Assessment of Neonicotinoid Insecticide Imidacloprid LC50 And Their Toxicity Parameters Against Earthworm (Eisenia Fetida)

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Research Article

Keywords: Eisenia fetida, Imidacloprid, Mortality, Neonicotinoid, LC50, Toxicity

DOI: https://doi.org/10.21203/rs.3.rs-587464/v1

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Abstract

Because of their high biomass in the soil, earthworms are used as bio-indicator species for assessing soil toxicity against pesticides. The regular observed sensitivity to relatively low pesticide concentrations exits in soils is a significant ecological observation. Insecticide residues harm the flora of beneficial invertebrates and harm the physiological functions of earthworms, resulting in death. They affect morphological parameters as well as internal organs, and eight different imidacloprid concentrations (0.050 µl/cm², 0.100 µl/cm², 0.150 µl/cm², 0.200 µl/cm², 0.250 µl/cm², 0.300 µl/cm², 0.350 µl/cm², 0.400 µl/cm²) were prepared with water during the procedure. To establish the LC50 value, earthworms of *Eisenia fetida* were exposed to various concentrations of imidacloroprid using the usual paper contact toxicity method, and their toxicity levels are established. The mortality percentage was estimated after 24 hours of imidacloroprid exposure, and a dosage of 0.195 µl/cm² resulted in 50% mortality of earthworms. When higher concentrations of imidacloroprid were used, negative effects were observed. For ecotoxicological evaluations, the following morphological and behavioural changes were observed during the experiment: Preclittelar bulging, body constriction, blackening of the body, segment swelling, oozing of coelomic uid, body constriction, cuticle rupture, and oozing of fluid from the body are all common side effects.

Introduction

Overuse of pesticides and fertilisers in cultivation has poisoned soil to dangerous levels in recent decades. This causes a difference in the aeration and fertility of the soil, resulting in an imbalance between the flora and fauna that live there (Van et al. 2009).

Soil is made up of minerals, organic substances, and the flora and fauna that dwell there. As a result, the ecosystem's principal consumers and decomposers, the fauna, are largely reliant on soil quality maintenance (Handrix 2000). Earthworms are a great bioindicator for assessing the state of the soil ecosystem's health. According to previous studies, pesticide application endangers their lives by exposing them to pesticide-contaminated soil (Jadhav and David 2017).

Imidacloroprid is systemic pesticides that acts as an insect neurotoxin and is a member of the neonicotinoid family of compounds that damage insects' nervous systems. To evaluate a pesticide's ecological risk, a projected non-effective concentration and predicted effect concentration values must be established in order to determine the toxicity exposure ratio.

With regard to target species, various toxicity assessment tests have been established, and effects have been characterised based on exposure time. Pesticide impacts on soil fauna can be assessed using a variety of methods. To examine the impact of pesticides on soil species, standard laboratory toxicity assays for *Hypoaspis aculeifer, Folsomia candida Eisenia andrei, Enchytraeus albidus,* and *Eisenia fetida,* were created in Europe (Jansch et al., 2006).
Imidacloprid, acetamiprid, nitenpyram, clothianidin, and thiacloprid are nicotinic acetylcholine receptor agonists with varying degrees of toxicity in various species. According to Kai Wang et al. (2015), acute toxicity studies demonstrated significant variations in lethal concentration 50 (LC50) values among clothianidin to *Eisenia fetida* after 14 days of exposure was significantly greater than the other four insecticides (thiacloprid, acetamiprid, imidacloprid, nitenpyram). Imidacloprid, acetamiprid, nitenpyram, clothianidin, and thiacloprid had LC50 values of 3.05 mg kg\(^{-1}\), 2.69 mg kg\(^{-1}\), 4.34 mg kg\(^{-1}\), 0.93 mg kg\(^{-1}\), and 2.68 mg kg\(^{-1}\) against *Eisenia fetida*, respectively. The LC50 values of imidacloprid in soil for earthworms on days 7 and 14 were 2.49 mg kg\(^{-1}\) and 2.26 mg kg\(^{-1}\), according to Xing Wang 2019.

