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Sublethal effect of synthetic fertilizers (urea and diammonium phosphate) on biomass accumulation and its histological perspicuity on the gut region of the earthworm, *Eisenia fetida*

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Abstract
Urea and Diammonium phosphate are commonly used fertilizers in almost every managed agro-ecosystem. The present study assessed the sublethal effect of synthetic fertilizers on biomass accumulation and its histological perspicuity on the gut region of the earthworm, *Eisenia fetida*. Earthworms, *E. fetida* **(**adult), were reared as per the recommendations of the Organization for Economic Co-operation and Development (OECD) guidelines (1984) number 207 for testing the sublethal effect of chemicals in the laboratory. The lethal concentration at which 50% of the worms were dead, i.e., (LC_{50}) was estimated for synthetic fertilizers, urea, and diammonium phosphate by log-probit analysis. The urea LC_{50} was 862.126 mg/kg, while that of diammonium phosphate was 2098.69 mg/kg in artificial soil. To explore the sublethal effect of synthetic fertilizers on biomass culture (n=10), 1/2 of the LC_{50} of urea and diammonium phosphate was used. Significant variation in biomass culture was observed in response to the amalgamation of synthetic fertilizers. With in 15 days, in the urea amalgamated culture, the weight of earthworms (n=10), unlike the control group, decreased from 2.86±0.142 g to 2.42±0.120 g, but the weight of the earthworms decreased to 2.72±0.135g after applying DAP. To investigate histological alterations in the gut of earthworms subsequent to exposure, specimens (n=5) were aseptically excised, embedded in paraffin, and subjected to section cutting (thickness- 5μm) after staining with hematoxylin and eosin stain and then examined. Notably, Histological damage was also registered in the chloragogen cells of the gut region of *Eisenia fetida* (earthworm) on days 15 and 60. Thus, synthetic fertilisers affect earthworms' ability to absorb and digest nutrients and soil health too.

Keywords: Diammonium phosphate, Earthworm, *Eisenia fetida,* Gut-region Histology, Lethal concentration (LC_{50}), Synthetic fertilizers, Urea

INTRODUCTION

The food production-food deficit gap poses a burden on the agriculture sector. The agricultural produce obtained with traditional methods was not sufficient to provide food to all in the early 80s. To combat this at present, farmers use more agrochemicals (chemical fertilizers, pesticides, hybrid seeds, Insecticides) to compensate for the deficit of food supply. The intermixing of chemicals into the soil alters the soil chemistry, which adversely affects the soil ecosystem (Liang *et al*., 2008). Furthermore, excessive fertilizer use contributes to both soil contamination and nutrient imbalance. Two common sources of nitrogenous fertilizer are urea and DAP (diammonium phosphate). Earthworms constitute more than 70% of the soil macrofauna and preserve the soil ecosystem's structure (Luan *et al*., 2020). Therefore, it is crucial to understand how these inorganic fertilizers affect the physiology and life characteristics (survival, cocoon production, nutrient absorption and cast production) of earthworms.

Earthworms act as soil pollution bioindicators for ecotoxicological studies. Soil pollution bio-indicators are essential to establish environmental standards (Huang *et al*., 2020; Esaivani *et al.,* 2017). Additionally, these worms are consumed by birds, which makes them a part of the food chain (Wang *et al*., 2016; KilIç, 2011; Huerta Lwanga *et al*., 2017a; Kesic *et al*., 2021).The residual chemicals in the soil may have a detrimental effect on the biology and physiology of the macrofauna, i.e, earthworms. The passage of sub–surface soil and leaf litter through the earthworm's gut significantly alters the physical structure of the material. The shredding increases the surface area of the litter, while transport makes it more accessible to microbes for decomposition. In addition, the feces of the macrofauna are rich in soluble carbon, nitrogen, phosphorus, potassium, calcium, and magnesium (Winsome, 2005; Hillel and Hatfield, 2005). According to Astaykina *et al*. (2022), changes in gut microbiota in response to chemical fertilizers might negatively impact the health and ability of earthworms to digest organic matter, thus decreasing soil fertility.

