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Research Article

A comparative study on lethal concentration (LC_{50}) of urea and diammonium phosphate to earthworms, *Eisenia fetida*

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Abstract
In managed agro-ecosystems, urea and diammonium phosphate (DAP) are widely used artificially synthesized fertilizers for crops as nitrogenous supplements. Overdose of chemical fertilizer poses a threat to various soil-dwelling microorganisms and earthworms, too. The present study aimed to determine the detrimental effect of nitrogen supplements (urea and diammonium phosphate) on earthworms (*Eisenia fetida*). To calculate LC_{50} for urea and DAP, the experimental set-up was maintained as per the recommendations of Organization for Economic Co-operation and Development (OECD) guidelines number 207 for testing the chemicals. Ten adult gut-cleaned earthworms were inoculated in artificial soil mixed with testing chemicals. Five sets of replica with each test chemical in different concentrations were used for 14 days for better estimation of mortality. Mortality was the endpoint. Median lethal concentration (LC_{50}) of urea and DAP was calculated through Finney Probit regression analysis. At 300 mg/kg concentration of urea, earthworms had no mortality and at 1000 mg/kg there was ≤50% mortality. The LC_{50} value for urea was 862.126 mg urea per kg of the soil at 95% confidence interval and at 500 mg/kg concentration of DAP, there was no mortality of earthworms; at 2400 mg/kg, there was ≤50% mortality. The LC_{50} value for DAP was 2098.69 mg DAP per kg of the soil at 95% confidence interval. Comprehending the LC_{50} value is crucial when determining an appropriate application rate of chemical fertilizers in an agricultural setting to avoid damaging earthworm populations, which are vital to the soil's fertility and overall health. More specifically, the diversity and population of earthworms indicate the health of the soil and evolve into a cost-effective component of the agro-managed ecosystem.

Keywords: *Eisenia fetida*, Earthworm, Chemical fertilizers, Diammonium phosphate, Urea

INTRODUCTION

The earthworms belong to subclass Oligochaeta, Class clitellata of the phylum Annelida. The subclass Oligochaeta has nearly 10,000 species, with wide habitats including freshwater, marine water, and terrestrial. Fifty per cent of the terrestrial species are earthworms (Reynolds and Wetzel, 2004). India is diverse in earthworms, with nearly 11% of the world's diversity. In India, there are nearly 67 genera of earthworms (Kathireswari, 2016; Prakash, 2019).

The use of fertilizer or pesticides has become critical to crop productivity and for financial gains. Applying fertilizer aids in restoring the nutrients that are absorbed by the earlier harvest. Every synthetic material, particularly insecticides and fertilizers, harms crop quality and soil fauna (Singh and Gupta, 2018). Earthworms are soil indicator species that can be used to diagnose environmental pollution in soil ecosystems (Shi *et al*., 2020; Raza *et al*., 2021). Various toxicological studies are carried out to evaluate the adverse effects of pesticides on earthworm. However, only a couple of studies have been done on the toxic effect of fertilizers. Earlier reports were conducted on NPK and superphosphate using the paper-contact method only. A certain viewpoint is that nitrogen promotes faster production, accelerating the rate at which organic matter replenishes the soil, increasing worm activity (Whalen *et al*., 1998). Various other studies concluded that tiny concentrations of urea do not harm earthworms (Rani, 2016) but larger amounts severely kill them (Rai *et al*., 2014; Rashid, 2019). Understanding LC_{50} is essential for figuring

out a safe application rate in an agricultural situation to prevent harm to earthworm populations, which are essential for the fertility and health of the soil. The present study aimed to assess the detrimental lethal concentration of two commonly used fertilizers urea and diammonium phosphate (DAP), on earthworms (*Eisenia fetida)*.

MATERIALS AND METHODS

Test material

Earthworms, *E. fetida*, were picked up from Godhan Unit, a private company that produces vermifertilizer in Bahadurgarh, Haryana. The earthworms were brought to the Toxicology lab of the Zoology Department, Maharishi Dayanand University, Rohtak, Haryana. The worms were kept in big plastic containers (Width-14", Height-5.5", Depth-20") to acclimatize to the laboratory condition for 5–6 months. Using an online taxonomy key and digital library, the identification of the worms *E. fetida* was confirmed again (Thakur and Yadav, 2018).

