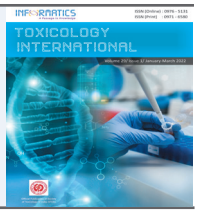
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The Toxicity of Imidacloprid on Early Embryonic Stages and Growth Rate of Hatchlings of Common Carp, *Cyprinus carpio*

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Abstract

In the present investigation, the toxicity of imidacloprid was tested on the embryos (fertilized eggs) and hatchlings of *Cyprinus carpio* in terms of mortality (%), hatching success (%) and abnormal/malformed hatchling along with their growth performance in toxicant medium. Reported data from acute toxicity tests of imidacloprid was statistically analyzed by Probit Analysis Method and 48 h (hour) LC50 was reported as 78 ppm for embryos of *C. carpio.* Three sublethal doses of imidacloprid i.e., 7.8 ppm (T1), 15.6 ppm (T2) and 23.4 ppm (T2), were selected for further experimentation along with control. The findings revealed that mortality of exposed eggs (%) was significantly ($p<0.05$) increased in dose dependent manner as a result hatching rate (%) was decreased as compared to control. Similarly, per cent of abnormal hatchling was also increased significantly ($p<0.05$) with increase in pesticide concentration. Various types of abnormalities has been reported in different body regions such as whole length curved body, stunted growth, hatchling with single eye, malformed head, flexure in the tail and deformed yolk sac etc. Growth rate was observed as length gain in centimeter (cm), weight gain in grams (g) and growth per cent gain in body weight of hatchling, which was also found to be declined significantly ($p<0.05$) with increase in pesticide concentration. The findings of the present investigation suggest that imidacloprid has significant effect on the early developmental stages, induces malformations and also decline the growth rate of hatchlings.

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1. Introduction

Pesticides are chemicals or mixture of chemicals manufactured and deliberately used in agriculture practice to eliminate insect pests, rodents, birds and other organisms that decline agricultural production[1]. During the last few years, large amount of pesticides has been released into the environment to increase the yield of important food crops like maize, wheat and rice by minimizing the loss caused by pests[2]. Unfortunately, large fraction of used pesticides never reaches the targets, but move to nearby lake, pond or river in the form of agricultural runoff along with rain water or some other way, causing high levels of pollution and ecological disturbance[3,4].

It is confirmed from evidences that direct or indirect prolonged exposure of pesticides inside water is harmful for fishes and other aquatic life[5], leading to acute and chronic health problems[6]. Hence, excessive, intensive and inappropriate practice of these chemicals is a matter of great concern. The problem becomes more severe in rainy season because it coincides with the spawning season of fishes and same is the periods of intensive use of pesticide in agricultural field. Inside water bodies, pesticides significantly decline the viability of fish eggs, reduce the hatching success, induce varieties of malformations in hatchlings, altered behaviour, decline growth performance as well as induces haematological and biochemical alterations in exposed fishes[7–9]. Early embryonic stages of fish are highly sensitive to pollutants even at low concentration, therefore, can be considered as ideal pollution indicators and may be used as tool in pesticidal toxicity assessment[10]. Investigations performed in laboratories have confirmed a correlation between toxicant exposure and toxicity related outcomes on early development of fish[11]. Fish embryo toxicity test (FET) is best ways to evaluate the toxicity of pesticides, therefore has been performed on various fish species such as *Cyprinus carpio*, *Paralichthys olivaceus*, *Oncorhynchus mykiss* and *O. latipes*[12–14].