Farmers are left with no choice but to use fertilisers to boost crop yields and replenish soil nutrients often called NPK, i.e, nitrogen (N), phosphorus (P), and potassium (K). All synthetic materials are detrimental to soil fauna and crop quality, especially fertilizers and insecticides (Singh and Gupta, 2018). Numerous toxicological investigations have been conducted earlier to assess the harmful impacts of pesticides on earthworms. Among soil invertebrates, earthworms comprises macrofauna (about 70–80%), making them "ecological engineers." Because of their short life span, sensitivity, and ease of cultivation due to their rapid growth, many earthworm species—including *Eisenia fetida* and *Lumbricus terrestris*—are used as model organisms to measure the ecotoxicity of emerging pollutants. The detrimental effects of fertilizers on earthworm is an emergent issue that requires careful consideration and research. The previously reported studies demonstrated dose-dependent toxicity of fertilizers, but they all agreed on reduction in growth rate, reproduction, and survival following urea (and to a lesser extent DAP) exposure (Bhattacharya and Sahu, 2015; Long *et al*., 2017; Li *et al*., 2025; Miglani and Bisht, 2019). Evaluation of hstological alterations helps to better understand the relationship between intestinal tissue damage and its biological effects on growth rate/biomass accumulation. However, there is a lack of sufficient data on tissue level damage, specifically chloragogenous cells of the gut region of *Eisenia fetida* after receiving a sublethal concentration of DAP and urea. The present study aimed to investigate histological changes in the gut region of *Eisenia fetida* after receiving a sublethal concentration of DAP and urea, as histological observations are important indicators in a toxicological context.

MATERIALS AND METHODS

Test animals and soil preparation

Earthworms, *Eisenia fetida* were obtained from Godhan unit, Bahadurgarh (Haryana), a private firm engaged in the production of vermicompost. Tropical artificial soil was prepared in the laboratory, containing 700g of industrial soil, 150g of clay, 50g of lime, and 100g of cocopeat per 1000g. Synthetic fertilizers used were solid and granular type (IFFCO) procured from local market.

Calculation of LC_{50}

Earthworms were exposed to urea or DAP dilutions, and the percentage of mortality was noted on the 14th day for each concentration. Initially, a range of 100 to 4000 mg of urea/kg of soil with a gap of 500 mg/kg of soil was employed. Finally, the LC_{50} calculations were performed at a regular interval of 100 mg/kg between 500 mg/kg and 1000 mg/kg. Likewise, DAP was also tested through a range-finding test. Final range of DAP was 300–4500 mg/kg of soil. On the log-probit plot, the percentage of earthworm mortality was plotted against the corresponding chemical fertilizer concentrations. The Finney (1952) log-Probit plot of the regression line was used to estimate the LC_{50} values.

Experimental design

For setting up an experiment, each replicate was placed in three different earthen pots with an internal radius of 6 cm and a depth of 8 cm (Volume ~900 cc), filled 3/4 full with artificial tropical soil. The pots were neatly labelled as 'T_0' Control, 'Urea T_1', and DAP 'T_2'. Ten adult *E. fetida* earthworms of approximate the same weight (280-300 mg) were randomly distributed in each pot. The artificial tropical soil was moistened (70%) before the transfer of earthworms and was watered on alternative days regularly. 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 1/2 of LC_{50} or either with 431.063 mg urea/kg soil, or 1049.345 mg DAP/kg of soil, per kg of artificial soil, as per the label on the pots. After thoroughly mixing the artificial soil, lime was added to adjust the pH to 6.0 ± 0.5 units. The earthworms were randomly harvested from each pot for observation after 1,15,30, 45, and 60 days of the treatment to study biomass accumulation and histological changes (on day 15 and 60) of the earthworms. As mentioned in OECD guidelines, (1984) the soil was watered every alternate day and dried cow dung was also supplemented weekly. To ensure adequate aeration and moisture levels and to prevent the earthworms from escaping the pots, each pot was covered with a net cloth. At first, pots were checked regularly for any aberrant activity for up to 7 days. The worms also underwenta thorough visual examination to document any morphological changes.

Biomass accumulation

The biomass accumulation was determined by weighing the earthworm as described by Senapati and Dash (1984). For this purpose, individual earthworms were removed from the culture pot and cleaned of any soil on their body surface, if present. To access the weight of earthworm culture, the weight of live earthworms was measured on 1st,15th,30th,45th and 60th day of post-exposure. The biomass accessed wasin a batch of 10 worms for total culture in each pot.

Statistical analyses

The results were statistically analyzed using Microsoft Excel on a computer. A regression equation was used to calculate LC_{50}. A two-way ANOVA was used to estimate the variance in biomass post-treatment.

