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RESEARCH ARTICLE

Biological monitoring of soil pollution caused by two different zinc species using earthworms

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Abstract

Zinc oxide nanoparticles (ZnO-NPs) are commonly used in both commercial and agricultural sectors. As a result, ZnO-NPs are extensively discharged into soil ecosystems, creating a significant environmental issue. Therefore, it is crucial to assess their influence on the soil ecology to ensure its secure and enduring utilization in the future. The exact degree of toxicity associated with ZnO-NPs and their ionic form is still uncertain. To address the challenges, the study used the soil bioindicator earthworm species *Eudrilus eugeniae* as an experimental model to evaluate the effects of two zinc species (ZnO-NPs and $ZnCl_2$) at 100, 250, 500, and 750 mg kg^{-1} and control (0 mg kg^{-1}) in garden soil over 28 days. The investigation also examined the impact of exposure on survival, reproduction, neuro-biomarker, avoidance behavior, and accumulation. The highest avoidance rates were 27.5% for ZnO-NP and 37.5% for $ZnCl_2$ at 750 mg kg^{-1}. $ZnCl_2$ treatment reduced juvenile production by 3.73 ± 1.73, while ZnO-NPs showed 4.67 ± 1.15. At 750 mg kg^{-1}, soils with $ZnCl_2$ (63.3%) demonstrated lower survival rates than those with ZnO-NPs (53.3%), likely because of higher Zn ion levels. After 28 days of exposure, $ZnCl_2$ (536.32 ± 11 mol min^{-1}) activated AChE enzymes more than ZnO-NPs (497.7 ± 59 mol min^{-1}) at the same dose, compared to control (145.88 ± 28 to 149.41 ± 23 mol min^{-1}). Nanoparticles and zinc ions bioaccumulated and reacted negatively with the neurotoxic marker AChE, affecting earthworm reproduction and behavior. However, earthworms exposed to $ZnCl_2$ exhibited less intestinal Zn than those exposed to NPs. The present work contradicts the finding that ZnO-NPs have hazardous effects on soil organisms. The results indicate that earthworm *E. eugeniae* may significantly affect soil metal uptake from metallic nanoparticles (NPs). This may help design NP soil pollution mitigation strategies. The study offers valuable information for establishing a relationship between the environmental toxicity of ZnO-NPs and soil ecosystems.

Keywords Avoidance behavior · Bioaccumulation · Neurotoxicity · Reproduction · Survivability · ZnO nanoparticles

Introduction

Zinc oxide nanoparticles (ZnO-NPs) are extensively used nanomaterials with a wide range of applications in various fields such as the food industry, agriculture, biosensors, pigments, industrial elastomers, solar panels, paints and coatings, and UV-protection sunscreens (Nejati et al. 2022). The various forms of zinc, like ZnO-NPs and $ZnCl_2$, are used as fertilizer to improve crop growth and alleviate crop deficiencies (Nejati et al. 2022). Furthermore, NPs have the potential to infiltrate soil via wastewater streams during their typical usage (Rajput et al. 2022; Saleem and Zaidi 2020). Disposal techniques exhibit variability across diverse residential practices, encompassing deposition in landfills, incineration, or conversion into biosolids for agricultural purposes, thereby leading to the emission of uncertain constituents. During the exploitation

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phase, the abundance of commercial products and nano-fertilizers being used may lead to the release of excessive nanoparticles (NPs) into the soil. It is anticipated that a significant portion of these NPs eventually accumulates in the terrestrial environment. The utilization of nanoparticles (NPs) in several sectors is likely to lead to an increase in NP levels in diverse environmental domains, such as soil ecosystems (Wigger et al. 2020). Nevertheless, NeDambiec et al. (2022) predicted that the concentration of metallic nanoparticles in the environment would be rather limited in comparison to naturally existing elements. Therefore, it is imperative to carefully investigate the impact of ZnO-NPs on soil organisms and their availability in soils, given the contentious nature of this scenario (Bao et al. 2020; Lahive et al. 2023).

Several studies have shown that nanomaterials have harmful effects on soil organisms at both the cellular and molecular levels, leading to reduced growth and reproductive activity as well as their behavior by interfering with physiological growth (Manna et al. 2023; Lahive et al. 2023). The nanoscale particle facilitates interaction, disruption, and penetration of neuro-membranes in the brain (Nho 2020). They have potential to enter the human body through various routes, including dermal penetration, respiratory inhalation, and circulatory inoculation. However, there exists a considerable amount of discordance regarding the ability of nanoparticles to traverse the blood–brain barrier, as posited by Tosi et al. (2020). Qiu and Smolders (2017) suggested that nanotoxicity is directly influenced by the metal ion activity released by ZnO-NPs. Zn ions can accumulate in the gastrointestinal (GI) tract of earthworms, alter physiological and behavioral responses, and have an effect on reproduction when released by ZnO-NPs or $ZnCl_2$ dissolution. Additionally, it modifies the structure of the gastrointestinal tract (Duo et al. 2022). More precisely, it affects the mucous membrane of the intestines and the chloragogenous tissues of earthworms, leading to amplification in the number of altered genes in their immune and digestive systems (Świątek and Bednarska 2019).

