International Journal of Current Science Research and Review
ISSN: 2581-8341
Volume 09 Issue 06 June 2026
DOI: 10.47191/ijcsrr/V9-i6-37, Impact Factor: 8.048
IJCSRR @ 2026
www.ijcsrr.org

# Reproductive Toxicity Effect of Isocycloserum 9.2% w/w DC on Earthworm, *Eisenia fetida*

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**ABSTRACT:** A laboratory study was conducted to find out the reproductive toxicity of Isocycloserum 9.2% w/w DC formulation on the inbred earthworm test species *Eisenia fetida* maintained in the laboratory. A preliminary range finding experiment was performed with concentrations of 50, 100, 250, 500 and 1000 mg/kg dry artificial soil of Isocycloserum 9.2% w/w DC. Based on the results of the range finding study, a full-fledged main experiment was conducted with concentrations of 50, 100, 250, 500, 750 and 1000 mg/kg dry artificial soil of Isocycloserum 9.2% w/w DC. Results of the main experiment revealed that there were no biomass changes observed in treated groups when compare to control group, indicating no adverse effects on growth or physiological condition. Similarly, cocoon production and juvenile emergence of earthworm were not significantly affected at any tested concentration of Isocycloserum 9.2% w/w DC, demonstrating that the test chemical does not have any potential adverse effect on the reproductive ability of earthworm.

**KEYWORDS:** Behavioural abnormality effects, Earhworms, *Eisenia fetida*, Isocycloserum 9.2% w/w DC, Mortality, IBM SPSS Statistics

## 1.0 INTRODUCTION

New insecticides gaining importance in crop protection is Isocycloseram, a member of the Isoxazoline chemical group. This insecticide acts on GABA-gated chloride channels in insects (Asahi *et al*., 2018; Nakao *et al*., 2015, Ozoe *et al*., 2010) leading to hyperexcitation, paralysis, and eventual death. It has demonstrated strong efficacy against a wide range of insect pests including thrips, mites, jassids, and borers.

Although these pesticides are highly effective against pests, limited information (Cassayre *et al*., 2021) is available regarding their effects on non-target organisms such as pollinators, soil invertebrates, and aquatic organisms. Since pesticides applied in agricultural fields may pollute ecosystems, it is essential to evaluate their potential ecological impacts.

However, its effects on non-target species and ecosystems are not well understood. This study aims to assess the ecotoxicological impact of Isocycloseram on selected species to provide data for environmental risk assessment and safe agricultural use. While effective on target pests, it is crucial to assess their potential impact on non-target soil organisms, particularly earthworms, which are vital to soil health and fertility.

Therefore, to better understand the ecological implications of such pesticide combinations on non-target soil organisms, further research is essential. As part of this effort, an acute and reproductive toxicity study of Isocycloseram 9.2% w/w DC was conducted on earthworms, specifically *Eisenia fetida*.

## 2.0 MATERIALS AND METHODS

### 2.1 Test Organism and Culture Conditions

An acute toxicity study on earthworms was conducted as a preliminary assessment to determine $LC_{50}$ and NOEC (based on biomass) to assess reproduction toxicity test. The acute toxicity study was conducted in accordance with OECD Test Guideline 207 using the

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earthworm species *Eisenia fetida* (Savigny 1826). The test organism was initially sourced from a GLP-certified laboratory in Germany and was subsequently maintained as a standardized laboratory culture in the Department of Ecotoxicology at International Institute of Biotechnology and Toxicology. Periodic identification has been undertaken to confirm the species status, with the latest taxonomic verification performed by the PG & Research Department of Zoology, The New College. In compliance with the guideline's requirements for physiological stability and reproducibility, the culture was maintained under a controlled temperature range of 20 ± 2°C and provided with continuous illumination of 400 - 800 Lux light intensity.

Earthworms (*Eisenia fetida*) at the age between five to six months old, possessed a well-developed clitellum, and had a body weight ranging from 300 mg to 600 mg per worm were selected from the same culture source used for both the treatment and control groups to maintain experimental consistency.