Study of histo-morphological alterations

The histology of the earthworm gut was studied by carrying out a routine paraffin method (Humason, 1979). For this purpose, the earthworms (n=5) were kept overnight in agarose gel to replace their gut content (Maenpaa *et al.,* 2002). The gut of the earthworms (post-exposure) was aseptically removed and washed, fixed in Bouin's fixative for 24 hours. The gut tissue was then dipped in 70% ethanol followed by successive ascending grades of ethanol to dehydrate the tissue. The tissue was dipped in xylene before paraffin embedding. The tissue was subjected to section cutting (thin sections-5 µm) after staining with a hematoxylin and eosin stain, and then mounted in DPX to prepare permanent slides. The slides of the microsections were observed under a Nikon microscope at 4x magnification, fitted with a camera on the eyepiece.

Ethical approval statement

The study employed an observational, experimental procedure on invertebrate earthworms, thereby not requiring clearance from the Ethics Committee.

RESULTS AND DISCUSSION

Urea and DAP are two synthetic fertilisers that are frequently used in almost all crops. Details of toxicity the LC_{50} of Urea and DAP against *E. fetida* are given in Table 1.

Assessment of biomass accumulation exposed to chemical fertilizer treatment

The weight of the 10 control group earthworms was 2.84 ± 0.14 g on day 1 and continued to increase over time, reaching 3.46 ± 0.17 g by 60th day of treatment (Table 2). The total weight of the culture reared on urea was 2.94 ± 0.14 g on day 1, which, unlike the control group, decreased with time and was reduced to 1.02 ± 0.05 g on the 60th day post-treatment. The earthworm reared on diammonium phosphate had a mean biomass of 2.88 ± 0.143 g and showed an insignificant increase/fluctuation in the biomass up to day 45 after which the biomass decreased to 2.04 ± 0.102 g. Two-way ANOVA revealed that the differences among the treatments were significant with an F value of 4.54 and a p-value <0.01. The loss of biomass in earthworms due to the use of chemical fertilizers, therefore, is a matter of grave concern.

The treatment with synthetic fertilisers was found to have a negative impact on the overall biomass of *E. fetida* earthworms in the soil. Synthetic fertilizers, such as diammonium phosphate and urea, when applied to soil harbouring earthworms, killed a large number of earthworms. The mortality-adjusted weight of the earthworms was also found to decline upon treatment with synthetic fertilisers, implying that the decline in their biomass was not solely due to mortality. Moreover, the loss in the biomass of the *E. fetida* earthworms was not observed in the control group (Table 2).

The longevity of the earthworms, being an important confounding factor for the biomass of the culture, was also studied in detail. Mortality of the earthworms was recorded from 3 different sets of 10 earthworms in each replicate. The recordings of three such experiments were pooled to calculate the mean mortality. Percent per day mortality (used in the study) is shown in Figure 1. The number of surviving earthworms decreased to 91 ± 3.30% of the starting number, whereas the surviving earthworms were only 28.5 ± 3.0% in the case of urea and 57.8 ± 4.20% in the case of DAP treatment.

In all treatment sets there were notable variations in the earthworms weight of *E. fetida* after 15 days. However, in all treatment sets with synthetic fertilizers and at time intervals, effect of the synthetic fertilizers on earthworm culture size might be as a result of a change in the soil pH. Urea is known to break down in the soil, releasing ammonia, thereby altering the soil chemistry. The change in the soil chemistry has been examined to be an antagonist to the soil micro-macroflora (Staley *et al*., 2018; Geisseler and Scow, 2014). In the current study, the discernible decline in earthworm biomass may be attributed to the worms' inability to directly consume the acidified soil following direct exposure to synthetic fertiliser. The worms' inability to directly consume the acidified soil while exposed to nitrogenous/synthetic fertilizer may bethe cause of the decline in earthworm biomass observed in the current study. Aouaichia *et al*., (2024) states that when a certain amount of contaminants exceeds ac-

Table 1. Median lethal concentration (LC_{50}) of *Eisenia fetida* (n=10) in urea and DAP fertilizer after 14 days.

