Nativa, Sinop, v. 11, n. 2, p. 241-250, 2023.
Pesquisas Agrárias e Ambientais
DOI: https://doi.org/10.31413/nativa.v11i2.15905

ISSN: 2318-7670

Effect of the balanced and nano-composite NPK fertilizer on the vitality of the earthworm *Octolasion cyanieum* and its effect on the histological composition of the middle of the body

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Submitted on 07/11/2023; Accepted on 08/04/2023; Published on 08/17/2023

ABSTRACT: This study was conducted to determine the effects of earthworm breeding *O. cyanieum* for 7, 14, 21, and 28 days in soil treated with concentrations of 800, 1000, 1500, and 2000 mg kg^{-1} of balanced NPK compound fertilizer and balanced NPK nano-compound fertilizer at concentrations of 30, 60, 120, and 240 mg kg^{-1}, in addition to the control treatment. It was found that the weight and relative growth rate of earthworms decreased with increasing concentrations of fertilizers. The highest significant decrease in weight (0.257 g) and relative growth rate (40.34%) was observed at a concentration of 1500 mg kg^{-1} of balanced NPK compound fertilizer. Meanwhile, the highest decrease in worm weight (0.225 g) and relative growth rate (86.31%) was observed at a concentration of 240 mg kg^{-1} of balanced NPK nano-compound fertilizer. Fertilizers also affected the protein content in the earthworms, which differed significantly from the control treatment for both fertilizers. In addition, the fertilizers affect the histological structure of the body, with clear disruption in the epithelium of the body, layers of circular and longitudinal muscle, the lining of the intestine, especially the typhlosole.
Keywords: chemical fertilizer; nano fertilizer; earthworm vitality.

Efeito do fertilizante npk balanceado e nanocomposto na vitalidade da minhoca octolasion cyanieum e seu efeito na composição histológica do meio do corpo

RESUMO: Este estudo foi conduzido para determinar os efeitos da criação de minhocas *O. cyanieum* por 7, 14, 21 e 28 dias em solo tratado com concentrações de 800, 1000, 1500 e 2000 mg kg^{-1} de solo de fertilizante composto NPK balanceado (20-20-20) e fertilizante nanocomposto NPK balanceado (20-20-20) nas concentrações de 30, 60, 120 e 240 mg kg^{-1} de solo, além do tratamento testemunha. O estudo mostrou que os fertilizantes químicos tiveram impacto sobre a vitalidade dessas minhocas, pois o peso e a taxa de crescimento relativo das minhocas diminuíram com o aumento das concentrações de fertilizantes. A maior queda significativa no peso (0,257 g) e na taxa de crescimento relativo (40,34%) foi observada na concentração de 1500 mg kg^{-1} de solo do fertilizante composto NPK balanceado. Já a maior redução no peso (0,225 g) e na taxa de crescimento relativo (86,31%) da minhoca foi observada na concentração de 240 mg kg^{-1} de solo do fertilizante nanocomposto NPK balanceado. Além disso, os tratamentos acima tiveram impacto no teor de proteína dos corpos das minhocas, que diferiu significativamente do tratamento controle, para ambos os tipos de fertilizantes. O estudo mostrou o impacto do fertilizante nas estruturas dos tecidos das minhocas, com clara ruptura na camada da pele e nas camadas musculares circulares e longitudinais abaixo dela, bem como um claro efeito no revestimento intestinal, especialmente o tiflosole.
Palavras-chave: fertilizante químico; nano fertilizante; vitalidade da minhoca.

1. INTRODUCTION

Earthworms are invertebrates and an essential part of the Earth's ecosystem since the pre-Cambrian era, which is about 600 million years ago. They belong to the phylum Annelida, subclass Oligochaeta (PIEARCE et al., 1990). these organisms act as natural plows for the soil and produce vermicompost (Antunes et al, 2021), which is a highly valuable material for meeting the nutritional needs of crops (GHOSH, 2018; ASLAM et al., 2021).

Earthworms are considered one of the most important large living organisms in the soil for maintaining and controlling soil fertility. They play a vital role in renewing the soil, and can only be found in soil with specific characteristics such as texture, sufficient moisture, appropriate acidity levels, and specific nutrients (KALE; KARMEGAM, 2010). Environmental degradation resulting from human activities can directly affect the quality of soil, and pose a threat to the biodiversity of living organisms (MASIN et al., 2020). According to some researchers, soil organisms that respond to changes in the environment are considered to be of great importance in maintaining soil quality, where non-vertebrate animals form the highest biomass in the soil (MIGLANI; BISHT, 2019; LUAN et al., 2020). Some researchers estimate that earthworms may contribute to about 40-90% of the large living biomass of soil organisms (AL-MALIKI et al., 2021; HUANG et al.,2021). And since earthworms have the ability to live in a variety of soil ecosystems, as well as their relatively large size and sensitivity to many pollutants, and their ease of cultivation due to their fast growth and short lifespan, they play an important role in maintaining soil quality (PODOLAK et al., 2020).

Earthworms can also be used for several generations to assess the potential effects of persistent pollutants over generations, as well as to study their ability to recover from or tolerate the studied compounds (SCHNUG et al., 2013; ŽALTAUSKAITĖ et al., 2020).

As increasing crop production largely depends on the type of fertilizers used, different types of agricultural chemicals such as chemical fertilizers are currently being used excessively, which negatively affects the soil, as well as soil plants and animals (MEENA et al., 2016; SENA et al., 2021). Negative effects include the loss of topsoil due to pollution from fertilizers, herbicides and pesticides, as well as the bioaccumulation of chemicals in food chains and webs, The use of excessive agricultural chemicals can have a negative impact on soil ecosystems by weakening the physical, chemical, and biological components of the soil, particularly beneficial non-target microorganisms and earthworms (DHANANJAYAN et al., 2020; GUNAWARDENA, 2022), Fertilizers also increase the concentration of nitrates in the water and this has a negative effect (AL-HUSSEIN et al., 2023)

This study was conducted to determine the effect of balanced NPK compound fertilizer and balanced NPK nano compound fertilizer on the vitality of the earthworm *Octolasion cyanieum*, as well as the effect on the tissue composition of the skin and the middle part of the intestine.