Tropical artificial soil preparation

The tropical artificial soil containing 700g industrial soil, 150g clay, and 100g cocopeat per 1000g was prepared in the laboratory. Calcium was supplemented to the artificial soil by adding 50g of crushed egg shells. The artificial soil was mixed well and the pH was adjusted to 6.0±0.5 units using lime. Cow dung cakes, nearly 37-40 days old, were crushed to a fine powder and supplemented with artificial soil to surplus the nutrition medium (Neuhauser and Callahan, 1990).

The tropical artificial soil thus prepared was moistened with water every second day to prevent desiccation. The laboratory-adapted earthworms were reared in earthen pots of 10 liters in size to mimic the natural soil conditions. The earthworms were acclimatized in the artificial soil placed at 27 ± 2^{o}C in culture pots for 24 hours before the onset of the experiment.

Chemical fertilizers

Chemical fertilizers used in the study were granular urea (N-46%) and diammonium phosphate-(DAP-P_2O_5-46%, 18%N). The fertilizers of Indian Farmers Fertilizer Cooperative Limited (IFFCO) were used in the study and purchased from the local market in the Rohtak district.

Culture maintenance

The experimental set-up was monitored as per the recommendations of Organization for Economic Co-operation and Development (OECD) guidelines number 207 (1984) for the testing of chemicals (OECD, 2004; 2015). To depurate gut, worms were given 1.5% sterile agarose gel dose overnight.

Experimental setup

For setting up an experiment, each replicate was set in 5 different earthen pots with an internal radius of 6 cm and depth of 8 cm (Volume ~900 cc) filled 3/4th with artificial tropical soil. The artificial tropical soil was moistened before the earthworm transfer and watered on alternative days after the transfer. Ten *E. fetida* earthworms of similar (approximate same weight) sizes were randomly distributed in the five pots each. Control set was also maintained side by side. The pots were kept in a laboratory maintained at 27 ± 2^{o}C for 24 hours. After 24 hours, the artificial tropical soil was supplemented with respective concentration either with urea or DAP/ kg of artificial soil. Each pot was masked with a net-cloth to ensure adequate aeration and moisture levels and to stop the earthworms from fleeing the pots. At first, pots were checked regularly for aberrant activity up to 7 days. The worms also underwent a thorough visual examination to document any morphological changes. Dates were carefully added to the remarks.

Ethical committee approval

The study was an observational, experimental procedure on invertebrate earthworms, thereby not requiring any clearance from the Ethical committee approval.

Estimation of LC_{50} of chemical fertilizers

For the synthetic fertilizers, urea and DAP, the lethal concentration (LC_{50}) at which half of the worms died was calculated. This experiment used an artificial soil testing approach on epigeic worms, *E. fetida* with urea and DAP, in varied concentration treatments to calculate LC_{50}. For this purpose, earthworms were exposed to different concentrations of urea or DAP and percent mortality was recorded after 14 days of exposure. Initially, a range of 100 to 4000 mg of urea/kg of soil with a regular interval difference of 500mg/Kg of soil was used to narrow down around the LC_{50}. Finally, the LC_{50} calculations were carried out in 300 mg/kg to 1500mg/kg with a regular 100 mg/kg gap for urea. Similarly, a range-finding test was also performed for DAP. To begin with a 100 mg/kg gap up to 600 mg/kg, and then up to 4500 mg/kg of soil with a 300 mg/kg regular break. The percent mortality of the earthworms was plotted against the concentrations of the respective chemical fertilizer on the log-Probit plot. The LC_{50} values were estimated using the regression line Finney (1952) log-Probit plot.

RESULTS AND DISCUSSION

Two widely used artificially synthesized chemical fertilizers in agriculture worldwide are urea and DAP. Details of the toxicity for LC_{50} are given in Fig.1 and Fig. 2. Mortality of adult *E. fetida* at different concentrations of

Table 1. Mortality of adult *Eisenia fetida* at different concentrations of urea fertilizer after 14 days.

