

Impact of chlorpyrifos on ammonia excretion in the earthworm *Eisenia fetida* under laboratory condition

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ABSTRACT

Background: Chlorpyrifos is a popular organophosphates pesticide that is said to pollute soil ecosystems and has negative impacts on non-target organisms including earthworms. Earthworms are very important in the fertility of soils and changes in their nitrogen metabolism especially ammonia excretion can be important in showing physiological stress and toxicity.

Methods: The current laboratory experiment tested the effect of sub-lethal levels of chlorpyrifos on ammonia release of the earthworm *Eisenia fetida*. Clitellated earthworms of healthy condition were subjected to various concentrations of chlorpyrifos treated artificial soil at a constant duration of exposure under controlled temperature and moisture conditions. To determine the rate of excretion of ammonia, standard colorimetric measurements were conducted at specific intervals and the findings were compared with that of a control group which was not treated.

Results: Earthworms exposed to chlorpyrifos showed a significant and concentration-dependent increase in ammonia excretion compared to the control group. The highest concentration resulted in marked elevation of ammonia levels, indicating disruption of nitrogen metabolism. Behavioral stress responses such as reduced activity and increased mucus secretion were also observed in treated groups.

Conclusion: The findings demonstrate that chlorpyrifos induces metabolic stress in *Eisenia fetida*, as evidenced by enhanced ammonia excretion. Ammonia excretion can serve as a sensitive biomarker for assessing pesticide toxicity in soil invertebrates. The study highlights potential ecological risks of chlorpyrifos usage and underscores the need for cautious application to protect soil health.

Keywords:- Chlorpyrifos, *Eisenia fetida*, Ammonia excretion, Nitrogen metabolism, Pesticide toxicity, Biomarker, Soil pollution.

Introduction

Soil is one of the most valuable natural resources and supports numerous biological processes that are essential for maintaining ecosystem stability. However human activities, in particular the excessive use of pesticides in

modern agriculture are increasingly influencing soil ecosystems. These pesticides are supposed to suppress pests but, in most cases, they contaminate non-target surroundings with some unwanted ecological effects on soil biota (**Shamim et al., 2024**). Earthworms are noteworthy organisms of soil; they influence soil structure, nutrient cycling and decay of organic matter. Their feeding and burrowing enhance soil aeration and fertility and their metabolism assists in the transformation and redistribution of organic and inorganic material through the soil profile (**Edwards & Arancon, 2022**).

Ecotoxicology commonly uses the common epigeic earthworm *Eisenia fetida* which is a sensitive earthworm, readily cultivable in the laboratory and is essential in the degradation of organic waste. Earthworms are continuously in touch with soil via ingestion and skin contact, thus being very effective in taking up of contaminants (pesticides) (**Gao et al., 2023**). Any interference with their physiology may disrupt the functions of soil and, hence, the productivity in the ecosystem (Y. Wang et al., 2012).

One of the most common insecticides that is used globally is the organophosphate pesticides like chlorpyrifos, because of their broad-spectrum action in agricultural pests. Chlorpyrifos (O,O'-diethyl-3,5,6-trichloro-2-pyridyl phosphorothioate) is primarily an acetylcholinesterase (AChE) inhibitor, an enzyme required to mediate the function of the nervous system in both the invertebrate and vertebrate nervous system (**Sami et al., 2025**). In addition to neurotoxicity, chlorpyrifos may also cause oxidative stress, destabilize metabolic pathways and change the activity of antioxidant enzymes in non-target soil organisms.

Even though a number of researches are based on lethal and sub-lethal impacts of chlorpyrifos on developing, reproducing, behavioural, as well as enzymatic functions of earthworms, there is a lack of studies that examine the effects of chronic intake on excretion patterns and nitrogen metabolism. One of the basic parameters under physiology is the excretion of ammonia which indicates the activity of protein breakdown and the general health of the body. Ammonia production variations may indicate a metabolic response to a stressor that is toxic, and is directly connected to the breakdown of amino acids and the excretion of nitrogen wastes. Therefore, ammonia concentrations can be a biomarker of metabolic dysfunction in case organisms have to deal with environmental pollutants (**Rasheed et al., 2020**), (**Chhaba et al., 2024**).