Worms avoided tropical artificial soil treated with insecticides imidacloprid and thiametoxam, as well as those treated with the fungicides captan and carboxin plus thiram, in an experiment conducted by Paulo et al., (2013), and at the lowest concentrations, more worms were found in the contaminated compartments than in the control, indicating that worms preferred low-pesticide soils to high-pesticide soils. Worms, on the other hand, preferred tropical fake soil treated with fipronil at all concentrations tested to tropical artificial soil treated with higher concentrations of these three pesticides.

**Material And Methods**

Fully developed clitellated earthworms were exposed to neonicotinoid insecticides viz. imidacloprid to assess toxicity parameters at the Department of Zoology, College of Basic Science and Humanities, Chaudhary Charan Singh Haryana Agricultural University, Hisar from July to September 2020.

**Selection of test earthworm**

Chaudhary Charan Singh Haryana Agricultural University, Hisar, has a vermiculture section in the Department of Zoology and from this vermiculture unit collected fully formed clitellated healthy earthworms of *Eisenia fetida* with an average body weight of 700 mg. The earthworms were brought to the vermiculture laboratory, where they were cultured in plastic tubs with a 60:40 mix of cow dung and organic manure, with adequate moisture (60–65 percent) maintained by sprinkling water on the substrate as required, and they were covered with gunny bags to protect them from pests and maintain moisture levels.

**Procurement of test neonicotinoid insecticides**

The technical grade of imidacloprid (95.3%) was obtained from the pesticide market of Hisar, Haryana. Treatments have been created. Table 1 shows the control and imidacloprid doses provided to the test earthworm *Eisenia fetida*.

**Experimental set up for assessing the LC50 of imidacloprid**

The earthworms were rinsed in distilled water and placed on wet filter paper for two hours to clean their gut contents. Whatmann filter papers were held to conform correctly to the size of the vials to prevent side overlapping, and flat bottomed glass vials 8 cm long, 3 cm diameter, medium grade 0.2 mm thick
were used. Eight different imidacloprid concentrations (0.050 µl/cm², 0.100 µl/cm², 0.150 µl/cm²) were tested. Each vial of filter paper was poured with one ml of insecticide and then rotated horizontally to ensure that the insecticide and control were evenly distributed throughout the filter paper (having 1 ml of deionized water only). They then inoculated one earthworm per vial with various concentrations of imidacloprid, as well as a control, and covered all vials with muslin. To reduce mortality, proper aeration, a temperature range between 20 and 25 degrees Celsius, and sufficient moisture levels were maintained during the experiment. To maximise the efficiency of the experiment, all treated vials were laid horizontally and shielded from light exposure. Eight replicates of each concentration, as well as a control, were held.

**Determination of LC50 of insecticides against adult *Eisenia fetida***

The corrected mortality was calculated after determining the percent mortality (Abbott, 1925), and the data collected during the experiment was subjected to Probit analysis (Finney, 1971) as suggested by the standard paper contact toxicity procedure.

\[
\text{Percent mortality} = \frac{\text{Total number of earthworms died}}{\text{Total number of earthworms released initially}} \times 100
\]

\[
\text{Corrected mortality} = \frac{\text{Mortality in treatment} - \text{Mortality in control}}{100 - \text{Mortality in control}} \times 100
\]