2. MATERIAL AND METHODS

2.1. Collecting earthworm samples:

This study was conducted at the University of Mosul, College of Education for Pure Sciences. Earthworms were collected in late August 2022 from home gardens in the Nimrud district south of Mosul. The samples were collected by digging the soil to a depth of 15 to 30 cm or more and were preserved and bred in plastic boxes with dimensions of (55 * 35 * 28 cm) (length * width * height). The boxes contained a piece of cloth placed at the bottom of the box and extending to its sides. Bird droppings with soil were also placed in these boxes for the purpose of feeding the worms during their rearing period according to the method (GARG et al., 2005; GARCZYŃSKA, 2020).

The soil was constantly moistened to keep the worms alive and maintain their viability for use in laboratory experiments. They were classified at the Research Center, Natural History Museum/University of Baghdad, Department of Insects and Invertebrates by Dr. Nabres Falih Jijan Al-Khafaji. The breed is: *Octolasion cyanieum* (Savigny, 1826) (Crassiclitellata, Lumbricidae)

2.2. Fertilizers used in the study and preparation of biological effects experiments on earthworms:

A completely randomized block design with three replicates and five treatments per fertilizer, including one control treatment and four other treatments, was used. The current study used two types of fertilizers: inorganic chemical fertilizer (balanced compound fertilizer NPK 20-20-20), which was obtained from local markets in Mosul, Iraq, and the following selected concentrations (800, 1000, 1500, and 2000 mg kg^{-1}). Also, a nano-fertilizer (the balanced compound nano-fertilizer 20-20-20-NPK), which was obtained from the Green Fertilizer Company, and the selected concentrations (30, 60, 120, and 240 mg kg^{-1}) were used. The concentrations used in the study were estimated based on the recommended amounts per fertilizer kilogram/ Dunam, as well as selecting concentrations higher than the recommended amounts. As well as, the necessary fertilizer ratio for one treatment was determined by calculating the amount of soil in kilograms at a depth of 15 centimeters per Dunam, which was estimated at 500,000 kilograms per Dunam. By performing the ratio and proportion operation for each selected concentration of fertilizers, the necessary amount of fertilizer in grams per kilogram of soil was determined. Several bioassays were conducted to determine the effect of fertilizers on the vitality of earthworms.

Plastic containers with a capacity of one kilogram were taken and filled with 500 grams of soil that had been previously sterilized at 90 degrees Celsius in an electric oven for five hours. The purpose of sterilization was to ensure that the soil was free of worm eggs or other organisms that could interfere with the experiment's results. The soil's pH was 7.8, and its moisture content was 17%. Before treating the earthworms with fertilizer, they were placed in cups containing distilled water for over two hours to clean their digestive tract of soil and expel their waste. The worms' weights were then measured using a sensitive electronic scale, with 5 worms per repeater, ensuring they were approximately the same size and age.

They were then placed in plastic containers containing soil treated with fertilizers, which had been mixed well. Cattle manure was used as food for the earthworms during the experiment period, added every week after weighing the worms. The plastic containers were then covered with a piece of transparent cloth to prevent the worms from escaping while being monitored for (7, 14, 21, and 28 days).

2.3. The effect of fertilizer concentrations on the weights of earthworms:

Adult worms (with a clitellum) were selected, which were similar in weight and age, and their weights were calculated every week using a sensitive electronic scale, and the difference in their weights was noted compared to the control group.

2.4. The effect of fertilizers on the relative growth rate of earthworms:

After measuring the weights of the worms before and after treatment with the aforementioned fertilizers to determine the extent of the effect of these fertilizers on the growth rate of the worms, their growth rate was calculated according to the method of Sogbesan; Ugwumba (2006) using the following equation:

Relative growth rate (RGR) = weight after treatment ÷ weight before treatment × 100.

2.5. Estimation of protein content in the bodies of earthworms:

The amount of protein in the bodies of earthworms treated with fertilizers was estimated using the method of (Lowry et al., 1951) and was modified by Schachterle; Pollack (1973).

2.6. Histological tests:

For the purpose of determining the effect of the fertilizers used in the experiments on the tissue structure of some areas in the bodies of the earthworms treated with fertilizers, histological tests were performed on these areas. The samples were stained using the hematoxylin-eosin and Delafield's hematoxylin stains based on the method of Luna (1968) and Culling et al. (1985).

2.7. Examination and microscopic imaging of tissue sections:

The tissue sections were examined using a compound light microscope to observe the tissue effects resulting from fertilizer treatment. After examination, the sections were imaged using a microscope equipped with a digital camera. The lens magnification power was calculated using the ratio of the objective lens magnification to the eyepiece lens magnification to determine the magnification power of the tissue dimensions in the section.

2.8. Statistical Analysis:

The results were statistically analyzed using the SAS program on a computer with a completely randomized design. A factorial C.R.D. experiment was conducted, and the differences between the means were selected using a multiple range test (Duncan's test) at a 5% probability level (L-ZUBAIDY; AL-FALAHY, 2016).

3. RESULTS

3.1. The effect of balanced NPK fertilizer

The results in Table (1) show that treating soil with concentrations of 800, 1000, 1500, and 2000 mg kg^{-1} of balanced NPK fertilizer for cultivation periods of 7, 14, 21, and 28 days had a significant effect on the weight rate of *O. cyanieum* earthworms. We find from the table that the different concentrations had a clear effect, as the weight rate significantly decreased, reaching 0.458 and 0.545 g at concentrations of 1500 and 2000 mg kg^{-1}, respectively. Both concentrations were significantly different from the weight rate of the control treatment, which was 0.832 g.

