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Impact Of Zinc Oxide Nanoparticles On The Growth And VermicompostingEfficiency Of Vermicompost Through *Eisenia fetida*

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Abstract

The wide application of zinc oxide nanoparticles (NPs) in agriculture, industry and household products has led to unintentional health hazards and environmental impacts.In the agriculture domain, their use in nano-fertilizer for crops has led to an accumulation in the soil. Earthworms being the megafauna of soil, are continuously exposed through contact and food. The present study is focused on the effect of zinc oxide NPs on the vermicomposting efficiency of *Eisenia fetida*. The worms were exposed for a duration of two weeks to artificial soil spiked with zinc oxide NPs of size 20 nm and 240 nm at different concentrations (250, 500, 750 and 1000 mg/kg of soil). The results revealed a maximum weight gain of 30.76% for the earthworms in the treatment containing zinc oxide NPs of size 240 nm (@750mg/kg of soil). The earthworms were very healthy for the treatment involving NPs of size 240 nm (@750 mg/kg of soil), whereas for the treatment involving NPs of size 20 nm (@750 mg/kg of soil),the earthworms were very thin. The shortest duration for the conversion of Farm Yard Manure (FYM) into vermicompost of 84 days was observed in this treatment involving a combination of NPs of sizes 20nm and 240 nm (@750 mg/kg of soil). The nutrient analysis of vermicompost revealed a general trend of an increase in the levels of total nitrogen, potassium and phosphorus (%) alongwith a decrease in pH levels and total organic carbon content (%). It is inferred that among the three treatments, vermicompost prepared by earthworms under the treatment involving a combination of 20 nm and 240 nm zinc oxide NPs is the best in terms of its nutrient value.

Keywords: Eisenia fetida, Life cycle, Reproduction, Vermicomposting, Zinc oxide Nanoparticles

Introduction

Technological advancements have touched every sphere of human development. Nanotechnology is one such emerging technology that has applications in various domains ranging from industrial and agricultural applicationsto biomedical uses extending to commercially available consumer products,which include transparent sunscreens, stain-resistant clothing, self-cleansing glass, paints and also as sports equipment (Rosi *et al.* 2005; Card *et al.* 2008). Nanomaterials, the building blocks of nanotechnology, are intentionally produced materials with a characteristic size between 1 and 100 nm. Since the last decade, nanomaterials have been used excessively in agricultural practices (Gogos *et al.* 2012) as fertilizers, applied as aerosols or directly to the soil (Sturikova *et al.* 2018), crop protection products, soil quality improvement, pollutant remediation (Parisi *et al.* 2015) and purification of water. The key factors that make them unique and different from their bulk counterparts are their tiny size and the high surface-to-volume ratio (Joudeh and Linke, 2022). The nanomaterials are used in the agriculture sector as organic or inorganic NPs to reduce the damage due to pests and diseases, to maintain the nutritional contents and to enhance the food shelf-life. Due to their peculiar properties, nutrients are frequently supplied to the crops in the form of nanocomposites for controlled release and to enhance the efficiency of use, leading to significant improvement in plant crops with lower environmental impacts (Elmer and White, 2018). Due to their widespread applications, NPs may intentionally or accidentally get discharged into the environment through activated sludge and their discharge into the wastewater stream. Eventually, these NPs get into the soil ecosystem through atmospheric deposition or when activated sludge is poured into fields to enhance soil fertility (Nowack and Buschelli, 2007; León-Silva et al., 2016). However, very little information about the dissociation behaviour, toxicity and risk of NPs to soil fauna in the natural soil environment is available.

