Toxicology International, *Vol 27(3&4), 158-167, 2020, DOI: 10.18311/ti/2020/v27i3&4/25707*

Original Research
ISSN (Print): 0971-6580
ISSN (Online): 0976-5131

Imidacloprid Induced Alterations in Behavioral and Locomotory Activity of Fingerlings of Common Carp, *Cyprinus carpio*

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Abstract

Pesticide imidacloprid is extensively used in agriculture field to kill variety of insect pests, from where it enters into surrounding aquatic ecosystem and results into toxicity related outcomes on non-target species. The present study was planned to evaluate the sublethal toxic effects of pesticide imidacloprid on behavior and locomotory activity of fingerlings of *Cyprinus carpio.* Initially, the LC_{50} (48 hrs) was calculated for fertilized eggs of *C. carpio* by probit analysis and was reported as 78 ppm. Further, the fingerlings of *C. carpio* were exposed to three different sublethal concentrations i.e. 7.8 ppm, 15.6 ppm and 23.4 ppm of imidacloprid along with control for 21 days and behavior of fish was supervised at regular intervals on days 2, 4, 7, 15 and 21. The control fish behaved normally but treated fishes demonstrated abnormal behavior like hyper excites, restless, loss of equilibrium, hyper activity, erratic swimming with jerky movements, excess mucus secretion, reduced skin pigmentation and loss of response to touch. Significant disruptions were also recorded in operculum beat frequency, surfacing movement and tail beat frequencies. All these outcomes are due to neurotoxic nature of imidacloprid as it blocks the receptor for neurotransmitter acetylcholine.

Keywords: Behavior, *Cyprinus carpio*, Imidacloprid, Toxicity

1. Introduction

Agricultural run-off includes harmful pesticides and other toxicants, which enters into surrounding water body and causes serious hazards to the aquatic biota[1-2]. Facts are confirming that incessant exposure of pesticides is not only harmful for aquatic organisms but also causes acute and chronic health problems in terrestrial organisms[3-5]. So there is an urgent need to assess the toxicity related responses of exposed organisms. There are broad range of responses of aquatic life depending on type of toxicant, exposure duration, water quality and the species[6]. Altered behavior is a most common response of stressed organism and such studies are playing important role in toxicity assessments in fishes and other organisms[7].

Fish being at top level of aquatic food chain (tertiary consumer), bio-magnification of pesticides occur which results into altered behavior and physiology of fishes[8]. The prime cause of behavioral disruption includes toxicant induced inhibition of cholinesterase, disruption of neural networks by altered neurotransmitter levels, sensory deprivation or impaired hormonal level[9-10]. In previous findings, behavioral alterations in pesticide exposed fish includes preference/avoidance, restlessness, social hierarchies, body tremors, less pigmentation, altered operculum movement, frequent surface movement, hanging vertical, tail beat frequency and excess mucous secretion[11-12]. Studies concerning the toxicity related behavioral alterations on the fish are lacking, due to difficult methodology but development of computer based softwares has made it possible[13].

Neonicotinoids are broad-spectrum systemic and synthetic insecticide used in agriculture fields to control variety of insect pests in various crops[14]. Insecticide imidacloprid is one among neonicotinoids and is most recommended insecticide all over world[15]. It is systemic and neurotoxic insecticide binds with acetylcholine's receptor in nervous system and blocks it irreversibly[16].

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Effectiveness of imidacloprid is more for those insects having piercing and sucking types of mouth parts and is effectively used in almost all crops like rice, maize, cotton, vegetables, sugarbeet, cereals, potato, citrus and other fruits[17-18] from where it gradually reach to nearby aquatic body via agricultural runoff and remain active for long time[19-20].

Cyprinus carpio is one of the important fresh water food fish of great economic importance being cultured all over India. Limited information is available regarding toxicity of imidacloprid for fishes, indicating that it is acutely toxic to mature fishes and also reduce the survival rate of embryos and larvae of rainbow trout as well as *C. carpio*[21-22]. In the present study attempts have been made to study the effects of insecticide imidacloprid on behavioral and locomotory aspects of *C. carpio*.

