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# EVALUATION OF AN ACUTE ORAL GAVAGE METHOD FOR ASSESSMENT OF IMIDACLOPRID TOXICITY IN TERRESTRIAL AMPHIBIAN *HOPLOBATRACHUS TIGERINUS*

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## ABSTRACT

Development of an acute oral toxicity test with a terrestrial-phase amphibian was considered necessary to remove the uncertainty within the field of agrochemical risk assessments. The present study intended to help fill the gap on the scarcity of information concerning the imidacloprid toxicity impact on bullfrog. The Indian bullfrog (*Hoplobatrachus tigerinus*) was selected for use as it is a representative of the family Dicroglossidae and historically this species has been used as an amphibian test model species. Prior to definitive study, oral gavage method was applied with Imidacloprid. The test pesticide subsequently tested with both male and female juvenile bullfrogs in comprehensive acute oral median lethal dose ($LD_{50}$) studies. The primary endpoint was mortality, whereas behavioral responses, food consumption and body weight were used to evaluate indications of sub-lethal toxicity (secondary endpoints). The results clearly indicates that the acute oral $LD_{50}$ (95% fiducial interval) for 24, 48, 72 and 96 h in static method obtained 213.8 mg/L, 195.34 mg/L, 172 mg/L, 165 mg/L. Therefore, our data suggest that the per cent mortality and probit mortality increased with the increase in concentration and the $LD_{50}$ values decreased with the increase in exposure period of toxicant. Based on the results of these studies, the methodology for the acute oral gavage administration of test items to terrestrial-phase amphibians was demonstrated as being a practical method of providing data for risk assessments.

**KEYWORDS:** Imidacloprid, Bullfrog, Acute oral toxicity, Risk assessment.

## 1. INTRODUCTION

Worldwide, many ecosystems around the world are being constantly challenged due to growing human and industrial pressure exerted upon them. The use of various biomarkers in local, easily available species can be of use to evaluate the response of the biota to such environmental pollutants (Larramendy, 2017a, 2017b). Several biological parameters mirror the interactions between toxic agents and biotic matrices. These are powerful tools that can be applied to monitor the quality of the environment. Their responses may reveal general deleterious effects to the organism in general, pinpointing alterations at cellular, biochemical and molecular level, as well as higher levels of organization (USEPA, 1975, 2002). In this sense, anthropogenic activities are continuously introducing extensive amounts of pesticides into the environment regardless of their persistence, bioaccumulation and toxicity. Furthermore, pesticides are able not only to affect target organisms, but concomitantly exert side effects on nontarget organisms (www.epa.gov/pesticides). Accordingly, the use of pesticides requires predictive, rapid and practical techniques for toxicity assessment, especially those concerning to their lethal and sub-lethal effects, including genotoxic and cytotoxic properties (OECD, 1997).

Amphibians are presently the most threatened and hastily declining group of vertebrates and this has raised concerns about their potential sensitivity and exposure to plant protection products and other chemicals. Current environmental risk assessment procedures rely on surrogate species (e.g. fish) to cover the risk to aquatic and terrestrial life stages of amphibians, respectively. At the same time as a recent meta-analysis has shown that in most cases amphibian aquatic life stages are less sensitive to chemicals than fish, little research has been conducted on the comparative sensitivity of terrestrial amphibian life stages. Therefore, in this paper we mainly focused on "What is the relative sensitivity of terrestrial amphibian life stages to acute chemical oral exposure when compared with other aquatic animals". Acute lethal oral amphibian toxicity data collected from the available scientific literature and eco-toxicological databases were

compared with toxicity data for amphibians risk assessment.

## 2. MATERIALS AND METHODS

### 2.1. Toxicity evaluation

**Collection of test organism**

The wild fresh water Indus Valley bullfrog or Indian bull frog *Hoplobatrachus tigerinus* of both sex were collected by hand net from their spawning ponds in un polluted and non-agricultural sites of Bhimavaram, West Godavari district Andhra Pradesh, India. The frogs were transported to the laboratory in covered baskets and acclimatized to the laboratory conditions for a period of 7 days. Adult frogs of the same size and almost same weight (35.87± 0.04 g) were acclimatized in glass tanks ($51\times32\times33cm^3$) containing two liters of dechlorinated tap water for seven days prior to the experiment (Vogiatzis and Loumbourdis, 1997). Tanks were placed on a slant to provide the option of both aqueous and dry environment. Water was changed for every two days and the tank was cleaned thoroughly. Frogs were fed with earth worms twice in a week. Uneaten earth worms and faecal wastes were removed and water replenished regularly (Allaran and Karasov, 2001). In any batch during acclimatization, if 5% mortality observed, the total batch was discarded.