Experimental Set up of earthen pots (Serial no)	No of Earthworms (Adult)	Dose mg/kg of soil	Mortality %
1	10	300	
2	10	400	10
3	10	500	14
4	10	600	18
5	10	700	22
6	10	800	38
7	10	900	42
8	10	1000	54
9	10	1100	68
10	10	1200	76
11	10	1300	86
12	10	1400	94
13	10	1500	100

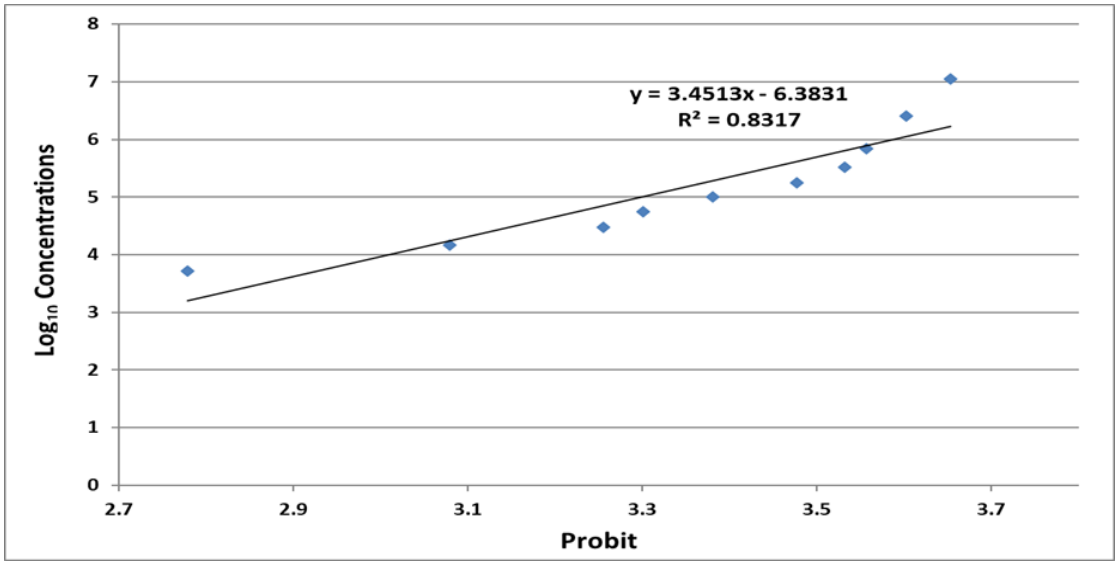

Fig. 1. *Graph depicting the Log-Probit analysis of the Urea fertilizer on the Eisenia fetida*

urea fertilizer and DAP fertilizer after 14 days is also presented in Table 1 and Table 2. The toxicity of nitrogenous fertilizers, urea and DAP to adult earthworms varied greatly.

Results of the present experiment revealed that (Table 1) the mortality of adult *E. fetida* earthworms on exposure to different treatment concentrations of urea ranged from 0.00 to 100%. At 300 mg/kg concentration of urea, there was no mortality of worms and at 1500 mg/kg there was 100% mortality. However, as the urea concentration in experimental set up of earthen pots (serial no 2-13) increased, the mortality rate among the test animals also increased. There was a positive correlation between earthworm mortality and the increased concentration of urea added to soil. At low urea concentration, earthworms show no death, but the mortality rate reached ≤50% at 1000 mg/kg of urea concentration treatment (experimental set up of earthen pot- serial no 8).