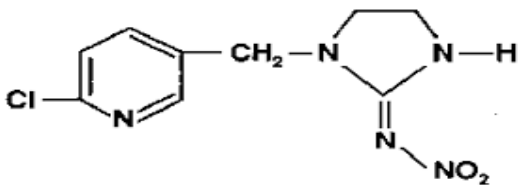
Currently, large varieties of pesticides are used in agricultural practice and imidacloprid is one among them. It is a type of neonicotinoid insecticide used to eradicate insect pest with sucking and piercing mouth parts[15]. Imidacloprid is a systemic, neurotoxic pesticide as it binds and blocks the nicotinic receptor for acetylcholine at post synaptic neural membrane[16]. The highly selective nature of imidacloprid for acetylcholine receptor of insect has encouraged its increased use in agriculture practice, from where it progressively move to surrounding aquatic bodies and persist for long period[17]. At present, imidacloprid is most frequently spotted neonicotinoid insecticide in water samples and various reported concentration are 89 µg/L[18] and 140 ng/L[19].

Fishes are most commonly used aquatic vertebrate as model organisms to assess the aquatic toxicity and related hazards assessment[20]. *Cyprinus carpio* is important freshwater fish species cultured throughout the world. Only few studies are available regarding toxicity of imidacloprid on early developmental stages of fishes, hence, its toxicological impacts still needs exploration. Therefore present study was planned to explore the toxic impacts of imidacloprid on mortality, hatching rate, larval deformities and growth rate of hatchling of *C. carpio*.

2. Material and Methods

2.1 Selection of Test Chemical

For the present investigation, Neonicotinoid insecticide imidacloprid (Maharaja, 17.8 % SL) (Manufactured and



Chemical structure of Imidacloprid[21]

marketed by Gharda Chemicals Ltd. Bandra, Mumbai) was purchased from pesticide's market of Kurukshetra, Haryana.

2.2 Collection of Eggs

Embryos (fertilized eggs) and hatchling of freshwater teleost, *Cyprinus carpio* were used as test organism, because of commonly cultured species, easy to handle and availability in nearby fish farm. The dead or unfertilized eggs become white/opaque due to precipitation and coagulation of proteins. Only fertilized eggs (transparent) were selected and procured from fish seed farm to the laboratory within 15 minutes of fertilization.

2.3 Determination of LC_{50} and Selection of Doses

LC_{50} or LD_{50} is the concentration of toxicant which is responsible for mortality of 50% population of test organisms with in fixed time period. For present investigation, semi static method was adopted to determine 48 h LC_{50} for embryos (fertilized eggs) of *C. carpio*. Initially, the procured eggs were divided into several groups (150 eggs/ group) and exposed to different doses of imidacloprid in the plastic tubs filled with 30 L (liter) dechlorinated water. Starting from 5 ppm/L, the dose of test chemical was increased continuously at interval of 10 ppm/L, to find out the suspected concentration for mortality of 50% of total number of eggs. Mortality in each group was recorded at regular interval of two hours till completion of hatching and data was analyzed by statistic software IBM SPSS. As per probit analysis, reported 48 h LC_{50} of imidacloprid for embryos of *C. carpio* was 78 ppm. For further experimentation, three different sublethal doses of imidacloprid such as 7.8 ppm ($\mathbf{T_1}$= 10% of LC_{50}), 15.6 ppm ($\mathbf{T_2}$= 20% of LC_{50}) and 23.4 ppm ($\mathbf{T_3}$= 30% of LC_{50}) were selected.

2.4 Experimental Set-up

The experiment was conducted according to OECD[22] guideline for testing the toxicity of chemicals on early life stage of fish. Experiment was conducted in two phages i.e., 1. Embryo toxicity test (from eggs to hatchlings) 2. Growth rate test (from hatchling to fingerling stage).

2.4.1 Embryo Toxicity Test

Another set of embryos were procured from fish seed farm and equal number of live eggs (100 eggs/ treatment) exposed separately to above selected doses of imidacloprid. The eggs were released in happa fitted in experimental tubs filled with respected concentrations of test chemical. Water was kept under circulation by using low pressure water pumps and aeration was maintained by aerators. Eggs were regularly monitored to record mortality if occurs, till completion of hatching. Dead eggs become opaque as a result of coagulation or precipitation of proteins. Mortality in each group was counted, recorded and dead eggs were removed immediately at regular interval of two hours.