As Table 1 shows, the weight rate was also affected by the duration of the earthworms' stay in the environment treated with NPK fertilizer. The weight rate decreased with the increase of the time period, with the weight being 0.662, 0.660, 0.625, and 0.570 g at time periods of 7, 14, 21, and 28 days, respectively.

As for the effect of the interaction between concentration and time period, we notice that all the earthworms died at a concentration of 2000 mg kg^{-1} starting from the time period of 14 days of treatment. As we can see from the table, in the first time period of treatment, which was 7 days, all concentrations showed a significant difference with the comparison group, which had a weight of 0.752 g. The lowest weight of the earthworms was 0.545 g at a concentration of 2000 mg kg^{-1}, and 0.645, 0.664, and 0.708 g at concentrations of 1500, 1000, and 800 mg kg^{-1}, respectively, and for the same time period.

In the time periods of 14 and 21 days, there was also a significant decrease in the weight of the earthworms, as shown in the table, compared to the comparison group, which had weights of 0.804 and 0.864 g in the mentioned time periods, respectively. The highest weight decrease was recorded in the 28-day period, which was 0.257 g at a concentration of 1500 mg kg^{-1}, and 0.571 and 0.544 g at concentrations of 800 and 1000 mg kg^{-1}, respectively, no significant difference between them. The weight of the comparison group was 0.911 g for the same time period, which was significantly different.

Table 1. The average weight (g) of the earthworm *O. cyanieum* raised in soil treated with different concentrations of NPK compound fertilizer for several time periods.
Tabela 1. Peso médio (g) da minhoca *O. cyanieum* criada em solo tratado com diferentes concentrações de fertilizante composto NPK por vários períodos de tempo.

Concentration (mg kg^{-1})	Exposure period (days)				Concentration effect rate
	7	14	21	28	
800	0.708 e	0.672 f	0.644 gh	* 0.571 j	0.648 b
1000	0.664 fg	0.621 hi	0.607 i	0.544 j	0.609 c
1500	0.645 gh	0.545 j	0.386 k	0.257 l	0.458 d
2000	0.545 j	…	…	** …	0.545 e
control	0.752 d	0.804 c	0.864 b	0.911 a	0.832 a
Rate (time period effect)	0.662 a	0.660 ab	0.625 b	0.570c	

*Numbers that carry different letters have different meanings according to the Duncan multiple range test at a significance level of 0.05%. ** (.....) means the death of all worms during the recorded period.

The results in Table 2 show that treating the soil with concentrations of 800, 1000, 1500, and 2000 mg kg^{-1} of NPK compound fertilizer for the cultivation periods of 7, 14, 21, and 28 days had a significant effect on the relative growth rate of the earthworm *O. cyanieum*. From the table, it can be seen that the different concentrations have a clear effect, with a significant decrease in the relative growth rate to 61.34% and 73.35% at concentrations of 1500 and 2000 mg kg^{-1}, respectively, and both concentrations differed significantly from the relative growth rate in the control treatment, which reached 113.60%.

As the table shows, the relative growth rate was also affected by the treatment period, with the lowest growth rate being 77.41% and 84.73% at the treatment periods of 28 and 21 days, respectively, meaning that the relative growth rate decreased with an increase in the exposure period. As for the effect of the interaction between concentration and treatment time, we notice that all worms died at a concentration of 2000 mg kg^{-1} and for treatment periods of 14, 21, and 28 days, respectively. The table also shows a significant difference in the relative growth rate during the first (7 days) of cultivation, as the lowest relative growth rate was 73.35% at a concentration of 2000 mg kg^{-1}. Similarly, the relative growth rates at the other concentrations showed a clear decrease compared to the comparison group which had a relative growth rate of 102.59% for the same time period.

Additionally, we observe a significant decrease in the relative growth rate of the worms during the second treatment period of 14 days and the third period of 21 days. The maximum relative growth rate during the second period was 91.42% at a concentration of 800 mg kg^{-1}, while the relative growth rate for the comparison group was 109.68% during this period. Furthermore, the maximum relative growth rate during the third period of 21 days was 87.61% at a concentration of 800 mg kg^{-1}, while the comparison group had a relative growth rate of 117.87% during the same period. As for the fourth time period of 28 days, we observe the highest decrease in the relative growth rate of 34.40% at a concentration of 1500 mg kg^{-1}, and 73.31% and 77.68% at concentrations of 1000 mg kg^{-1} and 800 mg kg^{-1}, respectively. All of the above results were significantly different from the comparison group, which had a relative growth rate of 124.28% during the same time period (28 days).

Table 2. Relative growth rate (%) of earthworm *O. cyanieum* raised in soil treated with different concentrations of NPK fertilizer for various periods of time.
Tabela 2. Taxa de crescimento relativo (%) da minhoca *O. cyanieum* criada em solo tratado com diferentes concentrações de fertilizante composto NPK por vários períodos de tempo.

Concentration (mg kg^{-1})	Exposure period (days)				Rate (concentration effect)
	7	14	21	28	
800	96.32 e	91.42 f	87.61 fh	* 77.68 jk	88.17 b
1000	89.48 fg	83.69 hi	81.80 ij	73.31 k	82.08 c
1500	86.34 gi	72.95 k	51.67 l	34.40 m	61.34 d
2000	73.35 k	…	…	…**	73.35 e
control	102.59 d	109.68 c	117.87 b	124.28 a	113.60 a
Rate (time period effect)	89.61 a	89.43 ab	84.73 b	77.41 c	

*Numbers that carry different letters have different meanings according to the Duncan multiple range test at a significance level of 0.05%. ** (.....) means the death of all worms during the recorded period.