Zinc species can generate reactive oxygen species (ROS) and alter biochemical factors such as lipid peroxidation (LPO) and enzymes like superoxide dismutase (SOD), catalase (CAT), acetylcholinesterase (AChE), and the antioxidant reduced glutathione (GSH). Aroniadou-Anderjaska et al. (2020) found that the biochemical marker AChE enzyme is responsible for the acetylcholine neurotransmitter's degradation in cholinergic synapses. However, their activity has been biochemically characterized in a variety of macro-invertebrates (Seleem 2019; Khalil 2015). In the long run, it can lead to neurotoxicity and deviant behavioral patterns. Additionally, it has been confirmed that avoidance behavior is a useful endpoint for evaluating pollution (Lowe et al. 2016). It might be just as sensitive to some chemicals as a reproduction test (Ardestani et al. 2020). It is still unclear whether there is an association between avoidance and metal bioaccumulation or because of the negative consequences of contaminant uptake or because of a sensory-based reaction (Lowe et al. 2016).

The aforementioned issue has elicited apprehension regarding the possible ecological ramifications, food chain accessibility, and potential human vulnerability resulting from the consumption of polluted crop yields (Garvey 2019). These concerns mentioned above bring attention to the restricted accessibility of data pertaining to nano-toxicity, despite its prominence as an investigation of the subject for the last 20 years. Earthworms are an appropriate experimental model for assessing toxicity including NPs due to their ubiquitous presence in various soil types and their significant contribution to the overall soil biomass (Frazão et al. 2019). Further, they are an appropriate choice for testing the ecological toxicity due to their ease of accessibility, fragile nature, and non-intrusive handling. In the present study, the earthworm *Eudrilus eugeniae* was chosen, since it is easily available at the most of the places. It exhibits a high level of aptitude for the bioconversion of organic waste, possesses greater resilience to environmental fluctuations in comparison to other species, and has been effectively integrated into agricultural fields through the implementation of organic farming approaches (Yesudhason et al. 2018).

According to few studies, the harmful effects of ZnO-NPs are brought on by the release of zinc ions, while other studies asserted that the toxic effects are brought on by the coating of the particles to the organism's surface or their internalization. The toxicity of ZnO-NPs is a subject of disagreement due to the uncertain surroundings whether the apparent effects are a result of the nanoparticle form or releasing its ions in different organisms. Therefore, the objective of the study was to assess the comparative toxicity of uncoated ZnO-NPs and $ZnCl_2$ (in its ionic form) on earthworms, *Eudrilus eugeniae*, in garden soil. The study analyzed Zn bioaccumulation, reproduction, avoidance behavior, and neurotoxicity caused by two species of Zn (ZnO-NPs and $ZnCl_2$). Additionally, this study assessed the toxic effects of ZnO-NP/$ZnCl_2$ on earthworm survival, weight, reproduction, and biochemical responses in their tissues, and established a correlation between these effects and Zn chemical measures in soil and earthworms. The exposure period for the earthworms was 56 days, representing a chronic exposure scenario. The aim of this investigation is to gain a thorough comprehension of the impacts of particle accumulation within the gastrointestinal system of

earthworms. It is imperative to ensure safe application and provide convincing evidence to establish the standard for the effective use of nanomaterials. The timely detection of ZnO nanoparticle contamination in a soil ecosystem may be facilitated by this approach.

Materials and methods

Nanoparticles and experimental model

The ZnO-NPs, with a size ranging from 35 to 40 nm (Sigma-Aldrich, ≥ 97% purity), and $ZnCl_2$ (Sigma-Aldrich, ≥ 98% purity) were used in the experiment. $ZnCl_2$ was used as the standard of reference to evaluate the possible toxicity associated with the presence of either zinc ions or nanoparticles in soil. Transmission electron microscopy (TEM) was used to examine the morphology and size of ZnO-NPs at the Sophisticated Instrumentation Facility Centre (SIFC), All India Medical Institute, New Delhi, India. In Fig. 1, the size distribution curve of nanoparticles is shown in TEM photograph.

Sexually mature clitellate earthworms *Eudrilus eugeniae* (~ 300 to 450 mg) with muscular clitella were obtained from vermicomposting unit. The earthworms were managed to maintain in the natural soil samples (mixture of 5% decomposed cattle manure and 40% of moisture) at 22 °C for at least 2 weeks prior subjected to the experiment. Before conducting the experiment, earthworms were reared for 24 h on a wet filter paper at 20 °C in the dark to clean their guts for impurities.

Experiment 1: Chronic exposure of ZnO nanoparticles and Zn ion ($ZnCl_2$)

The chronic exposure assay was carried out in a soil microcosm with garden soil collected from the nursery. To remove root debris, fresh soil was sieved through 2-mm mesh sieve. Before being used in assays, the soil was air dried, ground, and sieved through a 1-mm sieve after stones and plant residues were removed.

Three treatments, i.e., ZnO-NPs, $ZnCl_2$, and control (0 mg kg^{-1}), were used to represent treatments at dosage of 100, 250, 500, and 750 mg kg^{-1} ZnO-NPs (Yausheva et al. 2016; Zhang et al. 2022) and equivalent concentrations for $ZnCl_2$ were applied by thorough mixing the spiked soil (1 kg) by using a kitchen robot to attain a homogenous form of spiked soil. After the dosing procedure of the soil, to achieve at least 50% water holding capacity (WHC) in soil, demineralized water was mixed thoroughly to all treatments. Before inoculating worms, water was applied to the prepared soils to achieve a weight percentage of 25%. Entire earthen pots ($n = 26$) were kept in the dark at 22 °C up to 56 days of experiment. The experiment was conducted in a climate-controlled room (20 ± 2 °C) with a photoperiod of 16 h:8 h (light:dark). There were triplicates in each concentration/group and two control (control 1 and control 2), and twelve worms were used in each concentration of both treatments. All these pots were covered with perforated plastic film and kept for 28 d. Then, these pots were arranged in a large tray filled with water to maintain appropriate moisture conditions of pots. They were watered manually to increase the moisture condition. Adult worms were taken out on the 28th day of experiment, then washed with purified water, cleansed for 24 h on a wet filter paper, and then weighed. The earthworms in each test pot were chosen randomly: Four worms were removed for zinc uptake and accumulation, and three worms were used for the biochemical analysis. After another week (56 days since the inception of the experiment), the test pots were kept under the same circumstances, and young worms were then removed. These were manually removed from the same pot, noted, and put in a moist Petri plate to avoid undisturbed totalling. The total number of juvenile offspring was used to determine fertility in order to evaluate the effects on reproduction. A concise summary of the complete experimental design, including all parameters, is given in Fig. 2.