**2.2 Experimental Conditions**

The earthworm acute toxicity study was conducted under controlled environmental conditions, with test room temperature maintained between 18 - 20°C and light intensity ranging from 400 to 800 lux. Activities such as earthworm handling, sorting, and observations of behavioural abnormalities and mortality were performed under these controlled conditions. However, procedures including artificial soil preparation, pre-moistening, preparation and application of test concentrations, and earthworm weighing were carried out at ambient laboratory temperature (18-28°C).

**2.3 Artificial Soil Preparation and Acclimatization of Earthworms**

The earthworms were acclimatized under laboratory conditions for one day in the artificial soil prior to the experiment start. Artificial soil was prepared prior to experiment initiation according to the OECD Test Guideline No.: 207 entitled on "Earthworm, Acute Toxicity Tests", ISO Guideline No.11268 -1 entitled on "Soil Quality-Effects of pollutants on earthworms - Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei" (2012)". The artificial soil was prepared by mixing the ingredients 10% Sphagnum-peat air-dried and finely ground, 20% Kaolin clay, 70% air-dried quartz fine sand between 50 microns to 200 microns and thoroughly mixed using homogenizer for about 20 minutes. The pesticide residues analysis of artificial soil ingredients was done by an external agency. The pH of the artificial soil was checked after homogenization and maintained between pH of 6.0 ± 0.5 as per OECD Test Guideline No.: 222 entitled on "Earthworm Reproduction Test (Eisenia fetida/ Eisenia Andrei).

Prior to start of the experiment, five to six months old earthworms all within four weeks of the same age, well clitellate were collected from the breeding box and acclimatized for one day in artificial soil (approximately 100 worms / kg dry artificial soil) in a glass tank of size dimension of 23 cm length × 15 cm width × 16 cm height and covered with nylon net to avoid escapism of earthworms. The number of earthworms collected for acclimatization was based on the number of treatments. Enough earthworms were collected to avoid shortage due to over / under weight. These glass tanks were kept in the test room with the same testing controlled environmental conditions.

**2.4 Test Container and Test Medium**

Glass beakers of approximately 1.5 L capacity in size with a cross-sectional area of approximately 130 $cm^2$) was used as test containers. Each beaker was covered with a perforated transparent lid for the gaseous exchange between the medium and the atmosphere and to enable the required access of light, to enable exchange of air and to minimize evaporation of the artificial soil. The containers were filled with approximately 570 g dry artificial soil and added 200 mL of deionised water resulting in a total wet weight of approximately 750 g (water content calculated based on 35 mL/100g dry artificial soil as per OECD Test Guideline : 207 and ISO Test Guideline (ISO 11268-2, (1998). Soil moisture was maintained throughout the experiment duration according to OECD Test Guideline 207.

**2.5 Preparation and Application of Test Chemical Isocycloserum 9.2% W/W Dc**

On the day of the acute toxicity experiment, the test chemical stock solution was prepared by weighing test chemical 6.75 g of Isocycloserum 9.2% w/w DC using a calibrated weighing balance and diluted it to 25.0 mL with deionised water, and considered as stock solution. The stock solution was homogenized using a magnetic stirrer for 30 minutes to ensure uniformity of test chemical. Test chemical concentrations of 50, 100, 250, 500, 750, and 1000 mg/kg dry artificial soil were achieved from the stock solution of the test chemical by pipetting out the required test chemical solution from the test chemical stock solution and added to the artificial

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soil taken. Application of the test chemical was conducted in two batches, each consisting of two replicates (1.14 kg dry artificial soil per batch). Each treatment was treated as two batches (two replicates together). Each batch was divided into two replicates, thus four replicates altogether at each test chemical concentration level.

The pH and moisture content were measured at test start (day 0) and at test end (day 14) by following OECD Test Guideline No.: 222 (2016) in control and test chemical treatments.