Since chlorpyrifos is widely used and persistent, the mechanism by which chronic exposure to chlorpyrifos changes fundamental physiological functions like ammonia excretion in earthworms is important. The sub-lethal metabolic effects may have an impact on the ecological effects, such as a lower soil fertility or change in the nutrient cycle, which may not be noticeable until a longer period of exposure. Earthworms are also one of the first soil organisms to directly face pesticides by coming in direct contact with treated soil and ingesting polluted organic matter and hence; they are at the forefront of detecting soil contamination (**Wolejko et al., 2022**).

In spite of these insights, there are gaps in research. The connection of the biochemical endpoints such as the excretion of ammonia and pesticide dose and duration of exposure is critical in the sophisticated risk assessment and biomarker-based monitoring. This type of data enhances the characterization of ecological risks because chronic alterations in the metabolism of nitrogen can impair the development of the earthworm, its reproduction, and survival in the long run.

In this context, the present study investigates the impact of sub-lethal concentrations of chlorpyrifos on ammonia excretion in *Eisenia fetida* under controlled laboratory conditions. By examining dose- and time-dependent changes in ammonia excretion, this research aims to elucidate metabolic disturbances induced by chlorpyrifos exposure and assess the potential of ammonia excretion as a sensitive ecotoxicological biomarker.

Materials and Methods

Study Design

The present experimental research was conducted in a controlled laboratory environment to assess the impact of the sub-lethal levels of chlorpyrifos on the excretion of ammonia in the earthworm *Eisenia fetida*. The experiment was controlled in a completely randomized study with three experimental groups, including control, 1.5ml L⁻¹ chlorpyrifos, and 2.5ml L⁻¹ chlorpyrifos. These were observed over five exposure ranges, 15, 30, 45, 60 and 75 days to determine the effects with respect to time.

Experimental Organism

The earthworm *Eisenia fetida* were taken as adult specimens to carry out the experiment. The uniformity in the size and body weight used to select earthworms to reduce biological variability. The earthworms were previously acclimated to the laboratory conditions in pesticide-free soil having the necessary amount of moisture and organic matter in a period of seven days before experimentation. The acclimatization period saw the dumping of unhealthy or damaged earthworms.

Chemicals

As a test pesticide, commercial grade chlorpyrifos was employed. All the reagent materials in estimation of ammonia such as phenol, sodium nitroprusside, sodium hydroxide, sodium hypochlorite and sodium chloride were of analytical grade and were obtained through normal suppliers. The experiment was carried out using distilled water.

Preparation of Test Solutions

Preparation of test solutions can be carried out in various ways, notably through the use of standard reagents. Preparation of test solutions can be done in many forms, however use of standard reagents is the most common. Stock solutions of chlorpyrifos were made by dissolving the necessary amount of pesticide by using distilled water. Two sub-lethal concentrations were freshly prepared as 1.5 ml/L⁻¹ and 2.5 ml/L⁻¹ of the stock solution

as the concentrations of the two sub-lethal concentrations prior to each exposure time. The control group was left untreated of the pesticide and given the same conditions of an experiment.

Experimental Setup

Earthworms were separated into three categories, which are control, 1.5 ml/L, and 2.5 ml/L chlorpyrifos treatments. The groups were kept in different containers with moist soil that was not covered with pesticide. The chlorpyrifos solutions were uniformly applied to the soil so as to expose them evenly. Periodic addition of distilled water kept the soil moist at optimum levels. The experiment was carried out at the natural photoperiod at room temperature.



a



b



c

Figure 1 (a–c): Laboratory setup and colour development during ammonia estimation using the phenol–hypochlorite method in control and chlorpyrifos-treated *Eisenia fetida* samples.

Exposure Duration

The duration of exposure was made 15, 30, 45, 60 and 75 days to determine both acute and chronic effects of chlorpyrifos. Earthworms were retrieved from the soil with a lot of care at the end of every exposure time, washed with distilled water to remove any soil particles that were stuck on them, and dried using filter paper, after which they were subjected to analysis.

Collection of Excreta

To estimate the abundance of ammonia, the earthworm excreta were collected using clean containers in which the earthworms were kept at a fixed time. Quantitative estimation of ammonia was done using the excreta obtained. Caution was followed in the collection of samples.

Estimation of Ammonia

Ammonia excretion was estimated using the phenol–hypochlorite colorimetric method. The reagents were prepared as follows:

Phenol–sodium nitroprusside solution:

Sixty-two grams of phenol and 0.25 g of sodium nitroprusside were dissolved in one litre of distilled water.