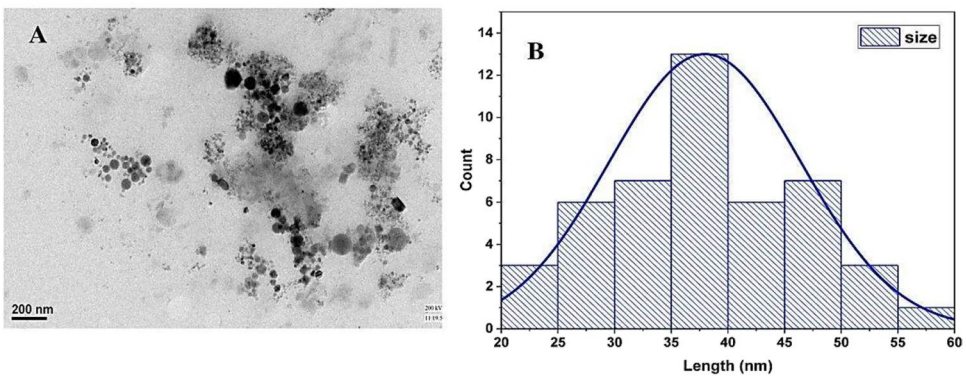

Fig. 1 (**A**) TEM image of ZnO nanoparticles; (**B**) corresponding to the histogram showing nanoparticle size distribution

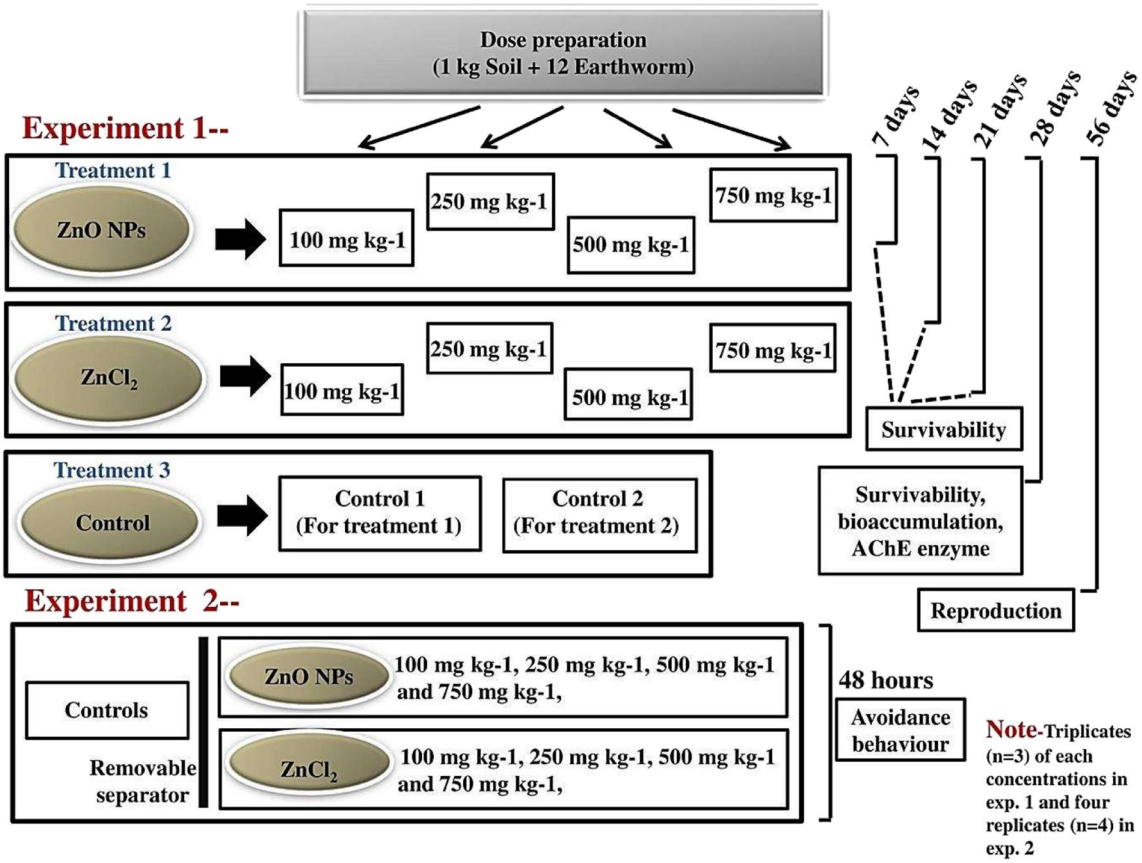

Fig. 2 A brief overview of the experimental design of the study

Bioassay method

Determination of survivability of earthworms

At the end of the experiment, surviving worms in the microcosms were collected by hand sorting and counted in each group of ZnO and $ZnCl_2$ treatment at days of 7th, 14th, 21st, and 28th, respectively. Earthworms were considered to be dead if they did not perform any physiological activity after providing the mechanical stimuli on the prostomium region, and if they were not found in the pot, it was considered died (Ge et al. 2018). The Kaplan–Meier approach was used to determine the survival probability, which provides a graphic representation of the rates at which a group of individuals survive over a particular time period. The survival rate (%) of worms was calculated by:

$$\text{Survivability} = \frac{N - D}{N} \times 100$$

where N represents the total number of earthworms at the start of the experiment and D represents the number of dead earthworms after 28 days.