2. Material and Methods

2.1 Selection and Acclimatization of Fish

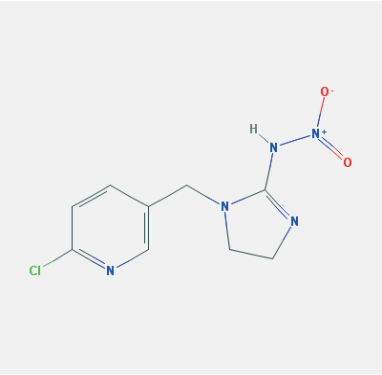
For the present investigation, fingerlings of *C. carpio* (10 ± 1 gm body weight) procured from National Fish Seed Farm, Kurukshetra and used as model organism. The fingerlings were bathed with solution of KMnO4 (0.05%) to evade dermal infection and acclimatized in dechlorinated water for three weeks in plastic tubs of 50 L capacity under laboratory conditions at Department of Zoology, Kurukshetra University, Kurukshetra. The experiment was conducted at 28 ± 1°C of water temperature under standard lighting schedule (12 hrs light: 12 hrs darkness). Throughout experimentation, fingerlings were provided a self-prepared diet (Table 1) having high protein content (40%) @ 4% BW/day at regular interval of 08:00 and 16:30 hrs.

Table 1. Ingredients of composition of dietary the feedous groups was analyzed by unpaired two-tailed students t-test and statistically significant when p< 0.05.

Ingredients	Quantity in grams/Kg
Groundnut oil cake	650.0
Rice bran	42.0
Processed soybean	276.0
Wheat flour	32.0
Mineral Mixture	10

2.2 Pesticide Used

Insecticide imidacloprid (17.8% SL) made by Gharda Chemicals Ltd., Bandra, Mumbai was purchased from the local market and used as test chemical for toxicity evaluation.



Molecular structure of Imidacloprid
(https://pubchem.ncbi.nlm.nih.gov/)

2.3 Determination of LC_{50}

The 48 hrs LC_{50} or LD_{50} was calculated using semi-static method for fertilized eggs of *C. carpio,* which were procured from National Fish Seed Farm, Kurukshetra. For this, fertilized eggs were exposed to increasing concentrations of imidacloprid with starting dose of 5 ppm. Further, dose of imidacloprid was increased continuously upto the concentration responsible for mortality of 50% of total eggs. Live fertilized eggs remain transparent and dead eggs become white due to coagulation and precipitation of proteins. Mortality of eggs was recorded by counting the dead eggs at each dose and data was evaluated for probit analysis using computerized software IBM SPSS Statics Ver. 20. The recorded LC_{50} of imidacloprid was 78 ppm for fertilized eggs of *C. carpio*. For further experimentation, three different sublethal doses of test chemical i.e 7.8 ppm coded as T_1, 15.6 ppm coded as T_2 and 23.4 ppm coded as T_3 (10%, 20% and 30% of LC_{50} respectively), were selected for embryo toxicity test as well as behavioral study.

2.4 Experimental Set-up

The acclimatized fingerlings were exposed to above selected concentrations of imidacloprid in glass aquarium having 15 L dechlorinated water. Low pressure aerators were used to maintain oxygen level. The experiment was conducted in triplicates along with control and alterations in behavior and locomotory activity of fingerlings were monitored on day 2, 4, 7, 15 and 21 by following the standard method as described by Rao *et al.*[13]. On day of response recording, one fingerling from each treatment

group was taken and released in aquaria (15×15×15 cm) having respective concentration of imidacloprid. Before video recording each fingerling was acclimatized for ten minutes and behavior was recorded for 5 min with a HD CCD camera (HDR-XR550E, SONY Handycam) fixed at leftover plain side, 25 cm away from recording aquarium. Three sides and bottom of aquarium were made opaque by placing white thermocol sheet to avoid visual disturbances and mirror image of fish. A minimum of 5 fishes from each concentration were selected individually for evaluation of altered behavior. Inside recording aquaria, all activities of fingerlings were observed carefully and video recordings were saved for further analysis of altered locomotory behavior of exposed fish. Alterations in behavior of control and imidacloprid treated fingerlings (T_1-T_3) were recorded and evaluated in terms of their general behavior and specific behavior.