Zinc Oxide (ZnO) NPs are considered as one of the most important metal oxide NPs having the third largest annual production in volume (Merdzan *et al.* 2014; Romero-Freire *et al.* 2017). They are used in many applications, from antibacterial agents (Ma *et al.* 2013) to plant fertilizers (Parisi *et al.* 2015; Segatto *et al.* 2018). Zinc plays a vital role in animal and plant physiology by acting as a cofactor for a variety of macromolecules, enzymes, cell signalling proteins and also protects biological membranes from oxidative stress (Borkert *et al.* 1998). In soil, the bioavailability and distribution of zinc arecontrolled by many factors like pH, total organic carbon, the surface charge of colloids and redox potential (Donner *et al.* 2010; Romero-Freire *et al.* 2017), which influence the interactions between NPs and the soil matrix, modifying their availability and toxicity potential (Pan and Xing 2012). Earthworms are a significant part of soil ecosystem services. Being ecological bioindicators, they are used widely to study the accumulation and toxicity of various chemicals (Liu *et al.* 2019; Song *et al.* 2019). The studies reported by Hooper *et al.* 2011; and García-Gomez *et al.* 2019 concluded that zinc oxide NPs cause changes in the activities of various enzymes like superoxide dismutase (SOD) and malondialdehyde (MDA) which leads to metabolic changes resulting in tissue damage. These changes are often employed as biomarkers to determine the biological changes in organisms due to environmental toxicity (Hackenberger *et al.* 2018). The iron oxide NPs result in morphological damage and colour change to *Eisenia fetida*. The nutrient content of vermicompost obtained for the treatment containing combination of 30 nm and 100 nm size iron oxide NPs was higher compared to the control and treatments involving 30 nm and 100 nm size iron oxide NPs separately (Goyal *et al.* 2023).

In this present study, *Eisenia fetida,* was exposed to an artificial soil system containing different concentrations (250 mg/kg, 500 mg/kg, 750 mg/kg and 1000 mg/kg) and different sizes (20 nm and 240 nm) of zinc oxide NPs. The effect of these NPs on the reproduction and vermicomposting efficiency of *Eisenia fetida* was evaluated to understand the implication of using NPs.

Material And Method

Zinc oxide NPs of sizes 20 nm and 240 nm were purchased from Otto Chemie private limited, Mumbai (India). The morphology and size of NPs were examined using Transmission Electron Microscopy (TEM Hitachi H-7650) at Electron Microscopy & Nanoscience Laboratory, Punjab Agricultural University, Ludhiana. The stock of the *Eisenia fetida* was obtained from Mahavir Organic Farm, Phillaur. The FYM was obtained from the dairy farms of the School of Organic Farming, Punjab Agricultural University, Ludhiana. The work on the standardization of zinc oxide NPs and the study on the impact of standardized dose on *Eisenia fetida* was carried out in the Department of Zoology, Punjab Agricultural University, Ludhiana. The nutrient analysis of the vermicompost was performed at School of Organic Farming, Punjab Agricultural University, Ludhiana.

Maintenance Of Stock

The stock of *Eisenia fetida* was maintained in the animal dung substrate obtained from the dairy unit of the School of Organic Farming, Punjab Agricultural University, Ludhiana. Prior to its use for the study, the dung was dried in the sunlight for 15 days. For using dung as a substrate, it was crumbled and sprayed with water to maintain the moisture content at a level of 70%. The stock was regularly sprayed with water to preserve the culture of earthworms. Adult clitellate worms were selected from the stock for carrying out laboratory experiments.The analysis of the chemical properties of the substrate is given in Table 1.

Table 1: Basic Analysis Of The Substrate Used For Study

Substrate	pH	N	P	K	C
		(%)			
Farm Yard Manure	8.87±0.18	1.42±0.02	0.46±0.05	0.58±0.03	0.50±0.02

2.2 Preparation Of Artificial Soil

The artificial soil prepared according to the OECD guideline no. 222 (OECD 2016), was composed of finely ground sphagnum peat (10%), kaolin clay (30%) and air-dried quartz sand (70%). The sand was obtained from the local market, kaolin clay from the pottery supplier, and the sphagnum peat from the nearby nursery. All the soil contents were appropriately mixed, and the pH of the soil was adjusted to 6.2 by adding calcium carbonate. Adult clitellate worms weighing 200-300 mg were selected randomly from the stock of *Eisenia fetida* and placed in the artificial soil for seven days before the experiment so that earthworms could acclimatize to the artificial soil.

Characterization Of Zinc Oxide NPs

The zinc oxide NPs were morphologically characterized using Transmission Electron Microscopy (TEM) with Hitachi H-7500 TEM at Electron Microscopy & Nanoscience Laboratory, Punjab Agricultural University, Ludhiana. The zinc oxide NPs were dispersed in acetone for 30 minutes inside a sonicator before fixing them on a carbon coated copper grid for imaging. The TEM analysis (Figs. 1- 2) revealed that the zinc oxide NPs are tetragonal and have averagesizes of 20 nm and 240 nm, respectively.