Serial No	Chemicals used	Regression equation	LC50 value	Statistically significant (95% confidence Interval)
1	Urea	Y=5.037x-9.789	862.126 mg/kg	Yes
2	DAP	Y=3.451x-6.383	2098.69 mg/kg	Yes

Table 2. Biomass(g) of earthworm, *Eisenia fetida* culture (post exposure) in synthetic fertilizers (Urea & DAP)

Days	Biomass control (T_0) Mean(g)±SD	Biomass urea (T_1) Mean(g)±SD	Biomass DAP (T_2) Mean(g)±SD
Day 1	2.84±0.141	2.94±0.146	2.88±0.143
Day 15	2.86±0.142	2.42±0.120	2.72±0.135
Day 30	2.88±0.143	1.98±0.099	2.96±0.147
Day 45	3.02±0.150	1.96±0.098	2.98±0.148
Day 60	3.46±0.172	1.02±0.051	2.04±0.102

*P value significant at 0.01

ceptable levels, earthworms have been shown to starve rather than eat as a natural physiological response. Earthworms overall lose in biomass of culture in response to synthetic fertiliser exposure, is a reflection of chemical stress that involves reducing food intake to prevent membrane system disorders or the disruption of cell membrane integrity by contaminants after exposure. Remarkably, in present study the decrease in weight gain on DAP (T_2) day 15 and 60 (Table 2) suggests that they had difficulty recovering in soil containing synthetic fertilizers, which impacted their growth/ mass accumulation capacity. The current study's findings corborates with Zhang *et al*. (2022) findings on Zinc oxide nanoparticles (ZnO NPs) and polyethylene microplastics (PE MPs) coexposure to *Eisenia fetida* results in significant weight loss after 28 days compared to 14 days. In treatment T_2, an inflammatory response may be the cause of weight gain on days 30 to 45, as it was declining again on 60th day(Table 2**)**. The results of thepresent study showed that inoculation of synthetic fertilizers, such as urea and DAP, impact earthworm biomass in treatment T_1 and T_2, the ultimate reason may be the change in pH of soil, the soil has become acidic (Curry, 2004; Pfiffner, 2014; Rashid, 2019; Rai *et al*., 2014; Passi *et al*., 2021). Another study by Mekersi *et al*. (2022) found that high salinity levels, including the high acidic pH of olive mill wastewater (OMWW) and olive mill pomace (OMP), significantly inhibit growth rate in earthworm, *A. trapezoids*. The results of present study also correlate with Aouaichia *et al.* (2024) experiment with ammonium sulfate fertilizer on *A. trapezoids*, and found that growth inhibition rate was higher on 4th week period as compared to 2nd week. Nitrogenous substances may cause harmful alterations to earthworms at individual and community levels (Marco and Ortiz-Santaliestra, 2009).

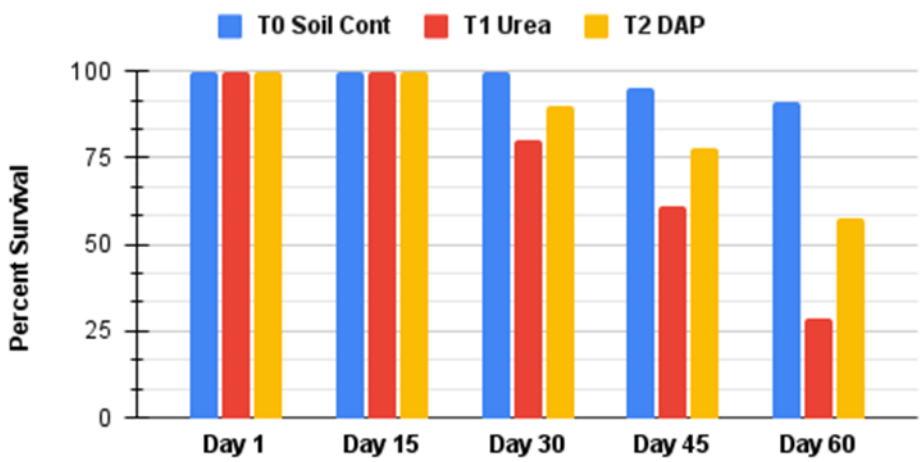

Fig. 1. *Survival of earthworms,Eisenia fetida culture (post exposure) in synthetic fertilizers (T_0-Control, T_1-Urea ;T_2- DAP)*