Reproduction behavior assay

To evaluate reproductive efficiency, the numbers of juvenile earthworms were collected from the soil by the stepwise wet-sieving method. Only units that retained a complete set of live earthworms after the experiment completion were considered. The fertility percentage was calculated by comparing the number of juvenile worms in treated units to the average number of control units. The EC50 value was calculated, which is the median effective concentration where the response was 50% of the maximum probable response and IC50 value was also recorded which represents the inhibitory concentration of 50% maximal response.

Biochemical assay

Three earthworms were weighed (±0.009 g) and positioned individually in test tubes for homogenization on ice in 1 ml of homogenizing buffer containing 50 mM potassium phosphate buffer (pH = 7.4) with 0.9% NaCl and 1 mM EDTA solution. Then the homogenate was transferred into

a micro-centrifuge tube and centrifuged for 30 min at a speed of 25,000 × g using a cooling centrifuge (REMI CR-24 Plus, Mumbai, India). If an analysis could not be completed immediately, the homogenates and supernatants were kept for later use at − 80 °C. Subsequently, the supernatant was collected and transferred into fresh tubes and subjected to AChE assay (Askar et al. 2010).

Determination of AChE (acetylcholinesterase) Acetylcholinesterase enzyme activity was measured by following Ellman et al. (1961). A glass test tube was filled with a reaction mixture that contained potassium phosphate buffer (0.1 M, pH 7.4), with DTNB (5 mM) and acetylthiocholine iodide (156 mM). To initiate the reaction, 5 µl of sample supernatant was added and the tube was maintained at 25 °C for 5 min. Following a brief incubation time, the tissue sample was introduced, and a 5-min kinetic reading was obtained at 405 nm using a UV spectrophotometer (Shimadzu UV-1800 UV/Vis spectrophotometer). The enzyme activity was expressed as n mol of acetylthiocholine and calculation was done by using 13.6×10^3 $M^{-1}C^{-1}$ as an absorption coefficient.

Zn accumulation and gut response

Transmission electron microscopy (TEM) was used to investigate the structural changes in gut cells as well as cytoplasmic organelles of the GI tract. Uptake and accumulation were observed at lowest (100 mg kg^{-1}) and highest (750 mg kg^{-1}) concentration of ZnO-NPs and $ZnCl_2$ including control after 28 days of exposure. For this context, the exposed earthworm tissue samples were preserved for 2 h at 4 °C in 4% paraformaldehyde with 1.25% glutaraldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.4). After three times washing in 0.1 M sodium phosphate buffer, the tissues were post-fixed for 2 h in 2% osmium tetraoxide. Thenafter, the fixed tissues followed three more washes in 0.1 M sodium phosphate buffer, were dehydrated in an alcohol or acetone gradient of 15–100%, and were then embedded in epoxy resin. Following heating at 60 °C, the blocks were cut (> 80 nm thick) using an Ultramicrotome (Leica EM UC7) and sections were examined under a TEM (FEI Philips Morgagni 268D) (Lichstein et al. 2000). After the exposure, the structural alterations in cytoplasmic organelles, i.e., mitochondria, endoplasmic reticulum, and cytosolic appearance of the worms, were observed and compared to control.

Experiment 2: Avoidance behavior assessment

A short-term experiment to determine earthworm avoidance behavior was performed in a rectangular plastic container (32 cm long, 16 cm wide, and 16 cm height) with a punctured lid. A removable plastic split (separator) was used to divide each container into two equal parts (Shoults-Wilson et al. 2011). The experimental soil (1 kg) containing ZnO-NPs or $ZnCl_2$ was placed in one-half of the container, while the other half 1 kg was used as the control soil. All the concentrations of both treatments (ZnO-NPs and $ZnCl_2$), which corresponded to 100, 250, 500, and 750 mg kg^{-1}, were observed for 48 h, as described in Fig. 2. After removing the separator, ten earthworms weighing 350–500 mg were positioned in the container's center (buffer zone). The experimental design was fully randomized with four replicates per group following Amorim et al. (2005). The experiment was executed in a dark chamber at 22 ± 2 °C temperature. After 48 h, the separator was introduced again and each section's earthworms were counted. According to ISO (2005), the counting results were represented as net response (NR):

$$\mathrm{NR} = \frac{(C - T)}{N} \times 100$$

where C represents the number of worms in the control soil, T represents the number of worms in the treated soil, and N represents the total number of worms in the beginning (Amorim et al. 2005).

Statistical analysis

Results are presented as the mean values ± standard deviation. The mean values were estimated of triple data set ($n = 3$) for reproduction and AChE activity, and four data set ($n = 4$) for avoidance behavior. At the 95% confidence level ($p < 0.05$), the significant differences among all the studied parameters were examined in the different treatments of reproduction, behavioral response, and neuro-biomarker (AChE), as well as its potential applications as a toxicity tool. Using IBM® SPSS® Statisticsv26 software, a one-way analysis of variance (ANOVA) with a Tukey post hoc test ($p < 0.05$) was used to assess the differences between and within treatments as well as between each treatment with the control. Microsoft Excel was used for calculation of AChE, fertility, and inhibition percentage of reproduction, along with avoidance net response. The graphical representation of AChE enzyme and avoidance behavior was created by GraphPad Prism v9 software. The figures and tables with different alphabetical letters (a, b, c…) were agreeing the significance of study, while few parameters were also shown not significant. The EC50/logIC50 values of reproduction (estimated values capable of affecting 50% of reproduction of earthworm) were estimated through nonlinear regression models by the software GraphPad Prism v9.