Standardization Of Zinc Oxide NPs dose

Four doses of each NPs (20 nm and 240 nm size) were applied to the artificial soil as 250 mg/kg (250 mg of zinc oxide NPs in 1 kg of artificial soil); 500 mg/kg (500 mg of zinc oxide NPs in 1 kg of artificial soil); 750 mg/kg (750 mg of zinc oxide NPs in 1 kg of artificial soil) and 1000 mg/kg (1000 mg of zinc oxide NPs in 1 kg of artificial soil). For the treatment involving a combination of 20 nm and 240 nm zinc oxide NPs, doses were prepared with equal ratios of both NPs. The experiment was run in triplicate for each treatment and compared with the control. Ten clitellate worms each weighing about 300 mg were introduced into each artificial soil inside plastic trays (22 cm x 17.5 cm x 5 cm) for 14 days. Five grams of dried FYM was added to the soil at an interval of 7 days. The final weight of the earthworms was observed. The concentrations that resulted in the highest gain in weight and nil mortality were selected as optimum doses for the three treatments and used for further testing of the physio-chemical characteristics of the vermicompost. The standardized doses determined in this way for various treatments are 500 mg/kg (for 20 nm NPs) and 750 mg/kg (each for 240 nm NPs and the combination of 20, 240 nm NPs) of soil. Table 2 summarizes various treatments under study.

Table2: Treatments Applied To Soil Amended With Increasing Concentration Of Zinc Oxide NPs.

Treatment	**Characteristics**
Control	**Artificial Soil + FYM + Earthworms**
T_1	**Artificial Soil + 20 nm zinc oxide NPs at concentrations 250, 500, 750, 1000 mg/kg of soil + FYM + Earthworms**
T_2	**Artificial Soil + 240 nm zinc oxide NPs at concentrations 250, 500, 750, 1000 mg/kg of soil + FYM + Earthworms**
T_3	**Artificial Soil + 20, 240 nm zinc oxide NPs in equal ratio at concentrations 250, 500, 750, 1000 mg/kg of soil + FYM + Earthworms**

Vermicompost Preparation

One kg of FYM was taken as substrate in plastic trays (22 cm x 17.5 cm x 5 cm). Adequate quantities of water were sprayed to adjust the moisture content and the mixtures were turned over manually every day for 15 days to remove volatile gases that may be potentially toxic to the earthworms. After 15 days, ten adult *Eisenia fetida* individuals of known biomass were introduced into each container having substrate spiked with standardized doses of NPs treatments: Control (no NPs), T_1 (20 nm@500 mg/kg of soil), T_2 (240 nm@750 mg/kg of soil), and T_3 (20 nm+240 nm@750 mg/kg of soil). The substrate was covered with a moist jute cloth to retain moisture and avoid intrusion by the pests. The experiment was replicated thrice for the control and each treatment along with regular monitoring. The substrate samples were drawn from each tray on the 1^{st}, 30^{th}, 60^{th}, and 90^{th} day. The time taken by earthworms under different treatments to prepare vermicompost from the substrate was compared with the control.

Nutrient Analysis

Samples of the vermicompost prepared from FYM were drawn every 30 days after the onset of the experiment for a maximum of 90 days. The methodology adopted to analyze the nutrient composition of the vermicompost are as per Table 3.

Table 3: Various Methods used For Determining The Nutrient Composition Of Vermicompost.

Sr. No.	Parameter	Method
1.	pH	Potentiometric method (Jackson 1973)
2.	Total Nitrogen Content	Micro-Kjeldahl method (Jackson 1973)
3.	Total Phosphorus Content	Vanadomolybdo-phosphoric yellow colour method (Jackson 1967)
4.	Total Potassium Content	Flame photometer (Jackson 1967)
5.	Total Organic Carbon	Rapid titration method (Walkley and Black 1934)

Results And discussion

The growth rate of *Eisenia fetida* were observed (Table 4). There was a significant increase in the body weight of earthworms by the end of the experiment. The percentage change in the body weight of earthworms is presented in Fig. 3. For the treatment T_1 involving zinc oxide NPs of size 20 nm, the highest increase in weight of 21.05% was observed at a concentration of 500 mg/kg. For the treatment T_2 involving 240 nm size NPs, the highest increase in weight of 30.76% was observed at 750 mg/kg of soil, whereas for the treatment T_3 involving thecombination 20 nm+240 nm size NPs, the highest increase in weight of 16% was observed at the concentration 750 mg/kg of soil. The earthworms in the treatment T_2 were very healthy and had large, long and prominent clitellum, while the earthworms in the treatment T_1 had excellent mobility but they looked very thin. However, no such changes were observed in the control.

Table 4: Effect Of Zinc Oxide NPs On Growth Of *Eisenia fetida* In Different Treatments.