The results in Table (3) show the treatment of soil with concentrations of 800, 1000, 1500, and 2000 mg kg^{-1} of balanced NPK compound fertilizer and its effect on the protein content in the body of earthworms *O. cyanieum* after 28 days, except for the concentration of 2000 mg/ kg^{-1} (protein was measured after seven days). There was a significant difference in the amount of protein recorded, which was 0.142, 0.166, and 0.216 mg g at concentrations of 800, 1000, and 1500 mg kg^{-1}, respectively. And all of these concentrations showed a significant decrease compared to the control group (0.250 mg g), The protein content of the worm increased, reaching 0.233 mg g at a concentration of 2000 mg kg^{-1}. Despite its increase, there was no significant difference with the control group.

Table 3. Protein content (mg g) in the body of earthworms *O. cyanieum* raised in soil treated with different concentrations of NPK compound fertilizer and for different periods of time.
Tabela 3. Teor de proteína (mg g) no corpo de minhocas *O. cyanieum* criadas em solo tratado com diferentes concentrações de fertilizante composto NPK e por diferentes períodos de tempo.

	control	800	1000	1500	2000
The amount of protein	0.250a	0.14d	0.166c	0.21b	*0.233ab

*Numbers that carry different letters have different meanings according to the Duncan multiple range test at a significance level of 0.05%

The results in Table (4) indicate that soil treatment with concentrations of 30, 60, 120, and 240 mg kg^{-1} of balanced nano NPK fertilizer for cultivation periods of 7, 14, 21, and 28 days had a significant effect on the weight of earthworms *O. cyanieum*. From Table 4, it is clear that different concentrations had a distinct effect, as the weight significantly decreased to 0.683 and 0.451 g at concentrations of 120 and 240 mg kg^{-1}, respectively. Both treatments were statistically different from the weight average. The weight at the comparison treatment was 0.833 g.

The table also shows that the weight rate was also affected by the length of time the worm stayed in the soil treated with balanced nano NPK fertilizer. The weight rate decreased with an increase in the time period, and the weight was 0.749, 0.714, 0.663, and 0.592 g for the time periods of 7, 14, 21, and 28 days, respectively. There was a significant difference between these treatments.

As for the effect of the interaction between concentration and time period, we notice from the table that in the first time period of 7 days, all concentrations recorded a significant difference from the comparison group, which had a weight of 0.782 g, except for the weight at a concentration of 60 mg kg^{-1}, which did not record a significant difference. We note that the weight at a concentration of 30 mg kg^{-1} was higher than that in the comparison group for the same period. In the 14- and 21-day periods, there was also a significant decrease in the weights of the worms, as shown in Table 4. The lowest weight was 0.530 and 0.417 g at a concentration of 240 mg kg^{-1}, compared to the comparison group, which weighed 0.812 and 0.844 g in the respective periods. As for the 28-day period, we note that there was no significant difference in weights between the concentrations of 30 and 60 mg kg^{-1}, but both recorded a significant difference from the comparison group, which weighed 0.894 g in the same time period.

3.2. The effect of balanced NPK nanocomposite fertilizer

The results in Table 5 show that treating the soil with concentrations of 30, 60, 120, and 240 mg kg^{-1} of balanced NPK nanocomposite fertilizer for cultivation periods of 7, 14, 21, and 28 days had a significant effect on the relative growth rate of *O. cyanieum* earthworms. We can see from the table that the different concentrations had a clear significant effect, as the relative growth rate significantly decreased. The growth rate percentage was 103.06% at a concentration of 30 mg kg^{-1}, while it was 63.87% at a concentration of 240 mg kg^{-1}. Table 5 also shows that the relative growth rate was significantly affected by the treatment period. The lowest growth rate was 94.55% and 84.46% for treatment periods of 21 and 28 days, respectively, indicating that the relative growth rate decreased with an increase in exposure time.

As for the effect of the interaction between concentration and treatment period, we can see from the table that there was a significant difference in the relative growth rate in the first period of cultivation, which was (7 days), All concentrations showed a significant difference in the growth rate compared to the control group, which was 111.23%. We also notice that the concentrations 30 and 60 mg/ kg^{-1} did not significantly differ in the relative growth rate. Similarly, we observe a significant decrease in the relative growth rate during the second period of cultivation, which was (14 days). The growth rate ranged from 109.42% to 75.07% in concentrations of 30 and 240 mg/ kg^{-1}, respectively, compared to the control group of 115.50%. During the third period of cultivation, which was 21 days, there was a significant decrease in the relative growth rate of the earthworms. The maximum growth rate during this period was 99.14% at a concentration of 30 mg/ kg^{-1}, while the control group had a growth rate of 120.05%. During the fourth time period of (28 days), we observed a significant difference between the concentrations and the comparison group, was the highest decrease in relative growth rate being 31.86% at a concentration of 240 mg/ kg^{-1}, while the comparison group recorded a growth rate of 127.16% during the same time period.

Table 4. The average weight (g) of *O.cyanieum* earthworms raised in soil treated with different concentrations of NPK nanocomposite fertilizer for several periods of time.
Tabela 4. Peso médio (g) de minhocas *O. cyanieum* criadas em solo tratado com diferentes concentrações de fertilizante nanocompósito NPK por vários períodos de tempo.

Concentration (mg kg^{-1})	Exposure period (days)				Rate (concentration effect)
	7	14	21	28	
30	0.788 bd	0.766 df	0.690 gh	* 0.636 i	0.720 b
60	0.781 ce	0.734 dg	0.709 fg	0.626 i	0.712 c
120	0.764 df	0.730 eg	0.656 hi	0.582 j	0.683 c
240	0.632 i	0.530 j	0.417 k	0.225 l	0.451 d
control	0.782 ce	0.812 bc	0.844 b	0.894 a	0.833 a
Rate (time period effect)	0.749 a	0.714 b	0.663 c	0.592 d	

*Numbers that carry different letters have different meanings according to the Duncan multiple range test at a significance level of 0.05%.