Newly hatched hatchlings were also counted and hatching rate (%) was calculated for each group separately. Among hatchlings, the deformities or malformations were recorded, examined under light microscope and classified in different categories according to Jezierska and co-workers [23]. Deformed hatchlings were also removed from experimental tubs and photographs were recorded using camera attached light microscope. Abnormal hatchling (%) was calculated with respect to total number of hatchlings reported in each concentration group.

2.4.2 For Growth Rate

The experiment was further maintained for 90 days to evaluate the effect of imidacloprid on growth rate of hatchling. For this, 10 healthy hatchlings were randomly selected from each treatment group and further exposed to similar concentration of test chemical for 90 days. All the experimental groups were maintained in triplicates along with control. Water in all experimental tubs was renewed on alternate days with the water having same concentrations of imidacloprid. Growth was monitored in terms of total length gain (cm), total weight gain (g) and growth per cent gain in body weight (BW), along with survival of fingerlings at the end of 90 days. Simple centimeter scale was used to measure the length of each fish. Weight of individual fish was measured in grams with weighing balance (AFCO SETEX-1200). Growth percentage gain was calculated using standard formulae given by Garg and co-workers[24] i.e.,

$$\text{Growth \% gain in body weight} = \frac{W_2 - W_1}{W_1} \times 100$$

where, W_1= Initial Weight taken as zero (0) and W_2= Final Weight (g) gain by fish at end of experimental period.

2.5 Statistical Analyses

The statistical software IBM SPSS (Version 20 for Window) was used for statistical analyses. Data obtained from imidacloprid toxicity tests were analyzed and significant differences among different treatment groups were evaluated by ANOVA (Analysis of variance) followed by Duncan's multiple range tests. Probability value for statistical significance was settled at $P<0.05$.

3. Results

3.1 Mortality of Eggs (%)

Mortality of eggs (%) exposed to selected concentrations of test chemical has been presented in Table 1, which was found to be increased significantly ($p\leq0.05$) with increase in pesticide dose as compared to control. Total mortality (%) within 48 h of exposure period was reported as 10.00±1.44 in control group, which was significantly increased upto 25.84±0.83 in T_3 treatment group. However, the difference was insignificant among control and T_1 group, significant among T_1 and T_2 group, and again insignificant among T_2 and T_3.

3.2 Hatching Rate (%)

On exposure of imidacloprid, the per cent hatching success was found to be decreased significantly ($p<0.05$) in dose dependent manner (Table 1). Maximum hatching was observed in control group (90.00±1.44), decreased insignificantly in T_1 treatment group (85.83±2.20) and declined significantly in T_2 and T_3 treatments (78.33±1.66 and 74.16±0.83 respectively).

3.3 Abnormal Hatchling (%)

In the present investigation, frequency of malformed hatchlings was increased significantly ($p\leq0.05$) in dose dependent manner (Table 1). Some abnormalities were also reported in control group (3.63±0.81), which was significantly increased as 6.75±0.78, 10.62±0.95 and 19.08±0.92 in T_1, T_2 and T_3 treatment group respectively.

3.4 Types of Abnormalities/Malformations

Present investigation demonstrated that exposed hatchling exhibited a variety of deformities in various body parts. Seven most common types of malformations has been observed among different treatment groups including curved vertebral axis, malformed head, lack of one or both eyes, malformed tail, abnormal yolk sac, stunted growth and immature hatchlings (Figure 1). Spinal and vertebral curvature was the most common type of malformation reported in all the treatment groups. The malformed hatchlings with one or more deformities were died after a short survival period. Hatchlings with greater

Table 1. Effects of imidacloprid on mortality of embryos (%), hatching rate (%) and abnormal hatchlings (%) of *Cyprinus carpio*