Concentration (mg/kg; size of zinc oxide NPs)	**Initial weight (g)**	**Final weight (g)**	**Change in weight (g)**
Control	0.52 ± 0.02	0.53 ± 0.03	0.01
T_1 (500; 20 nm)	0.57 ± 0.10	0.69 ± 0.08	0.12*
T_2 (750; 240 nm)	0.52± 0.02	0.68 ± 0.02	0.16*
T_3 (750; 20+240 nm)	0.50±0.23	0.58±0.06	0.08

Values are mean± S.E. of three replicates.

Indicates significant difference between values ($p<0.05$)

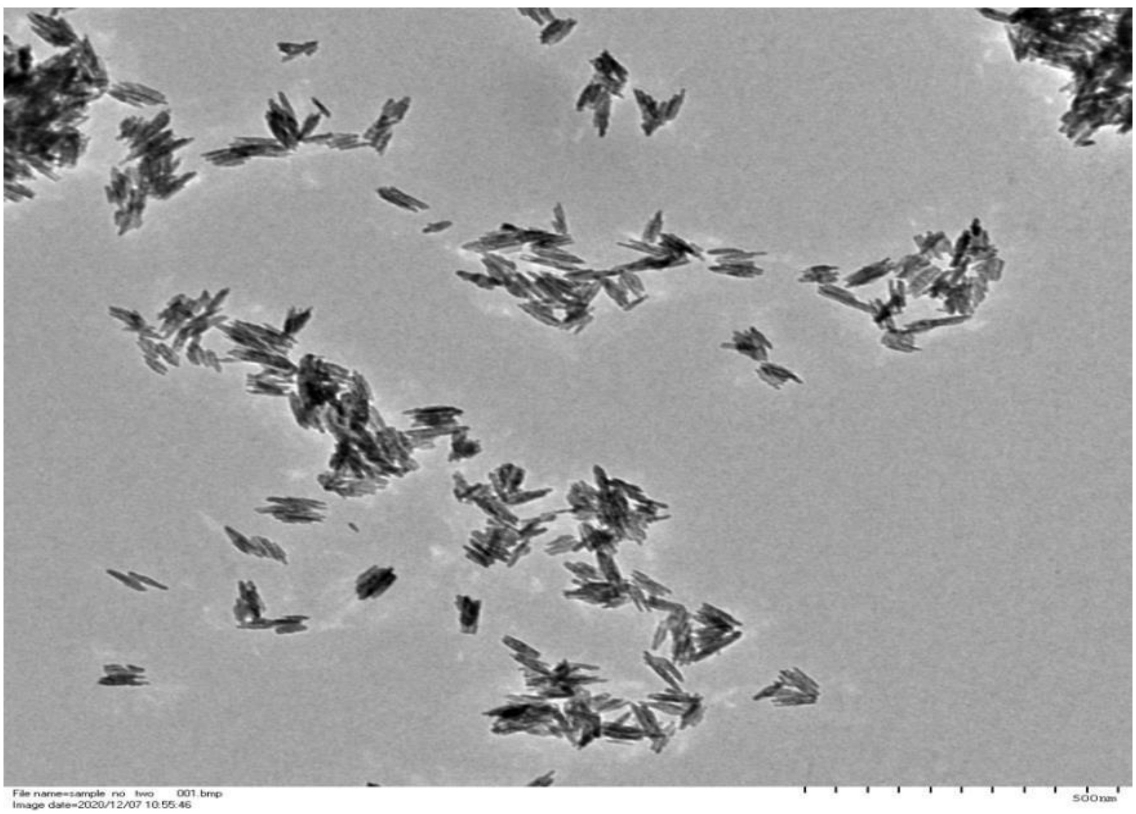

Fig 1: TEM image of zinc oxide NPs of size 20 nm.

