# Delineating the ecotoxic effects of Chlorpyrifos and Dimethoate on Eisenia fetida

**Vaibhav Srivastava**
University of Petroleum and Energy Studies
**Sinha Sahab**
BHU IESD: Banaras Hindu University Institute of Environment & Sustainable Development
**Rajeev Pratap Singh** ( rajeevprataps@gmail.com )
Instituite of Environment and Sustainable Development

**Research Article**

**Keywords:** Eisenia fetida, Dimethoate, Chlorpyrifos, Avoidance response, LC50, Acute and Chronic toxicity

**Posted Date:** April 21st, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1516313/v1

## Abstract

Acute (14 DOE) and chronic toxicity (56 DOE) experiments were conducted to compare the lethal and sub-lethal effects of chlorpyrifos and dimethoate on earthworms (*Eisenia fetida*). Earthworms were cultured in test soil (agricultural soils + cow dung) spiked with various concentrations of these two organophosphorus pesticides of different half-life. The determined effects were avoidance response, biomass change, growth inhibition, mortality, biomass gain per unit feed, cocoon and hatchling production. The resulted order of effects for acute and chronic toxicity exposure periods for *E. fetida* was dimethoate > chlorpyrifos. The reported median lethal concentration ($LC_{50}$) in present study was 17.45 and 228.06 mg $kg^{-1}$ dry test soil for dimethoate and chlorpyrifos, respectively. Growth inhibition was more critically influenced in dimethoate as compare to chlorpyrifos. Rather than lesser growth inhibition in chlorpyrifos, cocoon and hatchling production is influenced greatly at higher concentrations. The cocoon and hatchling production is significantly affected at lower concentrations of dimethoate. All the tested parameters were found to be dose dependent. The present results showed that even a small concentration of dimethoate is potentially dangerous to earthworms than higher concentrations of chlorpyrifos. In comparison the chlorpyrifos was found safer than dimethoate at sub-lethal concentrations for the development and reproduction of *E. fetida*. Our study provides a clear insight of different antagonistic effects of two organophosphate pesticides with different half-life.

## 1. Introduction

In the present scenario land use paradigms are shifting drastically worldwide, which is one of the major concerns followed by the burgeoning population. The availability of arable land resources is becoming scarce due to some of the burning environmental issues such as climate change, environmental pollution, globalization, industrialization, unsustainable development etc. (Suhani et al. 2020). Some of the major factors governing land resource deterioration includes, soil erosion, natural and man-made disasters, soil pollution (fertilizers, pesticides, heavy metals, etc.), and irrelevant extensive farming techniques. According to projections by 2050, the global population count will cross 9 billion and there will be demand to increase food supply by 70% to fulfill the global food demands (**Rattan 2015**). The projections raise a justified concern to the future food security. In agroecosystems, the growing crops needs a healthy soil-plant environment to attain its maximum growth and produce optimal yield. But the presence of plethora of environmental issues (biotic and abiotic stresses) diminishes the crop growth and yield (**Sahab et al. 2020**). Biotic stresses that include plant pathogens, diseases, pests and weeds, that are responsible for reducing 20–40% crop production and it cost the economy nearly US$ 290 billion annually on a global scale (Savary et al. 2019; **Gautham and Bhardwaj 2020**). Regarding the global crop production losses due to insects and pests, the use of pesticides is imperative to combat the rising food demand (Dasgupta et al. 2012).

In India, nearly 15–20% yield of principal food and cash crops lost to production apparently due to pests (Rathee and Dalal 2018; Kumar et al. 2021). Dealing with variety of pest and weed infestations, there are number of solutions available which majorly include chemical agents, popularly known as pesticides, insecticides, weedicides, herbicides, etc. Pesticides are the chemical compounds or mixture of chemicals or agents (either biological or man-made) which are mainly used for pest eradication purposes (Lackmann et al. 2021). Organophosphate pesticides (OPPs) are widely used because of their broad spectrum of activity and short residual time in comparison with persistent organochlorine pesticides (**Aswathi and Sukumaran 2019; Omwenga et al. 2020**). OPPs are well known acetylcholinesterase (AChE) inhibitors, it disrupts normal functions of nervous system (**Omwenga et al. 2020**). AChE is a cholinergic enzyme especially found in nerves and muscles, its inhibition can cause neurotoxicity, developmental impairments and alterations from lower to higher biological organization levels (Gravato et al. 2021). OPPs are broad spectrum pesticide which are used to control a variety of boring, chewing and sucking insects, spider, mites, aphides and pest that attacks crops like cotton, sugarcane, vegetables and fruits (Dong et al. 2020). Unfortunately, the extensive and unmannered use of OPPs is found hazardous to many non-target organisms residing in soil biota including earthworms (Dasgupta et al. 2012; Sahab et al. 2021). Earthworms live in a proximate contact with soil and constitute up to 90% of the invertebrate biomass depending on different soil conditions (**Gupta et al. 2011;** Kumar et al. 2020). Due to the remarkable abundance and vital contributions of earthworm in ecologically complex processes in soil biota (i.e., nutrient cycling, debris decomposition, soil formation), they can be well defined as an ecological engineer (Dasgupta et al. 2012; Sahab et al. 2021; Srivastava et al. 2021). Because of their higher proportion out of total invertebrate biomass they become victims of predation by organisms of different trophic level such as, mammals, reptiles, birds and amphibians (**Gupta et al. 2011;** Martinuzzi et al. 2020). Since many literatures reported about the ability of earthworms to accumulate pesticides and other contaminants, it renders a possible risk of contaminant transmission in higher trophic levels of ecosystems (Singh et al. 2020; Bošković et al. 2021).

In many literatures, the exposure of pesticides has been found to cause alteration in biomarkers at several levels of organizations in earthworm such as, cellular, biochemical, physiological and behavioral (Sanchez-Hernandez 2006; Reinecke and Reinecke 2007; **Morcillo et al. 2013;** Tiwari et al. 2016). Pesticide exposures are known to alter enzymatic functions and metabolism, reduce biomass, inhibit growth, affect reproduction, increase mortality and change behavioral responses in different species of earthworms (Rico et al. 2016; García-Gómez et al. 2019) (Fig. 1). There are numerous studies revealed reduction in gut enzymatic activities, imbalance in antioxidant enzyme levels, inhibition of AChE, carboxylesterase and other cholinesterase activities in earthworms due to pesticide exposure (Table 1). Histopathological studies revealed that acute and chronic exposures of pesticides produce morphological deformities such as damage to longitudinal and circular muscle layers, epidermal cells, cuticular membrane in earthworms (Saxena et al. 2014; Solé 2021).

Table 1
List of some biochemical and other activity responses of earthworms towards pesticides.

| **Pesticide and their group** | **Doses** | **Earthworm Species** | **Experimental duration** | **Tested biochemical and other activities** | **Importance of tested activities** | **Responses** | **Reference** |
|---|---|---|---|---|---|---|---|
| Chlorpyrifos and azinphos methyl (OPP) | Chlorpyrifos- 0 to 15µgkg$^{-1}$; azinphos methyl- 0 to 2.5 µgkg$^{-1}$ | *Aporrectodea caliginosa* | Chronic (6 months) | Cholinesterase activity | - | Cholinesterase inhibition | Reinecke and Reinecke 2007 |
| Lindane (organochlorine) and deltamethrin (pyrethroids) | Lindane- 20, 50, 80, 120, 150 mgkg$^{-1}$; Deltamethrin- 5, 25, 50, 100, 150 mgkg$^{-1}$ dry soil | *E. fetida* | Acute (14 day) and subchronic (42 day) | Cellulase activity | Cellulase plays important role in litter and other cellulosic plant material degradation | Lindane was found to increase cellulase activity in earthworms at both acute and subchronic test, while deltamethrin exerted effect only at acute toxicity bioassay. Deltamethrin decreased cellulase activity. | Shi et al. 2007 |
| Methomyl (Carbamate) | 0.86, 1.37, 2.19, 3.51, 5.62, 8.98, 14.4, 23 mgkg$^{-1}$ | *E. andrei* | Acute (48 h) | AChE activity | - | The inhibition of AChE activity is shown by *Eisenia andrei* in reponse to increasing concentration of methomyl. | Pereira et al. 2010 |
| Chlorpyrifos and fenvalerate (pyrethroid) | 10, 20, and 40 mgkg$^{-1}$ dry soil | *E. fetida* | Acute (7 day) | Enzyme activities (cellulase, SOD, CAT) | Cellulase is one of the digestive enzymes which is apparently important in organic matter degradation. SOD is an important component of the earthworms antioxidative-defense system. | The cellulase activity decreased with increasing chlorpyrifos and fenvalerate concentrations. SOD activity significantly inhibited in initial 3 days of experiment in case of both pesticides, while it gets stimulated with increasing exposure period. The CAT activity increased with increasing concentration and exposure period in case of fenvalerate. In case of chlorpyrifos, CAT activity increased in initial 3 days and decreased with increasing exposure period. | **Wang et al. 2012 a** |

| Pesticide and their group | Doses | Earthworm Species | Experimental duration | Tested biochemical and other activities | Importance of tested activities | Responses | Reference |
|---|---|---|---|---|---|---|---|
| Carbaryl (carbamate), chlorpyrifos, and endosulfan (organochlorine) | Carbaryl- 0, 0.75, 1.51, 3.03 $mgkg^{-1}$; chlorpyrifos- 0, 0.91, 1.82, 3.65 $mgkg^{-1}$ , and endosulfan- 0, 3.75, 7.50, 15.0 $\mu gkg^{-1}$ | *Perionyx excavatus* | Acute (28 day) and subchronic (56 day) | Rate of respiration | Respiration is an important indicator of metabolism and growth of earthworms. | $CO_2$ evolution is found to be increasing with increasing concentrations of pesticides. Highest $CO_2$ production is reported in chlorpyrifos and lowest in endosulfan bioassay. The increased respiration rate revealed the increased stress due to pesticide exposure. | Dasgupta et al. 2012 |
| Dimethoate (OPP) | 0.1, 0.5, 1, 2.5, 5, 7.5, 10, 15, 25, 50 µg $cm^{-2}$ | *Octolasion lacteum* and *E. andrei* | Acute (24 h) | AChE activity, carboxylesterase activity, efflux pump activity, CAT activity | Carboxylesterase is a hydrolytic enzyme which metabolizes xenobiotics (that contains thioester, ester, amide bonds). Its inhibition is considered to be a stoichiometric mechanism for reducing pesticide concentration at target sites. Efflux pump activity play a major role in removal of toxicants from cells. | AChE activity significantly inhibited in *O. lacteum* at lower concentration, while increased in *E. andrei*. Carboxylesterase activity was found to be significantly inhibited in *E. andrei*, while in *O. lacteum* a slight inhibition is seen in last two concentrations. Efflux pump activity was majorly affected in *E. andrei* at higher concentration of dimethoate. CAT activity is inhibited significantly in both species of earthworms. | Velki and Hackenberger 2012 |
| Chlorpyrifos (OPP) | 0.6, 3, 15 $mgkg^{-1}$ dry wt. | *Lumbricus terrestris* | Acute (72 h) | Esterase activities | - | ChE activity was significantly inhibited (upto 87%) at 48 h in all the concentrations used. AChE activity is not significantly inhibited. | **Morcillo et al. 2013** |