Observations	Imidacloprid Concentrations			
	Control	T_1	T_2	T_3
Mortality of Embryos (%)	10.00±1.44 ^{A}	14.17±2.20 ^{A}	21.67±1.66 ^{B}	25.84±0.83 ^{B}
Hatching Rate (%)	90.00±1.44^A	85.83±2.20^A	78.33±1.66^B	74.16±0.83^B
Abnormal Hatchlings (%)	3.63±0.81^A	6.75±0.78^B	10.62±0.95^C	19.08±0.92^D

All values are Mean ± S.E of mean

Values with different capital alphabets superscripts differ significantly ($P<0.05$) with in the same column

(Data were analyzed by Duncan's Multiple Range test)



Figure 1. Imidacloprid induced larval deformities of *C. carpio*. **A.** Hatchling with stunted growth **B.** whole length curved body **C.** hatchling with single eye **D.** stunted growth and coma shaped body **E.** malformed head **F.** oval shaped underdeveloped body, **G.** flexure in the tail region **H.** deformed yolk sac and tail **I.** edema in yolk sac and c shaped body.

body curvature observed at higher concentration of test chemical (T_3), were unable to swim and settled at bottom of tub.

3.5 Growth Rate

The toxic effects of imidacloprid on various growth parameters are summarized in Table 2. The observation clearly revealed significant differences ($P<0.05$) in the growth parameters of pesticide treated hatchlings (C, T_1 and T_2) as compared to control hatchlings. No data (ND) was reported for treatment group T_3 because of 100 per cent hatchling's mortality with in first month of experimentation. Growth parameters like length gain, weight gain and growth per cent gain in body weight

Table 2. Growth performed by hatchlings of *C. carpio* exposed to selected doses of pesticide Imidacloprid

Growth Parameters	Imidacloprid Treatments			
	Control (C)	T_1	T_2	T3
Length gain (cm)	6.13±0.07^A	5.12±0.06^B	4.01±0.01^C	ND
Weight gain (g)	1.75±0.03^A	1.42±0.017^B	0.87±0.01^C	ND
Growth per cent gain in BW	175.66±3.48^A	142.91±1.68^B	87.37±1.48^C	ND
Survival rate (%)	86.66±3.33^A	56.67±3.33^B	46.66±1.67^B	ND

All values are Mean ± S.E of mean

Values with different capital alphabets superscripts differ significantly (P<0.05) with in the same column

Data were analyzed by Duncan's Multiple Range test.

ND: No data, because of 100% mortality with in 1st month of observation.

(BW), along with survival rate were significantly (P<0.05) high in control group.

3.5.1 Length Gain (cm)

After 90 day's exposure duration, maximum length gain (cm) was reported in hatchling of control group (6.13±0.07), which was decreased in dose dependent manner upto 5.12±0.06 in T_1 and 4.01±0.01 in T_2 treatment groups (Table 2). Findings clearly indicates that length gain was significantly (P<0.05) different in pesticide treatment groups as compared to control.

3.5.2 Weight Gain (g)

Similar to length gain, weight gain by pesticide exposed hatchlings was also decreased significantly (P<0.05) with increase in pesticide concentration (Table 2). At the end of experimentation, weight gain (g) by hatchlings was again maximum in control group i.e 1.75±0.03 g, which was decreased as 1.42±0.017 g and 0.87±0.01 g in T_1 and T_2 groups respectively.

3.5.3 Growth Per cent Gain in Body Weight (BW)

The exposed hatchling also exhibited significant (P<0.05) decline in growth per cent gain in body as compared to control. The reported growth per cent gain in body weight was 175.66±3.48, 142.91±1.68 and 87.37±1.48 per cent for control, T_1 and T_2 treatment groups respectively (Table 2).

3.5.4 Survival Rate (%)

After trial period of 90 days, reported survival rate was 86.66±3.33 per cent in control group whereas, it was 56.67±3.33 per cent and 46.66±1.67 per cent in T_1 and T_2 groups respectively (Table 2). The survival rate was significantly (P<0.05) higher in control group as compared to treated groups. No survival per cent was reported for T_3 treatment because of 100 per cent mortality due to continuous exposure of pesticide from initial stage (fertilized eggs).