**Post treatment assessment of earthworms exposed to imidacloprid insecticide**

At various time intervals after treatment, morphological and behavioural changes were reported. After 24 hours of exposure, the mortality rate was tested. Earthworms that did not respond to stimuli were deemed dead, and the observed mortality was utilised to calculate the percent mortality rate. The adjusted mortality was calculated after subtracting the control mortality. The LC50 value was calculated using the collected data.
Table 1
Description of treatments (sprayed) given to the test earthworm species along with control

| S.N. | Treatment | Description       |
|------|-----------|-------------------|
| 1.   | Control   | No dose           |
| 2.   | T1        | Imidacloprid 0.050 µl/cm² |
| 3.   | T2        | Imidacloprid 0.100 µl/cm² |
| 4.   | T3        | Imidacloprid 0.150 µl/cm² |
| 5.   | T4        | Imidacloprid 0.200 µl/cm² |
| 6.   | T5        | Imidacloprid 0.250 µl/cm² |
| 7.   | T6        | Imidacloprid 0.300 µl/cm² |
| 8.   | T7        | Imidacloprid 0.350 µl/cm² |
| 9.   | T8        | Imidacloprid 0.400 µl/cm² |

Results

Experiments were carried out on the *Eisenia fetida* to measure the toxic effects of neonicotinoid insecticide imidacloprid from various pesticide groups. The LC50 values of imidacloprid insecticides on *E. fetida* were measured, and post-treatment behavioural and morphological changes caused by imidacloprid insecticide exposure were recorded at various intervals of time. It was discovered that as pesticide concentrations increased, the mortality of earthworms increased as well. Low doses of imidacloprid at a concentration of 0.050 µl/cm² resulted in the lowest mortality, and such concentrations are considered negligible. At a concentration of 0.400 µl/cm² of imidacloprid, 100 percent mortality was observed (Table 2 & Fig. 1). Table 3 shows the LC50 values of imidacloprid, which are 0.195 µl/cm². The LC50 value of a pesticide is described as the concentration at which it kills 50% of the earthworms under investigation.
Table 2
Description of *E. fetida* mortality after exposure to different doses of imidacloprid after 24 hour

| Imidacloprid doses | Earthworms | Before treatment count | Live after 24 hr | Mortality (%) |
|-------------------|------------|------------------------|-----------------|--------------|
| 0.050 µl/cm²      | 8          | 7                      | 12.50           |
| 0.100 µl/cm²      | 8          | 6                      | 25.00           |
| 0.150 µl/cm²      | 8          | 5                      | 37.50           |
| 0.200 µl/cm²      | 8          | 5                      | 37.50           |
| 0.250 µl/cm²      | 8          | 4                      | 50.00           |
| 0.300 µl/cm²      | 8          | 3                      | 62.50           |
| 0.350 µl/cm²      | 8          | 2                      | 75.00           |
| 0.400 µl/cm²      | 8          | 0                      | 100.00          |

Table 3: LC$_{50}$ value of imidacloprid against fully developed clitellum *Eisenia fetida*

| Insecticide | Expose time | Number | Slope(b) | $\chi^2$ | LC50     | LC95     |
|-------------|-------------|--------|----------|----------|----------|----------|
| Imidacloprid| 24 hr       | 8      | 1.743    | 1.59     | 0.195 µl/cm² | 0.207 µl/cm² |

Morphological and behavioral alterations induced by imidacloprid insecticide on exposure to *Eisenia fetida*

Imidacloprid, a neonicotinoide insecticide, has been shown to cause morphological and behavioural changes in stable earthworms (Table 4). For ecotoxicological evaluations, morphological and behavioural changes are instant symbols to be reported during the experiment.

**Morphological observations**

When earthworms were given a dose of imidacloprid at a concentration of 0.050 µl/cm², their bodies curled and coiled, as opposed to the control, which moved slowly. It was discovered that increasing the concentration of imidacloprid caused further damage to the earthworm’s body.

At higher concentrations of imidacloprid, morphological changes such as swift, aggressive, slow and sluggish movement, coiling around itself, curling of the body, hyperactivity, raising of the body, and at
0.400 µl/cm² the earthworms displayed restlessness, zigzag, and curling movement were observed. Table 4 and Fig. 2 show the morphological observations made in relation to imidacloprid.

**Behavioural observations**

According to behavioural research, imidacloprid at a concentration of 0.050 µl/cm² did not cause any changes in earthworms. In the case of control, similar observations were made.