The prepared test medium was then aerated before introducing the test organism. Healthy adult earthworms, selected based on the presence of a well-developed clitellum, were collected from the acclimatized soil, and gently washed twice with tap water to remove the adhering soil particles without causing injury to the earthworms. The washed earthworms were blotted carefully by placing on filter paper to remove excess moisture. Earthworm weight was then recorded by placing it on a Petri dish lined with filter paper. Earthworms were placed individually on the Petri dish during the weight assessment to ensure that the weight of each worm passes the specified range (300 mg to 600 mg/worm). The earthworms which were not within the specified range while weighing, were removed and not used. There were four replicates (10 worms/replicate) altogether at each test chemical concentration level and for control. The test containers were covered with perforated plastic lids after release of earthworms to permit gaseous exchange between the test chemical and the atmosphere and access to light whilst preventing the worms from escaping. Moisture content of the artificial soil in each glass container was monitored by weight on day 7. Any reduction in weight of glass container compared to day 0 was corrected by adding deionised water, ensuring that soil moisture loss remained within 10% of the initial moisture level throughout the test period.

The light intensity and test room temperature were maintained in the range of 400 – 800 lux and 18 - 22ºC temperature. The light intensity and temperature were monitored regularly using the calibrated instruments of Lux Meter.

Behavioral abnormalities, including inability to burrow into the soil, immobility and oozing, were assessed 15 minutes after the release of earthworms on Day 0. Following 14 days of exposure, mortality, behavioral abnormalities, and biomass changes were evaluated. Earthworms were carefully sorted from each test container, gently washed with tap water to remove adhering soil particles, and blotted dry with filter paper. Individual body weights were then recorded using a calibrated analytical balance.

Based on the findings of the acute toxicity test, a reproductive toxicity test was subsequently conducted with 56 days of duration. On the day of the experiment, the test chemical stock solution was prepared by weighing test chemical 9031.19 g of Isocycloserum 9.2% w/w DC diluted it to 25.0 mL with deionised water. Test application procedure, moisture maintenance, and pH estimation, mortality and behavioral observation were followed as followed in acute toxicity study except feed provision and consumption assessment.

**View of Experimental Set Up – Reproduction Toxicity Study -Isocycloserum 9.2% w/w DC**

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**Measurement of Biomass -Single earthworm**


**10 Earthworms together**


## 3.0 PARAMETERS OBSERVED FOR REPRODUCTION TOXICITY EXPERIMENT

### 3.1 Mean Biomass Change (Body Weights)

The total and mean body weights of all live earthworms in each test container were recorded at the start of the experiment (day 0) and on day 28. The percent biomass changes of the earthworm between start on day 0 and on day 28 was calculated by using the following formula:

$$\text{Mean Biomass Change (\%)} = \frac{\text{Weight of worm after treatment (mg/worm)} - \text{Weight of worm before treatment (mg/worm)}}{\text{Weight of worm before treatment (mg/worm)}} \times 100$$

### 3.2 Mortality and Abnormal Behavioral Abnormalities of Adult Earthworms

On day 28, the number of dead adult earthworms in each replicate was assessed. The earthworms that failed to respond to gentle mechanical stimulation were considered as dead. Behavioral abnormalities such as inability to burrow into the soil, immobility, coiling, surface avoidance, discoloration, fragmentation and oozing were observed.

### 3.3 Assessment of Feed

On Day 0, an initial quantity of 11.4 g of cow dung feed (@10 g/kg soil) was thoroughly incorporated into the artificial soil prior to the application of the test chemical concentration. One day after the introduction of the test chemical, an additional 5 g of finely ground cow manure moistened with 7 mL of deionised water (totally 12 g) was provided per container. This feed was uniformly distributed over the soil surface to ensure even availability.

Feeding was subsequently carried out at weekly intervals during the first three weeks of the experiment, specifically on days 1, 8, 15, and 22. Care was taken to ensure that the feed added during the previous week was almost entirely consumed before the next feeding event.