The present findings corroborate with the findings of Xiao *et al.* (2004) confirming that when applied in a concentration lower than 500 mg/kg, the urea was reported to be beneficial to the earthworms, *E. fetida*. However, urea in a concentration higher than 1000mg/Kg also had strong toxic effects in the present experiment. The earthworms in the present experiment died at 1500 mg/Kg of urea concentration. Rai *et al.* (2014) also stated that as the dose of urea increased from 0.75gm/kg to 1.5gm/kg and 2.25gm/kg, mortality among the test animal *E. fetida* increased. In another experiment, the mortality rate *of E. fetida* reached 100% when the dose of urea reached 3.48gm/kg

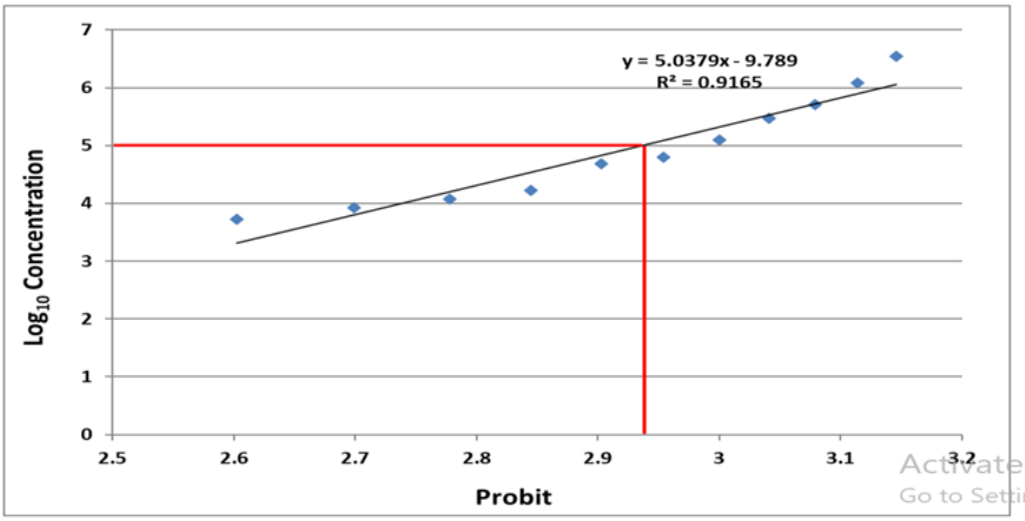

Fig. 2. *Graph depicting the Log Probit analysis of the DAP fertilizer on the Eisenia fetida*

(Rashid, 2019). The detrimental impact of artificial fertilizers, i.e. urea, on the *E. fetida,* earthworms biomass/ weight (culture and individual), cocoon production, juveniles was also supported by earlier findings of Rashid (2019) and Rai *et al.* (2014) . This could be understandable because every chemical pesticide and fertilizer bioaccumulates in the body of animals and plants to cause ecotoxicity in them. In another study reported by Long *et al*. (2017) soil supplemented with urea induces the mortality of ~48% of *E. fetida* earthworms when given in the concentration of 250 mg/kg. Long *et al.* (2017) noted alterations in soil replicate's chemical properties before and after urea treatment. With each rise in urea concentration, he observed that the pH decreased. Furthermore, the treatment with the largest proportion of adult earthworm death is the one with elevated urea content. This result was consistent with the findings of Rani (2016) and Rai *et al*. (2014). The present experiment also demonstrates a positive relationship between the higher concentrations of urea applied to soil and earthworm mortality. The present study differs from previous studies in terms of soil type, application of dosage time of fertilizers and methodology. The present investigation was a preliminary step to find LC_{50} values of urea fertilizer and the impact of urea's deadly concentration on earthworms, *E. fetida*. Determining the LC_{50} values provides fundamental knowledge for developing essential information to create more intricate poisoning disposal strategies for the animals involved. It is also useful in determining the safe threshold tolerance for any chemical. Earthworm i.e *E. fetida* is epigeic in nature and shows more sensitivity than other endogeic species (Yahyaabadi *et al.*, 2018).

In this study Finney Probit analysis indicated LC_{50} value of urea on *E. fetida* earthworms was 862.126 mg per kg of the soil (Fig. 1**)**. In comparison, the LC_{50} value for the toxicity of DAP on the *E. fetida* earthworms was 2098.69 mg DAP per kg of the soil (Fig. 2**)**. Experimental set up of earthen pots at different concentrations of urea or DAP fertilizer on mortality of *E. fetida* earthworms after 14 days is also presented in Table 1 and Table 2.