### 2.2. Preparation of imidacloprid (17.8% SL)

Imidacloprid, a soluble pesticide was dissolved in acetone without any agitation immediately prior to use. Doses of Imidacloprid were prepared and incubated into the experimental animals according to the design of the experiment.

### 2.3. Route of Administration

Imidacloprid was given orally to all the experimental animals. At sub-lethal doses after every test period of 24 h, the pesticide was administered orally with the help of a syringe fitted with a 16 gauze oral blunt feeding needle. The oral feeding needle was placed into the mouth and passed back into the stomach; this is called oral intubation. Control animals of were treated with distilled water without giving pesticide.

### 2.4. Selection of sub-lethal concentrations

The lethal concentrations ensure death even before noticing the behavioral abnormalities. Anderson and Peterson (1969) reported that sub-lethal exposures to longer periods may be more dangerous than lethal concentrations to the organisms. Even when the animal is exposed to low doses continuously, many behavioral abnormalities and physiological alterations will occur. In the present study, $1/10^{th}$ of 96 h $LD_{50}$ value was taken as sub-lethal concentration to study the behavioral alterations and physiological alterations (As per the recommendations of committee on toxicity studies – Anon, 1975). The data on the mortality rate of frogs were recorded. The dead frogs were removed immediately. The toxicity tests were conducted to choose the mortality range from 10% to 90% for 24, 48, 72 and 96 h. Finney's probit analysis (Finney, 1971) as recorded by Roberts and Boyce (1972) was followed to calculate the $LD_{50}$ values. The respective probit values were taken from Table IX of Fisher and Yates. For the determination of the 95% confidence limits, $LD_{50}$ values and a normal variant of 1.96 were taken into consideration.

## 3. RESULTS AND DISCUSSION

The per cent mortality and probit mortality increased with the increase in concentration of imidacloprid. The 24, 48, 72 and 96 h $LD_{50}$ values of imidacloprid for the frog *Hoplobatrachus tigerinus* obtained in static method 213.8 mg/L, 195.34 mg/L, 172 mg/L, 165 mg/L. In general, *Hoplobatrachus tigerinus* is sensitive towards the test toxicant. These findings are in agreement with Jaffery and Keizer, (1995) on *Rana sphenocephala;* Feng *et al.* (2004) on *Rana hallowell.;* Feng *et al* (2004) on *Rana linocharis.* However, the present study showed that the relationship between amphibian declines and exposure to insecticides can be complex. Test toxicant kills 90–100% of exposed tadpoles, and is considered a strong contributing factor to amphibian population declines and extirpations (Lesbarrères et al., 2012). Future studies could explore the potentially beneficial effects of exposure of amphibians to anti-inflammatory compounds.

In the present study it was observed that the $LD_{50}$ values decreased with the increase in exposure period. The toxicity of imidacloprid has been well studied in mammals, birds, terrestrial invertebrates and aquatic organisms, and the mechanism of action is fairly well known. In all species, the toxicity of imidacloprid metabolites is equivalent to or less than that of the parent compound. The nitrosoimine metabolite, a contaminant of imidacloprid metabolism, is of low toxicity to mammals. The predominant metabolites associated with toxicity in insects are olefinic dihydroxy – and hydroxyl – imidacloprid.

In mammals, the primary toxic effects of imdacloprid are on body weight and the thyroid. In birds, imidacloprid causes neurotoxicity and effects advers on hatching growth, and there is evidence that birds learn to avoid imidaclopprid – treated seed. Birds appear to be more sensitive to imidacloprid than mammals. According to Label review manual, 2007, Imidacloprid is moderately toxic (Toxicity Category II) if ingested. Oral $LD_{50}$ values in rats were found to be 450 mg/kg for both sexes in one study and 500 and 380 mg/kg in males and females, respectively in another study (Tomlin,2006; WHO, 2004) and the $LD_{50}$ values in mice were noticed to be 130 mg/ kg for males and 170 mg/kg for females(WHO, 2004). The dermal $LD_{50}$ in rats was estimated at greater than 5000 mg/ kg (very low in toxicity via dermal exposure) (Tomlin, 2006 and WHO, 2004). Eiben and Rinke (1989) studied the chronic toxicity of Imidacloprid in rats. In their experiment they have given doses of 14, 61, and 300 mg/kg/day for males and 20, 83, and 420 mg/ kg/day for females for three months in their diet.

They noticed reductions in body weight gain, liver damage, and reduced blood clotting function and platelet counts at 61 mg/kg/day in males and 420 mg/kg/day in females and found the NOAEL at 14 mg/kg/day.