2.5 General Behavioral Study

General fish behavior was observed in terms of consciousness, feeding activity, swimming pattern, mucus secretion, schooling behavior, shifting towards bottom or upper surface of aquaria, abnormal hanging pattern, skin pigmentation, response to stimulus, jumping behavior and wide opening of the mouth, redness in body parts and mortality if occur in any group.

2.6 Specific Behavior

Specific behavior such as Operculum Beat Frequency (OBF), Surface Movement (SM) and Tail Beat Frequencies (TBF) per minute was recorded in present study.

2.6.1 Operculum Beat Frequency (OBF) (min^{-1})

It was recorded in term of total number of movement or beats performed by operculum in one mint. For this, video recordings of three fish form each concentration were reviewed many times and OBF was counted as number of operculum beats performed in one mint.

2.6.2 Surfacing Movement (min^{-1})

Fishes escapes themselves from direct contact of toxicant present in water by coming to the uppermost water layer which is known as surface movement. Like OBF, surface movement was recorded in term of number of movement made by fish to upper surface in one mint.

2.6.3 Tail Beat Frequency (min^{-1})

It is a way to measure swimming performance of fish as it help to avoid predators, to gain food and escape from toxic medium. Tail beat frequency was recorded in term of number of tail beats or movement done by fish in one mint.

2.7 Water Quality Characteristics

Water samples from all the experimental aquaria, were collected at regular interval and analyzed for temperature, Dissolved Oxygen (DO) and pH following the standard procedure described in APHA[23], Temperature was maintained around 28 ± 1^{0}C, pH was 7 to 7.5 and dissolved oxygen level was ranged between 6 to 6.15 mg/l in all experimental groups.

2.8 Statistical Analysis

Significant differences among treatment and control group were tested by Analysis of Variance (ANOVA) and Duncan's multiple range tests. Statistical significance was established at probability of $P<0.05$ and statistics was performed using computerized software (IBM SPSS Statics-20 for Windows).

3. Result

3.1 General Behaviour

3.1.1 General Behaviour of Control Fish

Fingerlings maintained in control group behaved in usual manner covering almost entire water tank. These fingerlings were fully conscious, aware even at least disturbance, well active with controlled and coordinated movement. They were actively feeding, showing normal operculum movement without any extra ordinary activities. All these activities remained same in the control groups throughout experimental period, therefore, are taken as standard for the entire study.

3.1.2 General Behaviour of Exposed Fish

Imidacloprid induced disrupted behavior was recorded in the fishes exposed to different sublethal concentrations. The exposed fishes were hyper activity, displaced erratic swimming, jerky movements and even loss of equilibrium from the very first day of pesticide exposure. Hyper excites, restless and excess mucus secretion was commonly reported in all treatment groups. Increased operculum activity and wide mouth opening for excess air intake was also observed in fishes exposed to higher concentration. Disrupted school behavior, shifting towards bottom of aquaria and independent swimming pattern was also exhibited by exposed fingerlings. Significant decline in feeding activity (loss of appetite) was also reported with increase in exposure duration in treated fishes resulting in slow growth rate and reduced fitness. Exposed fishes also displaced irregular hanging vertically, spending time at the

bottom and loss of response to touch and sound from mid of experimentation. Exposed fingerlings also demonstrated oblique side walk and tried to jump out from pesticide treated aquaria, commonly known as escaping behavior to avoid the touch with toxic medium.