Feed consumption was first assessed after the feed application on day 1 using an indigenously developed measurement device. This device consisted of a circular acrylic disc covering 360°, divided into 20 equal segments of 18° each, with each segment representing 5% of the total area. The disc was gently placed over the soil surface where the feed had been applied. The amount of uneaten feed present within each segment was visually estimated, and observations were used to calculate the percentage of feed consumed in each container.

Subsequent assessments of feed consumption were conducted on days 8, 15, 22, and 28 following treatments. During each assessment, the quantity of fresh feed added to each container was adjusted based on the level of consumption observed in that specific replicate. Both the amount of feed consumed by the earthworms and the quantity of fresh feed added were recorded and

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throughout the study. At the end of the exposure, the earthworms were carefully removed, sorted, and weighed. After the removal of adult earthworms on day 28, 5 g of cow manure per container was carefully mixed into the artificial soil to provide nourishment for the developing juveniles, irrespective of prior consumption levels. No additional feeding was carried out for the remainder of the experimental period.

**View of Feed Consumption on Day 0, Day 3 and Day 8**


### 3.4 Assessment of Cocoons

On Day 28, all cocoons produced in each replicate during this period were counted and then promptly returned to their respective containers to allow for hatchability assessment. The test containers, now containing the cocoons, were maintained under the same experimental conditions.

To support the development of juveniles emerging from the cocoons, approximately 5 g of cow dung was gently mixed into the artificial soil in each container as a food source. The containers were then incubated for an additional four weeks under identical test conditions to evaluate reproductive output based on juvenile production.

### 3.5 Juveniles Assessment

At the end of the experimental period on day 56, the artificial soil from each test container was carefully removed and processed for juvenile extraction. Initially, the soil was emptied from the containers onto a sieve and manually sieved to separate larger particles. The juveniles retained on the sieve were then gently transferred into a plastic tray, where they were counted using a hand tally counter to ensure accuracy.

Following this, the remaining soil from each container was transferred into an enamel tray for further examination. To extract any additional juveniles that might still be embedded within the soil, the tray was placed in a calibrated hot water bath maintained at approximately 60°C. This heat treatment stimulated the juveniles to wriggle out of the soil matrix, making them easier to collect. All emerging juveniles were promptly removed and counted. Finally, the total number of juveniles collected from both the control and treated groups was compiled and subjected to statistical analysis. These results were then compared with data obtained from the control worms to assess the effects of the treatments.

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**4.0 STATISTICAL ANALYSIS**

Body weight change data of the earthworms were evaluated for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively. The results confirmed that the data were normally distributed and met the assumption of homogeneity. Consequently, a parametric one-way ANOVA followed by Dunnett's t-test (two-sided) was conducted to compare the biomass of worms in the control group with those exposed to treatment concentrations of 50, 100, 250, 500, 750, and 1000 mg/kg dry artificial soil. Statistical analysis was performed using Statistics software (IBM SPSS Statistics, Version 29). As the data for food consumption, Cocoon production and Juvenile emergence was non normally distributed, nonparametric test (Dunn's) was performed.

**5.0 RESULTS AND DISCUSSION**

Based on the results from the preliminary acute toxicity test, reproductive toxicity study was conducted using *Eisenia fetida* exposed to Isocycloserum 9.2% w/w DC at concentrations of 50, 100, 250, 500, 750, and 1000 mg/kg dry artificial soil. Mortality and behavioural abnormalities observations were recorded on Days 7, 14, and 28, while reproductive parameters, including cocoon production was assessed on Day 25 and juvenile emergence was assessed at the end of the exposure period (Day 56). The results demonstrated that exposure to Isocycloserum 9.2% w/w DC did not induce mortality, visible behavioural abnormalities, or reproductive impairment in earthworms at any of the tested concentrations.

Earthworms are widely recognized as important bioindicators of soil health because of their direct contact with soil contaminants and their critical role in nutrient cycling, organic matter decomposition, soil aeration, and maintenance of soil structure. Therefore, assessment of both survival and reproductive performance provides a comprehensive evaluation of potential ecological risks posed by pesticides in terrestrial ecosystems.