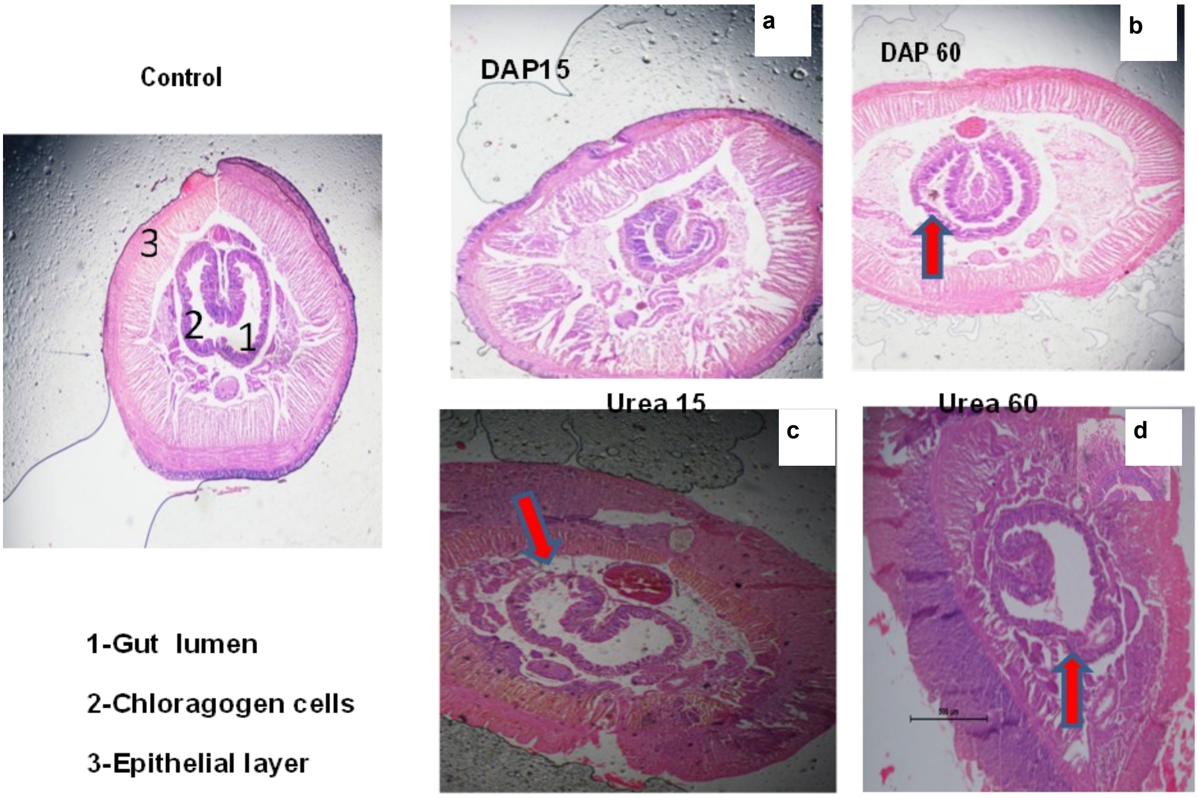

Fig. 2. *Histology sections of the gut of Eisenia fetida under the influence of synthetic fertilizer (Urea & DAP***);** *Control day 1; a) DAP 15 day b)DAP 60 day c)Urea 15 day d)Urea60 day; *red arrows point to the region of damaged Chloragogen cells*

Mosa Mohammed (2024) observed that the weight of *Octolasion cyanieum* earthworms in the treatment group decreased as urea level increased, affecting their vitality. Rai *et al*. (2014) claimed a dose-dependent correlation in weight reduction of *Eisenia fetida* worms when the urea dose was escalated from 0.75gm/kg to 2.25gm/kg over a duration of 60 days. The present study findings confirm that, the epigeic earthworm *E. fetida* was more vulnerable to soil acidity (pH <6.5) by synthetic fertilizers (urea and DAP). Passi *et al*. (2021) in their experiment observed higher LC_{50} value of urea in comparison to DAP on 14-day post-exposure period to *Eisenia fetida.*

Effect of the chemical fertilizers on the gut of *Eisenia fetida*

A common method for evaluating the environmental effects of potential exogenous pollutants on various living organisms, including earthworms, is histology (Jung *et al*., 2012 ; Gowri and Thangaraj, 2020; Li *et al*., 2020).

In the present study, extensive histopathological alterations **(**Fig. 2) were observed in the epithelial layer, width of central gut vesicle/lumen and chloragogenous cells of the gut region of *E. fetida* exposed to Urea and Diammonium phosphate. The earthworms from the control set showed structured epithelial layer, well defined gut vesicle/lumen and chloragogen tissue throughout the study period. The histopathological examination revealed that the exposure to increased concentrations of synthetic fertilizer affected not only the epidermal layer but also chloragogen cells, which further increased with time.

However, after 15 days of synthetic fertiliser treatment, there was a noticeable decrease in the width of the gut lumen or central vesicle Fig.2 (a), and after 60 days of exposure to diammonium phosphate, there was a noticeable breakdown of the chloragogenous cells Fig.2 (b). On the other hand, in the case of urea fertiliser, damage to chloragogen cells was shown to cause a loosening effect in the central vesicle's cavity after 15 days Fig.2 (c), and by the 60th day, the damage to chloragogen cells had/get more intensified Fig.2 (d) in gut region.