Results

Survivability and reproduction

After being exposed to ZnO-NPs and $ZnCl_2$, the survivability of *E. eugeniae* was affected on 7th, 14th, 21st, and 28th days. In contrast, after 21 days of the experiment, 40% of earthworms were dead in $ZnCl_2$ exposure. Additionally, 100% survival was observed in ZnO-NPs and $ZnCl_2$ concentrations of 100 mg kg^{-1} during 14 days (Table 1). After 28 days, survivability in both ZnO-NPs and $ZnCl_2$ treatments was reduced to 63.3% and 53.3% at 750 mg kg^{-1} concentration, respectively. Figure 3 depicts the survival patterns of earthworms when exposed to different doses, as illustrated by the Kaplan–Meier curve. The results indicate a significant decrease in survival rates in $ZnCl_2$ compared to ZnO-NPs.

In order to observe the effectiveness of reproduction, juvenile worms in the soil were carefully collected at day 56 and sorted by hand as well as using a sieve (Fig. 4).

Table 1 Survivability of earthworm over the course of the exposure of ZnO-NPs and $ZnCl_2$ (at 7th, 14th, 21st, and 28th days). All values expressed in percent (%)

Nominal [Zn] concentration (mg kg^{-1})	Survival % Day 7	Survival % Day 14	Survival % Day 21	Survival % Day 28
ZnO-NPs				
0	100	100	100	100
100	100	96.7	93.3	93.3
250	93.3	90.0	86.7	86.7
500	90.0	86.7	83.3	80.0
750	86.7	73.3	70.0	63.3
$ZnCl_2$				
0	100	100	96.7	96.7
100	93.3	90	86.7	86.7
250	86.7	86.7	80.0	76.7
500	83.3	80.0	60.0	60.0
750	66.7	60.0	56.7	53.3

Following 56 days of exposure, the production of juvenile worms was found to be lower in the ZnO-NPs (4.67 ± 1.15 mean number) and $ZnCl_2$ (3.00 ± 1.73 mean number) treatments when compared to the control soil. Notably, the juvenile worm in the $ZnCl_2$ treatment showed a higher reduction. Additionally, fertility was also used as a sublethal indicator of reproductive efficacy, expressed as juvenile production in units of the entire population of surviving worms (Fernández et al. 2021), as shown in Fig. 5. The ZnO-NPs caused a varying degree of inhibition of reproduction, ranging from 22.22 to 61.11%. The lowest concentration showed the least inhibition, while the highest concentration showed the most inhibition. The reproductive suppression caused by $ZnCl_2$ ranged from 31.42 to 74.28%. The EC50 represents the concentration at which both ZnO-NPs and $ZnCl_2$ produce a response that is half of the maximum. On the other hand, the IC50 corresponds to the concentration at which the response that occurs was inhibited by half. The EC50 values for ZnO-NPs and $ZnCl_2$ were 533.4 mg kg^{-1} and 268.6 mg kg^{-1}, respectively, indicating the concentration at which 50% of the observed effect was achieved. The highest EC50 concentration of ZnO-NPs indicates that the nano form of zinc was less harmful than the ionic zinc form with reference to reproduction. Furthermore, the logarithmic IC50 value (2.72) of ZnO-NPs was higher than that of $ZnCl_2$ (2.42), suggesting that $ZnCl_2$ had a higher level of detrimental effects in comparison to ZnO-NPs.

The average number of juvenile worms produced during the course of the 56-day test period is summarized in Table 2. The average juvenile production in the control (control 1 and control 2) soil for both treatments (ZnO-NPs and $ZnCl_2$) was 12.00 ± 3.61 and 11.67 ± 2.89, respectively. The average output was very low (Table 2) per unit in soil treated with ZnO-NPs and $ZnCl_2$ at a highest concentration of 750 mg kg^{-1}. Nevertheless, there was no significant difference in the average values of juvenile worms between the ZnO-NPs and $ZnCl_2$ treatments (one-way ANOVA, $p > 0.05$). However, there were significant differences observed among the different concentrations (100, 250, 500, and 750 mg kg^{-1}) of both treatments, indicating that the different concentrations

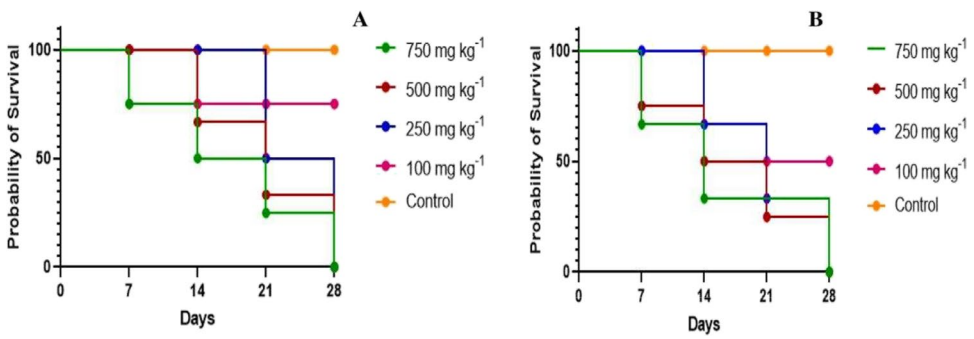

Fig. 3 Kaplan–Meier survival analysis (**A**) ZnO-NPs; (**B**) $ZnCl_2$ at different concentrations (100 mg kg^{-1}, 250 mg kg^{-1}, 500 mg kg^{-1}, and 750 mg $kg.^{-1}$)