No mortality was observed in any treatment group throughout the 28-day exposure period. The absence of mortality even at the highest tested concentration of 1000 mg/kg dry artificial soil indicates that Isocycloserum 9.2% w/w DC possesses very low acute toxicity toward *E. fetida*. Furthermore, no behavioural abnormalities responses such as inability to burrow into the soil, immobility, coiling, surface avoidance, discoloration, fragmentation and oozing were observed.

Such observations suggest that the test substance did not cause measurable physiological stress in earthworms under the experimental conditions.

Biomass change is considered a sensitive indicator of physiological stress and feeding impairment in earthworms. The percentage biomass changes recorded in treated groups ranged from –12.10% to –14.61% at the tested concentration of 50, 100, 250, 500, 750 and 1000 mg/ kg dry artificial soil, while the control group exhibited a biomass change of –12.13% (**Table 3**). The close similarity between treated and control groups demonstrates that the observed weight loss was likely attributable to normal experimental variation and environmental factors rather than treatment related toxicity. Statistical analysis further confirmed that there were no significant differences between treated and control groups. The maintenance of body weight comparable to controls indicates that Isocycloserum 9.2% w/w DC did not interfere with feeding activity, nutrient assimilation, or energy metabolism.

Reproduction is regarded as one of the most ecologically relevant endpoints in chronic earthworm toxicity studies because it reflects long-term impacts on population sustainability. The total number of cocoons produced in the control group was 155, while cocoon production in treated groups ranged from 140 to 155 (**Table 3)**. Although minor numerical variations were observed among treatment groups, these differences were not statistically significant. The absence of treatment-related reductions in cocoon production indicates that exposure to Isocycloserum did not adversely affect sexual maturity, mating behavior, gamete development, or reproductive physiology of *Eisenia fetida*.

Similarly, juvenile emergence was not significantly affected by treatment. The control group produced 483 juveniles, whereas treated groups produced between 455 and 492 juveniles. The comparable numbers of juveniles across all treatment levels indicate that embryonic development, cocoon viability, hatching success, and juvenile survival remained unaffected. These findings suggest that the test item does not impair reproductive success or early developmental processes in earthworms even at concentrations substantially higher than those likely to occur under normal environmental exposure conditions (**Figure 1**).

The present findings are consistent with previous reports regarding the environmental safety of compounds belonging to the Isoxazoline class. Camilo Romero *et al*. (2021) reported that Sarolaner, another Isoxazoline insecticide, did not adversely affect

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IJCSRR @ 2026
www.ijcsrr.org

earthworm growth or reproduction during a 30-day exposure period. The similarity between the present study and previous findings may indicate that Isoxazoline compounds generally exhibit low toxicity toward non-target soil invertebrates, particularly earthworms. This selective toxicity may be attributed to the mode of action of Isoxazolines, which primarily target ligand-gated chloride channels in arthropods, resulting in a greater affinity for insect nervous systems than for annelid species.

The current results also support information reported by the World Health Organization (WHO, 2025), which indicated that the $LC_{50}$ of Isocycloserum technical material in artificial soil exceeds 1000 mg/kg dry soil for *E. fetida*. The absence of mortality in the present study up to the highest tested concentration supports this classification of low acute toxicity. Furthermore, the WHO report identified a No Observed Effect Concentration (NOEC) of 10 mg Technical Isocycloserum/kg for chronic exposure. Although the present study tested concentrations considerably above this value, no statistically significant effects were observed on biomass, cocoon production, or juvenile emergence. These findings provide additional evidence supporting the low hazard potential of Isocycloserum toward earthworms under laboratory conditions.

From an ecological perspective, the lack of adverse effects on survival, growth, and reproduction suggests that Isocycloserum 9.2% w/w DC is unlikely to negatively impact earthworm populations when present in soil at environmentally relevant concentrations. Since earthworms contribute significantly to soil fertility and ecosystem functioning, preservation of their reproductive capacity is particularly important in evaluating environmental safety. The results therefore indicate a favorable ecotoxicological profile of the test substance toward this representative soil invertebrate species.