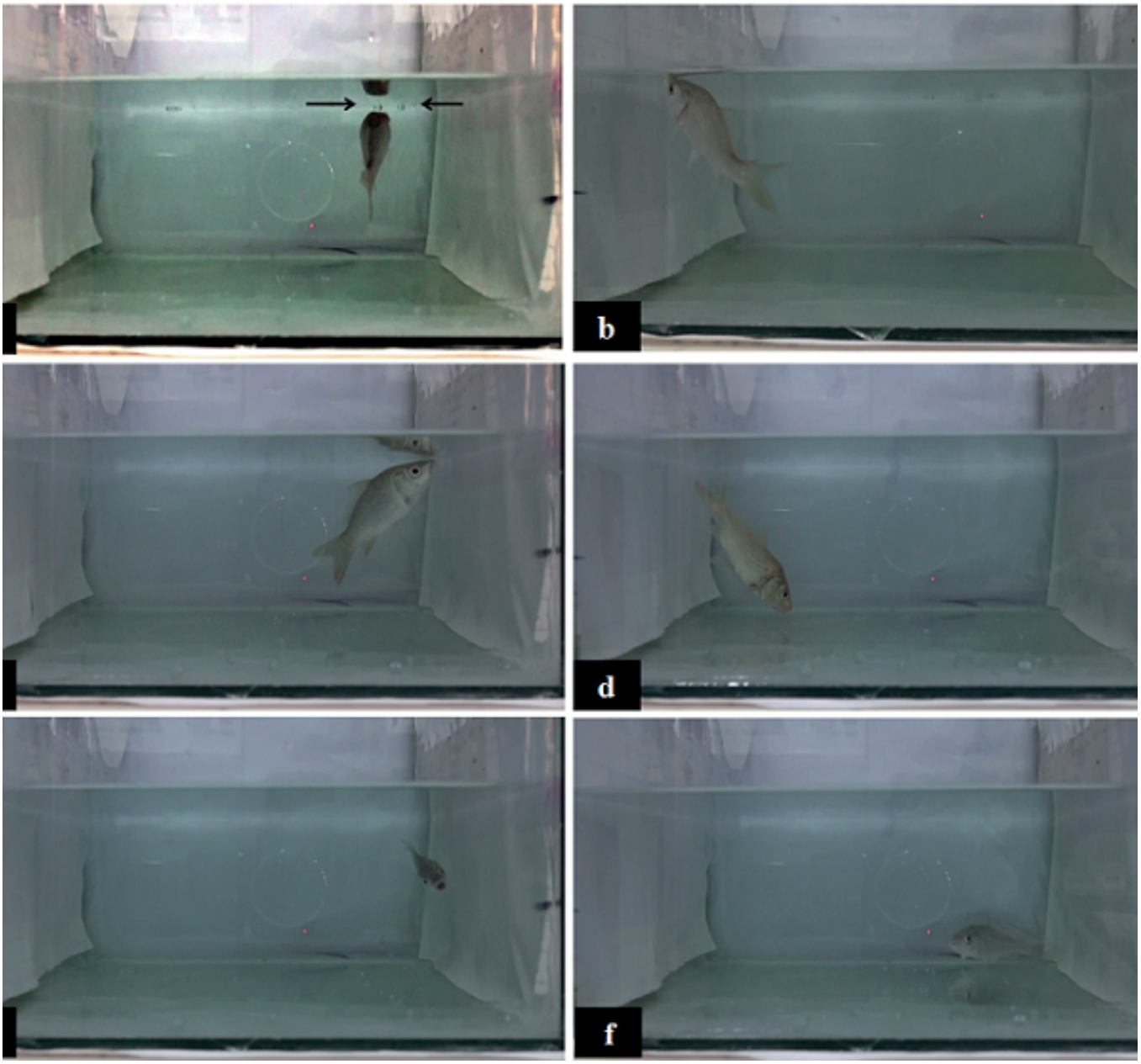


Figure 1. Imidacloprid induced alterations in general behaviour of *C. carpio.* (a) Bubble generated on gulping of mouth, (b-c) Vertically upward hanging, (d) Vertically downward hanging, (e) Oblique side walk, (f) shift to bottom.

Respiratory distraction in the form of wide opening of the mouth was mostly observed at high concentration (T_2 and T_3 group). Redness in certain region due to blood coagulation was also seen in some fishes after one week

exposure to pesticide. At the end of experiment, mobility of some of the exposed fish was decreased greatly, fish sunk to the bottom with very least movements and finally died with open mouth. However, the mortality was recorded only in T_3 treatment group. Abnormal locomotory activities such as hyper excites, restless and excess mucus secretion were not much common in fingerlings exposed to lowest concentration of imidacloprid (T_1) and found to be increased in dose dependent manner, which is supposed to be a result of stress induced by neurotoxic nature of imidacloprid.