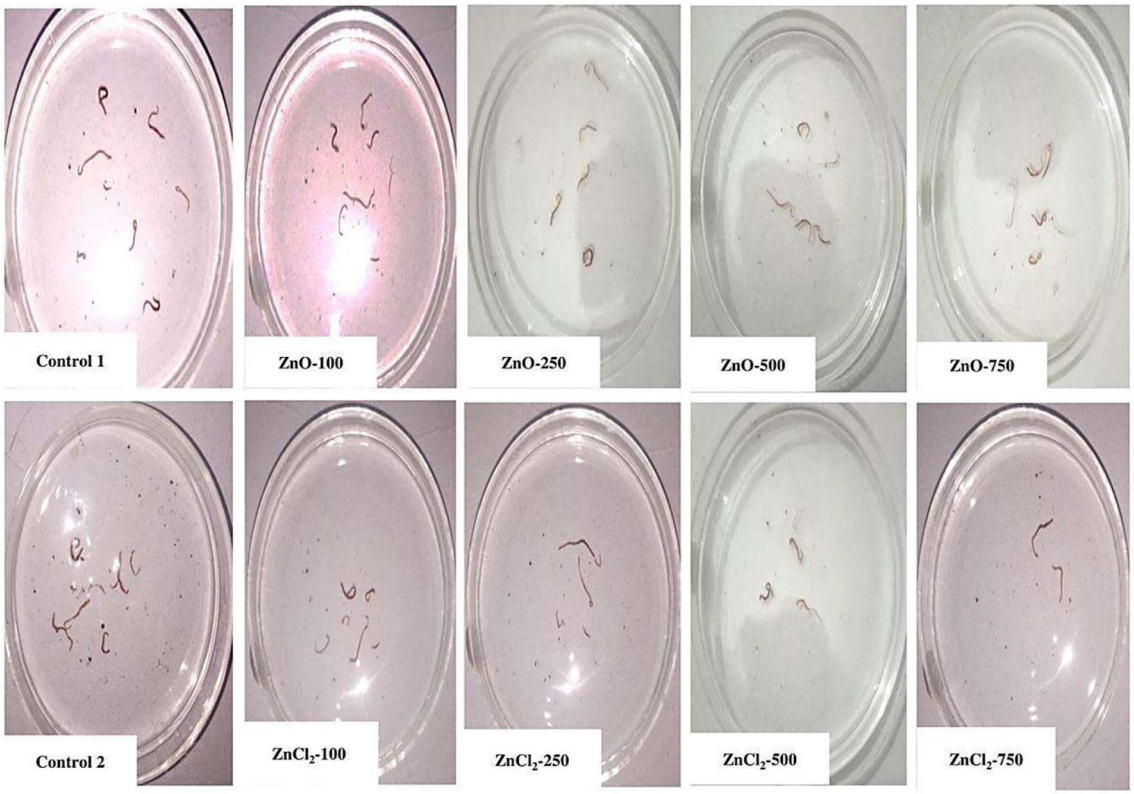

Fig. 4 Number of juvenile worms produced by *E. eugeniae* in soils amended with ZnO-NPs and $ZnCl_2$ of 100, 250, 500, and 750 mg kg^{-1} after 56 days of experiment

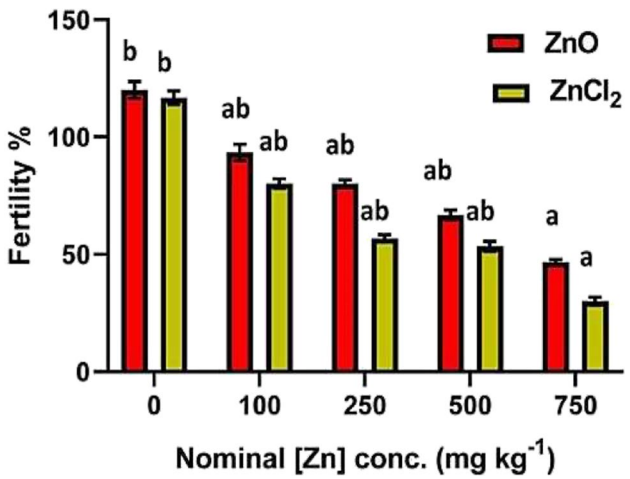

Fig. 5 Fertility of *E. eugeniae* in soils amended with ZnO-NPs and $ZnCl_2$ of different concentrations (0, 100, 250, 500, and 750 mg kg^{-1}) after 56 days. **Note**. The same alphabetical letters demonstrate that there are no statistical differences between the ZnO and $ZnCl_2$ treatments ($p < 0.05$) at each concentration

of ZnO-NPs/$ZnCl_2$ had an immediate impact on juvenile production. In treated soils, excess zinc has an impact on overall juvenile production over a range of exposure concentrations.