Alkaline hypochlorite solution:

Twenty grams of sodium hydroxide and 43 ml of sodium hypochlorite were dissolved in one litre of distilled water.

Sodium chloride solution:

A 10% (w/v) sodium chloride solution was prepared, and the pH was adjusted to 2.

Analytical Procedure

Two millilitres of earthworm excreta were combined with 30ml of 10 percent sodium chloride solution and shaken in a mechanical shaker during 30 minutes. The mixture was then filtered and filtrate estimated by use of ammonia.

Phenol sodium nitroprusside solution (1 ml) was added to 1 ML of the filtrate and then 1 ML of alkaline hypochlorite solution was added. The colour development was done by letting the reaction mixture stand after 5 minutes. Then, 2 ml of the distilled water was introduced, and the absorbance of the resulting complex of blue colour was measured at 625nm using the spectrophotometer.

A reagent blank was prepared using distilled water instead of the sample. Ammonia concentration was determined by comparing the absorbance values with a standard calibration curve prepared using known concentrations of ammonia. The results were expressed as $\mu\text{gNH}_3/\text{g/hr}$.

Results

The effect of chlorpyrifos on ammonia excretion in the earthworm *Eisenia fetida* was evaluated over different exposure durations (15, 30, 45, 60, and 75 days) at two sub-lethal concentrations (1.5 ml/L and 2.5 ml/L), along with an untreated control. Ammonia excretion values were expressed as $\mu\text{g NH}_3/\text{g/h}$ and are presented in Table 1.

Ammonia Excretion under Chlorpyrifos Exposure

The findings indicate an evident and constant rise in the excretion of ammonia on earthworms treated with chlorpyrifos relative to the control at all exposure periods. At the initial time point of 15 days, the levels of both pesticides gave more ammonia than the control. The control group showed similar ammonia excretion during the experiment which was indicative of normal metabolic activity that was not stressed by the pesticides.

Control group had $(0.204 \pm 0.001) \mu\text{gNH}_3/\text{g/h}$ at the 15 days of exposure. On the contrary, earthworms that received 1.5ml/L and 2.5ml/L chlorpyrifos displayed higher values of $(0.214 \pm 0.003) \mu\text{gNH}_3/\text{g/h}$ and $(0.264 \pm 0.007) \mu\text{gNH}_3/\text{g/h}$, respectively. The early increase may indicate a premature metabolic reaction to the exposure to pesticides.

At 30 days, a gradual rise in ammonia excretion was observed in the treated groups. The control value remained almost unchanged at $(0.208 \pm 0.001) \mu\text{gNH}_3/\text{g/h}$, whereas the 1.5 ml/L and 2.5 ml/L treatments showed increased ammonia excretion $(0.234 \pm 0.009) \mu\text{gNH}_3/\text{g/h}$ and $(0.283 \pm 0.016) \mu\text{gNH}_3/\text{g/h}$, respectively. This trend indicates a time-dependent accumulation of metabolic stress due to prolonged chlorpyrifos exposure.

A further increase in ammonia excretion was recorded at 45 days. The control group exhibited a value of $(0.213 \pm 0.011) \mu\text{gNH}_3/\text{g/h}$, while the 1.5ml/L and 2.5ml/L treated groups showed values of $(0.277 \pm 0.020) \mu\text{gNH}_3/\text{g/h}$ and $(0.325 \pm 0.007) \mu\text{gNH}_3/\text{g/h}$, respectively. The difference between treated and control groups became more pronounced at this stage, highlighting the chronic impact of chlorpyrifos on nitrogen metabolism.

At 60 days of exposure, ammonia excretion showed a marked elevation in treated earthworms. The control group recorded a value of $(0.230 \pm 0.002) \mu\text{gNH}_3/\text{g/h}$, while ammonia excretion increased to $(0.344 \pm 0.003) \mu\text{gNH}_3/\text{g/h}$ in the 1.5ml/L group and $(0.374 \pm 0.002) \mu\text{gNH}_3/\text{g/h}$ in the 2.5ml/L group. The continued rise in ammonia excretion with prolonged exposure indicates sustained metabolic disruption.