| Pesticide and their group | Doses | Earthworm Species | Experimental duration | Tested biochemical and other activities | Importance of tested activities | Responses | Reference |
|---|---|---|---|---|---|---|---|
| Dimethoate, and trichlorfon (OPP) | Dimethoate- 5, 11, 25, 57, 128 $mgkg^{-1}$; Trichlorfon- 33, 50, 75, 113, 169, 253 $mgkg^{-1}$ | *E. fetida* | Acute (14 day) | Histopathological examinations and biomarker analysis- cholinesterase (ChE), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activities. | Cholinesterase is an enzyme which is required in functioning of nervous system, it break down acetylcholine. LDH is a glycolytic enzyme found in nearly all tissues of earthworm. | The ChE and LDH activities are significantly inhibited by pesticides exposure at lower and higher concentrations. Both pesticides resulted in significant decrease in ALP activity in *E. fetida*. Histopathological assessment revealed that higher concentrations of both pesticides seriously damaged the longitudinal and circular muscular layers and affected epidermis. | Rico et al. 2016 |
| Dinotefuran (neonicotinoid) | Acute- 1, 2, 4, 6, 8, 10, 12, 14, 16, and 18 $mgkg^{-1}$; Subchronic- 0.1, 0.5, 1 and 2 mgkg-1 | *E. fetida* | Acute (14 day) and subchronic (28 day) | Antioxidant enzymes- superoxide dismutase (SOD), catalase (CAT), ROS ($OH^-$ radical), lipid peroxidation, DNA damage, Gene expression | $OH^-$ radical (ROS) play a major role in mediating $O_2$ toxicity (*in vivo*). SOD is an effective $O_2^-$ radicle remover. CAT enzyme is an important $H_2O_2$ scavenger. Lipid peroxidation is a process which disrupts cellular functions and was considered as a cell damaging process. | SOD activity increased with increasing pesticide concentrations. Higher concentrations enhanced oxidative stress inside *E. fetida* cell, as a result CAT activity also enhanced with time. The higher doses of dinotefuran induced lipid peroxidation in *E. fetida*. At 14th and 28th day 8-OHdG content has increased indicating that higher concentrations induced DNA damage. | Liu et al. 2018 |
| Chlorpyrifos (OPP) | 0, 20, 40, 80, 160, 320 $mgkg^{-1}$ | *E. andrei* | Subchronic (28 day) | Acetyl cholinesterase activity (AChE), Glutathione-S-transferase (GST), CAT activity, and MDA content | Alteration in MDA content and antioxidant enzyme activity show the degree of cell injury inside earthworms. | There is a significant reduction in AChE activity caused by chlorpyrifos exposure, which implicates the neurotoxicity of the pesticide. MDA content, CAT, and GST activities are decreased with increasing chlorpyrifos concentrations. | García-Gómez et al. 2019 |

In last few decades, chlorpyrifos (O, O -diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate) and dimethoate (O, O-dimethyl S-methylcarbamoylmethyl phosphorodithioate) insecticides are massively used in agriculture (Anwar et al. 2009; Dasgupta et al. 2012; Dong et al. 2020; Martinuzzi et al. 2020). Both OPP insecticides are non-persistent in nature and possess a lower half-life in environment. The half-life of dimethoate ranges from 4 to 16 days and that of chlorpyrifos ranges from 7 to 120 days in environment (Anwar et al. 2009; Srivastava et al. 2010; Yadav et al. 2020). In most of the European countries as well as in USA, chlorpyrifos and dimethoate pesticides are banned from the past few years (López-Dávila et al. 2021; Yadav et al. 2021).

As per our extensive literature review, we found that there is plenty of research works which comprises of avoidance response, acute and chronic toxicity of OPP pesticides on earthworms. In this study we have tried for a wholesome approach to provide a comparative study about the short and long term effects of two organophosphorus (OPP) insecticides of different persistence (half-lives) time on earthworms.

## 2. Materials And Methods

## 2.1. Procurement of earthworms

Earthworms were collected from vermicomposting unit of Institute of Environmental and Sustainable Development, Banaras Hindu University, India. The earthworm species, *Eisenia fetida* was chosen for the study because it was robust, easy to control and easily available. The specimens were brought to the laboratory 2 weeks prior to experiment in culture pots supplemented with finely ground soil and cow-dung (preconditioned) mixed in the ratio of 1:1 (Dasgupta et al. 2012). The culture pots were covered with lid (fine meshed iron net) and kept inside the BOD incubators at 28 ± 1°C and an optimal moisture content (50–60% moisture level) was maintained by adding double distilled water (DDW) into the culture medium (Dasgupta et al. 2012). Only the adult (having clitellum) and healthy earthworms of weight ranging between 400–500 mg were selected for the toxicity tests. Before the commencement of experiments, the earthworms were removed carefully from the culture, and rinsed with DDW, after that placed on blotting sheets to remove soil and litter particles. After surface cleaning the worms then placed in moistened filter paper for next 12 hours (h) to clear the food content present in gut of earthworms.

## 2.2. Procurement of chemicals and experimental containers

The insecticides used in the experiments were OPP insecticides, i.e. chlorpyrifos and dimethoate. Insecticides and acetone were of analytical reagent grade (98% purity) was ordered from Sigma-Aldrich, India. Plastic containers of two different size selected for the experiments, big containers (dimensions are 25cm × 17cm × 8cm) are subjected to avoidance response, acute and chronic toxicity assay and small containers (dimensions are 8cm × 4cm × 3cm) are selected for cocoon and hatchling study.

## 2.3. Test soil preparation and pesticide spiking

The upper layer soil (5cm) with no pesticide application history was used in experiments which were collected from the field of Institute of Environmental and Sustainable Development, Banaras Hindu University, India. 25 days preconditioned cow dung (CD) was procured from agricultural farm, Institute of Agricultural Sciences (IAS), BHU. The collected soil and CD were screened manually and air dried under shadow. After drying passed through a 10-mesh (2mm) sieve and stored for further use. The test soil (TS) was prepared by mixing sieved soil and CD in 3:1 ratio (Kavitha et al. 2020). General physico-chemical properties of prepared TS, was measured, pH and EC (electrical conductivity) was analyzed using water parametric analysis kit (Model 371, systronics, India), percent nitrogen using kjeldahl method (Khjeltron, tulin, India), and organic carbon was done by Walkley and Black method (Walkley and Black 1934). The physico-chemical characteristics of TS used in this study were mentioned in Table 2.

For desired concentrations, a particular amount of insecticides (active ingredient, a.i.) was dissolved in 10 ml of acetone (solvent). The dissolved solution was spiked and mixed thoroughly in 100 gm of dry TS. The spiked TS was placed overnight (12 h) under an exhaust hood to evaporate the solvent and then mixed meticulously with the desired amount of TS according to the different experimental assay. Similarly, the control TS was prepared by mixing only 10 ml acetone without insecticides.

Table 2
Physicochemical characteristics of test soil (TS) used in study

| Characteristic | Value |
|---|---|
| pH | 7.4 ± 0.2 |
| EC (mS $cm^{-1}$) | 1.27 ± 0.23 |
| Organic carbon (%) | 1.82 ± 0.30 |
| Organic matter (%) | 3.13 ± 0.52 |
| Nitrogen (%) | 0.38 ± 0.10 |
| Soil texture | Sandy loam (sand 57%, silt 20%, and clay 20%) |

## 2.4. Avoidance response test

Avoidance response test was designed following the recommendations of the ISO protocol (ISO 17512-1, 2008). The concentrations of insecticides used in the avoidance response test are mentioned in Table 3. Rectangular plastic containers (25cm × 17cm × 8cm) were divided into two equal sections with the help of a removable plexiglass divider. One half of the container was filled with 0.5 kg of insecticide spiked TS and another half was filled with 0.5 kg control TS (Bernardino et al. 2021). Then the plexiglass was removed, and 10 healthy earthworms with well-developed clitellum (weighing between 400 to 500 mg) were placed on the separating line of each test containers. To prevent the earthworms escaping from containers, it was covered with perforated plastic lid which was permeable to light and air. Then, the test containers were incubated for 48 h at 23 ± 2°C under continuous light exposure (Light intensity: 700–800 lux).

Table 3
Concentrations of the active ingredient (a.i.) of insecticides used in avoidance response test, acute and chronic toxicity.

| Active ingredient | Tested concentrations (mg $kg^{-1}$ dry soil of a.i.) | | |
|---|---|---|---|
| | Avoidance response test | Acute test | Chronic toxicity test |
| **Dimethoate** | 1; 5; 10; 25; 50 | 1; 5; 10; 15; 20; 50; 100 | 1; 5; 10; 15; 20 |
| **Chlorpyrifos** | 5; 10; 25; 50; 100 | 5; 10; 25; 50; 100; 175; 200; 300 | 5; 10; 25; 50; 100 |

After fulfillment of incubation period, the control and spiked TS were separated by introducing plexiglass divider and the number of alive earthworms was counted throughout each section. Earthworms which are getting divided due to introduction of plexiglass divider were counted as 0.5 independently to the remaining body length. Each insecticide was tested at varying concentrations with four replicates. To guarantee that the earthworms were distributed randomly throughout the container, a double-control (control-control) test was carried out in which control TS was placed in both compartments, in four replicates (Bernardino et al. 2021). The simplified experimental set up is explained diagrammatically in Fig. 2.

After fulfillment of 48 h of incubation period the avoidance response (%) was calculated following Rico et al. (2016), using the following equation:

**Avoidance response (%) = [** $\frac{(\mathbf{C}-\mathbf{T})}{\mathbf{N}} \times 100$**]**

Where, C is the numbers of earthworm present in the control TS containing section, T is the number of earthworm present in spiked TS containing section, and N is the total number of earthworms introduced in the test container. A positive (+ ve) value indicates avoidance of earthworms to spiked TS, a zero (0) represents no avoidance, and a negative (-ve) value indicates an attraction of earthworms towards spiked TS (Amorim et al. 2005).

## 2.5. Acute toxicity

The acute toxicity test was designed by following the protocol of ISO 11268-1 (ISO, 2012a). The TS selected for the acute toxicity test consists of sieved soil and cow dung in 3:1 ratio, for maintaining the same condition as agricultural fields (Kavitha et al., 2020). The information about the selected concentrations of insecticides for acute toxicity assay were detailed in Table 3. Rectangular plastic containers (25cm × 17cm × 8cm) were used for the acute toxicity test. Each container contains 0.5kg spiked TS (60% soil moisture) and 10 adult earthworms (with well-developed clitellum ranging between 400–500 mg weights). Additionally, a control with no pesticide spike was carried out. To prevent the worms from escaping, containers were covered with perforated plastic lid. The containers were kept in an incubation chamber (23 ± 1°C, 70–90% relative humidity with continuous illumination of 500–700 lux) throughout the 14 days of exposure (14 DOE). Three replicates were used for each dose and control.

After 14th DOE the number of dead earthworms is counted in each experimental container and mortality at various concentrations were determined. Earthworms were considered as dead if they don’t move and did not respond to any external stimulus. Because earthworms disintegrate very fast after death, they were considered dead if missing (Yang et al. 2015). The $LC_{50}$ values were calculated in the study to compare the acute lethal effects of chlorpyrifos and dimethoate separately on earthworms. For the calculation of $LC_{50}$ probit regression modeling was used (Shi et al. 2007; **ISO 2012a**). The test endpoint was mortality.