4. Discussion

Toxicity is the innate property of toxicants to kill or adversely affect physical or physiological process of any organism. Acute toxicity of pesticides is generally evaluated and expressed in the form of LC_{50} and is the best way for toxicity assessment[25]. Toxicants having higher LC_{50} count are generally less toxic because high dose is required to cause mortality of 50% population of model organisms[26].

In the present investigation, semi static method was used to assess acute toxicity of imidacloprid on fertilized

eggs of *C. carpio* and 48 h LC_{50} was found to be 78 ppm, indicating moderate toxic nature of imidacloprid. Similarly, Islam *et al.*[27] also conducted acute toxicity test of imidacloprid on embryo of common carp and reported 48 h LC_{50} was 611 µg/L. In addition, Koprucu and Aydın[28] recorded 48 h LC_{50} of deltamethrin for embryo of common carp as 0.213 μgL^{-1}. After that, Aydın and Koprucu[28] also conducted acute toxicity test of diazinon on embryo of common carp and 48 h LC_{50} was found to be 0.999 mgL^{-1}. It is clear from the above observations that imidacloprid is moderately toxic for fish embryo as compared to other pesticides because high count of LC_{50} value.

Our observation also includes a significant ($p<0.05$) increase in mortality of eggs (%) and decrease in hatching per cent with increase in imidacloprid concentration. Recently, Islam *et al.*[27] also reported significant decline in hatching per cent of carp embryo exposed to imidacloprid. The reported hatching success was 81% in control group, which was significantly, declined upto 29% when embryos were exposed to 1000 µg/L of imidacloprid. Our observations also supported by the finding of Aydın and Koprucu[28], who reported significant increased mortality and decreased hatching success of carp embryos (*C. carpio*) exposed to various concentration of diazinon. Similarly, significant decline in hatching success has been reported previously in carp embryos exposed to cypermethrin and deltamethrin[13,29]. Increased mortality of exposed embryos indicates that chorion is not effective barrier against toxicants as lipophilic nature helps toxicants to penetrate the chorion and cause toxicity to embryos[30,31]. Present findings also suggest that chorion is inefficient barrier as it allows imidacloprid to penetrate.

The decreased hatching rate of pesticide exposed embryos may be a result of toxicant induced disruption in the activity of hatching enzymes protease and chorionase[32]. However, neurotoxic pesticides decrease the hatching success by blocking the receptors for acetylcholine, required for normal developmental and hatching process[16]. According to Ansari *et al.*[33], most of pesticides inhibit neural transmission and also disturb the standard metabolic processes of exposed embryo resulting in their increased mortality and decreased hatching rate. Evidence also confirms that pesticides induce embryo toxicity by acting at cellular level, interact with DNA and alter the synthesis of important protein vitellogenin, required for embryonic development of fish[34,35].

In present investigation, frequency of abnormal hatchlings (%) was significantly high in imidacloprid treated groups as compared to control. The reported malformations includes, hatchling with stunted growth, curved body, hatchling with single eye, coma shaped body, malformed head, oval shaped underdeveloped body, flexure in tail region and deformed yolk sac. In the same direction, Islam *et al.*[27] also observed a number of malformations including deformed notochord, deformed caudal fin, lordosis and pericardial edema in hatchlings of common carp exposed to 300 µg/L and 1000 µg/L of imidacloprid. Recently, Velisek and Stara[36] also reported similar abnormalities such as spinal curvature, deformed yolk-sac and pericardial edema in the hatchlings of *C. carpio* exposed to neonicotinoid pesticide thiacloprid. Our findings are in accordance with the earlier observations Kanazawa[37] who reported significant increase in malformed embryos as well as hatchlings of fish *Lepomis macrochirus* and *O. mykiss* due to toxicity of pesticide diazinon. Pesticide induces teratogenic effects on embryos and larvae of fish resulting into body malformations like edema in pericardial and yolk sac, deformed tail and curved notochord[38]. The possibility behind deformed spinal and notochord is neurotoxic nature of pesticides which blocks the neural transmission at neuro-muscular junction and disrupt the coordination which decline the collagens content in the notochord and spinal column[39]. Similar deformities have been previously reported in hatchlings zebra fish exposed to pesticide atrazine, lindane and deltamethrin[40], in embryos of *O. niloticus* exposed to organophosphate trichlorfon[41], in larvae of pink snapper (*P. auratus*) treated with diuron[42] and hatchlings of *Danio rerio* exposed to pesticide chlorpyrifos[43].