Table 5. Relative growth rate (%) of *O.cyanieum* earthworms raised in soil treated with different concentrations of NPK nanocomposite fertilizer for several periods of time.
Tabela 4. Taxa de crescimento relativo (%) de minhocas *O. cyanieum* criadas em solo tratado com diferentes concentrações de fertilizante NPK nanocomposto por vários períodos de tempo.

Concentration (mg kg^{-1})	Exposure period (days)				Rate (concentration effect)
	7	14	21	28	
30	112.57 dc	109.42	99.14 hi	* 91.14	103.06 b
60	112.21 dc	105.45	101.86	89.94 jk	102.36 bc
120	107.90 dh	103.10	92.65 ij	82.20 k	96.46 c
240	89.51 jk	75.07 l	59.06 m	31.86 n	63.87 d
control	111.23 de	115.50	120.05	127.16 a	118.48 a
Rate (time period effect)	106.68 a	101.70	94.55 c	84.46 d	

*Numbers that carry different letters have different meanings according to the Duncan multiple range test at a significance level of 0.05%.

The results in Table (6) show the effect of soil treatment with concentrations of 30, 60, 120, and 240 mg kg^{-1} of nano NPK compound fertilizer on the protein content in the body of *O. cyanieum* earthworms after 28 days. We observe a significant decrease in protein content, with the lowest protein percentage of 0.043 mg g occurring at a concentration of 30 mg kg^{-1}. The protein content was 0.075, 0.116, and 0.193 mg g at concentrations of 60, 120, and 240 mg kg^{-1}, respectively, and all showed a significant difference from the control group (0.250 mg g).

Table 6. Protein content (mg g) in the body of *O. cyanieum* earthworms raised in soil treated with different concentrations of NPK nano compound fertilizer for various time periods.
Tabela 3. Teor de proteína (mg g) no corpo de minhocas *O. cyanieum* criadas em solo tratado diferentes concentrações de fertilizante NPK nanocomposto e por diferentes períodos de tempo.

	control	30	60	120	240
The amount of protein	0.25a	0.043e	0.075d	0.116c	*0.193b

*Numbers that carry different letters have different meanings according to the Duncan multiple range test at a significance level of 0.05%

4. DISCUSSION

4.1. Effect of balanced NPK compound fertilizer and balanced NPK nano compound fertilizer on the weight and relative growth rate of earthworms

Table 1 shows that the weight of earthworms decreased as the concentration and duration of exposure to balanced NPK fertilizer increased over periods of 7, 14, 21, and 28 days. Similarly, the relative growth rate of earthworms decreased as the concentration and duration of exposure increased, as shown in Table 2. These results demonstrate that treating soil with NPK compound fertilizer concentrations of 800, 1000, 1500, and 2000 mg kg^{-1} for cultivation periods of 7, 14, 21, and 28 days resulted in a decrease in the relative growth rate of earthworms. This decrease in relative growth rate was more pronounced with increased concentration and duration of exposure.

According to the results shown in Table 4, the treatment of soil with concentrations of 30, 60, 120, and 240 mg kg^{-1} of balanced nanoscale NPK composite fertilizer, and for the cultivation periods of 7, 14, 21, and 28 days, resulted in an increase in worm weight of 0.788 g in the first period of 7 days at a concentration of 30 mg kg^{-1} compared to the control group, as previously mentioned in the results. Also, the relative growth rate for the same fertilizer and the same cultivation period of 7 days increased at concentrations of 30 and 60 mg kg^{-1}, reaching 112.57% and 112.21%, respectively. This is similar to the results obtained by RAI et al. (2014) and is consistent with Long et al. (2017) when treating worms with urea at concentrations of 50, 100 and 150 mg kg^{-1}. There may be two explanations for this: first, the bodies of the earthworms exposed to the fertilizers swelled, leading to an increase in weight. The other explanation is that the increase in the weight of the earthworms was a healthy increase (RAI et al., 2014).

Despite what was mentioned, the weight of the worms for the above concentrations and other concentrations of 120 and 240 mg kg^{-1} decreased with an increase in the time period. Also, the results in Table 5 showed that the relative growth rate of worms treated with balanced nano NPK compound fertilizer for the same concentrations and for a period of 28 days decreased with an increase in the concentrations and exposure time, except for what was mentioned above.

The reason for the decrease in the weight and relative growth rates of the worms may be due to the physiological stress that the worms were exposed to as a result of the changes that occurred in the environment due to the addition of fertilizers. It has been found that adding urea fertilizer to the soil leads to changes in the soil chemistry, such as changes in the soil pH and differences in ammonia levels. It has also been shown that these changes impact soil organisms, including earthworms (STALEY et al., 2018). Our results are consistent with the study by ZHANG et al. (2022) in their study on the effect of polyethylene nanoparticles (PE MPs) and zinc oxide nanoparticles (ZnO NPs) on the earthworm *E. fetida*. The application of these materials led to a decrease in worm weights. Our results also match Yan et al. (2021) in their study on the combined effect of neonicotinoid pesticides and heavy metals on earthworms, which had a negative impact on growth rate. Our current study's results are similar to QIAO et al. (2019) in their study on the earthworm *E. fetida*. Treating earthworms with five concentrations of the insecticide cyantraniliprole for 28 days resulted in a significant effect on the growth rate of the worms.