## 2.6. Chronic toxicity

### 2.6.1. Determination of growth inhibition ($GI_t$) and net biomass gain per gram feed ($NBGPGF_T$)

For determining the chronic effects of chlorpyrifos and dimethoate on growth attributes of *E. fetida*, bioassays were installed same as in the acute toxicity test, differed only in the experimental duration, concentrations used and assessment methods. According to acute toxicity test results, 5 sub-lethal concentrations of chlorpyrifos and dimethoate were selected for chronic toxicity test (Table 3). Ten adult earthworms (with developed clitellum) weighing between 400–500 mg were taken from the cultured earthworm stock. For voiding the gut content earthworms were placed on damp filter paper inside petri dishes and put in the dark at 23 ± 1°C. Before introduction, the earthworms were rinsed with DDW, blotted dry on filter paper and biomass of 10 earthworms was determined by weighing. Then they were released onto the soil surface of each experimental container containing 0.5kg of TS either spiked or control (feed matter). The test containers were maintained at 23 ± 1°C inside an incubator and to prevent earthworms from starvation a total of 5 g (dry weight) of finely sieved cow dung (moistened to 60% w/w) was added each week on the TS upper surface. Moisture loss from the test soil was checked by weighing the test containers at the weekly intervals and replenished by adding DDW if needed. Bioassay run for 56

DOE with three replicates used for each dose and control groups. The earthworms were weighed on the 0th, 7th, 14th, 21st, 28th, 35th, 42nd, 49th and 56th DOE to determine the change in biomass and weekly growth rates.

Mosleh et al. (2003) found growth rates as important biomarkers of toxicity of pesticides (endosulfan and aldicarb) to *Lumbricus terrestris*. The $GI_t$ was analyzed according to the increase or decrease in biomass of earthworms in each dose group at different exposure periods. The $GI_t$ was calculated following Shi et al., (2007), using this equation:

$$\textbf{Growth Inhibition \% (GI}_t\textbf{)} = \frac{(B_0 - B_t)}{W_0} \times 100,$$

Where $GI_t$ is the growth inhibition percentage for the dose or control groups at $t^{th}$ DOE, $B_0$ is the mean initial biomass of earthworms at start of the experiment and $W_t$ is the mean biomass of earthworms at time, t (DOE). Positive value of $GI_n$ indicates growth inhibition of earthworms due to experimental exposure and negative value represents increase in growth of earthworms.

For calculation of earthworm net biomass gain per gram feed ($NBGPGF_T$) at time (T), a formula was designed which is as follows:

$$\textbf{Net biomass gain per gram feed (NBGPGF}_T\textbf{)} = \frac{B_T - B_i}{W} \times N \text{ (mg/g)}$$

Where, $B_T$ is mean biomass of individuals at a time (T) (in mg), $B_i$ is mean initial biomass of individuals (in mg), $W$ is the total weight of feed mixture (in gram) and $N$ is the total number of individuals. Using this formula, the net biomass gain of earthworms per gram feed at 28th DOE ($NBGPGF_{28\ DOE}$) and 56th DOE ($NBGPGF_{56\ DOE}$) were calculated.

## 2.6.2. Determination of cocoon and hatchling production

Reproduction tests with *E. fetida* were carried out in accordance to the ISO 11268-2 guideline (**ISO 2012b**). The cocoon production determines the reproductive success of test specimens. For determination of cocoon and hatchling production, the spiked and control TS were examined carefully under a magnifying glass, every week throughout 56 DOE of chronic toxicity experiment. After every week cocoons and hatchlings were hand sorted and transferred in small experimental containers (8cm × 4cm × 3cm) containing same TS. At the last day of experiment, the survived hatchlings were rinsed with DDW, blotted with blotting paper, then weighted and biomass per hatchling were calculated.

## 2.7. Statistical significance

Statistical analysis was performed using the SPSS (version 16) software for windows program. Treatments were compared using analysis of variance (ANOVA) and Duncan's multiple ranged tests (DMRT) to test the significance of difference (at $P < 0.05$) between the treatments. The graphs were drawn using Sigma Plot version 10 software.

## 2.8. Chemicals and quality control

All the chemicals used in this study were of analytical grades and used without further purification. The analytical quality control was guaranteed through the use of laboratory quality assurance and quality control protocols and standards. Standard operating procedures were followed.

### 3. Results And Discussions

## 3.1. Avoidance response test

Avoidance response test is relatively a recent technique in terrestrial eco-toxicological assessments, offering rapid and cost-effective analysis of pesticide contamination in soil (Garcia et al. 2008; Bernardino et al. 2021). The data generated from avoidance response tests are helpful in defining acceptable concentration of pesticides in soil (Nunes and Espíndola 2012; Bernardino et al. 2021). In our study, the results of the test performed with the double control TS (control–control) showed that *E. fetida* were randomly distributed among both soil compartments. A significant avoidance response showed by *E. fetida* in TS spiked with higher concentrations of insecticides, except first two low concentrations. At first two low concentrations of insecticides, more earthworms were found in compartment containing spiked TS (Figs. 3 and 4). Apparently, *E. fetida* preferred the first two low concentrations of insecticides chlorpyrifos and dimethoate.

On the highest concentration (50 mg $kg^{-1}$ dry TS) of dimethoate, an avoidance of 93.33 ± 3.33% was observed. According to ISO guidelines (ISO, 2008), the concentrations that reported avoidance higher than 80% can be concluded as the pesticide concentration which caused reductions in habitat functions in the soil.

In the case of chlorpyrifos a significantly low avoidance response was reported as compared to dimethoate. At the highest concentration (100 mg $kg^{-1}$ dry TS) of chlorpyrifos, an avoidance of 60% was reported. Besides the higher concentrations (upto 100 mg $kg^{-1}$ dry TS) of chlorpyrifos used in the avoidance response study, *E. fetida* reacted more sensitively in dimethoate spiked TS than in chlorpyrifos spiked TS. The avoidance response for different earthworms varies with the differences in type and composition of feed matter and pesticide (Table 4).

Table 4
Avoidance responses of different earthworm species to different pesticides

| Test medium | Pesticide used | Concentrations | Earthworm Species | Avoidance response | Reference |
|---|---|---|---|---|---|
| OECD artificial soil | Chlorpyrifos (OPP) | 0, 5, 10, 20, 40, 60 $mgkg^{-1}$ | *E. fetida* | A significant avoidance response by *E. fetida* was found in last two higher concentrations. | **Zhou et al. 2007** |
| OECD artificial soil | Dimethoate (OPP) | 1, 3, 10, 100, 300, 900 $mgkg^{-1}$ dry soil | *E. andrei* | There was no significant avoidance was shown in first two concentrations. At 30 $mgkg^{-1}$ dimethoate there is 35% avoidance was shown and it increased to 85% at 100 $mgkg^{-1}$. | **De Silva and Amarasinghe 2008** |
| OECD artificial soil | Lambda-cyhalothrin (pyrethroid) | 0.32, 1, 3.16, 10, 31.6, 100 $mgkg^{-1}$ dry soil | *E. fetida* | *E. fetida* started avoiding Lambda-cyhalothrin from 3.16 $mgkg^{-1}$ concentration and showed 100% avoidance at pesticide concentration 10 $mgkg^{-1}$ dry OECD artificial soil. | Garcia et al. 2008 |
| Tropical artificial soil (TAS) | Lambda-cyhalothrin | 0.32, 1, 3.16, 10, 31.6, 100 $mgkg^{-1}$ dry soil | *E. fetida* | In TAS *E. fetida* started avoidance from the first lowest concentration. More than 60% avoidance reported at 0.32 $mgkg^{-1}$ concentration and it increased to 80% at 3.16 $mgkg^{-1}$. An avoidance of 100% reported at 31.6 $mgkg^{-1}$. | Garcia et al. 2008 |
| The European natural field soil (LUFA 2.2) | Lambda-cyhalothrin | 0.32, 1, 3.16, 10, 31.6, 100 $mgkg^{-1}$ dry soil | *E. fetida* | In LUFA soil, an avoidance of 40% is reported at lowest concentration (1 $mgkg^{-1}$), which increased to 70% at 1 $mgkg^{-1}$, 80% at 10 $mgkg^{-1}$, 90% at 31.6 $mgkg^{-1}$ and more than 90% at 100 $mgkg^{-1}$. | Garcia et al. 2008 |
| LUFA 2.2 | Methomyl (99.5% purity) | 0.86, 1.37, 2.19, 3.51, 5.62, 8.98, 14.4, 23 $mgkg^{-1}$ dry soil | *E. andrei* | An increasing net avoidance response was showed by E. andrei in response to increasing concentration of methomyl. | Pereira et al. 2010 |
| TAS | Imidacloprid (Gaucho® 600 FS), fipnoril (Standak® 250 SC), thiamtoxam (Cruiser® 350 FS), captan (Captan® 480 SC), carboxim + thiram (Vitavax® 200 SC) | Imidacloprid- 0.125, 0.25, 0.5, 1, 2 $mgkg^{-1}$; fipnoril- 0.1, 1.25, 2.5, 5, 10 $mgkg^{-1}$; thiamtoxam- 1.25, 2.5, 5, 10, 20 $mgkg^{-1}$; captan- 0.625, 1.87, 5.62, 16.87, 50.62 $mgkg^{-1}$; carboxim + thiram- 0.313, 1.25, 5, 20, 80 $mgkg^{-1}$ dry soil | *E. andrei* | There is no sign of avoidance shown towards fipnoril concentrations used in study, as earthworms are attracted towards this pesticide. Significantly higher avoidance was shown at lower concentrations of Imidacloprid, 100% at 1 $mgkg^{-1}$. An avoidance of 80% was shown at 50.62 $mgkg^{-1}$ for captan, 60% at 80 $mgkg^{-1}$ carboxim + thiram, and 20% at 10 $mgkg^{-1}$ thiamtoxam concentration. | Alves et al. 2013 |
| Agricultural soil | Chlorpyrifos | 0.6, 3, 15 $mgkg^{-1}$ dry soil | *Lumbricus terrestris* | There is no significant avoidance response was observed in *L. terrestris* at any concentration of chlorpyrifos used in study. | **Morcillo et al. 2013** |

## 3.2. Acute toxicity test

Acute toxicity test has been widely used to evaluate the short-term toxicity of pesticides to earthworms (**Zhou et al. 2007;** Cang et al. 2017; García-Gómez et al. 2019). Earthworm's mortality can be used as a predictable indicator of environmental pollution (Shi et al. 2007). The most probable end point measured when evaluating acute toxicity of chemicals to earthworms was $LC_{50}$ (the concentration that is lethal to 50% of individuals of a population). The endpoint "$LC_{50}$" shows effects at relatively high pesticide concentrations and indicates the maximum casualty likely to earthworms. According to Mosleh (2009**)**, those pesticides which have $LC_{50}$ value higher than 1000 mg $kg^{-1}$ were safe and harmless to earthworms in the agricultural field.