AChE enzyme activity

Figure 6 shows the impact of ZnO-NPs/$ZnCl_2$ on the AChE enzyme activity in earthworms. The AChE enzymes were more activated by ZnO-NPs and $ZnCl_2$ treatment after 28 days of exposure, despite $ZnCl_2$ treatment having a greater activating response than ZnO-NPs. The increased activity of AChE in both treatments shows a dose-dependent pattern. Moreover, AChE activity was directly proportional to the concentration of ZnO-NPs and $ZnCl_2$. When compared to the control (145.88 ± 28 to 149.41 ± 23 mol min^{-1}), the AChE enzymes were more activated by $ZnCl_2$ (536.32 ± 11 mol min^{-1}) treatment after 28 days of exposure, as opposed to ZnO-NPs (497.7 ± 59 mol min^{-1}) at higher concentrations (750 mg kg^{-1}). Both $ZnCl_2$ and ZnO-NPs treatments had higher levels of activity than the control (> approximately 3.6-fold higher in $ZnCl_2$ treatment and 3.0-fold higher in ZnO-NPs treatment compared to control). In contrast to ZnO-NPs treatment, it was much higher in the $ZnCl_2$ treatment (> approximately onefold higher). Consequently, at the end of exposure, $ZnCl_2$ causes the significant change in AChE enzyme and eventually is responsible for the neurological alterations in earthworms Fig 6.

Table 2 Effects of ZnO-NPs and $ZnCl_2$ exposure on reproduction of *Eudrilus eugeniae* after 28-day of exposure

Nominal [Zn] concentration (mg kg^{-1})	Mean number of juveniles per test unit ± SD	Reproduction inhibition (%) with respect to control	Log IC50 value	EC50 value (mg kg^{-1})
ZnO-NP			2.72	533.4
0	12.00 ± 3.61^{b}	_		
100	9.33 ± 3.51^{ab}	22.22		
250	8.00 ± 1.73^{ab}	33.33		
500	4.33 ± 2.08^{ab}	44.45		
750	4.67 ± 1.15^{a}	61.11		
$ZnCl_2$			2.42	268.6
0	11.67 ± 2.89^{b}	_		
100	8.00 ± 2.00^{ab}	31.42		
250	5.67 ± 1.53^{ab}	51.43		
500	5.33 ± 2.08^{ab}	54.29		
750	3.00 ± 1.73^{a}	74.28		

SD standard deviation, *IC* inhibitory concentration, *EC* effective concentration

Note. All values are mean ± SD ($n = 3$). The same superscript letters indicate statistically non-significant between the different concentration/groups in both ZnO and $ZnCl_2$ treatments ($p < 0.05$) ($n = 12$ worms per group)

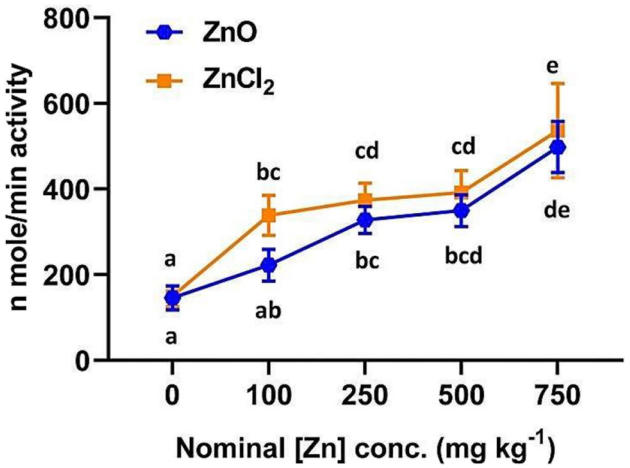

Fig. 6 AChE activity in tissue expressed as nanomoles per minute of *E. eugeniae* after 28 days of exposure to ZnO and $ZnCl_2$ (0, 100, 250, 500, and 750 mg kg^{-1}). The values are presented as the means ± SD; $n = 3$. Different alphabetical letters represent the significant differences between the concentrations of the ZnO and $ZnCl_2$ treatments, as well as between the control and treated groups: $p < 0.05$. *SD* standard deviation

Zn accumulation and gut response

The Zn content in body tissue was estimated using an atomic absorption spectrophotometer (AAS, Model: 55B AA, Agilent Technology, Australia) after 28 days of exposure and results shown in our previous study (Singh et al. 2022), which certified that Zn content in tissues increases in a dose-dependent manner in both ZnO-NPs and $ZnCl_2$ treatments; however, more Zn content accumulated in ZnO-NPs exposure compared to $ZnCl_2$. In the present study, the gut tissue was examined under a transmission electron microscope (TEM) for qualitative observation after being exposed to NPs and ions. After 28-day exposure to ZnO-NPs, the overflow of particles at the portal of entry in the gut of worm was observed (Fig. 7A). And, cytoplasmic organelles also indicated an abnormal appearance, including breakage, reduction, expansion, and disorganisation, as well as cytosolic disorder (Fig. 7B, C). The gut of worms did not accumulate Zn in control condition (Fig. 8A), while an increase in bioaccumulation was observed (Fig. 8B–E), with lowest (100 mg kg^{-1}) and highest (750 mg kg^{-1}) dose of ZnO-NPs and $ZnCl_2$ Fig. 8. The highest Zn accumulation was found at exposure of 750 mg kg^{-1} in ZnO-NPs and appeared as an abnormal structure of mitochondria (Fig. 8C). The result proposes that the earthworm can uptake and accumulate Zn by increasing the dose of NPs and ions in their body tissue, while higher accumulation was seen in NPs exposure than ionic form. Thus, earthworms exposed to soil treated with ZnO-NPs accumulated noticeably higher zinc than earthworms exposed to soil treated with $ZnCl_2$, suggesting that ZnO-NPs have a higher bioavailability compared to $ZnCl_2$.