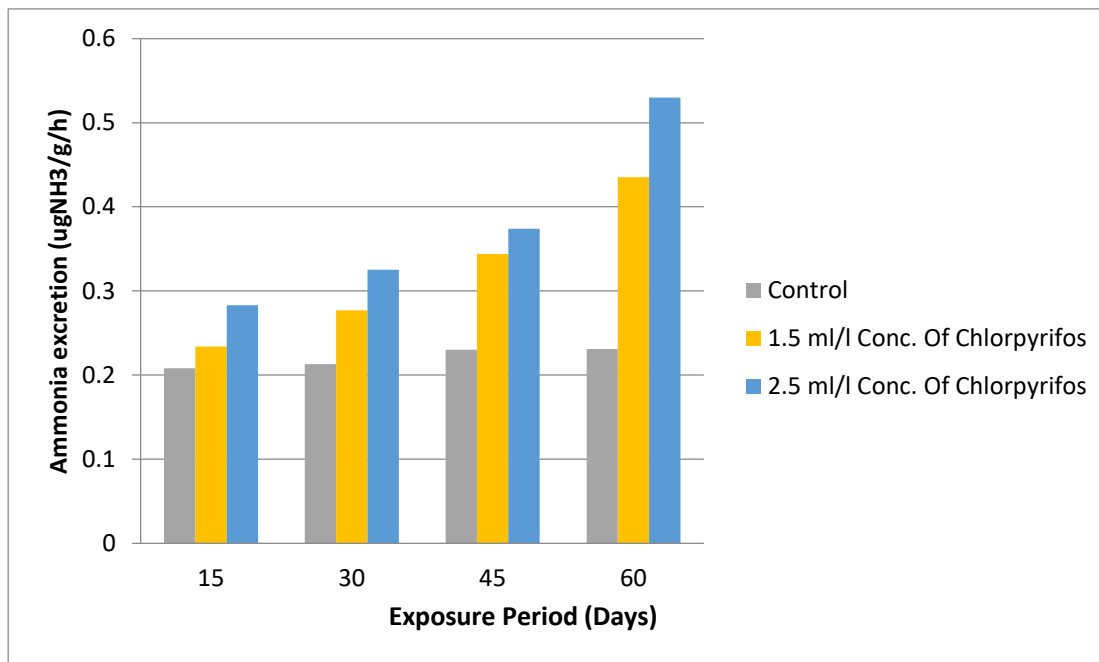
The highest ammonia excretion values were observed at 75 days of exposure. The control group showed a slight increase to $(0.231 \pm 0.005) \mu\text{gNH}_3/\text{g/h}$, whereas earthworms exposed to 1.5ml/L and 2.5ml/L chlorpyrifos exhibited significantly higher values of $(0.435 \pm 0.056) \mu\text{gNH}_3/\text{g/h}$ and $(0.530 \pm 0.008) \mu\text{gNH}_3/\text{g/h}$, respectively. This substantial increase demonstrates a strong dose- and duration-dependent effect of chlorpyrifos on ammonia excretion.

Overall, ammonia excretion increased progressively with both increasing chlorpyrifos concentration and exposure duration. Among the treated groups, the 2.5ml/L concentration consistently resulted in higher ammonia excretion compared to the 1.5ml/L concentration at all exposure periods, suggesting greater physiological stress at higher pesticide levels.

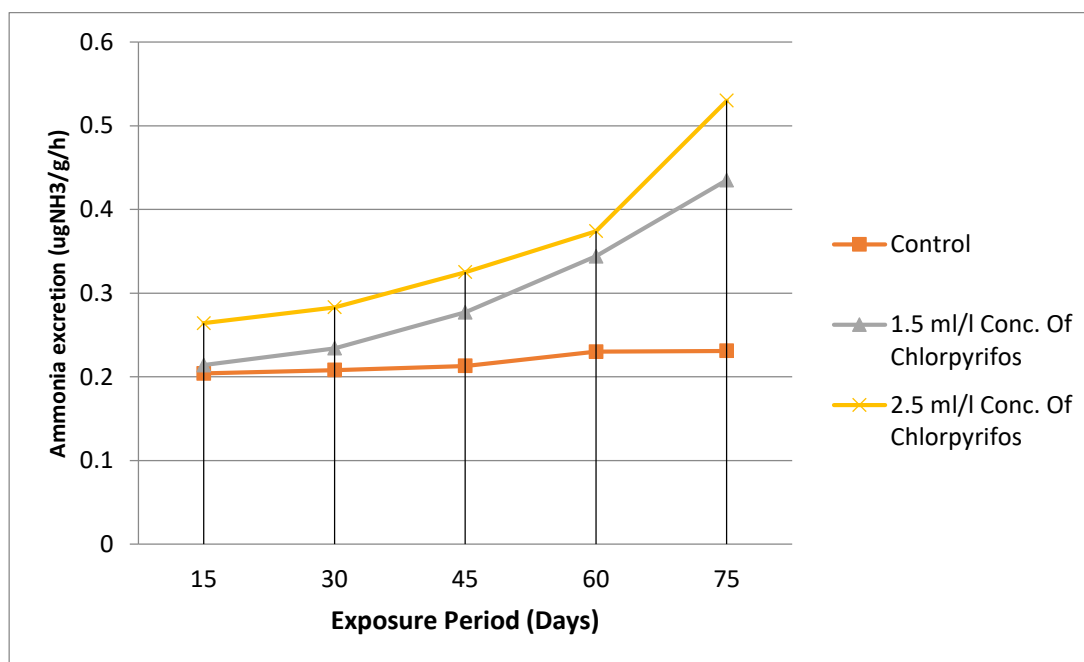
Table 1. Ammonia excretion ($\mu\text{gNH}_3/\text{g/h}$) in *Eisenia fetida* exposed to chlorpyrifos.