Table 5
Previous studies from different countries on the effects of pesticides on earthworm

| Country | Test medium | Pesticide used | Concentrations | Earthworm Species | $LC_{50}$ value | Response | Reference |
|---|---|---|---|---|---|---|---|
| The Netherlands | OECD artificial soil (characteristics- 70% fine sand, 20% kaolinite clay and 10% ground sphagnum peat), Clayey soil, Humus soil | Dimethoate (97–99% purity) | 0, 3, 9, 27, 81, and 243 $mgkg^{-1}$ | *Aporrectodea caliginosa tuberculata* | In OECD artificial soil- 56 $mgkg^{-1}$; Clayey soil- 40 $mgkg^{-1}$, and Humus soil- 65 $mgkg^{-1}$ | The biomass changes and $LC_{50}$ values are found different in different soil type. The soil $CO_2$ production was found increased with increasing dimethoate concentration. | **Martikainen 1996** |
| India | Black soil and Cow dung (3:1) | Dimethoate (Rogor 30 EC) | 0.4, 1.6 $mgkg^{-1}$ dry soil | *E. fetida* | - | Application of dimethoate showed negative impact on biomass and reproductive output of earthworms. | **Yasmin and D'Souza. 2007** |
| China | OECD artificial soil | Lindane and deltamethrin | Lindane- 20, 50, 80, 120, 150 $mgkg^{-1}$; Deltamethrin- 5, 25, 50, 100, 150 $mgkg^{-1}$ dry soil | *E. fetida* | Lindane- 162.1 $mgkg^{-1}$; Deltamethrin- 432.9 $mgkg^{-1}$ (14 day) | Both the pesticides exerted significant effects on cellulase activity and growth of *E. fetida*. | Shi et al. 2007 |
| China | OECD artificial soil | Chlorpyrifos | 75, 90, 100, 125, 150, 200 $mgkg^{-1}$ dry soil | *E. fetida* | At 14 day- 118.5 $mgkg^{-1}$, and 21 day- 91.78 $mgkg^{-1}$ | The biomass of *E. fetida* was significantly decreased with increasing concentration of chlorpyrifos. The decrease in biomass, cocoon number, and cocoon viability is seen to be dose dependent. | **Zhou et al. 2007** |
| Germany | OECD artificial soil, Tropical artificial soil (TAS), The European natural field soil (LUFA 2.2) | Lambda-cyhalothrin | 0.32, 1, 3.16,10, 31.6, 100 $mgkg^{-1}$ dry soil | *E. fetida* | In OECD soil- 99.8 $mgkg^{-1}$, TAS- 23.9 $mgkg^{-1}$, and LUFA- 139.9 $mgkg^{-1}$. | Different soil composition resulted in different $LC_{50}$ values. | Garcia et al. 2008 |
| Germany | OECD artificial soil, TAS, LUFA 2.2 | Carbendazim | 1, 3.2, 10, 31.6, 100, 316, 1000 $mgkg^{-1}$ dry soil | *E. fetida* | In OECD soil- 5.8 $mgkg^{-1}$, TAS- >1000 $mgkg^{-1}$, and LUFA- 4.1 $mgkg^{-1}$. | Different soil composition resulted in different $LC_{50}$ values. | Garcia et al. 2008 |
| Germany | OECD artificial soil, TAS, LUFA 2.2 | Benomyl | 1, 3.2, 10, 31.6, 100, 316, 1000 $mgkg^{-1}$ dry soil | *E. fetida* | In OECD soil- 22 $mgkg^{-1}$, TAS- 633 $mgkg^{-1}$, and LUFA- 14.6 $mgkg^{-1}$. | Different values of $LC_{50}$ in different soil type resulted due to different composition and characteristics of soil. | Garcia et al. 2008 |
| India | Grassland soil and FYM (1:1) | Chlorpyrifos (20 EC) | 5.75, 11.5, 17.25, 23 $mgkg^{-1}$ dry soil | *E. fetida* | 28.58 mg $kg^{-1}$ (96 h) | Chlorpyrifos found moderately toxic to *E. fetida* and environmentally less hazardous than other insecticides such as, endosulfan, carbamates, and cypermethrin. | **Gupta et al. 2011** |

| Country | Test medium | Pesticide used | Concentrations | Earthworm Species | $LC_{50}$ value | Response | Reference |
|---|---|---|---|---|---|---|---|
| India | Grassland soil and FYM (1:1) | Chlorpyrifos (20 EC) | 0.91, 1.82, 3.65 $mgkg^{-1}$ dry soil | *Perionyx excavats* (Perrier) | 7.3 $mgkg^{-1}$ | Highest concentration of chlorpyrifos rendered 24.84% hatching reduction. A slight decrease in cocoon number, hatching success and body weight reported with no mortality of earthworms. | Dasgupta et al. 2012 |
| China | Filter paper test | Chlorpyrifos (97% pure) | 0.1, 1.0, 10, 100, 1000 μg $cm^{-2}$ | *E. fetida* | 14.19 μg $cm^{-2}$ (48 h) | - | **Wang et al. 2012 b** |
| China | OECD artificial soil | Chlorpyrifos (97% pure) | 0.1, 1.0, 10, 100, 1000 $mgkg^{-1}$ dry soil | *E. fetida* | 7-day (421.3 mg $kg^{-1}$) and 14-day (384.9 mg $kg^{-1}$) | The $LC_{50}$ value of chlorpyrifos showed that it is less toxic to the earthworms in soil as compared to other pesticides. | **Wang et al. 2012 b** |
| Croatia | Filter paper test | Dimethoate (Chromgor 40) | 0.1,<br>0.5, 1, 2.5, 5, 7.5, 10, 15, 25, 50 μg $cm^{-2}$ | *Octolasion lacteum* and *E. andrei* | *O. lacteum* (1.98 ± 0.25 μg $cm^{-2}$) and *E. Andrei* (10.36 ± 1.39 μg $cm^{-2}$) | - | Velki and Hackenberger 2012 |
| Brazil | TAS | Imidacloprid (Gaucho® 600 FS), fipnoril (Standak® 250 SC), thiamtoxam (Cruiser® 350 FS), captan (Captan® 480 SC), carboxim + thiram (Vitavax® 200 SC) | Imidacloprid- 6.25, 12.5, 25, 50, 100 $mgkg^{-1}$; fipnoril- 62.5,125,250, 500, 1000 $mgkg^{-1}$; thiamtoxam- 62.5,125,250, 500, 1000 $mgkg^{-1}$; captan- 62.5,125,250, 500, 1000 $mgkg^{-1}$; carboxim + thiram- 62.5,125,250, 500, 1000 $mgkg^{-1}$ | *E. andrei* | Imidacloprid- 25.53 $mgkg^{-1}$, fipnoril- >1000 $mgkg^{-1}$, thiamtoxam- >1000 $mgkg^{-1}$, captan- >1000 $mgkg^{-1}$, carboxim + thiram- >1000 $mgkg^{-1}$ | At tested concentrations only imidacloprid caused mortality in earthworms. Except fipronil, all other pesticides induced negative effect on E. andrei in chronic toxicity and avoidance test. | Alves et al. 2013 |
| India | Laterite type soil and Cow dung (9:1) | Dimethoate (Rogor 30% E.C.) | 1,2,3,5,7,9,11,13,15,17 $mgkg^{-1}$ dry soil | *Drawida wilsi* (Michaelsen) | LC50 for adult, immature and juvenile worms were 9.0, 6.7, and 5.5 $mgkg^{-1}$ (96 h) | Mortality started at 3 $mgkg^{-1}$ and 100% mortality was achieved at 17 $mgkg^{-1}$ dry soil. | **Bhattacharya and Sahu. 2013** |
| Spain | OECD artificial soil | Dimethoate (Citan 40 EC) | 5, 11, 25, 57, 128 $mgkg^{-1}$ dry soil | *E. fetida* | 28 mg $kg^{-1}$ (14 day) | The insecticide dimethoate showed higher toxicity than other tested insecticides such as, prochloraz, trichlorfon, and tebuconazole. | Rico et al. 2016 |
| China | Filter paper contact test | Chlorpyrifos (96% technical grade) | - | *E. fetida* | LC50 at 24 h and 48 h were 5654 mg a.i. $L^{-1}$ and 1015 mg a.i. $L^{-1}$ respectively | Based on 24 h $LC_{50}$ results authors conclude that chlorpyrifos was 968.2 times less toxic than insecticide imidacloprid. | Cang et al. 2017 |

| Country | Test medium | Pesticide used | Concentrations | Earthworm Species | $LC_{50}$ value | Response | Reference |
|---|---|---|---|---|---|---|---|
| China | OECD artificial soil | Chlorpyrifos (96% technical grade) | - | *E. fetida* | At 7-day (421.3 $mgkg^{-1}$) and 14-day (384.9 $mgkg^{-1}$) | Based on 14 day $LC_{50}$ results authors conclude that chlorpyrifos was 137.5 times less toxic than imidacloprid. | Cang et al. 2017 |
| Spain | Agricultural soil | Chlorpyrifos (FOSTAN, a commercial product containing 480 $gL^{-1}$) | 20, 40, 80, 160 or 320 $mgkg^{-1}$ soil | *E. fetida* | 148 $mgkg^{-1}$ dry soil | Chlorpyrifos resulted in reduction of acetylcholinesterase (AChE) activity, which revealed the neurotoxicity of this insecticide. | García-Gómez et al. 2019 |

In present study, the toxicity of dimethoate on *E. fetida* after 14 DOE was higher than chlorpyrifos. The $LC_{50}$ (14 DOE) of dimethoate and chlorpyrifos were 17.45 and 228.06 mg $kg^{-1}$ dry TS, respectively. Dimethoate caused physiological and morphological alterations in earthworms at higher concentrations (15 and 20 mg $kg^{-1}$ dry TS), surviving worms had ruptured skin surface, deformed body and decreased body weight. Rico et al. (2016), found that high concentration of dimethoate and trichlorfon insecticides resulted in internal impairment and some serious damage to longitudinal and circular muscle-layers of *E. fetida*. Saxena et al. (2014), reported the disintegration of muscle layers, epidermal cells, cuticular membrane thus leading to bleeding, fragmentation and loss in typical architecture of body wall in earthworms *Metaphire posthuma* and *E. fetida* exposed to higher concentration of pesticides, carbofuran, carbaryl, fenvalerate and cypermethrin. The calculated $LC_{50}$ values in this acute toxicity test were not similar to those reported elsewhere in the literature. As Rico et al. (2016), reported $LC_{50}$ value of 28 mg $kg^{-1}$ after 14 DOE to dimethoate in OECD soil. Wang et al. (**2012 b**), reported $LC_{50}$ (14 DOE) of 384.9 mg $kg^{-1}$, whereas Zhou et al. (2007), reported $LC_{50}$ (14 DOE) of 118.5 mg $kg^{-1}$ for chlorpyrifos using *E. fetida* in OECD soil. According to **De Silva and Amarasinghe (2008)**, dimethoate became more toxic for earthworms in natural soils rather than OECD soil. In the study by Zhang et al. (1985), feed matter is positively correlated with the $LC_{50}$ value of pesticides. Previous studies give a clear insight that the $LC_{50}$ values varies with the type and composition of feed matter, pesticides and earthworm species (Table 5). Difference observed in the $LC_{50}$ values in our study and previous studies may be because of the difference in composition of TS (feed matter) used.

# 3.3. Chronic toxicity test

## 3.3.1. $GI_t$

$GI_t$ of earthworms at 7th, 14th, 21st, 28th , 35th, 42nd, 49th and 56th DOE for dimethoate and chlorpyrifos were shown in Figs. 4 and 5. For controls, the $GI_t$ value was negative, with mean earthworms biomass increasing throughout the exposure period 0 to 56th DOE. Negative values of $GI_t$ indicates the increase in growth of earthworm biomass (Shi et al. 2007).