When higher concentrations of imidacloprid were used, negative effects such as preclitellar bulging, body constriction, blackening of the body, segments swelling, oozing out of coelomic fluid, body constrictions, cuticle rupture, and oozing out of fluid from the body were observed at 0.400 µl/cm² had showed blackening of body, oozing out of coelomic fluid and dark pigmentation. Table 4 and Fig. 2 show the behavioural changes in response to imidacloprid.

**Discussion**

The LC50 value is used to express the chemical analysis of mortality caused by imidacloprid exposure. In ecotoxicological assessments, morphological and anatomical changes in earthworms caused by imidacloprid insecticide exposure can be used as indicators for the presence of toxicants in top soil. Earthworm activity, development, reproduction, and organisational structure are all affected by sub-lethal pesticide doses, which are more closely associated to long-term harmful consequences in ecological systems (Capowiez et al., 2005; Reinecke and Reinecke, 2007).

The filter paper contact test and artificial soil test results of Wang et al (2012a) on 24 pesticides revealed that contact toxicity varied greatly among insecticides, and that various insecticides within the same chemical class had varying toxicity to Eisenia fetida.

**Toxicity of neonicotinoid imidacloprid insecticide against *Eisenia fetida***

Because of their easy capacity and higher reproduction rates, the Organisation for Economic Cooperation and Development (OECD, 2004) recommended *E. fetida* are the best earthworm species for ecotoxicological research. The artificial soil test is preferred to the filter paper contact test in the natural habitat of earthworms because pesticides are absorbed mostly via the gut of earthworms in soil (Zhang et al., 2014). Earthworm mortality increased as pesticide concentrations increased, with an LC50 value of 0.195 µl/cm² for imidacloprid, which was close to the findings of Zang et al., (2000), who found that the neonicotinoid insecticide imidacloprid (LC50 of 0.1 µl/cm² at 24 hr and 0.034 µl/cm² at 48 hr) was highly sensitive to earthworms.

Wang et al., (2012) published similar findings when 24 pesticides were tested using a filter paper contact test and an artificial soil test. They used a contact filter paper toxicity test to test five neonicotinoid
insecticides and found that the LC50 values for acetamiprid (97%) was 0.0088 µg/cm², clothianidin (96.5%) was 0.28 µg/cm², imidacloprid (95.3%) was 0.027 µg/cm², nitenpyram (95%) was 0.22 µg/cm², and thiacloprid (97.75%) was 0.45 µg/cm². In a soil toxicity test on *Eisenia fetida*, imidacloprid, acetamiprid, nitenpyram, clothianidin, and thiacloprid exhibited LC50 values of 3.05 mg kg⁻¹, 2.69 mg kg⁻¹, 4.34 mg kg⁻¹, 0.93 mg kg⁻¹, and 2.68 mg kg⁻¹, respectively.

**Morphological and behavioral changes**

Imidacloprid treatment of earthworms resulted in morphological and behavioural alterations. Slow movements were observed in the control group, although increased imidacloprid concentrations resulted in a variety of morphological changes in the treatment groups. At 0.050 µl/cm² and 0.100 µl/cm², curling and coiling of the body, as well as rapid movement, coil around itself, were observed. A concentration of 0.150 µl/cm² resulted in hyperactive, curling of the body, while a concentration of 0.200 µl/cm² resulted in worms moving violently and coiling around themselves. Fast movement by lifting the body, slow sluggish movements, restlessness, zigzag curling movement and highly lethargic reduced movements were recorded at 0.0250 µl/cm², 0.300 µl/cm², 0.350 µl/cm² and 0.400 µl/cm² concentrations of imidacloprid respectively.
| S. No. | Treatments IMD | Morphological changes | Behavioural change |
|--------|----------------|-----------------------|-------------------|
| 1      | Control        | Slow movement         | No changes observed |
| 2      | 0.050 µl/cm²   | Curling and coiling of body | No changes observed |
| 3      | 0.100 µl/cm²   | Fast movement, coil around itself | Preclittlar bulging, body constriction |
| 4      | 0.150 µl/cm²   | Hyperactive, curling of the body | Oozing out of coelomic fluid, clitellar bulging |
| 5      | 0.200 µl/cm²   | Violent movement, coil around itself | Blackening of body, segments swelling |
| 6      | 0.250 µl/cm²   | Fast movement, lifting the body | Blackening of body, constrictions, oozing out of coelomic fluid |
| 7      | 0.300 µl/cm²   | Slow, sluggish movement | Body constrictions, swelling in segments |
| 8      | 0.350 µl/cm²   | Restlessness, zigzag, curling movement | Cuticle rupture, oozing out of fluid |
| 9      | 0.400 µl/cm²   | Highly lethargic, reduced movements | Blackening of body, oozing out of coelomic fluid and dark pigmentation, |