Days	Control (Mean $\pm$ SD) n=3	1.5 ml/l conc. Chlorpyrifos (Mean $\pm$ SD) n=3	2.5 ml/l conc. Chlorpyrifos (Mean $\pm$ SD)n=3
15	0.204 $\pm$ 0.001	0.214 $\pm$ 0.003	0.264 $\pm$ 0.007
30	0.208 $\pm$ 0.001	0.234 $\pm$ 0.009	0.283 $\pm$ 0.016
45	0.213 $\pm$ 0.011	0.277 $\pm$ 0.020	0.325 $\pm$ 0.007
60	0.230 $\pm$ 0.002	0.344 $\pm$ 0.003	0.374 $\pm$ 0.002
75	0.231 $\pm$ 0.005	0.435 $\pm$ 0.056	0.530 $\pm$ 0.008

- Ammonia excretion increased with exposure duration.
- Chlorpyrifos exposure caused a dose-dependent elevation in ammonia excretion.
- The 2.5 ml/L concentration consistently induced higher ammonia levels than 1.5 ml/L.
- Control values remained relatively stable throughout the experiment.
- Maximum ammonia excretion was observed at 75 days, indicating chronic metabolic stress.



Graph 1:- Time- dependent changes in ammonia excretion of *Eisenia fetida* following exposure to sub-lethal concentrations of chlorpyrifos.



Graph 2:- effects of exposure duration and chlorpyrifos concentration on ammonia excretion *Eisenia fetida*.

Discussion

The present study investigated the impact of chronic exposure to sub-lethal concentrations of chlorpyrifos on ammonia excretion in the earthworm *Eisenia fetida* under laboratory conditions. The findings indicate that there is a significant, evident rise in excretion of ammonia which is dependent on concentration and time. These alterations provide insights into metabolic alterations due to pesticide stress and give insight into the earthworms and the physiological reactions to soil contamination.

Ammonia excretion in the control group was rather stable during the experiment. Such stability suggests that there is normal metabolic activity and good nitrogenous waste management in the absence of pesticides. It also establishes that the experimental set up did not stress the earthworms. On the other hand, earthworms exposed to chlorpyrifos showed an increasing trend of excretion of ammonia between days 15 and 75 days, and this is an indication that a long period of pesticide exposure affects the normal nitrogen metabolism.

The increased ammonia excretion in chlorpyrifos-treated groups could be explained by the increased protein catabolism in the toxic conditions. Organophosphates pesticides, e.g. chlorpyrifos, disrupt enzyme systems, to affect energy metabolism, raise protein and amino-acid breakdown. The primary nitrogen product of earthworms, ammonia, piles up when the deamination of amino acids increases. The slow, progressive rise in excretion rate of ammonia with increasing exposure of the organism means that there is cumulative metabolic overload but not an acute one.

The toxicological effects of chlorpyrifos are also confirmed by the dose dependent reaction. Earthworms in the higher concentration (2.5mL/L^{-1}) always exhibited higher ammonia excretion compared to 1.5mL/L^{-1} at all-time intervals. This tendency indicates the increased loads of pesticides lead to an increased physiological load, which is beyond the power of the detoxification and excretion processes. Other dose-dependent metabolic variations have been observed in earthworms that were exposed to different pesticides and soil pollutants, in which increased generation of nitrogenous wastes was an indicator of stress that caused a metabolic disequilibrium.