Nevertheless, certain limitations should be acknowledged. The study was conducted under controlled laboratory conditions using artificial soil and a single test species, *E. fetida*. Natural field environments are considerably more complex and may influence pesticide bioavailability, degradation, and interactions with soil characteristics such as pH, organic matter content, moisture, and microbial activity. Furthermore, information regarding the effects of Isocycloserum and other Isoxazoline compounds on additional soil organisms, including other members of the Lumbricidae family, soil arthropods, nematodes, and microbial communities, remains limited. Therefore, further investigations involving multiple soil organisms, field-based assessments, and long-term ecological studies would provide a more comprehensive understanding of the environmental fate and ecological safety of Isocycloserum.

## 6.0 CONCLUSION

The reproductive toxicity assessment of Isocycloserum 9.2% w/w DC in *Eisenia fetida* demonstrated no treatment related mortality, behavioural abnormalities at concentrations ranging from 50 to 1000 mg/kg dry artificial soil. Biomass changes observed in treated groups were comparable to those recorded in the control group, indicating no adverse effects on growth or physiological condition. Similarly, cocoon production and juvenile emergence were not significantly affected at any tested concentration, demonstrating the absence of reproductive toxicity.

These findings indicate that Isocycloserum 9.2% w/w DC exhibits very low toxicity toward earthworms under laboratory conditions and does not impair population-relevant reproductive endpoints. Results of the present investigation obtained with Isocycloserum 9.2% w/w DC are consistent with the findings of Camilo Romero *et al*., (2021), who reported that the Isoxazoline group insecticide Sarolaner did not affect the growth and reproduction of earthworms upto 30 days post-administration and also align with the World Health Organization (WHO 2025) report that the $LC_{50}$ of Isocycloserum technical is greater than 1000 mg/kg in dry artificial soil for the earthworm *Eisenia fetida*, and NOEC for chronic study was10 mg/kg.

Overall, the study demonstrates that Isocycloserum 9.2% w/w DC presents a low ecological risk to earthworms and is unlikely to adversely affect earthworm survival, growth, or reproductive performance in soil ecosystems under anticipated exposure conditions. However, additional studies involving other soil organisms and field conditions are recommended to further strengthen the environmental risk assessment of this compound.

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**Table 1. Mortality and Behavioural abnormalities of the Earthworms after Exposure to Isocycloserum 9.2% w/w DC (Range Finding Experiment)**

| Concentration (mg/kg dry artificial Soil) | Mortality on Day 7 (%)# | Mortality on Day 14 (%)# | Behavioural abnormalities on Day 7 & Day 14 (%) # |
|---|---|---|---|
| Control (Deionised water) | 0 | 0 | 0 |
| 50 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 |
| 250 | 0 | 0 | 0 |
| 500 | 0 | 0 | 0 |
| 750 | 0 | 0 | 0 |
| 1000 | 0 | 0 | 0 |

% mean of 4 replicates

**Table 2. Biomass Changes observation of the Earthworms after Exposure to Isocycloserum 9.2% w/w DC (Range Finding Experiment)**

| Concentration (mg/kg dry artificial Soil) | Biomass loss (%)# | Mortality # | Significance |
|---|---|---|---|
| Control (Deionised water) | -12.90 | 0 | - |
| 50 | -12.73 | 0 | - |
| 100 | -12.85 | 0 | - |
| 250 | -12.65 | 0 | - |
| 500 | -13.90 | 0 | - |
| 750 | -13.56 | 0 | - |
| 1000 | -14.02 | 0 | - |