IMD = Imidacloprid

In the control group, no behavioural changes were observed. When earthworms were handled with imidacloprid at a concentration of 0.050 µl/cm², similar results were observed. Behavioral changes such as preclittlar bulging with body constrictions and oozing out of coelomic fluid with symptoms of clittelar bulging were reported at 0.100 µl/cm² and 0.150 µl/cm², respectively.

Imidacloprid concentration of 0.200 µl/cm² had showed blackening of the body with segment swelling. At 0.250 µl/cm², 0.300 µl/cm², 0.350 µl/cm², and 0.400 µl/cm² imidacloprid concentrations, additional behavioural changes such as blackening of the body associated with oozing out of coelomic fluid, body constrictions with swelling in segments, oozing out of fluid due to cuticle rapture, and blackening of the body with concomitant oozing out of coelomic fluid causing dark pigmentation were observed.

The first exposure to insecticide caused morphological changes such as rigid body movements. The earthworms' bodies were instantly shocked and exhibited coiling, which was due to an adapted technique to limit insecticide exposure by curving the body surface area. The insecticide-coated filter paper was less vulnerable to movement like lifting the earthworm's body. The body's surface was reduced as a result of
Imidacloprid exposure, resulting in hyper coiling. Lack of energy and unnecessary stress caused by insecticide exposure result in extreme lethargy and decreased activity.

Earthworms used swift and zigzag movements to shield themselves from epidermal rapture. Worms behaved violently in order to reduce the insecticide effect. The earthworms eventually displayed slow restless movements, sluggish movements due to tiredness, and a low energy level in the body as a result of the insecticide effect.

When an insecticide comes into contact with the earthworm's skin, it enters the body's coelomic fluid, causing toxic symptoms. Imidacloprid penetration tears the cell membranes, causing the segments to swell. Dark pigmentation occurs in worms when body wall tissue is traumatised or microscopic damage is caused by toxicants migrating from the touch surface area. In line with the current research, Reinbeck and Reinecke (2007) discovered that sub-lethal pollutant concentrations can affect earthworm behaviour, development, reproduction, and organisational structure, which are more akin to long-term harmful consequences in the natural environment.

Imidacloprid insecticide causes morphological and behavioural changes in stable *E. fetida* earthworms, making it a significant symbol for worm ecotoxicology. The alterations which were observed due to imidacloprid were also reported by the study of Suneel and Singh, (2017) in earthworm *Eisenia fetida* after 48 hour exposure to phorate showed curling, coiling and rupture of body wall, increased mucus secretion, coelomic fluid ejection, sluggishness, and body surface lesions. Due to atrophy, the earthworm's body wall began to thin, eventually leading to necrosis and erosion of the entire body wall, although no such changes were observed in the control. There were significant epidermal, morphological changes observed after 48 hours of exposure to phorate concentrations of 20–40 µl/cm².

Earthworms subjected to the greatest amounts of phorate developed constrictions, swelling in the clitellar area with many ruptures, and punctures in the body wall with many lesions, resulting in body fragmentation and cuticle shedding, comparable to ecdysis in insects and snakes.