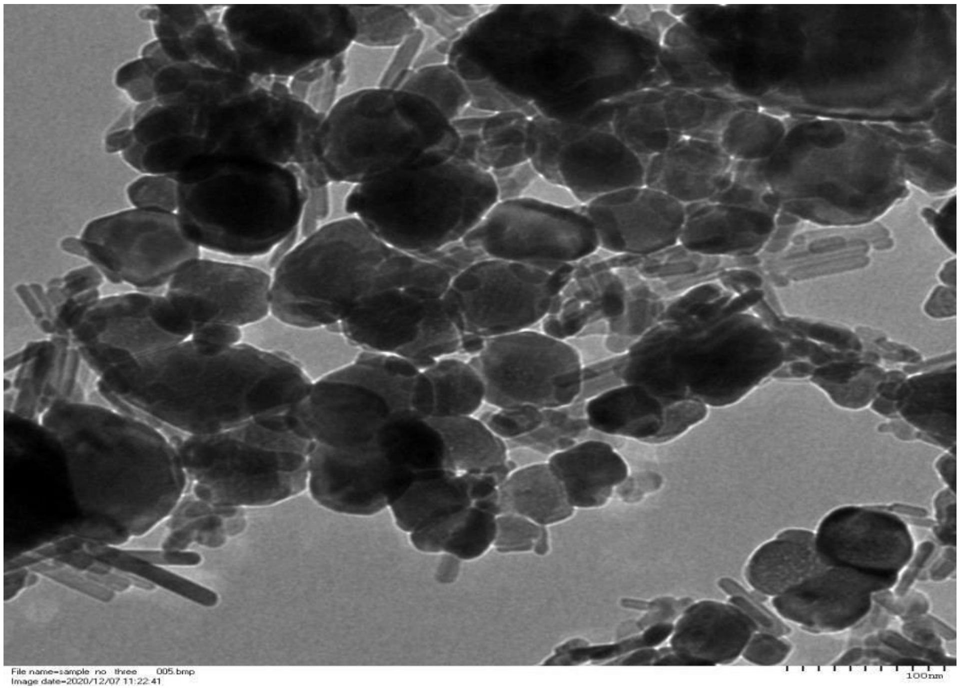

Fig 2: TEM image of zinc oxide NPs of size 240 nm.

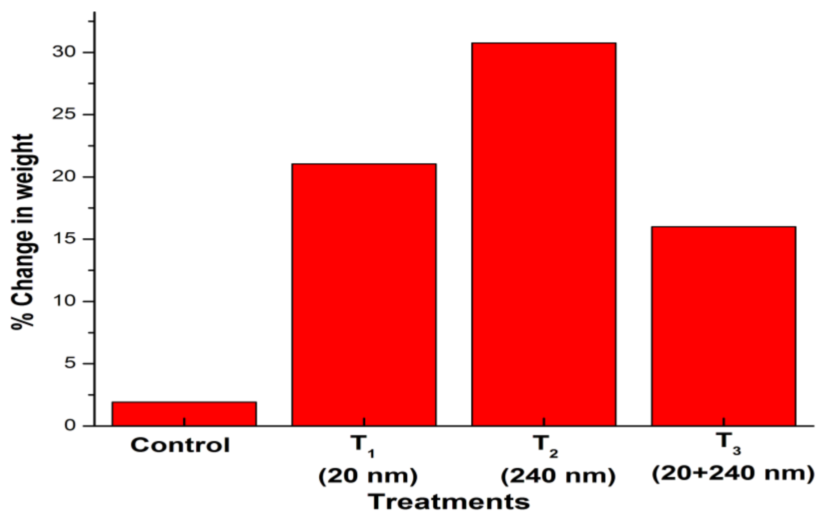

Fig 3: Percentage Change In The Weight Of Earthworms.

The fastest substrate conversion into vermicompost was recorded in the combination treatment $T_{3,}$ *i.e.* 84 days and the slowest for treatment T_1 (87 days) as presented in Fig. 4. This signifies that the size as well as dose of different NPs affect the vemicomposting efficiency of *Eisenia fetida* significantly.

Physio-Chemical Parameters Of FYM

The vermicompost obtained at the end of the experiment for different treatments were assessed for pH, total organic carbon (OC), total N, total P, and total K content.

pH

The pH of the substrate changed to nearly neutral after vermicomposting (Table 5). The final vermicompost's pH was observed to be lower than the initial substrate (control) for all the treatments. The possible reason behind pH-shift is the higher mineralization of nitrogen and phosphorus into nitrites or nitrates and orthophosphates, respectively. The formation of intermediate organic acid species while bioconversion of organic matter may also cause pH-shift. Similar trends of reduction in pH were reported by Bhat et al. (2015) during vermicomposting of various substrates such as sugarcane bagasse and cattle dung. The study revealed that another possible reason of pH-shift can be the production of acids such ashumic and fulvic during the composting process.

Table 5: Effect Of Different NPs Concentrations On pH Of Vermicompost.

