecidine and Ultineem. After one month of exposure, three sets of one fully matured female along with two males were placed in 25L glass aquaria containing 10L dechlorinated water to observe the fecundity, viability, hatchability and survival of eggs laid by fishes by the method used by Ansari and Kumar (1986). The parents were returned to the stock culture. The viable eggs were transparent while the unfertilized/unviable eggs were white in colours which were removed from the aquaria. The hatching starts from the 24hr of fertilization and nearly all the eggs get hatched in 72hr. Hence, the numbers of hatchling were counted after

every 24hr. The experiment was terminated after 96-hr and the remaining eggs were supposed to be dead/unhatched. The data was compared with the control and Student's t-test was applied to test its significance by using StatPlus version 2009 computer software.

RESULTS AND DISCUSSION

After one month continuous exposure of fishes to both the pesticides (Nimbecidine and Ultineem), they were brought back to normal water for breeding purpose. During the present investigation we observed significant ($p < 0.05$) reduction in fecundity and hatchability (Table 1). This experiment revealed that the mean numbers of eggs laid by zebrafish are 376 ± 11.02 under normal condition, whereas the mean number of eggs laid by treated zebrafish was remarkably reduced. It was observed that the fecundity was reduced by 21% (297 ± 2.08) in Nimbecidine and 37.77% (234 ± 10.02) in Ultineem treated fishes as compared to the untreated control considered to be 100%. A significant ($p < 0.05$) decrease in hatchability up to 21.69% in Nimbecidine and 26.99% in Ultineem treated fishes were also observed respectively. The number of unhatched/dead eggs increased significantly ($p < 0.05$) in both the treated groups (Table 1). Chemicals in effluents from waste water treatment plants (WWTRS) can affect the normal endocrine homeostasis and development, which can lead to the reproductive disorders and population fall (Gracia *et al.*, 2008). The xenobiotic pollutants may disrupt reproductive endocrine functions by acting at the hypothalamus, pituitary, gonads and liver dysfunctions may result in rate of gonadal development and viability of gametes (Sebire *et al.*, 2009). Even the organophosphorus pesticides (Malathion) have also been found to cause abortions in zebrafish (Ansari and Kumar, 1986), moreover, seven days exposure to 0.9 mg/L malathion resulted in marked atresia with loss of the characteristic spherical shape of the ovarian follicles. This means that development and growth of follicles were abruptly affected by malathion (Ansari and Kumar, 1986). Pyrethroids are widely used in rice fields in some countries (Bao *et al.*, 2007) but their application in paddy fields has been prohibited in China because of their high toxicity to aquatic animals (Koprucu and Aydin, 2004; Cakmak and Gorgon, 2003). Researchers have shown that exposure to Lambda-cyhalothrin even at some lethal concentrations induces behavioral and biochemical changes in fish (Bao *et al.*, 2007). Literatures on the toxic effect of neem based formulations to fish especially *Danio rerio* are scanty. Recently, Ansari and Sharma (2009) reported

Table 1: Effect of Nimbecidine and Ultineem on the fecundity, fertility, hatchability and survival of zebrafish embryos

Test condition	Mean no. of eggs laid by 3 females	Mean no. of unfertilized eggs	Mean no. of viable eggs	Mean no. of hatched eggs			Mean no. of unhatched/dead eggs
				24hr	48hr	72hr	
Control(00)	$376 \pm 11.02^*$ (100)	11.67 ± 0.88	364.33 ± 11.41 (100)	90 ± 5.29 (24.70)	201.33 ± 4.91 (55.17)	57.33 ± 2.19 (15.74)	$15.67 \pm 0.88^*$ (4.30)
Nimbecidine Treated(0.27 µg/L)	$297 \pm 2.08^*$ (78.99)	45 ± 2.31	252 ± 4.36 (100)	71.33 ± 4.10 (28.31)	88.33 ± 2.03 (35.05)	37.67 ± 3.38 (14.95)	$48.67 \pm 2.03^*$ (19.31)
Ultineem Treated(0.08 µg/L)	$234 \pm 10.02^*$ (62.23)	53.67 ± 3.29	180 ± 7.86 (100)	55.67 ± 3.38 (30.87)	61.33 ± 1.86 (34)	14.67 ± 1.20 (8.14)	$48.66 \pm 4.37^*$ (26.98)

*Significant ($p < 0.05$) change in the fecundity rate after pesticidal stress as compared to the control mean (100%) when Student's t-test was applied between the control and treated fishes. Figures in parentheses show the per cent values.