$GI_t$ of the earthworms exposed to dimethoate spiked TS were significantly higher than chlorpyrifos spiked TS as well as controls (Figs. 5 and 6). For concentrations upto 5 mg $kg^{-1}$ dry TS of dimethoate, negative value of $GI_t$ was observed. This indicates that exposure of concentration level upto 5 mg $kg^{-1}$ dry soil of dimethoate in agricultural field soils was safer for the biomass growth of *E. fetida.* The $GI_t$ started at 10 mg $kg^{-1}$ dry TS of dimethoate was observed with a significant increase till 35th DOE. A significantly higher $GI_t$ was reported at last two concentrations of dimethoate i.e., 15 and 20 mg $kg^{-1}$ dry TS (Fig. 5). This sharp decrease in biomass might be due to the higher toxicity, residual nature of pesticides and decrease in feed matter with time. The $GI_t$ at 20 mg $kg^{-1}$ dry TS of dimethoate found to be highly harmful for the biomass growth, this may be due to its lower $LC_{50}$ value (17.45 mg $kg^{-1}$ dry TS) observed in the study. Reduction in growth of *E. fetida* at sub-lethal concentrations of insecticides has been observed when exposed to carbendazim, glyphosphate and dimethoate (**Yasmin and D'souza. 2007**).

In our study, chlorpyrifos was found capable of retarding the growth of *E. fetida* only at two highest concentrations i.e. 50 and 100 mg $kg^{-1}$ dry TS (Fig. 6). $GI_t$ at concentrations upto 25 mg $kg^{-1}$ dry TS was observed to be negative indicating increase in biomass. The growth was observed reducing manner upto 28th DOE at 50 mg $kg^{-1}$ dry TS, and followed an increase from 35th to 56th DOE. Biomass changes can be a good indicator of chemical stress, which can be linked to the chemical effects in energy dynamics (Rico et al. 2016). The slight increase in biomass of control earthworms suggested that the soil nutrients were just sufficient to sustain the survival of earthworms (Fig. 7). Dimethoate produced most severe effects on the growth after exposure to concentration greater than 5 mg $kg^{-1}$ dry TS, while earthworms affected least after exposure to 50 mg $kg^{-1}$ dry TS of chlorpyrifos. **Gupta et al. (2010)**, also reported that chlorpyrifos and monocrotophos were moderately toxic to *E. fetida* and environmentally less hazardous than the carbamates, endosulfan and cypermethrin. Reductions in growth of earthworms by sub-lethal concentrations of insecticides have been observed in *E. fetida* exposed to carbendazim, glyphosphate and dimethoate (Yasmin and D'Souza., 2007). Changes in biomass through progression of experimental duration for both insecticides have shown in Fig. 7.

### 3.3.2. $NBGPGF_T$

A significant reduction in $NBGPGF_T$ was found in earthworms feeding on TS spiked with dimethoate (Fig. 8A). The negative values depict the loss in $NBGPGF_T$ due to the exposure to higher concentration of insecticides. A sharp reduction was observed in the last three concentrations i.e. 10, 15 and 20 mg $kg^{-1}$ dry TS, with negative values of $NBGPGF_T$. This sharp decrease in $NBGPGF_T$ indicate the extent of toxicity of dimethoate to *E. fetida*.

$NBGPGF_{56\ DOE}$ was found greater than $NBGPGF_{28\ DOE}$ for concentrations upto 10 mg $kg^{-1}$ dry TS and 50 mg $kg^{-1}$ dry TS for dimethoate and chlorpyrifos, respectively. A sharp decrease in $NBGPGF_T$ was observed in last two sub-lethal concentrations of chlorpyrifos (50 and 100 mg $kg^{-1}$ dry TS) (Fig. 8B). The decrease in $NBGPGF_T$ is found to be dose dependent for both insecticides. Garg and Gupta (2011**)** and Daam et al. (2020) reported that the rate of net biomass gain per gram feed in *E. fetida* was mainly depend on available feed matter (substrate composition and soil quality) and its population density. In the present study it was observed that $NBGPGF_T$ of *E. fetida* was higher in chlorpyrifos in comparison with dimethoate spiked TS.

### 3.3.3. Cocoon production

The number of cocoons and hatchlings produced in control and different treatments of the two insecticides have been presented in Fig. 9. The cocoon production started from the 14th DOE in both control and dose groups. The number of cocoon at concentration 1 mg $kg^{-1}$ dry TS of the dimethoate was approximately equal to that of controls and there was slight increase at 5 mg $kg^{-1}$ dry TS as compared to controls. At concentrations above 5 mg $kg^{-1}$ dry TS of dimethoate, it was observed that cocoon production was significantly reduced as compared to controls (Fig. 9A**)**. The results indicated that the reproductive performance of *E. fetida* was significantly affected in concentrations higher than 5 mg $kg^{-1}$ dry TS dimethoate. The reduction in cocoon production is found to be dose dependent. The cocoon production was negatively affected in the last two concentrations i.e. 15 and 20 mg $kg^{-1}$ dry TS.

In case of chlorpyrifos, there was a reducing trend observed in the cocoon production with increasing concentration (Fig. 9B). The cocoon production started from the 2nd week of DOE as same in dimethoate treatment. The mean number of cocoon produced at 5 mg $kg^{-1}$ dry TS was found to be near about the controls. This indicates the concentration below 5 mg $kg^{-1}$ of chlorpyrifos was safer for reproductive performance of *E. fetida*. The cocoon production got severely reduced at highest concentration i.e. 100 mg $kg^{-1}$ dry TS of chlorpyrifos. A comparatively lower decrease in cocoon number was found at sub-lethal concentrations of chlorpyrifos than dimethoate. However, the selected concentrations of dimethoate (upto 20 mg $kg^{-1}$ dry TS) was lower than the chlorpyrifos (upto 100 mg $kg^{-1}$ dry TS), this indicates that dimethoate is more harmful for *E. fetida* than chlorpyrifos at sub-lethal concentrations.

### 3.3.4. Hatchling production

Hatchling production is adversely affected with increasing concentrations for both insecticides, while dimethoate produced the most severe effects on the hatchling production of *E. fetida.* A significant decrease was observed in hatchlings production at the lowest concentration (1 mg $kg^{-1}$ dry TS) of dimethoate as compared to controls (Fig. 10A). Average weight of hatchlings was significantly decreased with increasing concentration of dimethoate as compared to control (Fig. 10A). The results suggests that reproductive performance of *E. fetida* was negatively influenced even at the lowest concentration of dimethoate. In case of chlorpyrifos, there was slight decrease in hatchling production with increase in concentration (Fig. 10B). Major reduction in hatchling production was found at the concentration (100 mg $kg^{-1}$ dry TS). At 5 and 10 mg $kg^{-1}$ dry TS, the average weight of hatchlings was almost equal to controls. Whereas, at higher concentrations i.e. 25, 50 and 100 mg $kg^{-1}$ dry TS, there was slight decrease in the average weight of hatchlings as compared to controls.

The obtained results suggest that dimethoate is more lethal than chlorpyrifos for *E. fetida*. This might be due to the high stress of dimethoate than chlorpyrifos on reproductive system of *E. fetida*. Reports from earlier studies have shown effects on reproductive system, histological disorders in spermatheca, alteration of cell proliferation in seminal vesicle and DNA fragmentation in spermatogonia as a result of sub-lethal toxicity of OPP insecticides causing anomalous reproduction in *E. fetida* (Bustos-Obregón and Goicochea 2002; **Espinoza-Navarro and Bustos-Obregón 2005;** Zou et al. 2018; Zhao et al. 2021).

## 4. Conclusion

Our study provides a clear insight of different antagonistic effects of two OPP insecticides with different half-life. The avoidance response at highest concentrations of dimethoate (50 mg $kg^{-1}$ dry TS) was 93.33 ± 3.33% and for chlorpyrifos (100 mg $kg^{-1}$ dry TS) it was 60%. The resulted $LC_{50}$ values at 14 DOE for dimethoate and chlorpyrifos was 17.45 and 228.06 mg $kg^{-1}$ dry TS, respectively. Acute toxicity experiments show the level of toxicity of dimethoate is approximately 13 times higher than that of chlorpyrifos. A significantly higher $GI_t$ was reported at 10, 15 and 20 mg $kg^{-1}$ dry TS concentration of dimethoate and for chlorpyrifos the growth retarded at two highest concentrations i.e. 50 and 100 mg $kg^{-1}$ dry TS. The results of cocoon and hatchlings production strongly suggests that even through the population density not immediately affected on exposures to sub-lethal concentrations but prolonged exposure might result in reproductive changes that ultimately reduces population density of *E. fetida*. In comparison the chlorpyrifos was safer than dimethoate at sub-lethal concentrations for the development and reproduction of *E. fetida*.

# Declarations

**Acknowledgement**

The authors are grateful to the Dean & Head, Department of Environment and Sustainable Development and Director, Institute of Environment and Sustainable Development, Banaras Hindu University, for providing institutional support. Rajeev Pratap Singh is grateful to the authorities of Banaras Hindu University, Varanasi for providing support under the Institute of Excellence (IOE) scheme under Dev scheme No. 6031.

**Competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Ethical Approval:** Not Applicable

**Consent to Participate:** Not Applicable

**Consent to Publish:** Not Applicable

**Data Availability:** All data generated or analyzed during this study are included in this published article.

**CRediT roles**

**Vaibhav Srivastava:** Conceptualization, visualization, data analysis, illustrations, writing original draft, review & editing

**Sinha Sahab:** Visualization, investigation, data analysis, illustrations, writing original draft, review & editing

**Rajeev Pratap Singh:** Conceptualization, visualization, review & editing, resources, supervision, funding acquisition

# References

1. Alves PRL, Cardoso EJBN, Martines AM et al (2013) Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions. Chemosphere. https://doi.org/10.1016/j.chemosphere.2012.11.046
2. Amorim MJB, Römbke J, Soares AMVM (2005) Avoidance behaviour of Enchytraeus albidus: Effects of Benomyl, Carbendazim, phenmedipham and different soil types. https://doi.org/10.1016/j.chemosphere.2005.01.057. Chemosphere
3. Anwar S, Liaquat F, Khan QM et al (2009) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by Bacillus pumilus strain C2A1. J Hazard Mater. https://doi.org/10.1016/j.jhazmat.2009.02.059
4. Aswathi A, Pandey A, Sukumaran RK (2019) Rapid degradation of the organophosphate pesticide – Chlorpyrifos by a novel strain of Pseudomonas nitroreducens AR-3. Bioresour Technol. https://doi.org/10.1016/j.biortech.2019.122025
5. Bernardino MM, Alves PRL, de Santo FB et al (2021) Ecotoxicity of imidacloprid to soil invertebrates in two tropical soils with contrasting texture. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-021-12562-0
6. Bhattacharya A, Sahu SK (2013) Acute toxicity of dimethoate on soil health: A study of its impact on earthworm. Int J Biosci. https://doi.org/10.12692/ijb/3.7.98-106
7. Bošković N, Bílková Z, Šudoma M et al (2021) Conazole fungicides epoxiconazole and tebuconazole in biochar amended soils: Degradation and bioaccumulation in earthworms. https://doi.org/10.1016/j.chemosphere.2021.129700. Chemosphere
8. Bustos-Obregón E, Goicochea RI (2002) Pesticide soil contamination mainly affects earthworm male reproductive parameters. Asian J Androl 4(3):195–200
9. Cang T, Dai D, Yang G et al (2017) Combined toxicity of imidacloprid and three insecticides to the earthworm, Eisenia fetida (Annelida, Oligochaeta). Environ Sci Pollut Res. https://doi.org/10.1007/s11356-017-8627-z
10. Daam MA, Garcia MV, Scheffczyk A, Römbke J (2020) Acute and chronic toxicity of the fungicide carbendazim to the earthworm Eisenia fetida under tropical versus temperate laboratory conditions. https://doi.org/10.1016/j.chemosphere.2020.126871. Chemosphere
11. Dasgupta R, Chakravorty PP, Kaviraj A (2012) Effects of carbaryl, chlorpyrifos and endosulfan on growth, reproduction and respiration of tropical epigeic earthworm, Perionyx excavatus (Perrier). J Environ Sci Heal - Part B Pestic Food. https://doi.org/10.1080/03601234.2012.616771. Contam Agric Wastes
12. Dong Y, Wang Y, Zhu Q et al (2020) Dimethoate blocks pubertal differentiation of Leydig cells in rats. https://doi.org/10.1016/j.chemosphere.2019.125036. Chemosphere
13. Espinoza-Navarro O, Bustos-Obregón E (2005) Effect of malathion on the male reproductive organs of earthworms, Eisenia foetida. Asian J Androl. https://doi.org/10.1111/j.1745-7262.2005.00005.x
14. Garcia M, Römbke J, de Brito MT, Scheffczyk A (2008) Effects of three pesticides on the avoidance behavior of earthworms in laboratory tests performed under temperate and tropical conditions. Environ Pollut. https://doi.org/10.1016/j.envpol.2007.08.007