The results of the present study are similar to the work done by Qi *et al*. (2018) on earthworm, *Eisenia fetida* gut, with the neonicotinoid insecticide cycloxaprid, which results in damage to the chloragogenous cells. Reports available on histopathological examination showed loss of structural integrity and vacuolated chloragogenous cells in the earthworm, *Lumbricus terrestris*, isolated from heavy metal-contaminated soil

(Kılıç, 2011). The changes observed in the dilatation of the intestinal tract and degradation of chloraogen cells in *E. fetida* (Wang *et al*., 2015; Wang *et al*., 2020) were also on the same line. In the present study dilation of central gut vesicle and damaged chloragogen cells indicated that synthetic chemicals interact with the receptors present on the epithelium of the midgut lumen of earthworms resulting in the alteration of the midgut permeability ultimately leading to the rupture of the chloragogen cells (Mohssen, 2000; Samal *et al.,* 2017). Chloraogen tissue act as food reserve and helps in detoxification process upon pollutant exposure (Akat and Arman, 2016; Paul, *et al*., 2021; Hussain, *et al*., 2021). Furthermore, Kamat (2000) stated that tissue damage alters earthworm bioenergetics, which eventually disrupts the overall energy budget and reduces energy for physiological functions including growth and repair mechanism. The same findings were supported by Aquichaia *et al*. (2024).

In the present study, it was observed that earthworm from treatment pots T_1 and T_2, showed less weight at the end of study, i.e on 60th day, unlike the control group. It could be assumed that the primary reason for the reduced feeding activity of earthworms was the presence of synthetic fertilizers in the culture medium i.e, treatment pots T_1 and T_2. Starvation appeared to be a defensive physiological response of earthworms against the toxic effects of synthetic fertilizers. Due to prolonged starvation, the gut cavity of the worms exhibited either loosening i.e Fig. 2(c) or/and a noticeable reduction in size Fig. 2(a). The possible explanation for this: earthworms exposed to DAP exhibited an initial swelling of body tissues between the 30th and 45th day, resulting in a temporary increase in body weight. As further, a subsequent decline in weight was observed by the 60th day may indicate the onset of physiological stress, tissue degradation, or impaired metabolic function due to prolonged exposure of the fertilizer. The other explanation is that: synthetic fertilizer exposure led to alterations in the permeability of the midgut and caused significant damage to the chloragogen tissue.

In light of the present findings, available data and literature information it can assumed that due to this prolonged starvation, the gut cavity of the worms exhibited loosening i.eFig.2(c) or a noticeable reduction in size of central vesicle/gut in Fig. 2(a). The other explanation is that: synthetic fertilizer exposure led to alterations in the permeability of the midgut epithelium and caused significant damage to the chloragogen tissue. The chloragogen cells of earthworms are comparable to liver of vertebrates, as they function in the storage of nutrients and metabolic intermediates (Prento,1979). During periods of physiological stress, these stored components are released into the body fluids to support osmoregulation, detoxification, and tissue repair processes (Molnar,1992).

The results of the present study indicated that the LC_{50} value of urea was higher than that of DAP. Thereby, the toxic effects of urea manifest more early and chloragogen tissue damage becomes evident only after 15 days of exposure, as illustrated in Fig. 2(c). Chloragogen cells can restore damaged areas with their regenerative ability (Vogel,1992). Consequently, on the 60th day, with longer exposure duration, chloragogen cells show complete disintegration due to inability of exposed earthworms to rebuild tissue (Morgan 1981,1982).

Conclusion

The study concluded that *E.fetida*earthworms, one of the important soil fauna, are vulnerable to the sublethal dose(LC_{50}) of two widely used nitrogenous fertilizers, urea and DAP. Severe damage to chloragogen tissue emphasize the sensitivity of *E. fetida* earthworms to synthetic fertilizers. Furthermore, physiological changes in body mass and chloragogenous tissue caused by synthetic fertilizers carried out in vitro research reveal on how the earthworms adjust to shifting environmental conditions that may serve as important indicators for early detection and diagnosis of soil contamination. The experiment needs to be replicated under field conditions to ascertain the toxic effects of chemical fertilizers on the biology and physiology of earthworms in natural settings. The results of this investigation could help to understand how overuse of chemical fertilizers used in fields hamper the biological attributes of earthworms.

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Conflict of interest

The authors declare that they have no conflict of interest.

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