Treatment	**1st Day**	**30th Day**	**60th Day**	**90th Day**
Control	8.87±0.016^{cD}	8.47 ±0.02^{dC}	8.38±0.008^{cB}	7.72±0.006^{dA}
T_1 (20 nm@500 mg/kg of soil)	8.78±0.002^{abD}	8.16±0.03^{bC}	7.92±0.017^{aB}	7.48±0.016^{bA}
T_2 (240 nm@750 mg/kg of soil)	8.83±0.017^{bcD}	8.27 ±0.024^{cC}	8.03±0.021^{bB}	7.59±0.012^{cA}
T_3 (20nm+240nm@750 mg/kg of soil)	8.74±0.03^{aD}	7.97±0.016^{aC}	7.87±0.016^{aB}	7.41±0.008^{aA}

Values are mean± S.E. of three replicates.

Values with superscript (a, b, c, d) indicate significant difference between treated and untreated soil samples ($p<0.05$) , Values with superscript (A, B, C, D) indicate significant difference between values at different days ($p<0.05$)

Total Organic Carbon Content

The total organic carbon decreased over time during vermicomposting processes in control and other treatments. The maximum lowering in value of total organic carbon content was observed in treatment T_3 and minimum in the control (Table 6). The microbial degradation of organic matter at the time of the vermicomposting and action of earthworms leads to lowering of the total organic carbon values. Carbon is the building block of all living organisms and also a major component of organic molecules. It needed as the primary source of energy for the process of composting (Ansari and Rajpersaud 2012). Another reason behind decrease in the trends of organic carbon values includes the loss of organic carbon amount as CO_2 through microbial respiration and mineralization of organic matter, causing an increase in total nitrogen. A part of the carbon in the decomposing residues is released as CO_2, and a part is assimilated by the microbial biomass.

Table 6: Effect Of Different NPs Concentrations On total organic carbon content of vermicompost.

Treatment	1st Day	30th Day	60th Day	90th Day
Control	0.62 ± 0.02^{aD}	0.60 ± 0.005^{bC}	0.52 ± 0.009^{bB}	0.42 ± 0.041^{cA}
T_1 (20 nm@500 mg/kg of soil)	0.6 ± 0.008^{aD}	0.52 ± 0.017^{aC}	0.46 ± 0.019^{abB}	0.35 ± 0.004^{bA}
T_2 (240 nm@750 mg/kg of soil)	0.61 ± 0.004^{aD}	0.53 ± 0.008^{aC}	0.48 ± 0.02^{abB}	0.40 ± 0.006^{cA}
T_3 (20nm+240nm@750 mg/kg of soil)	0.59 ± 0.002^{aD}	0.51 ± 0.004^{aB}	0.44 ± 0.017^{aC}	0.30 ± 0.016^{aA}

Values are mean± S.E. of three replicates.

Values with superscript (a, b, c, d) indicate significant difference between treated and untreated soil samples ($p<0.05$)

Values with superscript (A, B, C, D) indicate significant difference between values at different days ($p<0.05$)

Nitrogen, Phosphorus, Potassium

There was an increase in total nitrogen, phosphorus, and potassium levels in the final vermicompost, which might be due to the mineralization of the organic matter. The maximum increase in total nitrogen, phosphorus and potassium levels was recorded in the treatment T_3 containing a combination of 20 nm and 240 nm ZnO NPs (Tables 7-9). According to Atiyeh *et al.* 2002, the conversion of ammonium-nitrogen into nitrate increases nitrogen content. The possible reason behind the increase in total P and K levels is the biological grinding of the organic matter while passing through the earthworm's gut leading to the physical decomposition by the enzymatic activities (Goswami *et al.* 2013). The percentage changes in the pH, organic carbon, N, P, and K content are presented in Fig. 5. The results of the present study are consistent with the findings of Panday and Yadav (2009), Ansari and Jaikishun (2011) and Goswami *et al.* (2013). In a nutshell, the nutrient content of the final vermicompost obtained from the combination treatment T_3 (20+240 nm; 750 mg/kg) had the highest nutritional value as compared to the rest of the treatments.

Table 7: Effect of different NPs concentrations on the nitrogen content of vermicompost.