Achook, a neem based pesticide to be toxic to zebrafish. With the present study it was concluded that the "safe" neem based products are not so safe to the zebrafish. At higher concentration of pesticides the eggs of *Cyprinus carpio communis* Linn. died before hatching because the pesticide affects the activity of hatching enzymes. Mortality of hatchlings in higher concentrations immediately after hatching indicates that they are more sensitive to pesticides than the embryonic stages (Kaur and Toor, 1977). According to Dave and Xiu (1991) low concentrations of copper (0.25 µg/L), lead (30 µg/L), mercury (0.2 µg/L) and nickel (80 µg/L) can interfere in hatching and survival of the zebrafish. Von Westernhagen's review (1988) cites numerous studies which show that exposure of mature female fish to contaminants may reduce ovary weight and egg size, and increase teratological and pathological effects on larvae. Schmitz et al. (2001) studied the effect of a neem based pesticide, Neemazal on the life cycle of Zebrafish and found a significant reduction in fecundity after exposure of 0.63 mg/L Neemazal. It is evident from present observations that both the pesticides are neem based formulations even then Ultineem is more toxic than Nimbecidine because the reduction in fecundity and hatchability observed is more in Ultineem treated fishes at very low concentration (0.08 µg/L). This may be due to the different adjuvant added to both the pesticides. That means the percent Azadirachtin and the type of adjuvant decides the potency of the pesticide. Both the pesticides are found to cause alteration in reproductive ability of zebrafish at very low concentration. Thus from the above discussion, it is concluded that neem pesticides should be used with caution and in a sustainable way, as it could be hazardous to aquatic biota, domestic animals as well as human beings. Also, extensive researches should be established to provide information for the suitable methods of application of neem based pesticides in aquatic environment to be fully explored in future for its safe use in aquaculture.