In the 2nd phase of investigation, it has been reported that pesticide imidacloprid has significant adverse effect on the growth performance of hatchlings. Highest growth rate was recorded in hatchlings of control group, while it was found to be decreased on prolonged pesticide exposure in dose dependent manner. The study clearly revealed that weight gain and length gain were declined significantly ($P<0.05$) in treated hatchlings as compared to control hatchlings. Our observations are supported by earlier findings of Ponmani and Logaswamy[44], who also reported reduced feeding activity and growth performance of *C. carpio* exposed to sublethal doses

of monocrotophos. In our previous findings various histopathological and behavioural alterations has been reported in the fingerlings of *C. carpio* exposed to the sublethal concentrations of imidacloprid[8,45]. Similar, Velisek and Stara[36] also observed declined growth rate of common carp exposed to neonicotinoid pesticide thiacloprid, which could be a result of increased utilization of fat and stored protein for extra energy required for increased physical and physiological activities of fish under stress condition.

Our observations also include significant decrease in survival rate of hatchlings in treated groups as compared to control. All the hatchlings exposed to highest concentration of imidacloprid (T_3 group) die with in 1st month of exposure due to continuous exposure of pesticide from initial stage (eggs). Our findings are further supported by Sweilum[46], who also observed significant declined feed utilization, growth performance and survival rate of fish (Tilapia) exposed to pesticides. The declined performance in toxic medium may be a result of poor food conversion efficiency, increased protein utilization and reduced deposition of proteins as well as lipid in the fish muscles[47]. Similar results have been obtained in the growth rate of fingerlings of catfish *C. gariepinus* on exposure to pesticides[48]. Toxicants induce oxidative stress and anemia, which could be one of major factor responsible for reduced growth rate as well as reduced carcass composition of lipids and protein[49]. Pesticide induced hormonal disruption (declined level of thyroxin, insulin like growth factor-I (IGF-I), growth hormone, estradiol-17β and testosterone), followed by decreased growth rate of *Barbus stigma* exposed to malathion has been reported by Lal *et al.*[50].

Most of pesticides decrease the feeding activity by suppressing the appetite as a result burning of storage nutrients (fat and proteins) provides required energy, leading to reduce growth rate and survival of fish in the natural habitat[51]. Probiotic bacteria present in intestine act as exogenous source of enzymes and help in digestion, decreased population of these bacteria in toxic conditions might a cause of decreased digestibility and also growth performance of fish[52]. According to Ganeshwade[53], decreased growth performance of pesticide exposed fish might be a result of histopathological alterations in the digestive tract caused by pesticide. Poor food quality is another possible cause of declined growth rate. Although same feed at same ratio was provided to all the fish during the investigation, still the reported growth performance of treated fishes was low, that all could be a result of imidacloprid toxicity. Thus, our findings suggest that increased fish mortality, malformed hatchlings and declined growth rate of fish in wild might be outcome of repeated and overuse of pesticides including imidacloprid.

5. Conclusion

It is concluded that pesticide imidacloprid has significant effect on mortality of eggs, hatching rate and also induces a variety of malformations in exposed hatchling. Declined growth rate is another issue related to health of fish, indication the toxicity of used pesticides on non-target organisms. Thus over and repeated use of pesticides is matter of great concern; hence we should stop or minimize the use of these pesticides immediately and try to adopt some other eco-friendly method of pest control like IPM (Integrated Pest Management).

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