15. García-Gómez C, Babín M, García S et al (2019) Joint effects of zinc oxide nanoparticles and chlorpyrifos on the reproduction and cellular stress responses of the earthworm Eisenia andrei. Sci Total Environ. https://doi.org/10.1016/j.scitotenv.2019.06.083
16. Garg VK, Gupta R (2011) Optimization of cow dung spiked pre-consumer processing vegetable waste for vermicomposting using Eisenia fetida. Ecotoxicol Environ Saf. https://doi.org/10.1016/j.ecoenv.2010.09.015
17. Gautam HR, Bhardwaj ML (2020) International Year of Plant Health 2020 to focus on threat of pests on food security. Curr Sci 118:857
18. Gravato C, Abe FR, de Oliveira DP et al (2021) Acetylcholinesterase (AChE) Activity in Embryos of Zebrafish. In: Methods in Molecular Biology. https://doi.org/10.1007/978-1-0716-1091-6_10
19. Das Gupta R, Chakravorty PP, Kaviraj A (2010) Studies on relative toxicities of six insecticides on epigeic earthworm, perionyx excavatus. Bull Environ Contam Toxicol. https://doi.org/10.1007/s00128-010-0038-5
20. Das Gupta R, Chakravorty PP, Kaviraj A (2011) Susceptibility of epigeic earthworm Eisenia fetida to agricultural application of six insecticides. https://doi.org/10.1016/j.chemosphere.2011.03.016. Chemosphere
21. ISO 11268-1 (2012a) Soil quality - Effects of pollutants on earthworms - Part 1: Determination of acute toxicity to *Eisenia fetida/ Eisenia andrei*
22. ISO 11268-2 (2012b) Soil quality - Effects of pollutants on earthworms - Part 2: Determination of effects on reproduction of *Eisenia fetida/ Eisenia andrei*
23. ISO 17512-1 (2008) Soil Quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*)
24. Kavitha V, Anandhan R, Alharbi NS et al (2020) Impact of pesticide monocrotophos on microbial populations and histology of intestine in the Indian earthworm Lampito mauritii (Kinberg). https://doi.org/10.1016/j.micpath.2019.103893. Microb Pathog
25. Kumar J, Murali-Baskaran RK, Jain SK et al (2021) Biotic stresses of agricultural crops in India: re-visiting national status and mitigation strategies. Curr Sci 120(2):264
26. Kumar R, Sharma P, Gupta RK et al (2020) Earthworms for Eco-friendly Resource Efficient Agriculture. Resources Use Efficiency in Agriculture. Springer, Singapore, pp 47–84. https://doi.org/10.1007/978-981-15-6953-1_2
27. Lackmann C, Velki M, Bjedov D et al (2021) Commercial preparations of pesticides exert higher toxicity and cause changes at subcellular level in earthworm Eisenia andrei. Environ Sci Eur. https://doi.org/10.1186/s12302-021-00455-5
28. Liu T, Zhang X, Wang X et al (2018) Comparative toxicity and bioaccumulation of two dinotefuran metabolites, UF and DN, in earthworms (Eisenia fetida). Environ Pollut. https://doi.org/10.1016/j.envpol.2017.12.007
29. López-Dávila E, Houbraken M, De Rop J et al (2021) Pesticide traces in local crops of Sancti Spíritus, Cuba: risk assessment study. Int J Food Contam. https://doi.org/10.1186/s40550-021-00081-2
30. Martinuzzi CS, Attademo AM, Peltzer PM et al (2020) Comparative Toxicity of Two Different Dimethoate Formulations in the Common Toad (Rhinella arenarum) Tadpoles. https://doi.org/10.1007/s00128-019-02741-8. Bull Environ Contam Toxicol
31. Mosleh YYI (2009) Assessing the toxicity of herbicide isoproturon on Aporrectodea caliginosa (Oligochaeta) and its fate in soil ecosystem. Environ Toxicol. https://doi.org/10.1002/tox.20437
32. Martínez Morcillo S, Yela JL, Capowiez Y et al (2013) Avoidance behaviour response and esterase inhibition in the earthworm, Lumbricus terrestris, after exposure to chlorpyrifos. https://doi.org/10.1007/s10646-013-1051-3. Ecotoxicology
33. Mosleh YY, Ismail SMM, Ahmed MT, Ahmed YM (2003) Comparative toxicity and biochemical responses of certain pesticides to the mature earthworm Aporrectodea caliginosa under laboratory conditions. Environ Toxicol. https://doi.org/10.1002/tox.10134
34. Nunes MET, Espíndola ELG (2012) Sensitivity of Eisenia andrei (Annelida, Oligochaeta) to a commercial formulation of abamectin in avoidance tests with artificial substrate and natural soil under tropical conditions. https://doi.org/10.1007/s10646-012-0859-6. Ecotoxicology
35. Omwenga I, Kanja L, Zomer P et al (2021) Organophosphate and carbamate pesticide residues and accompanying risks in commonly consumed vegetables in Kenya. Food Addit Contam Part B Surveill. https://doi.org/10.1080/19393210.2020.1861661
36. Pereira JL, Antunes SC, Ferreira AC et al (2010) Avoidance behavior of earthworms under exposure to pesticides: Is it always chemosensorial? J Environ Sci Heal - Part B Pestic Food. https://doi.org/10.1080/03601231003613625. Contam Agric Wastes
37. Rathee M, Dalal P (2018) Emerging insect pests in Indian agriculture. Indian J Entomol. https://doi.org/10.5958/0974-8172.2018.00043.3
38. Lal R (2015) Restoring soil quality to mitigate soil degradation. Sustain. https://doi.org/10.3390/su7055875
39. Reinecke SA, Reinecke AJ (2007) The impact of organophosphate pesticides in orchards on earthworms in the Western Cape, South Africa. https://doi.org/10.1016/j.ecoenv.2005.10.006. Ecotoxicol Environ Saf
40. Rico A, Sabater C, Castillo M (2016) Lethal and sub-lethal effects of five pesticides used in rice farming on the earthworm Eisenia fetida. Ecotoxicol Environ Saf. https://doi.org/10.1016/j.ecoenv.2016.02.004
41. Sahab S, Suhani I, Srivastava V et al (2021) Potential risk assessment of soil salinity to agroecosystem sustainability: Current status and management strategies. Sci Total Environ. https://doi.org/10.1016/j.scitotenv.2020.144164
42. Sanchez-Hernandez JC (2006) Earthworm biomarkers in ecological risk assessment. Rev. Environ. Contam. Toxicol
43. Savary S, Willocquet L, Pethybridge SJ et al (2019) The global burden of pathogens and pests on major food crops. Nat Ecol Evol. https://doi.org/10.1038/s41559-018-0793-y

44. Saxena PN, Gupta SK, Murthy RC (2014) Comparative toxicity of carbaryl, carbofuran, cypermethrin and fenvalerate in Metaphire posthuma and Eisenia fetida -A possible mechanism. Ecotoxicol Environ Saf. https://doi.org/10.1016/j.ecoenv.2013.11.006
45. Shi Y, Shi Y, Wang X et al (2007) Comparative effects of lindane and deltamethrin on mortality, growth, and cellulase activity in earthworms (Eisenia fetida). https://doi.org/10.1016/j.pestbp.2007.02.005. Pestic Biochem Physiol
46. Singh SI, Singh S, Bhawana, Vig AP (2020) Earthworm-assisted bioremediation of agrochemicals. In: Agrochemicals Detection, Treatment and Remediation. pp. 307–327. Butterworth-Heinemann. https://doi.org/10.1016/B978-0-08-103017-2.00013-1
47. Solé M (2021) Biomarkers in Earthworms. Interaction and Fate of Pharmaceuticals in Soil-Crop Systems: The Impact of Reclaimed Wastewater, 311–337. https://doi.org/10.1007/698_2020_628
48. Srivastava AK, Mishra D, Shrivastava S et al (2010) Acute toxicity and behavioural responses of Heteropneustes fossilis to an organophosphate insecticide, dimethoate.Int J Pharma Bio Sci
49. Srivastava V, Squartini A, Masi A et al (2021) Metabarcoding analysis of the bacterial succession during vermicomposting of municipal solid waste employing the earthworm Eisenia fetida. Sci Total Environ. https://doi.org/10.1016/j.scitotenv.2020.144389
50. Suhani I, Sahab S, Singh RP (2020) Impacts of Land-use Paradigm Shifts on the Agricultural Economy: Sustainable Management. RASSA J Sci Soc 2(3):177–181
51. Tiwari RK, Singh S, Pandey RS, Sharma B (2016) Enzymes of Earthworm as Indicators of Pesticide Pollution in Soil. Adv Enzym Res. https://doi.org/10.4236/aer.2016.44011
52. Velki M, Hackenberger BK (2012) Species-specific differences in biomarker responses in two ecologically different earthworms exposed to the insecticide dimethoate. https://doi.org/10.1016/j.cbpc.2012.05.001. Comp Biochem Physiol - C Toxicol Pharmacol
53. Walkley A, Black IA (1934) An examination of the degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. https://doi.org/10.1097/00010694-193401000-00003
54. Wang JH, Zhu LS, Liu W et al (2012a) Biochemical responses of earthworm (Eisenia foetida) to the pesticides chlorpyrifos and fenvalerate. Toxicol Mech Methods. https://doi.org/10.3109/15376516.2011.640718
55. Wang Y, Cang T, Zhao X et al (2012b) Comparative acute toxicity of twenty-four insecticides to earthworm, Eisenia fetida. https://doi.org/10.1016/j.ecoenv.2011.12.016. Ecotoxicol Environ Saf
56. Yadav S, Khan MA, Sharma R et al (2021) Potential of formulated Dyadobacter jiangsuensis strain 12851 for enhanced bioremediation of chlorpyrifos contaminated soil. Ecotoxicol Environ Saf. https://doi.org/10.1016/j.ecoenv.2021.112039
57. Yadav U, Kushwaha S, Anand V et al (2020) Chlorpyrifos degradation by plant growth-promoting Alcaligenes faecalis bacteria isolated from oil-contaminated soil. Bioremediat J 1–9. https://doi.org/10.1080/10889868.2020.1837066
58. Yang G, Chen C, Wang Y et al (2015) Joint toxicity of chlorpyrifos, atrazine, and cadmium at lethal concentrations to the earthworm Eisenia fetida. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-015-4097-3
59. Yasmin S, D’Souza D (2007) Effect of pesticides on the reproductive output of Eisenia fetida. Bull Environ Contam Toxicol. https://doi.org/10.1007/s00128-007-9269-5
60. Zhang RW, Li ZX, Bai QY et al (1985) Detection of the toxicity of pesticides on earthworms by standard method. Acta Sci Circum 5(3):327–333
61. Zhao S, Wang Y, Duo L (2021) Biochemical toxicity, lysosomal membrane stability and DNA damage induced by graphene oxide in earthworms. Environ Pollut 269:116225. https://doi.org/10.1016/j.envpol.2020.116225
62. Zhao S, Wang Y, Duo L (2021) Biochemical toxicity, lysosomal membrane stability and DNA damage induced by graphene oxide in earthworms. Environ Pollut. https://doi.org/10.1016/j.envpol.2020.116225
63. Zou X, Xiao X, Zhou H et al (2018) Effects of soil acidification on the toxicity of organophosphorus pesticide on Eisenia fetida and its mechanism. J Hazard Mater. https://doi.org/10.1016/j.jhazmat.2018.04.036