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REFERENCES

- Anonymous, 1975.** The EPA and the regulation of pesticides, Staff Report to the subcommittee on administrative practices and procedure on the committee on the judiciary of the US senate. pp. 32-34.
- Ansari, B. A. and Kumar, K. 1982.** A record of Zebra danio (*Brachydanio rerio*) (Cyprinidae) from Uttar Pradesh with notes on sexual dimorphism. *J. Advanced Zoology*. **3**: 88.
- Ansari, B. A. and Kumar, K. 1986.** Malathion Toxicity: Embryotoxicity and Survival of Hatchlings of Zebrafish (*Brachydanio rerio*). *Acta hydrochim. hydrobiol.* **14(6)**: 567-570.
- Ansari, B. A. and Kumar, K. 1988.** Cypermethrin toxicity Effect on the carbohydrate metabolism of the Indian catfish, *Heteropneustes fossilis*. *The Sci. Total Environ.* **72**:161-166.
- Ansari, B. A. and Sharma, D. K. 2009.** Toxic effect of synthetic Pyrethroid Deltamethrin and Neem based formulation Achook on zebrafish, *Danio rerio*. *Trends in Biosciences*. **2(2)**: 18-20.
- Bao, G. G., Wang, M. H., William, L. C., Dao, J. C. and Zheng, J. S. 2007.** Risk assessment of cyhalothrin on aquatic organisms in paddy field in China. *Regulatory Toxicology and Pharmacology*. **48**: 69-74.
- Cakmak, M. N. and Gorgon, A., 2003.** Toxic effect of a synthetic pyrethroid insecticide (Cypermethrin) on blood cells of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *On Line J. Biological Sciences*. **3**: 694-698.
- Dave, G. and Xiu, R., 1991.** Toxicity of mercury, copper, nickel, lead and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. *Arch. Environ. Cotan. Toxicol.* **21**: 126-134.
- Dikshit, T. S. S. and Dutta, K. K. 1973.** Endrin induced cytological changes in albino rats, *Bull. Environm. Cantam. Toxicol.* **9**:65-69.
- Fiuman, L. A. 1993.** Water quality and the early life stages of fishes. *American fisheries society symposium*. **14**: 172.
- Gracia, M., Jones, P. D., Higlly, E. B., Hilscherova, K., Newsted, J. L., Murphy, M. B., Chan, A. K. Y., Zhang, X., Hecker, M., Lam, P. K. S., Wu, R. S. S. and Giesy, J. P. 2008.** Modulation of Steroid genesis by coastal waters and Sewage effluents of Hongkong, China using the H295R, assay. *Environ. Sci. Pollut. R.* **15**: 332-343.
- Harikrishnan, R., Rani, M. N. and Balasundaram, C. 2003.** Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following treatment for *Aeromonas hydrophila* infection. *Aquaculture*. **221**: 41-50.
- Herric, E. E. 1991.** General Principal of toxicology, In: ILEC, Toxic substances management in lakes and reservoirs. *Guidelines of Lake Management*. **4**: 7-27.
- International Organization for Standardization, 1976.** Final (Revised) Proposal for Screening Chemicals and other Products for Acute Toxicity to fresh water fish, document ISO/TC 147/SC. 5/WG. 3 (Secrariat-6), 18 November.
- Islam, B. N. 1984.** Pesticidal action of neem and certain indigenous plants and weeds of Bangladesh, Proc. 2nd Int. Neem Conf. (Rauischolzhausen, 1983), 263-290.
- Isman, M. B., Koul, O., Luczynski, A. and Kaminski, J. 1990.** Insecticidal and antifeedant bioactivities of neem oils and their relationship to Azadirachtin content. *J. Agric. Food Chem.* **38**: 1406-1411.
- Jilani, G. and Su, H.C.F. 1983.** Laboratory studies on several plant materials as insect repellents for protection of cereal grains. *J. Econ. Ent.* **76**:154-157.
- Kashyap, S. K. 1984.** Report of PTI: International workshop on pesticide formulation, held at New Delhi.
- Kaur, K. and Toor, H. S. 1977.** Toxicity of pesticides to embryonic stages of *Cyprinus carpio communis* Linn. *Indian J. Experimental Biology*. **15**: 193-196.
- Kopruca, K., Aydin. 2004.** The toxic effects of pyrethroid deltamethrin on the common carp (*Cyprinus carpio* L.) embryos and larvae, *Pestic. Biochem. Physiol.* **80**:47-53.
- Lanno, R. P. and Dixon, D. G. 1994.** Chronic toxicity of water born thiocyanate to the fathead minnow (*Pimephalas promelas*): a partial life cycle study. *Environ. Toxicol. Chem.* **13(9)**: 1423-1432.
- Organization of Economic Cooperation and Development, 1992.** Guidelines for Testing Chemicals, Guidline 210 "Fish, Early-life Stage Toxicity Test."
- Saxena, R. C., Epino, P. B., Cheng-Wen, T. U. and Puma B. C. 1984.** Neem chinaberry and custard apple Antifeedant and insecticidal effects of seed oils on leaf hopper and plant hopper pests of rice, Proc. 2nd Int. Neem Conf. (Rauischolzhausen, 1983),403-412.
- Schmitz, A., Schafers, C., Trob, R., Ruch, B. and Kleeberg, H. 2001.** Effects of Neemazal on fish: Full life cycle test with the Zebrafish, *Danio rerio*, proceeding of the 2. Workshop "Neem and Pheromones" University of Uberaba, Brazil, May. **15-16**: 36-49.
- Sebire, M., Scott, A. P., Tyler, C. R., Gresswell, J., Hodgson, J. J., Morris, S., Sanders, M. B., Stebbing, P. D. and Katsiadeki, I. 2009.** The organophosphorous pesticides Fenithion acts as an anti-androgen

and alters reproductive behaviour of the male three-spined stickleback *Gasterosteus aculeatus*. *Ecotoxicology*. **18**: 122-133.

Singh, R., Singh, B. and Verma, R. A. 2001. Efficiency of different indigenous plant products as grain protectant against *Callosobruchus chinensis* Linn. on pea. *Indian J. Ent.* **63**:179-181.

Tripathi, A.K., Prajapati, B., Jain, D. C. and Saxena S. 1999. Antifeedant oviposition deterrent and growth inhibitory activity of *Andrographis paniculata* against *Spilaretea oblique*, *Insect Sci. Applic.*

19:211-216.

Tripathi, A. K., Prajapati, V., Agarwal, K. K., Khanuja, S. P. S. and Kumar, S. 2000. Repellency and toxicity of oil from *Artemisia annua* to certain stored product beetles, *J. Econ. Ent.* **93**: 43-47.

Von Westernhagen, H. 1988. Sub-lethal effect of pollutants on the fish egg and larvae. 253-346. In: W. S. Hoar and D. J. Randall (Eds.), *Fish physiology* vol. XI A- The physiology of developing fish. Academic press, San Diego, 546.