The significant escalation of the amount of ammonia released with longer duration of exposure, particularly in 60 and 75 days, highlights the relevance of chronic exposure in ecotoxicological tests. Although exposure to a moderate level of a substance might cause moderate physiological responses in short-term exposure, the effects of long-term exposure to a substance can cause long-term metabolic disturbance. These chronic stress responses are ecologically significant in the sense that earthworms in agricultural soils are not usually subjected to a one-time acute dose of pesticides; they are exposed to repeated or chronic pesticides.

The modified ammonia excretion can have more extensive role to play on earthworm health and survival. Internal ammonia levels are very high, which interferes with the cellular pH level and enzyme activity. Though

the mortality was not determined in this study, long-term metabolic stress may eventually cause growth, reproduction and fitness impairment. Because earthworms are one of the most important components of the soil structure, nutrient turnover, and decomposition of organic matter, the physiological stress caused by pesticides may have an indirect impact on soil fertility and the functioning of the ecosystem.

The fact that laboratory conditions permit a controlled assessment of the pesticide effects but do not necessarily reflect the complexity of representatives of the field settings with multiple stressors interacting should also be mentioned. The type of soil, microbial activity and changes in temperature, as well as combined with pesticide exposure may have an effect on the ammonia excretion patterns in natural environments.

In general, the excretion of ammonia increased in the post-exposure period of chlorpyrifos in the case of the earthworms emphasizes the vulnerability of the earthworms to the organophosphate pesticides and the significance of the physiological biomarkers usage in the ecotoxicology of soils. These findings contribute to the accumulating literature that sub-lethal exposure to pesticides may be severely disruptive to metabolic processes and may lead to serious metabolic disruption, even without instantaneous death.

Conclusion

The current experiment indicates that ammonia excretion in the *Eisenia fetida* during laboratory settings is largely affected by chronic exposure to chlorpyrifos. Exposure to sub-lethal levels of chlorpyrifos (1.5-ml L^{-1} and 2.5-ml L^{-1}) showed a progressive ammonia excretion of earthworms as compared to the control. The size of this growth is directly proportional to the concentration of chlorpyrifos and the length of exposure meaning that it is definitely a dose- and time-dependent reaction.

The constant ammonia excretion during the period of experiment in the group of controls indicates healthy metabolism, and the high level of ammonia in treated groups indicates protein catabolism and metabolic stress caused by pesticide exposure. The significant difference in the ammonia excretion at increased length of exposure, particularly at 75 days highlights the cumulative physiological effects of the continuous exposure of chlorpyrifos to earthworms.

These results show that ammonia excretion is one of the most sensitive physiological indices of a pesticide toxicity of sub-lethal concentrations in soils. The changes in nitrogen cycling in *Eisenia fetida* can possibly have ecological consequences on a larger scale since earthworms are vital in the soil nutrient cycling and soil fertility. The chronic metabolic stressing of earthworms may reduce the soil health and ecosystem performance.

Finally, the research highlights the environmental hazard of chlorpyrifos contamination of the soil environment and justifies the use of earthworms and metabolic biomarkers, including excretion of ammonia, as effective methods of ecotoxicology. The findings highlight the importance of use of chlorpyrifos in agriculture with

caution and the results are used to provide the background information on the study on future research on the metabolic imbalances caused by the pesticides on soil fauna.

Appendix

The soil used in the experiment process was prepared using pesticide free soil, saw dust and dried cow dung. Soil was taken from agricultural field and cow dung was at first dried and grounded properly then sieved through 2mm sieve. Saw dust was collected from nearby saw mill and soaked in water for 14 days with regular watering to facilitate partial decomposition. Then soil, cow dung and soaked saw dust was mixed in equal proportions to prepare the artificial soil.



a



b



c



d

fig 2:- (a-d) Preparation of artificial soil

Nomenclature

Symbol/abbreviation	description
1. AChE	Acetylcholinesterase
2. ANOVA	Analysis of Variance
3. EC	Emulsifiable concentrate
4. NH ₃	Ammonia
5. SD	Standard deviation
6. µg	microgram
7. <i>Eisenia fetida</i>	Earthworm species used in the study.

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