The acute $LD_{50}$ varied from species to species in birds; it was determined to be 31 mg/kg in Japanese quail and 152 mg/kg in bobwhite quail. Dietary $LC_{50}$ values for a five-day interval were estimated to be 2225 mg/kg/day for bobwhite quill and in excess of 5000 mg/kg for mallard ducks (Tomlin, 2006). $LC_{50}$ values for a 96-hour exposure were 237 mg/L for golden oriole (Leuciscus idus) and 21 mg/L for rainbow trout (*Oncorhyncus mykiss*) (Tomlin, 2006). For Daphnia it is 85 mg/L for 48-hour exposure (Tomlin, 2006). Oral $LD_{50}$ values for bees range from 3.7 to 40.9 ng per bee, and contact toxicity values ranged from 59.7 to 242.6 ng per bee. Based on these values, imidacloprid is considered to be highly toxic to bees (Label review manual, 2007).

On the basis of acute toxicity, amphibians are less sensitive than mammals, fish, and sensitive aquatic invertebrates. Acute NOEC values of 30 mg/L and 101.2 mg/L are used in this assessment for sensitive and tolerant amphibian species, respectively. For longer – term exposures, NOEC values of 17.5 mg/ L and 88 mg/L are used for sensitive and tolerant species, respectively.

Obtainable data indicate that toxicity studies on amphibia are less than pisces. The toxicity of Nuvacron and determined $LD_{50}$ of Nuvacron for *Rana cyanophlyctis.* Ravitchandirane (2007) studied the toxicity of Endosulfan and Ekalux EC25 and found $LD_{50}$ values for male green frog *Rana hexadactvla.* Ramanujm (1989) also determined $LD_{50}$ and effect of Metacid 50 on the biochemistry and male reproduction of green frog *Rana hexadactyla.*

**Table 1: Static 24 h amd 48 h per cent mortality, probit mortality of frog *Hoplobatrachus tigerinus* exposed to Imidacloprid.**

| | x axis | | | | y | | |
|---|---|---|---|---|---|---|---|
| Conc, C% | log(C%) | Alive | Dead | Prop.p | Corr.p | Logit(p) | Probit(p) |
| 175 | 2.27 | 8 | 2 | 0.2 | 0.296979 | -1.21664 | 4.283947 |
| 195 | 2.29 | 7 | 3 | 0.3 | 0.361979 | -0.65949 | 4.646826 |
| 215 | 2.33 | 5 | 5 | 0.5 | 0.611979 | 0.20209 | 5.284481 |
| 235 | 2.37 | 4 | 6 | 0.6 | 0.736979 | 0.611095 | 5.63406 |
| 255 | 2.41 | 2 | 8 | 0.8 | 0.986979 | 1.596332 | 6.68 |

| | |
|---|---|
| Slope: | 19.00876 |
| Intercept: | -44.2598 |
| Test value: | 0 |
| Log(C%): | 2.328388 |
| LC50: | 213.0041 |

**24 h**

| | x axis | | | | y | | |
|---|---|---|---|---|---|---|---|
| Conc, C% | log(C%) | Alive | Dead | Prop.p | Corr.p | Logit(p) | Probit(p) |
| 165 | 2.21 | 9 | 1 | 0.1 | 0.099537 | -2.20186 | 3.715806 |
| 185 | 2.26 | 7 | 3 | 0.3 | 0.321759 | -0.77728 | 4.537215 |
| 205 | 2.31 | 4 | 6 | 0.6 | 0.655093 | 0.493312 | 5.399106 |
| 225 | 2.35 | 2 | 8 | 0.8 | 0.877315 | 1.478549 | 6.161668 |
| 245 | 2.38 | 1 | 9 | 0.9 | 0.988426 | 2.290944 | 6.68 |

| | |
|---|---|
| Slope: | 26.1685 |
| Intercept: | -59.9832 |
| Test value: | 0 |
| Log(C%): | 2.292189 |
| LC50: | 195.9698 |

**48 h**

**Table 2: Static 72 h and 96 h per cent mortality, probit mortality of the frog *Hoplobatrachus tigerinus* exposed to imidacloprid.**

| Conc, C% | x axis log(C%) | Alive | Dead | Prop.p | y Corr.p | Logit(p) | Probit(p) |
|---|---|---|---|---|---|---|---|
| 145 | 2.16 | 8 | 2 | 0.2 | 0.236979 | -1.21664 | 4.283947 |
| 165 | 2.21 | 6 | 4 | 0.4 | 0.486979 | -0.20871 | 4.967356 |
| 185 | 2.26 | 4 | 6 | 0.6 | 0.736979 | 0.611095 | 5.63406 |
| 205 | 2.31 | 3 | 7 | 0.7 | 0.861979 | 1.055449 | 6.089255 |
| 225 | 2.35 | 1 | 9 | 0.9 | 1.111979 | 2.408727 | 6.68 |

Slope: 17.63245
Intercept: -39.2841
Test value: 0
Log(C%): 2.227943
LC50: 169.0218