4.2. Effect of balanced NPK compound fertilizer and NPK nano compound fertilizer on the protein content in the body of earthworms

From the results in Table 3, treating the soil with concentrations of 800, 1000, 1500, and 2000 mg kg^{-1} of balanced NPK compound fertilizer, and the results in Table 6, treating the soil with concentrations of 30, 60, 120, and 240 mg kg^{-1} of balanced NPK nanocomposite fertilizer, showed that the fertilizers affected the protein content in the body of earthworms. Similarly, our findings matched those of SAMAL et al. (2017) in their study of the effect of urea, phosphate gypsum, and paper mill sludge on the earthworms *Drawida willsi* and *Lampito mauritii*, as the protein content in the bodies of the earthworms decreased significantly with the decrease in the concentration of urea and phosphate gypsum. Our results were also consistent with those of MOSLEH et al. (2003), as exposure of the earthworms *E. fetida* to pesticides caused a significant decrease in their protein content. This was attributed to a lower food intake in the earthworms exposed to pesticides compared to the control group (RIBERA et al., 2001). We hypothesize that the same applies to the chemical fertilizers used in our current study.

4.3. Effect of balanced compound NPK fertilizer and balanced NPK nano compound fertilizer on the tissue composition of earthworm *O. cyanieum*.

Adeel et al. (2021) stated that any injury to the external skin and intestines may have harmful effects on the health of earthworms, which delays the normal performance of these tissues and may lead to earthworm mortality. Additionally, tissue injury affects the vital energy of earthworms, which ultimately leads to a disturbance in the overall energy budget, leaving less energy for physiological processes such as growth and reproduction.

Therefore, our results matched those of SAMAL et al. (2017) in their study, where damage occurred to the skin and surface tissues, separation between longitudinal and circular muscles, and decay occurred. Also, the connective tissues were affected when worms were treated with urea, phosphate gypsum, and paper mill sludge. Our results also matched those of WANG et al. (2020) in their study where tissue damage occurred in the body wall, intestine, and seminal vesicles of earthworms under a high level of exposure to nickel. Our results also matched those of Lahive et al. (2014) in their study on the effect of cerium oxide nanoparticles (NPs) on the earthworms *E. fetida*, the exposure led to significant damage to the skin and surface layer. Our study also matched that of Adeel et al. (2021), where the application of rare earth metals, including nano and bulk lanthanum (La_2O_3) and yttrium oxides (Yb_2O_3), on earthworms caused radical changes in the epithelium layer of the digestive system and typhlosol. The epidermis of the Earthworm treated with 200 mg kg^{-1} of nanominerals and ordinary metals has been sucked and peeled. Applying the same substances with the same concentration also led to edema in the layers of longitudinal muscle and circular muscle inflammatory cells in the intestinal tissue of the earthworm. Also, the occurrence of necrosis in mucous cells, compared with the untreated control group.

4.4. Figures The effect of balanced NPK compound fertilizer and NPK nano compound fertilizer on the tissue structure of *O. cyanieum* earthworms

To understand the mechanism of the effect of the fertilizer used in this study, tissue sections were made of the middle part of the earthworm body. Figures 1 and 2, show a section of an untreated earthworm, illustrating the composition of the body wall, which consists of the skin layer, circular and longitudinal muscles, and the wall part of the peritoneal membrane. The section also shows the middle part of the intestine and the blind area of the typhlosole.

Figure 3 shows the effect of breeding earthworms for 14 days in soil treated with a concentration of 1500 mg kg^{-1} of the balanced NPK compound fertilizer. This led to an increase in the thickness of the circular and longitudinal muscle layers, as well as the separation and detachment of fibers from these muscle layers. The treatment also resulted in the separation of the cuticle layer from the skin, causing congestion of blood vessels within the muscle layers and necrosis of skin cells.

Similarly, breeding these worms for 21 days in soil treated with the same previous concentration of fertilizer led to an inflammation in the intestinal lining and a clear separation of the peritoneal membrane from the muscle layer, with edema occurring in the connective tissue of the typhlosole. Folds were also present in the mucous membrane lining the intestines and typhlosole (Figure 4).

As shown in Figure 5, after 28 days of breeding with the same concentration of fertilizer, there was inflammation in the circular muscle layer and a separation between the fibers of the longitudinal muscle layer, as well as a separation of the peritoneal membrane from the muscle layer. There were also bulges in the skin layer and separation of the cuticle layer.

As shown in Figure 6, breeding *O. cyanieum* earthworms for seven days with a concentration of 2000 mg kg^{-1} of balanced NPK compound fertilizer led to a separation of the cuticle layer, with a clear increase in inflammation of the skin layer cells and a separation of the fibers of the circular muscle layer, as well as inflammation in the longitudinal muscle layer.

Figure 7 shows that the balanced nano NPK compound fertilizer also had an effect on the histological structure of the earthworms' body medium. Breeding earthworms for 28 days in soil treated with a concentration of 60 mg kg^{-1} of the fertilizer led to a separation of the cuticle layer from the skin layer, with an increase in the pigmentation of the connective tissue under the circular muscle layer, and a separation in the longitudinal muscle layer. In addition, there was an increase in the thickness of the skin layer in some areas.

Figure 1. A cross-sectional view of the control group of the earthworm midgut, showing (1) skin layer, (2) circular muscles, (3) longitudinal muscle layer, (4) peritoneal membrane. The image is colored with E and H stains and has a magnification of 40x.
Figura 1. Corte transversal do grupo controle do intestino médio da minhoca, mostrando: (1) camada da pele, (2) músculos circulares, (3) camada muscular longitudinal e (4) membrana peritoneal. A imagem é colorida com manchas E e H e tem uma ampliação de 40x.