## Figures

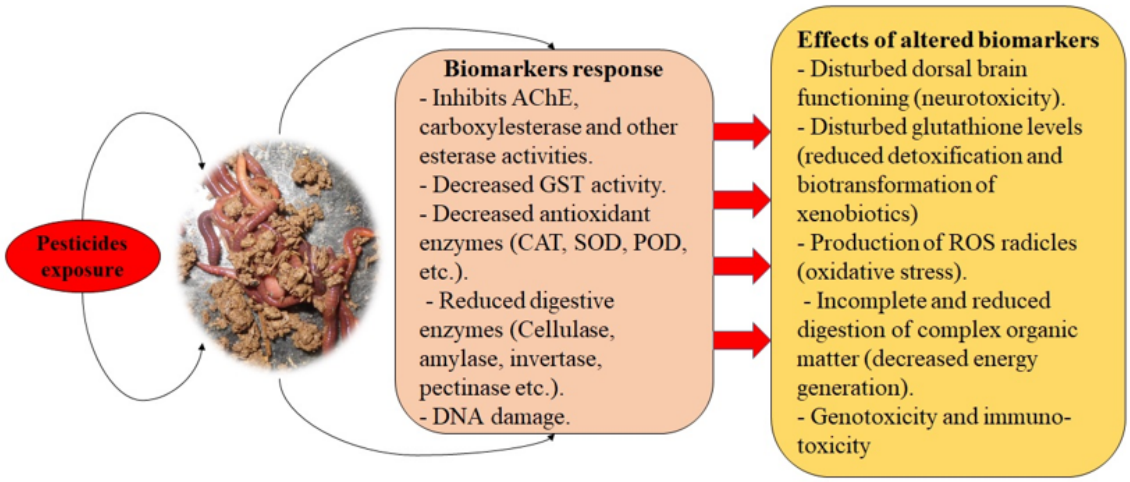



**Figure 1**

*Pesticide exposure leading to altered biomarker responses*

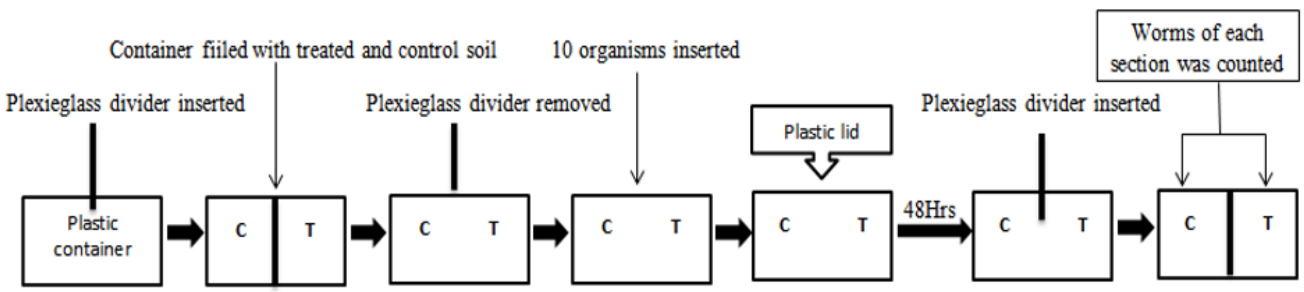



**Figure 2**

Experimental set up and procedures of avoidance response test. Where, side C contains control TS and side T contains insecticide spiked TS

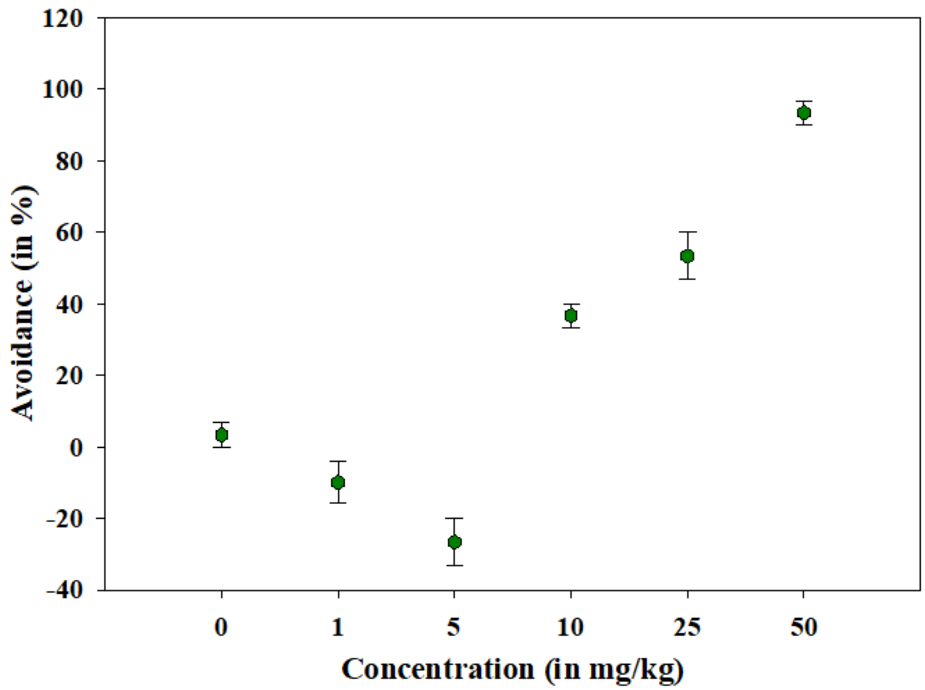



Figure 3

Avoidance response of *E. fetida* to dimethoate concentrations in TS

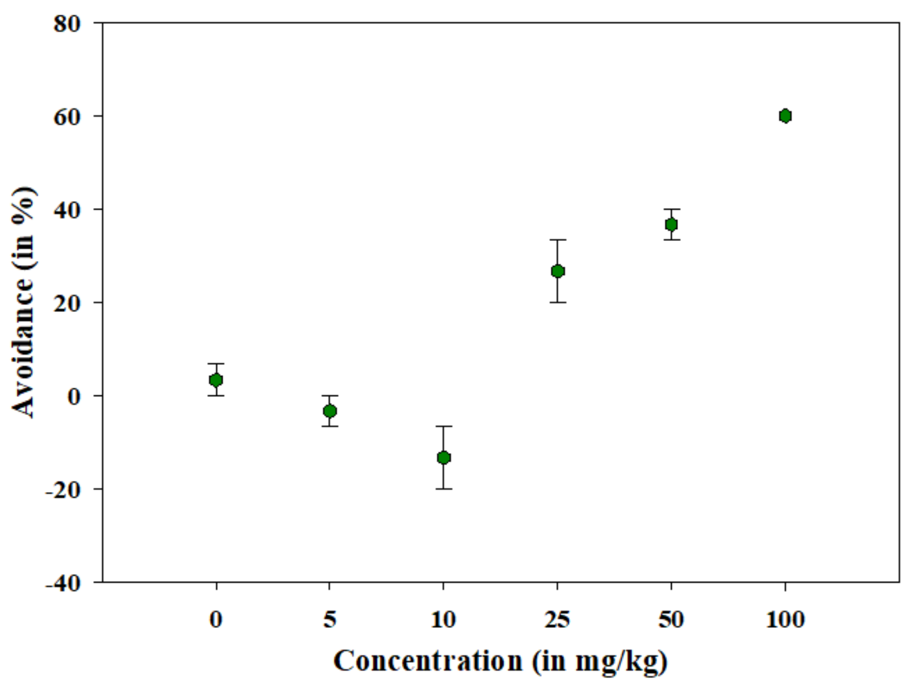



Figure 4

Avoidance response of *E. fetida* to various concentrations of chlorpyrifos spiked TS