Treatment	1^{st} Day	30^{th} Day	60^{th} Day	90^{th} Day
Control	1.64 ± 0.024^{aA}	1.92 ± 0.012^{aB}	2.58 ± 0.019^{aC}	2.98 ± 0.004^{aD}
T_1 (20 nm@500 mg/kg of soil)	1.76 ± 0.008^{bA}	2.06 ± 0.029^{cB}	2.72 ± 0.02^{cC}	3.20 ± 0.026^{cD}
T_2 (240 nm@750 mg/kg of soil)	1.69 ± 0.018^{aA}	1.97 ± 0.024^{bB}	2.67 ± 0.01^{bC}	3.03 ± 0.016^{bD}
T_3 (20nm+240nm@750 mg/kg of soil)	1.65 ± 0.31^{aA}	2.11 ± 0.012^{dB}	2.77 ± 0.017^{dC}	3.26 ± 0.012^{dD}

Values are mean± S.E. of three replicates.

Values with superscript (a, b, c, d) indicate significant difference between treated and untreated soil samples ($p<0.05$)

Values with superscript (A, B, C, D) indicate significant difference between values at different days ($p<0.05$)

Table 8: Effect of different NPs concentrations on the phosphorus content of vermicompost.

Treatment	1^{st} Day	30^{th} Day	60^{th} Day	90^{th} Day
Control	0.72 ± 0.02^{cA}	0.76 ± 0.022^{aB}	0.83 ± 0.006^{aC}	0.91 ± 0.012^{aD}
T_1 (20 nm@500 mg/kg of soil)	0.67 ± 0.034^{bcA}	0.77 ± 0.008^{aB}	0.9 ± 0.016^{bC}	1.05 ± 0.020^{bD}
T_2 (240 nm@750 mg/kg of soil)	0.66 ± 0.03^{aA}	0.73 ± 0.14^{aB}	0.87 ± 0.022^{bC}	0.95 ± 0.028^{aD}
T_3 (20nm+240nm@750 mg/kg of soil)	0.70 ± 0.031^{abA}	0.8 ± 0.044^{aB}	0.99 ± 0.027^{cC}	1.12 ± 0.032^{cD}

Values are mean± S.E. of three replicates.

Values with superscript (a, b, c, d) indicate significant difference between treated and untreated soil samples ($p<0.05$)

Values with superscript (A, B, C, D) indicate significant difference between values at different days ($p<0.05$)

Table 9: Effect of different NPs concentrations on the potassium content of vermicompost.

Treatment	1st Day	30th Day	60th Day	90th Day
Control	0.88 ± 0.008^{aA}	0.96 ± 0.009^{aB}	1.3 ± 0.01^{aC}	1.69 ± 0.008^{aD}
T_1 (20 nm@500 mg/kg of soil)	1.18 ± 0.01^{dB}	1.1 ± 0.042^{cA}	1.49 ± 0.22^{cC}	1.85 ± 0.034^{bD}
T_2 (240 nm@750 mg/kg of soil)	1.12 ± 0.021^{cB}	1.05 ± 0.035^{bA}	1.35 ± 0.03^{bC}	1.73 ± 0.039^{aD}
T_3 (20nm+240nm@750 mg/kg of soil)	1.08 ± 0.015^{bA}	1.13 ± 0.049^{cB}	1.60 ± 0.025^{dC}	1.93 ± 0.045^{cD}

Values are mean± S.E. of three replicates.

Values with superscript (a, b, c, d) indicate significant difference between treated and untreated soil samples ($p<0.05$)

Values with superscript (A, B, C, D) indicate significant difference between values at different days ($p<0.05$)