The epidermis and cuticle serve as a major barrier between the earthworm's body and the environment, allowing ion transfer and allowing or blocking xenobiotics (Clauss, 2001). *Eisenia andrei* avoidance behaviour due to methomyl (Carbamates chemical) toxicity was described by Pereira et al (2010). Insecticide exposure at higher levels resulted in a thick yellow discharge from the body. Worms most likely used extensive coiling to minimise their body surface area and thereby reduce insecticide exposure. Santos et al., (2012) and Ferreira et al., (2015) found that the earthworms curled up and became less responsive to touch. After exposure to imidacloprid and methyl tetra-butyl ether, similar findings were recorded in other earthworm species, *E. andrei* and *Perionyx excavates* (Youn, 2005; Yvan et al., 2005).

With growing concentrations of chlorpyrifos, morphological changes such as coiling, clitellar swelling, mucus release, and bleeding, followed by body segmentation, were observed in chlorpyrifos-treated earthworms. However, morphological changes were found to be more prevalent for cypermethrin than for
chlorpyrifos, whereas the influence of co-exposed pesticides on morphological changes was found to be intermediate (Rishikesh et al. 2019).

**Conclusion**

- The earthworm *Eisenia fetida* is extremely susceptible to imidacloprid insecticide.
- Imidacloprid insecticide causes morphological and behavioural changes in healthy earthworms, making it an important symbol for worm ecotoxicology.
- The amount of imidacloprid insecticide that harms earthworms is proportional to its concentration.
- The imidacloprid pesticide has a harmful potential for the organism being studied. The observed effects were tissue specific and dose based. Changes in a pesticide-contaminated climate may jeopardise the life of an eco-friendly non-target organism, the earthworm. The measured LC50 for the following doses of imidacloprid on earthworm *Eisenia fetida* is 0.195 µl/cm².

**Declarations**

**ACKNOWLEDGEMENTS**

The Chaudhary Charan Singh Haryana Agricultural University in Hisar, Haryana, offered financial support, and the Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS) in Hisar, Haryana, offered technical assistance.

**Declaration Section**

1. Ethical Approval : Not Applicable
2. Consent to Participate: Not Applicable
3. Consent to Publication: Not Applicable
4. Authors contributions:

   a) Parveen Gill (PG): PG is responsible for major part of the work such as growing earthworms, multiplication and carried out histopathological studies.

   b) Dommalapati Sudhakara Rao (DSR): Assessed the LC 50 values.

   c) Rajendra Kumar Gupta (RKG): Conducted earthworms mortality studies at different doses of imidacloprid.

   d) Dharambir Singh (DS): Morphological alterations were studied and identified.

   e) Tejpal Dahiya (TD): Behavioural observations were carried out and identified.

   f) Deepika Lather (DL): Assessed the toxicity of imidacloprid against earthworms.
g) Naresh Kumar (NK): Weather parameter during experiment.

5. Competing interests: Authors declare that there are no competing interests.

6. Availability of data and materials: This paper does not qualify for data sharing since no datasets were created or analyzed during the research.

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**Figures**
Figure 1

Eisenia fetida mortality at different doses of imidacloprid after 24 hr exposure
Figure 2

Impact of imidacloprid on integumentary morphology of E. fetida after 24 hr exposure (A through R) (A) Adult E. fetida under control condition (B) Loosened epidermis and segmentation (C) Mucus production (D) Swelling in clitellar region and prostomial region (E) Restless body (F) Deformed body (G) Multiple ruptures at body length and deformed segments (H) ziczag, curling movement (I) Oozed haemocoel (J) Escaping behavior (K) Coiling body behavior (L) Bulging of anterior end (M) Multiple ruptures at body
length and swelling in clitellar region (N) Fluid secretion (O) Swelling in clitellar region (P) In the anterior fragmented prostomial region, there is a self-protection mechanism. (Q) Dead worm with hyper coiling (R) Complete deformed segments.