3.2 Specific Behaviour

Specific behaviour was recorded in the form of opercular beat frequency, surface movement and Tail Beat Frequency (TBF). In present study, it is observed that imidacloprid has significant (P<0.05) effect on frequency of specific behavioral responses of *C. carpio*, which has been presented in Tables 2, 3 and 4. During early days of exposure, all the treated groups have higher frequency of alterations in specific behavioral parameters as compared to control. The frequencies of specific behavioral parameters were significantly (P<0.05) high during 1st day of exposure and later on decreased significantly (P<0.05) with duration of exposure.

3.2.1 Operculum Beat Frequency (OBF) (min^{-1})

Difference in operculum beat frequency (min^{-1}) of fingerlings exposed to imidacloprid has been presented in Table 2. Operculum Beat Frequency of control group was almost same throughout the experimentation, however it was recorded significantly (P<0.05) high during 7th and 15th day within treatment. However, OBF was decreased significantly (P<0.05) in treatment groups T_1 and T_3 from days 1st to 21st, except in T_2 treatment group where it was increased significantly (P<0.05) upto day 7th and decreased later on. Day wise comparison (with in row) between opercular beat frequency of difference concentration (along row) also showed significant (P<0.05) difference which was increased upto day 4th and decreased later on.

3.2.2 Surfacing Movement (min^{-1})

In order to avoid the contact with toxic medium, the impulse for coming up to surface recorded in almost all the experimental groups and is presented in Table 3. The reported frequency of surfacing movement recorded significantly (P<0.05) high in pesticide treated fingerlings as compared to control in does dependent manner (with in row). However, with increase in exposure duration, the surfacing movement was increased initially upto 15th day and remained almost constant later on. Maximum surfacing activity was recorded on day 15th in T_1 treatment group. Significant (P<0.05) difference with in the same concentration was also reported in time dependent manner except for control group.

Table 2. Operculum Beat Frequency (min^{-1}) of *C. carpio* exposed to 7.8 ppm (T_1), 15.6 ppm (T_2) and 23.4 ppm (T_3) concentrations of imidacloprid along with control

Exposure Duration	Treatments			
	Control	T_1	T_2	T_3
Day 2	89.00 ± 0.57^{Cb}	91.00 ± 0.57^{Ca}	100.00 ± 0.57^{Bc}	100.33 ± 0.88^{Ba}
Day 4	89.00 ± 0.57^{Db}	87.33 ± 0.33^{Db}	104.67 ± 1.45^{Bb}	94.00 ± 0.57^{Cb}
Day 7	91.67 ± 0.33^{Ba}	79.00 ± 0.57^{Dd}	112.00 ± 1.15^{Aa}	86.66 ± 0.88^{Cc}
Day 15	90.33 ± 0.33^{Aa}	81.67 ± 0.88^{Bc}	$79.00 \pm 0.57C^{d}$	77.66 ± 0.33^{Cd}
Day 21	89.67 ± 0.88^{Ab}	73.33 ± 0.88^{Be}	52.00 ± 1.15^{Ce}	53.33 ± 0.88^{Ce}

Values having different capital alphabets superscripts significantly (P<0.05) differ within the row.
Values with different small alphabet superscripts significantly (P<0.05) differ within the column.

Table 3. Surface activities (min^{-1}) of *C. carpio* exposed to 7.8 ppm (T_1), 15.6 ppm (T_2) and 23.4 ppm (T_3) concentrations of imidacloprid along with control

Exposure Duration	Treatments			
	Control	T_1	T_2	T_3
Day 2	0.33 ± 0.33^{Ca}	2.67 ± 0.33^{Bc}	0.33 ± 0.33^{Cc}	3.67 ± 0.33^{Bb}
Day 4	0.33 ± 0.33^{Da}	1.00 ± 0.00^{Dd}	2.67 ± 0.33^{Cb}	5.33 ± 0.33^{Ba}
Day 7	1.00 ± 0.00^{Da}	5.67 ± 0.33^{Bb}	3.33 ± 0.33^{Cb}	5.67 ± 0.33^{Ba}
Day 15	0.66 ± 0.33^{Ca}	8.66 ± 0.66^{Aa}	6.00 ± 0.57^{Ba}	5.00 ± 0.57^{Bab}
Day 21	0.33 ± 0.33^{Ca}	6.00 ± 0.57^{Ab}	6.33 ± 0.33^{Aa}	5.00 ± 0.57^{Aab}