**72 h**

| Conc, C% | x axis log(C%) | Alive | Dead | Prop.p | y Corr.p | Logit(p) | Probit(p) |
|---|---|---|---|---|---|---|---|
| 125 | 2.09 | 8 | 2 | 0.2 | 0.236979 | -1.21664 | 4.283947 |
| 145 | 2.16 | 7 | 3 | 0.3 | 0.361979 | -0.65949 | 4.646826 |
| 165 | 2.21 | 5 | 5 | 0.5 | 0.611979 | 0.20209 | 5.284481 |
| 185 | 2.26 | 3 | 7 | 0.7 | 0.861979 | 1.055449 | 6.089255 |
| 205 | 2.31 | 1 | 9 | 0.9 | 1.111979 | 2.408727 | 6.68 |

Slope: 16.36347
Intercept: -35.7398
Test value: 0
Log(C%): 2.18412
LC50: 152.799

**96 h**

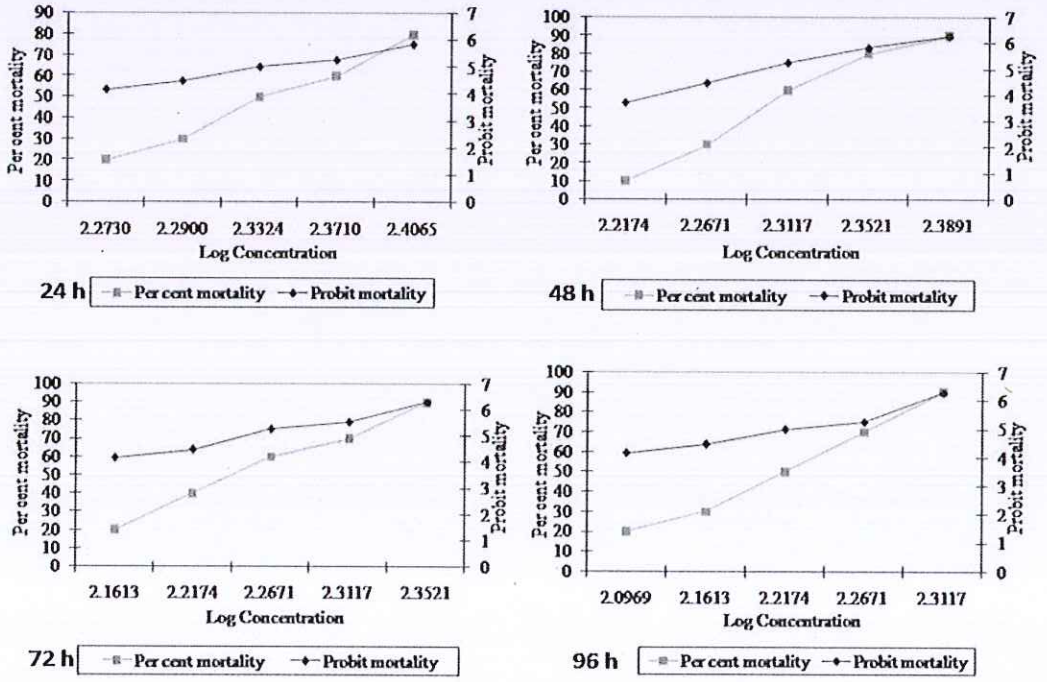


**Fig. 1: Static 24 h, 48 h, 72 h and 96 h per cent mortality and probit mortality of the frog, *Hoplobatrachus tigerinus* exposed to imidacloprid.**

**Table 3: Regression values and 95 per cent Confidence Levels for toxicant of imidacloprid exposed to frog, *Hoplobatrachus tigerinus* at different exposed periods.**

| Hours of exposure | Static method Regression equation Y=(y-bx) + bx | 95% Confidence levels Static method | |
|---|---|---|---|
| | | Lower | Upper |
| | y=15x+3 | 175.73 | 254.26 |
| 24 | $R^2$=0.9868 | | |
| | y=21x-9 | 165.73 | 244.26 |
| 48 | $R^2$=0.9757 | | |
| | y=17x+5 | 145.73 | 224.26 |
| 72 | $R^2$=0.9897 | | |
| | y=18x-2 | 125.73 | 204.26 |
| 96 | $R^2$=0.9878 | | |

## 4. CONCLUSIONS

The widespread distribution of bullfrog (*Hoplobatrachus tigerinus)* and perceptible occurrence in the habitats, reproduction along the year connected to heavy rainfall, forbearance of maintenance under laboratory circumstances, number of eggs per lay, larvae size, and the chance of maintaining both winter and spring larvae (long and short life cycles, respectively), make the species a excellent candidate for eco-toxicological studies at different levels of response. Our present results agree fighting fit with this concept. Thus, the species must be considered an excellent sign organism in environmental control programs of the region.

### Conflict of interest statement

The authors affirm that there are no conflicts of interest.


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