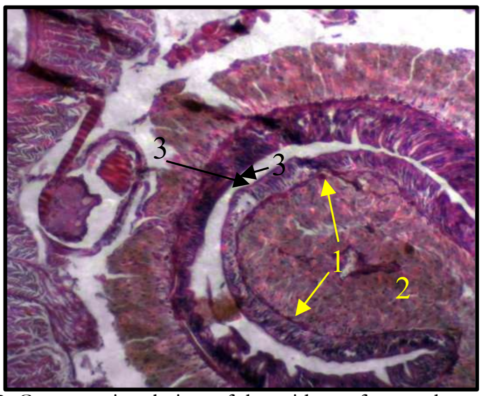
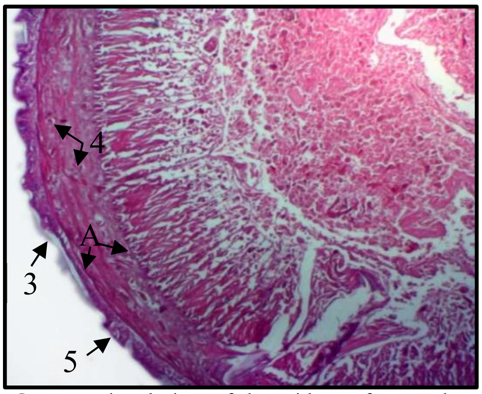


Figure 2. Cross-sectional view of the midgut of an earthworm from the control group. The image shows: (1) blind channel, (2) hepatic cells located within the blind channel's invagination, (3) lining of the digestive channel.
Figura 2. Corte transversal do intestino médio de uma minhoca do grupo de controle. A imagem mostra: (1) canal cego, (2) células hepáticas localizadas na invaginação do canal cego, (3) revestimento do canal digestivo.



Figue 3. Cross-sectional view of the midgut of an earthworm that was raised for 14 days in soil treated with a balanced NPK compound fertilizer at a concentration of 1500 mg kg^{-1}. The image shows: (1) an increase in the thickness of the circular muscle layer, (2) an increase in the thickness of the longitudinal muscle layer and the occurrence of gaps and separations between its fibers, (3) separation of the cuticle layer, (4) congestion of blood vessels within the circular muscle layer, and (5) erosion of skin cells.
Figura 3. Corte transversal do intestino médio de uma minhoca que foi criada por 14 dias em solo tratado com fertilizante composto NPK balanceado na concentração de 1500 mg kg^{-1}. A imagem mostra: (1) aumento da espessura da camada muscular circular, (2) aumento da espessura da camada muscular longitudinal e ocorrência de lacunas e separações entre suas fibras, (3) separação da camada de cutícula, (4) congestão dos vasos sanguíneos dentro da camada muscular circular e (5) erosão das células da pele.

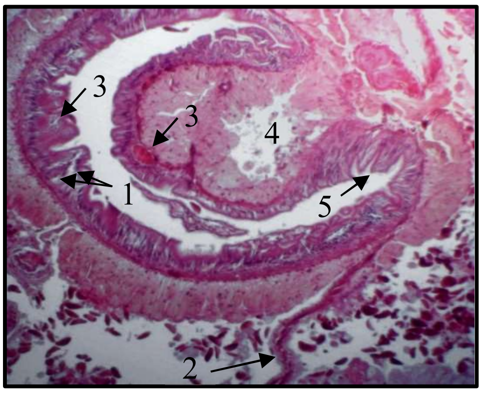


Figure 4. Cross-sectional view of the midgut of an earthworm that was raised for 21 days in soil treated with a balanced NPK compound fertilizer at a concentration of 1500 mg kg^{-1}. The image shows: (1) inflammation in the intestinal lining, (2) separation of the peritoneal membrane from the longitudinal muscle layer, (3) congestion in blood vessels, (4) edema within the connective tissue of the blind channel cells, and (5) the appearance of folds in the mucous membrane that lines the intestine and blind channel.
Figura 4. Visão transversal do intestino médio de uma minhoca que foi criada por 21 dias em solo tratado com fertilizante composto NPK balanceado na concentração de 1500 mg kg^{-1}. A imagem mostra: (1) inflamação no revestimento intestinal, (2) separação da membrana peritoneal da camada muscular longitudinal, (3) congestão nos vasos sanguíneos, (4) edema dentro do tecido conjuntivo das células do canal cego e (5) o aparecimento de dobras na membrana mucosa que reveste o intestino e o canal cego.

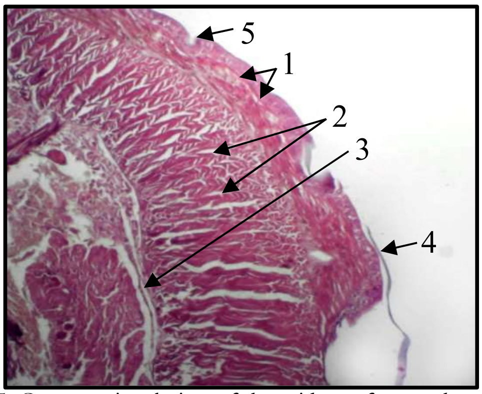


Figure 5. Cross-sectional view of the midgut of an earthworm that was raised for 28 days in soil treated with a balanced NPK compound fertilizer at a concentration of 1500 mg kg-1. The image shows: (1) inflammation in the circular muscle layer, (2) separation between the fibers of the longitudinal muscle layer, (3) separation of the peritoneal membrane from the muscle layer, (4) separation of the cuticle layer, and (5) the appearance of bulges in the skin layer.
Figura 5. Visão transversal do intestino médio de uma minhoca que foi criada por 28 dias em solo tratado com fertilizante composto NPK balanceado na concentração de 1500 mg kg-1. A imagem mostra: (1) inflamação na camada muscular circular, (2) separação entre as fibras da camada muscular longitudinal, (3) separação da membrana peritoneal da camada muscular, (4) separação da camada de cutícula e (5) o aparecimento de protuberâncias na camada da pele.