Values having different capital alphabets superscripts significantly (P<0.05) differ within the row.
Values with different small alphabet superscripts significantly (P<0.05) differ within the column

3.3.3 Tail Beat Frequency (TBF) (min^{-1})

Variations in Tail Beat Frequency (min^{-1}) are presented in Table 4. TBF values showed significant increasing trend during initial days, with increase in imidacloprid concentration from control to T_3 treatment group (with in row), however decreased significantly (P<0.05) from day 7^{th} onward. Within control group, no significant variations were recorded in TBF count at different days, however with in same concentration (T_1, T_2 and T_3 groups), the values increased significantly (P<0.05) from day 2^{nd} to 4^{th} and thereafter decreased significantly (P<0.05) from day 7^{th} to 21^{st}.

Table 4. Tail Beat Frequency (min^{-1}) of *C. carpio* exposed to 7.8 ppm (T_1), 15.6 ppm (T_2) and 23.4 ppm (T_3) concentrations of imidacloprid along with control

Exposure Duration	Treatments			
	Control	T_1	T_2	T_3
Day 2	59.33 ± 0.33^{Ba}	61.33 ± 0.66^{ABb}	61.33 ± 0.66^{ABb}	63.33 ± 0.66^{Ab}
Day 4	59.66 ± 0.33^{Ca}	63.33 ± 0.33^{Ba}	66.66 ± 0.33^{Aa}	66.66 ± 0.33^{Aa}
Day 7	59.33 ± 0.33^{Ba}	56.00 ± 0.57^{Cc}	67.00 ± 0.57^{Aa}	48.33 ± 0.88^{Dc}
Day 15	60.33 ± 0.33^{Aa}	51.33 ± 0.66^{Bd}	49.33 ± 0.33^{Bc}	41.66 ± 0.88^{Cd}
Day 21	59.66 ± 0.33^{Aa}	52.66 ± 0.33^{Bd}	47.00 ± 0.57^{Cd}	33.66 ± 0.88^{De}

Values having different capital alphabets superscripts significantly (P<0.05) differ within the row.
Values with different small alphabet superscripts significantly (P<0.05) differ within the column

4. Discussion

Behavioral studies are becoming promising tool in toxicity assessments on fishes because of such alterations are easily detectable as compared to complicated neural process behind them[24]. Similar to most of pesticides imidacloprid induce behavioral alterations in exposed fish by disrupting their nervous system as it binds irreversibly with acetylcholine's receptor at postsynaptic neural membranes and disrupt the normal signaling mechanism[25]. In present investigation acute toxicity (48 hrs LC_{50}) of imidacloprid was recorded as 78 ppm for fertilized eggs of *C. carpio*, indicating its moderate toxic nature for fishes. Still, in present investigation, exposure of various sublethal concentrations of imidacloprid induced significant alterations in the locomotory and behavioral response of *C. carpio*.

The fingerlings maintained in control group were active, fully conscious and exhibited well controlled activities and even alert at least disturbance. However, exposed fingerlings exhibited abnormal behaviour like erratic and jerky movements, restless, loss of equilibrium, hyper excitations, excess mucus secretion, increased operculum movement and gulping wide mouth for air. Excess mucus secretion over body is a defensive mechanism as mucus forms a barrier between skin and toxic media reducing the chances of contact, thus minimizes the irritating effects[26-27]. Excess mucous secretion is a result of pesticide induced dysfunction of pituitary gland leading to alterations in number and area of mucous glands[28]. According to Dutta and Chaudhary,[29] gills can metabolize and eliminate toxic compounds through heavy elution of mucous thus is a defensive measure against toxicant. But heavy elution of mucous is problematic as mucus is impermeable for oxygen, creates disturbance in gas exchange and considerably decline the rate of oxygen uptake leading to hypoxia[30].