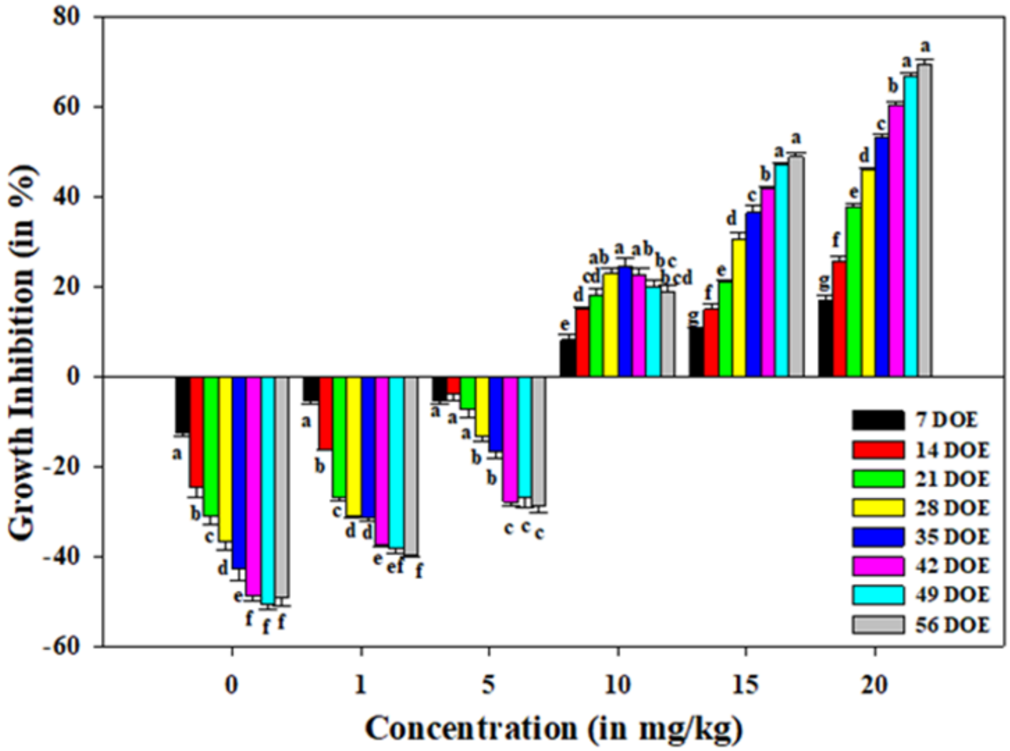



**Figure 5**

Growth inhibition of *E. fetida* in response to concentrations of dimethoate

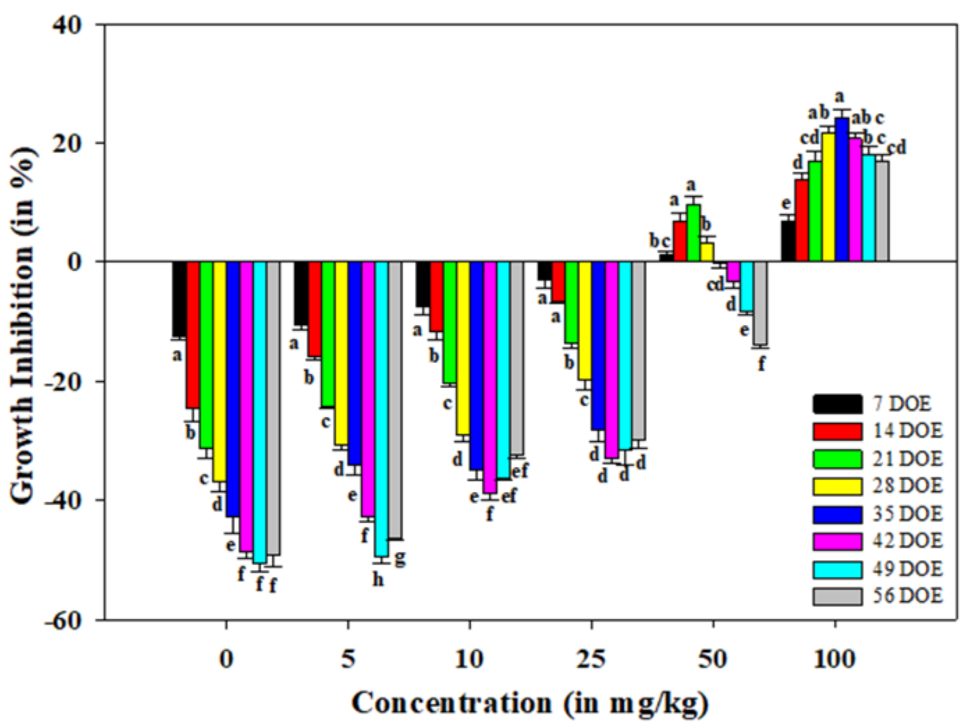



**Figure 6**

Growth inhibition of *E. fetida* in response to concentrations of chlorpyrifos

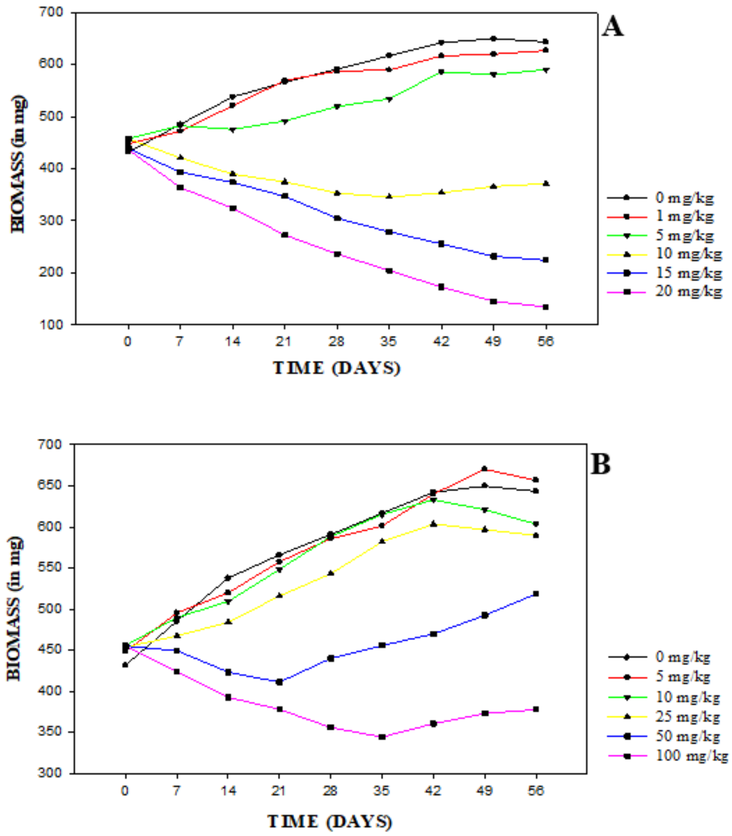
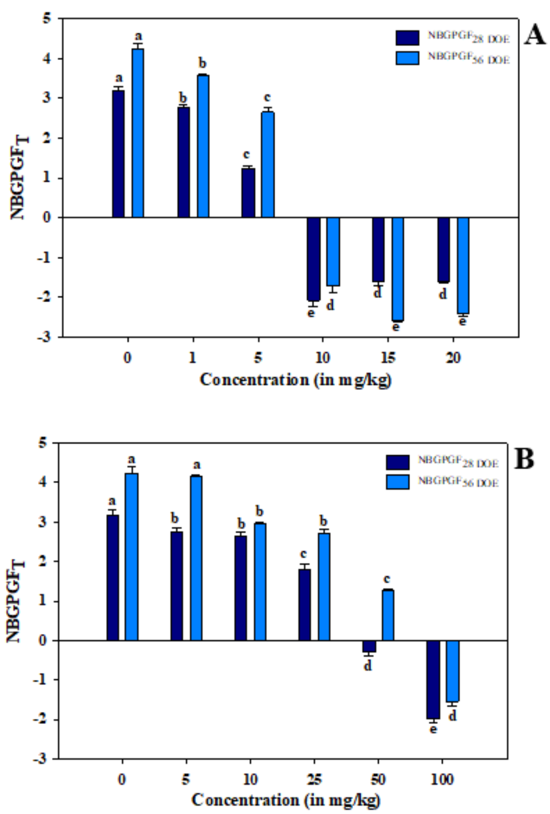


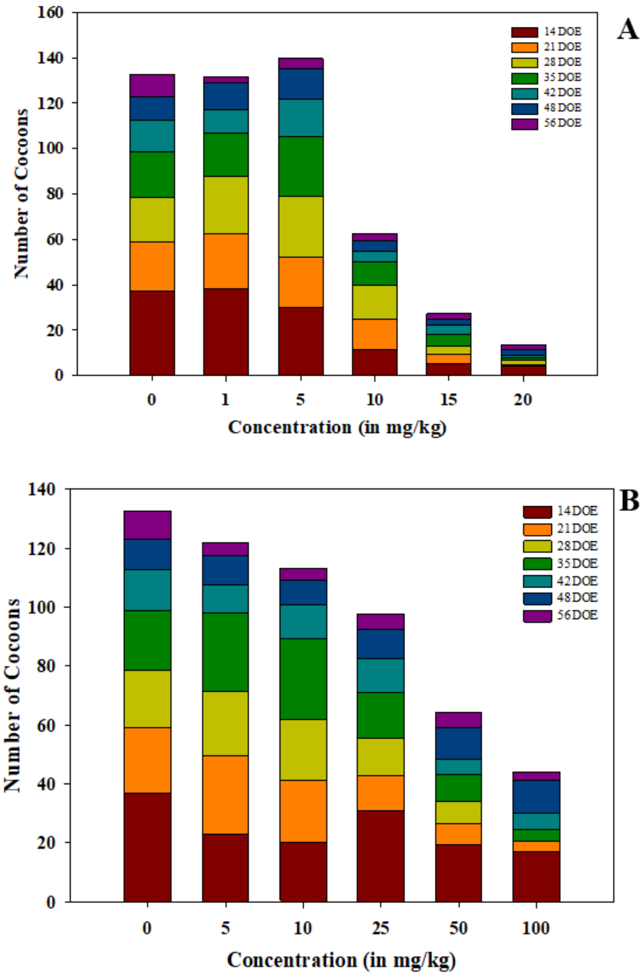

**Figure 7**

Change in biomass through progression of exposure period. (A- Dimethoate and B- Chlorpyrifos)



## Figure 8

NBGPGF$_T$ of E. fetida at 28 and 56 DOE. (A- Dimethoate and B- Chlorpyrifos)




## Figure 9

*Total number of cocoon produced by* E. fetida *exposed to various concentrations of insecticides. (A- Dimethoate and B- Chlorpyrifos)*

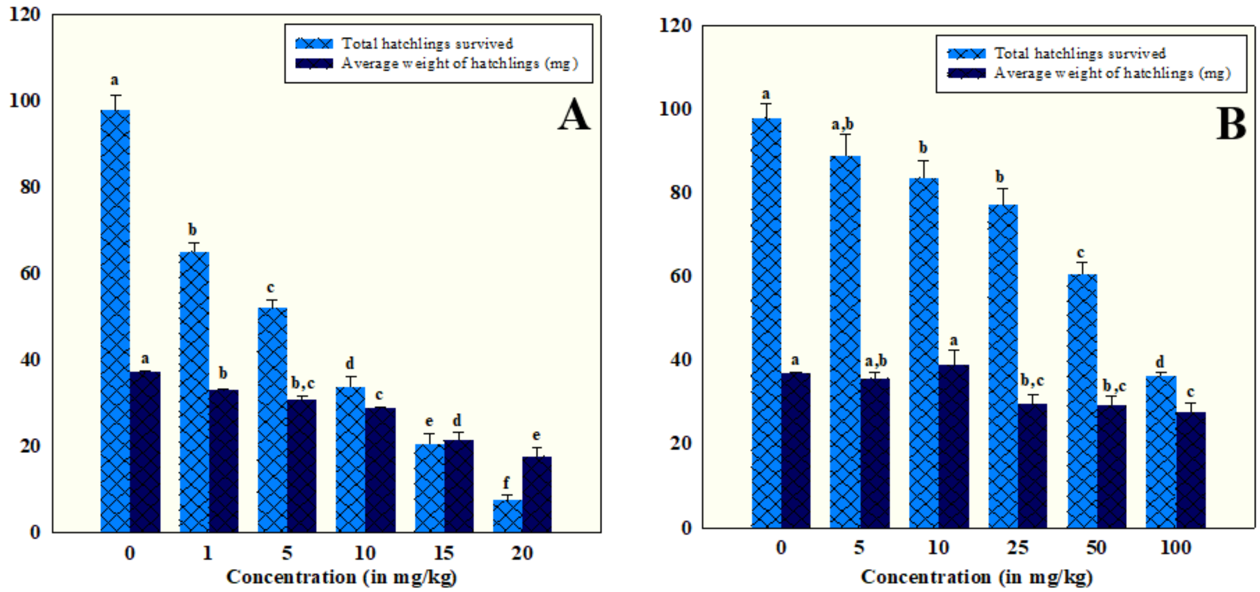



**Figure 10**

Total number of survived hatchlings and their average weight at 56 DOE

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.png