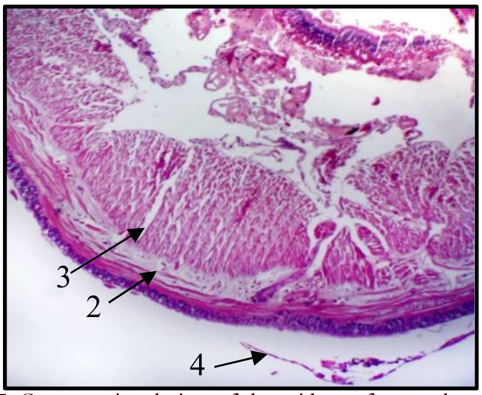


Figure 7. Cross-sectional view of the midgut of an earthworm that was raised for 28 days in soil treated with a balanced nanoscale NPK compound fertilizer at a concentration of 60 mg kg-1. The image shows: (1) separation of the cuticle layer, (2) an increase in connective tissue pigmentation under the circular muscle layer, (3) separation in the longitudinal muscle layer, and (4) an increase in the thickness of the skin layer in some areas.
Figura 7. Visão transversal do intestino médio de uma minhoca que foi criada por 28 dias em solo tratado com fertilizante composto NPK em nanoescala balanceado na concentração de 60 mg kg-1. A imagem mostra: (1) separação da camada da cutícula, (2) aumento da pigmentação do tecido conjuntivo sob a camada muscular circular, (3) separação da camada muscular longitudinal e (4) aumento da espessura da camada da pele em algumas áreas.

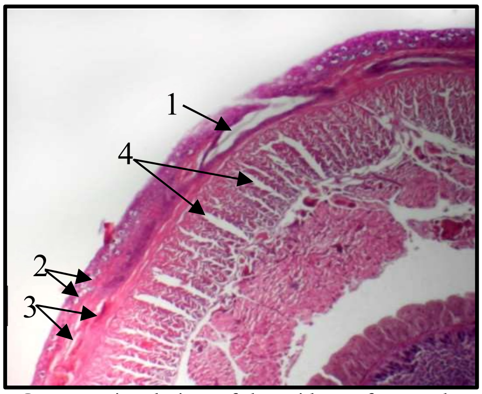


Figure 6. Cross-sectional view of the midgut of an earthworm that was raised for seven days in soil treated with a balanced NPK compound fertilizer at a concentration of 2000 mg kg-1. The image shows: (1) separation of the skin layer from the circular muscle layer, (2) clear inflammation in the skin layer cells, (3) separation in the circular muscle layer, and (4) separation and inflammation in the longitudinal muscle layer.
Figura 6. Visão transversal do intestino médio de uma minhoca que foi criada por sete dias em solo tratado com fertilizante composto NPK balanceado na concentração de 2.000 mg kg-1. A imagem mostra: (1) separação da camada da pele da camada muscular circular, (2) inflamação clara nas células da camada da pele, (3) separação na camada muscular circular e (4) separação e inflamação na camada muscular longitudinal.

Figure 8 shows that breeding earthworms for the same duration as before but at a higher concentration caused nibbling in the skin layer, as well as separation in some areas of the circular muscles with edema. There was also degradation and separation of the longitudinal muscle fiber layer. The intestines were also affected, with finger-like protrusions appearing in their lining, and separation from the surrounding muscle layer.

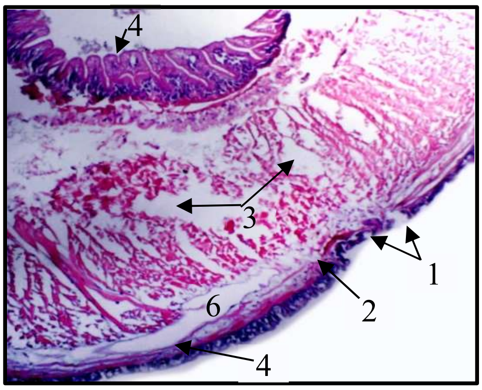


Figure 8. Cross-sectional view of the midgut of an earthworm that was raised for 28 days in soil treated with a balanced nanoscale NPK compound fertilizer at a concentration of 240 mg kg-1. The image shows: (1) presence of erosion in some areas of the skin layer, (2) presence of separation in some areas of the circular muscle layer, (3) degradation and separation of the fibers of the longitudinal muscle layer, (4) appearance of finger-like protrusions in the lining of the intestine of the blind channel, (5) separation of the intestinal lining from the muscle layer, and (6) appearance of edema between the fibers of the circular muscle layer.
Figura 8. Visão transversal do intestino médio de uma minhoca que foi criada por 28 dias em solo tratado com fertilizante composto NPK em nanoescala balanceado na concentração de 240 mg kg-1. A imagem mostra: (1) presença de erosão em algumas áreas da camada da pele, (2) presença de separação em algumas áreas da camada muscular circular, (3) degradação e separação das fibras da camada muscular longitudinal, (4) aparecimento de saliências semelhantes a dedos no revestimento do intestino do canal cego, (5) separação do revestimento intestinal da camada muscular e (6) aparecimento de edema entre as fibras da camada muscular circular.

5. CONCLUSIONS

The study showed that the improper use of chemical fertilizers causes soil pollution and has a clear impact on the vitality of earthworms. These fertilizers (conventional NPK and nano) had a significant effect on the relative growth, weight, and protein content of earthworms, as well as on the tissue structure of the skin, circular and longitudinal muscles, and intestinal lining.

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Acknowledgments: The authors would like to express their sincere thanks and appreciation to the presidency of the University of Mosul and the College of Education for Pure Sciences at the University of Mosul, for their support in presenting this research paper in the best possible way

Author Contributions: DH.H.M. – data collection, writing and draft writing; A.M.M. – methodology, statistical analysis, proofreading; management and supervision. All authors read and agreed to the published version of the manuscript.

Funding: *Not applicable.*

Institutional Review Board Statement: *Not applicable.*

Informed Consent Statement (Ethics Committee of the area): *Not applicable.*

Data Availability Statement (how the data can be made available): Raw and analyzed data can be obtained by request to the corresponding Author by e-mail.

Conflicts of Interest: The authors declare no conflict of interest. Supporting entities had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results