# % mean of 4 replicates
- not significantly different compared to the control

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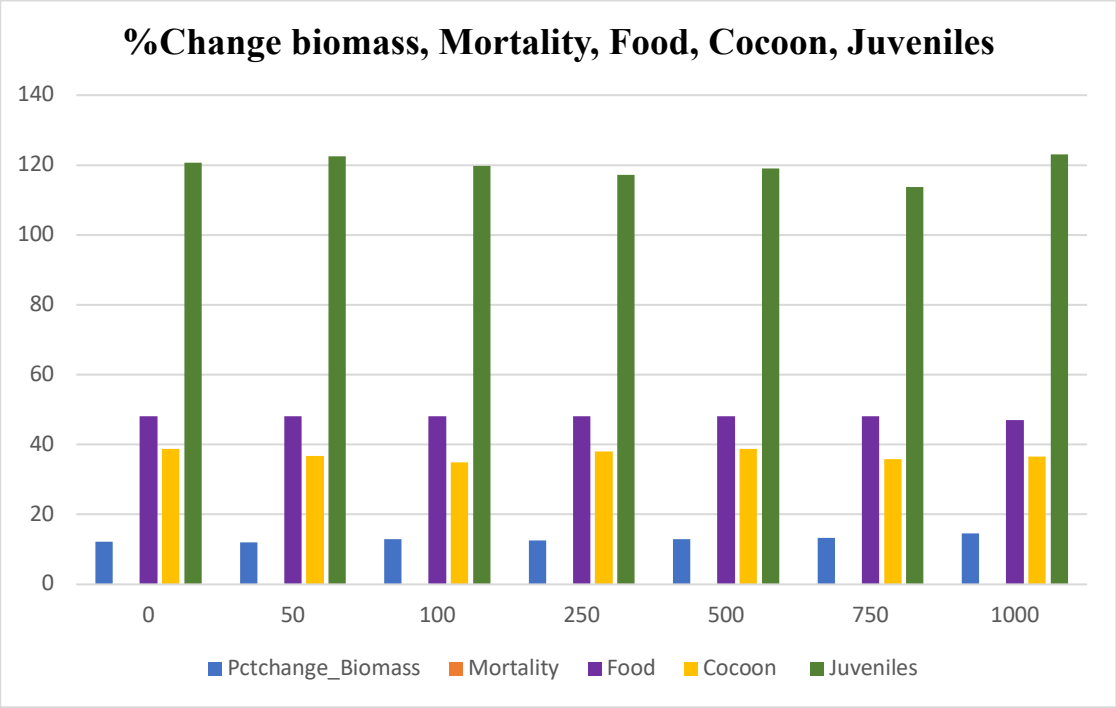
**Table 3. Mortality, Behavioural abnormalities and Biomass Changes of the Earthworms after Exposure to Isocycloserum 9.2% w/w DC (Main Experiment)**

| Concentration (mg/kg dry artificial Soil) | #Biomass loss (%) | #Mortality | #Cocoon Production | #Juvenile Emergence | Significance |
|---|---|---|---|---|---|
| Control (Deionised water) | -12.14 | 0 | 155 | 483 | - |
| 50 | -12.11 | 0 | 147 | 490 | - |
| 100 | -12.87 | 0 | 140 | 479 | - |
| 250 | -12.66 | 0 | 152 | 469 | - |
| 500 | -12.97 | 0 | 155 | 476 | - |
| 750 | -13.31 | 0 | 143 | 455 | - |
| 1000 | -14.61 | 0 | 150 | 492 | - |

# % mean of 4 replicates
- not significantly different compared to the control,
# Sum of four replicates

**Figure 1 : Graphical representation of % Biomass Change, Mortality, Food consumption, Coccon production and Juvenile emergence**



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**ACKNOWLEDGEMENTS**

Authors wish to thank to the Management of IIBAT, for their support, encouragement and providing the facility to conduct the experiment.

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***Cite this Article: Geetha, S., Chandrasehar, G., Newgilin Florence, F. (2026). Reproductive Toxicity Effect of Isocycloserum 9.2% w/w DC on Earthworm, Eisenia fetida. International Journal of Current Science Research and Review, 9(6), pp. 3314-3323. DOI: https://doi.org/10.47191/ijcsrr/V9-i6-37***