A distinct variation of adult earthworm mortality was also recorded with respect to different doses of DAP (Table 2). In DAP treated soil no mortality was observed (experimental set up of earthen pots serial no 1-3) on exposure upto 500 mg/kg (Table 2). Mortality rate reaches 50% at 2400 mg/kg (earthen pot serial no 10), ≤70% mortality at 3000 mg/kg (earthen pot serial no 12) and at 4500 mg/kg conc of DAP (earthen pot serial no 15), there was 100% mortality (Table 2). Bhattacharya and Sahu (2015) found no mortality of adult earthworms *Drawida willsi* when exposed to a superphosphate dose of 100 mg/kg and 100% mortality at 650 mg/kg for adult. In another study, Chaudhari (2016) observed 100% mortality at 650 mg/kg of DAP for *Eudrilus eugeniae*. However, the result of the present study showed no toxicity of DAP on *E. fetida* upto 500mg/kg and 100% mortality at 4500 mg/kg (Table 2).

Based on the calculated LC_{50} value in the present study, urea was more toxic to the *E. fetida* earthworms than the DAP. However, literature is available about the mortality rate of the earthworms (*Eudrilus eugeniae*) developing on urea and DAP mixed vermifeed. Shruthi *et al.* (2017) confirmed urea mixed vermifeed cause mortality of 94% worms at 5 g/kg and 100% at 10 g/kg. In contrast the, mortality rate of worms housed on a combination of DAP mixed vermifeed was 52% at 10 g/kg and 46% at 5 g/kg.

According to the Indian soil testing manual issued in 2011 by the Department of Agriculture (Ministry of Agriculture & Government of India) the advised urea dosage for agricultural fields is 120 kg/hectare (Roberts

Table 2. Mortality of adult *Eisenia fetida* at different concentrations of DAP fertilizer after 14 days.

Experimental Set up of earthen pots (Serial no)	No of Earthworms (Adult)	Dose mg/kg of soil	Mortality %
1	10	300	0
2	10	400	0
3	10	500	0
4	10	600	4
5	10	900	8
6	10	1200	10
7	10	1500	16
8	10	1800	38
9	10	2100	42
10	10	2400	52
11	10	2700	68
12	10	3000	78
13	10	3500	86
14	10	4000	94
15	10	4500	100

and Dorough, 1984; Velki *et al*., 2013). However, the actual concentration used in the field is higher than the recommended dose to achieve high productivity (Moonilall, 2015). In Indian fields where the recommended dose for nitrogen is 150 kg N/ha or more, all farmers use more nitrogen (Tripathi, 2023), whereas DAP application rate ranges from 11.5–40.9 kg/ha. Moreover, in the earthworm *E. eugeniae,* a decline in the growth/biomass is reported upon treatment with 0.5% and 1% chemical liquid fertilizers (Karthick *et al.*, 2015). A comparative study on life table attributes of *E. fetida* by Passi *et al.* (2021) observed that chemical fertilizers in soil, urea and DAP negatively affect earthworm biomass, survival and reproductive potential.

The results of the present study indicated that higher concentrations of urea and DAP had harmful effects, i.e. high mortality and low survival rate. DAP is not as deadly as urea; earthworms are still at serious risk from it at excessive concentrations. The findings highlight that reducing worm populations has detrimental effects on the soil-environment because it slows the rate at which organic matter breaks down and aerates the soil, two processes essential to the soil quality (Zhao *et al.,* 2022).

Conclusion

The present study's finding that fertilizers also induce toxicity to earthworm survival raises serious concern about artificially synthesised fertiliser use in agriculture. One of the significant soil fauna, earthworms, has been found to be susceptible to lethal dosage (LC50) of two commonly used nitrogenous fertilisers, urea and DAP. Therefore, the amount of nitrogenous fertilizers that can be applied to soil should be restrained to 862.126 mg of urea and 2098.69 mg of DAP per kg of soil, respectively. In the future, using enough organic manure instead of chemical fertilizers in crops will help to promote earthworm activity in the soil for a sustainable agro-ecosystem.

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Conflict of interest

The authors declare that they have no conflict of interest.

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