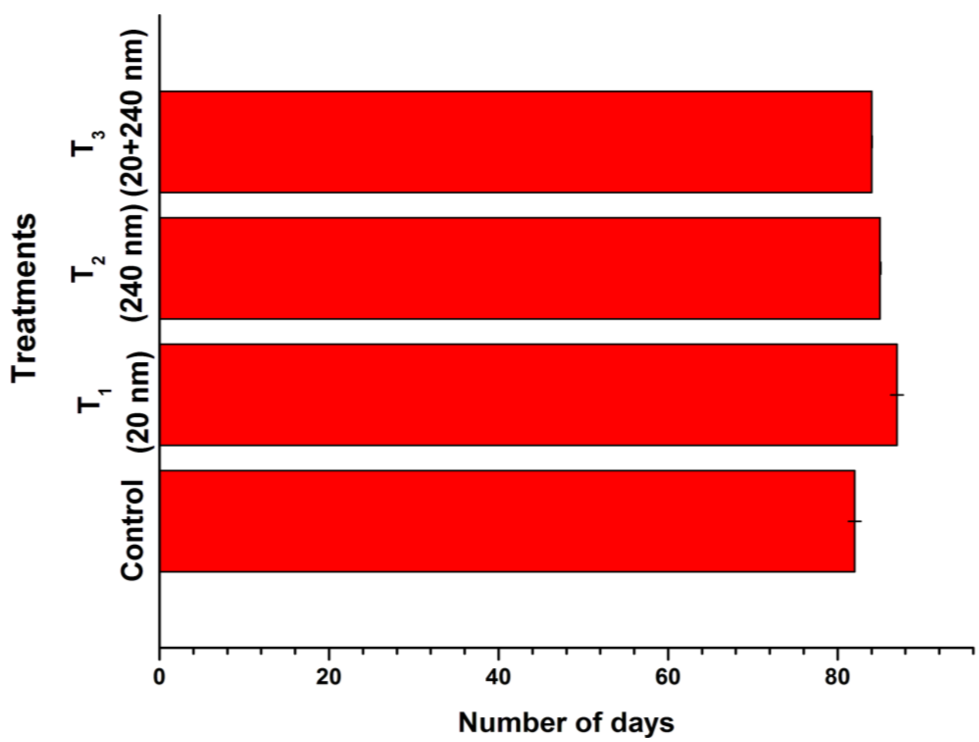

Fig 4: Days taken by *Eisenia fetida* for vermicompost preparation.

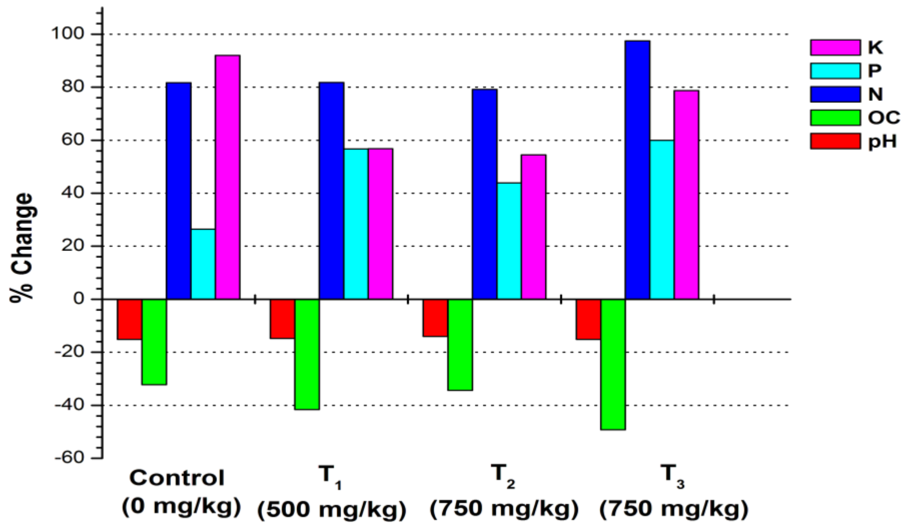

Fig. 5: Comparison of nutrients in vermicompost obtained under different treatments.

Conclusions

Earthworms respond variedly to different treatments of zinc oxide NPs and exhibit different efficacy rates for vermicomposting. The zinc oxide NPs of sizes 20 nm, 240 nm and their combination (20 nm+240 nm) have a dose-dependent effect on the vermicomposting efficacy of *Eisenia fetida*. The treatment T_3 involving a combination of zinc oxide NPs (20 nm+ 240 nm; 750 mg/kg) was the most favoured by *Eisenia fetida* compared to other treatments. The findings of the present study foreground the potential of earthworms to make value-added vermicompost even in the presence of NPs. The faster conversion rates and high nutrient content further accentuate that the substrate and nutrients are the primary and most important parameters while determining the potential of vermicomposting. In an attempt towards a greener environment, the study underlines the future prospects of vermicomposting and its use for improving the process of bio-management of agricultural wastes by converting them to valuable and nutrient-rich organic manure. Therefore, vermicomposting can potentially be explored by marginal farmers of developing countries as a support to farming practices.

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Statements And Declarations

Ethical approval and consent to participate

Not Applicable.

Consent to Publish

All the authors read and approved the final manuscript for publication.

Author Contributions

SSH and NR conceived the concept. AG, SS and NR executed the field experiments, data collection, investigation, analysis of data and interpretation. NR and ND prepared the manuscript. SSH, NR and ND edited the manuscript. All authors read and approved the final version of the manuscript.

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The authors declare no competing interests.

Availability Of Data And Materials

The data and materials can be accessed by writing to the authors.

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