During the present observation, fingerlings exposed to higher concentration (T_2 and T_3) of imidacloprid were found struggling for breathing, frequently moved to surface and demonstrated jumping behaviour to escape from toxic medium. Frequent surface movement, increased operculum beat and gulping wide mouth during the exposure period indicates a shift towards aerial respiration to full fill the demand of increased oxygen level[27-31]. Omitoyin *et al.*[32] also concluded that pesticide induced respiratory impairment is a result of alterations in respiratory organs. Increased operculum movements also minimize the chances of pesticide absorption through gills[33]. Observations recorded in present study are well supported by the findings of Lata *et al.*[34] who concluded that increased opercular activity is a response against toxicant shock received by fish or a sensational stimulus against hypoxia in order to ventilation of gills. Other possibility of increased opercular movement is to remove toxicant debris and deposited mucus from the gill region to maintain proper breathing process[35]. Lokhande[36] also found altered rate of oxygen consumption by *Rasbora daniconius* exposed to various doses of dimethoate.

In present observation, mortality was reported only at highest concentration (T_3) of imidacloprid, which could be a result of direct toxicity of toxicant or indirect by making medium unconductive. Similar type of mortality has been also reported by Ayoola and Ajani[37] at sublethal concentration of cypermethrin on juvenile catfish, *Clarias gariepinus*. Convulsions before the death of fish illustrate the signs of asphyxiation under toxic medium. Similar observations have been previously made on fish *Sarotherodon mossambicus* exposed to dimethoate[38]. Likewise, Kumar *et al.*[1] observed irritability, hyper-excitation subsequently mucus secretion and unbalancing in catfish, *Clarias Batrachus* exposed to copper sulphate. The hyper-excitability and unbalancing of stressed fish might be a result of altered functioning of enzyme acetylcholinesterase and accumulation of acetylcholine at synaptic region in nervous system[39]. In addition, pesticidal induces severe damage to peripheral sense organs of exposed fish, resulting in delayed response to mechanical disturbance[40]. Malathion induced behavioral alterations like irregular, darting swimming movements, erratic and loss of equilibrium has been previously reported in *Labeo rohita* which could be a result of inhibition of acetylcholinesterase enzyme[27]. Recently, Mintram *et al.*[41-42] observed that most of toxicants induce behavioral alterations in exposed fish by disrupting their endocrine system.

The finding of previous reports indicates that pesticide induces behavioral alterations like impaired swimming and spatial discrimination both in hatchlings as well as adult fish[43-44]. Observations recorded in present investigation has been well supported by the findings of Rao *et al.*[13] on mosquito fish, *Gambusia affinis* exposed to chlorpyrifos and reported behavioral alterations like loss of equilibrium, erratic swimming and jerky movements, excess mucous secretion and hyperactivity.

Earlier findings also indicate, the food deprivation, anaerobic conditions and inhibition of neural control are also responsible for disrupted behavioral and locomotor activity of organisms[45-46]. However, in the present experimentation, the exposed fishes were not facing any of

conditions like starvation or oxygen deficiency; therefore, overall impairments in behavioral response of exposed fingerlings were only due to toxicity of imidacloprid. As earlier described,[16] imidacloprid irreversibly binds and blocks the receptor for neurotransmitter acetylcholine, interrupt the control and coordination at neuro-muscular junction resulting in the behavioral disruption of exposed fish. So there is urgent need to decline the use of imidacloprid and other pesticides for the safety of non-targeted organisms.

5. Conclusion

It is concluded that all the reported behavioral alterations in exposed fingerlings are only because of toxicity of imidacloprid even at sublethal concentration (7.8 ppm), indicates its toxicity to fishes at very low concentration. Further, behavioral alterations may provide early warning signals for determination of toxicity of pesticides.

6. Acknowledgement

Authors are highly thankful to Kurukshetra University, Kurukshetra for providing the laboratory and other necessary facilities to conduct this part of research work. The authors are also thankful to UGC-SAP, New Delhi for providing the financial assistance.

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