Avoidance behavior

Earthworms were offered the option of soils amended with control soil as well as treated soil with ZnO-NPs and $ZnCl_2$ at equal concentrations of 100, 250, 500, and 750 mg kg^{-1} for both materials in a separate study. They showed a slightly less avoidance of ZnO-NPs treated soil in comparison of $ZnCl_2$ at each concentration ($p < 0.05$;

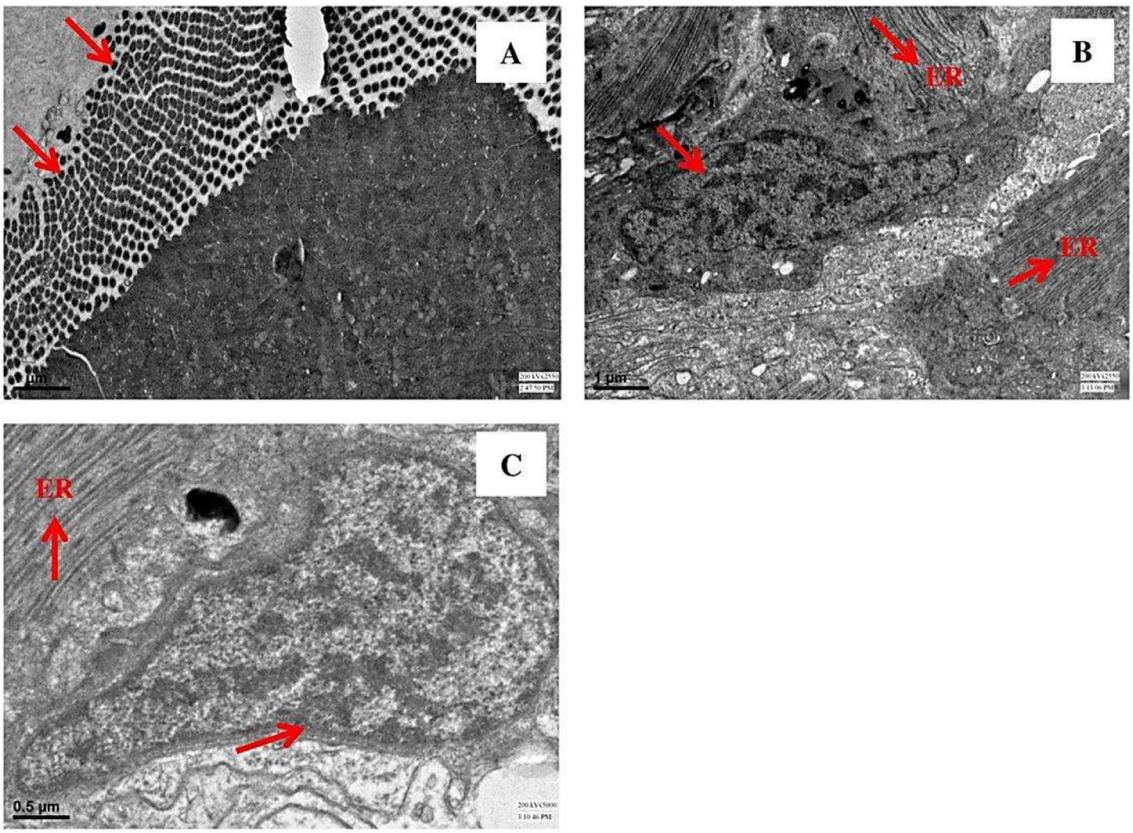

Fig. 7 Transmission electron microscopic (TEM) images (A–C) of gut of *E. eugeniae* after ZnO-NPs exposure at 28 days (**A**) showing entry of NPs and cytoplasmic disturbance (**B**, **C**) irregular endoplasmic reticulum (ER) and showing cytosolic abnormality

Fig. 9). The avoidance percentage was increased from 5 to 27.5% in the ZnO-NPs exposure, while increased from 7.5 to 37.5% in $ZnCl_2$ exposure from lowest (100 mg kg^{-1}) to highest (750 mg kg^{-1}) concentrations, respectively. Earthworms significantly avoided the contaminated soil spiked with 750 mg kg^{-1} concentration of both ZnO-NPs and $ZnCl_2$ treatment (Fig. 9). Although it has been proven that Zn contamination appears to have a significant effect on earthworm avoidance behavior, however, more investigation is needed using well-characterized Zn nanoparticles to evaluate the role that internal nanomaterials play in avoidance behavior.

Discussion

Toxicity of ZnO-NPs and $ZnCl_2$ to earthworm *E. eugeniae* in garden soil

There is a scarcity of information regarding the possible risks posed by ZnO-NPs to soil organisms, especially in terms of ecological changes that occur in the ecosystem (García-Gómez et al. 2020; Bao et al. 2020; Jośko et al. 2021). Nevertheless, few studies have demonstrated the harmful effects of ZnO-NPs on other organisms in particular residing in the aquatic ecosystem. A prior study indicated that the detrimental impacts of ZnO-NPs are caused by the liberation of zinc ions, while other studies have argued that the toxic effects are caused by the particles adhering to the organism's surface or being taken up internally. There are still knowledge gaps concerning the possible risks of ZnO-NPs compared to Zn ion through using *E. eugeniae*. The toxicological information can be improved by incorporating biochemical indicators, such as oxidative stress responses (Bao et al. 2020), for instance, although silver nanoparticles in sediment did not cause the lethality of *Bellamya aeruginosa*, they caused significant alteration to induce oxidative stress (Bao et al. 2020). Consequently, sub-lethal toxicity endpoints such as Zn bioaccumulation, behavior response, and the neuro-toxicity biomarker AChE enzyme received a great deal of attention in this work.

The study investigates the potential environmental impacts of ZnO-NPs, considering their escalating usage in diverse applications. The detrimental impact of varying concentrations of Zn nanoparticles and Zn ions on *E. eugeniae* highlights their toxic effects. Under certain conditions,

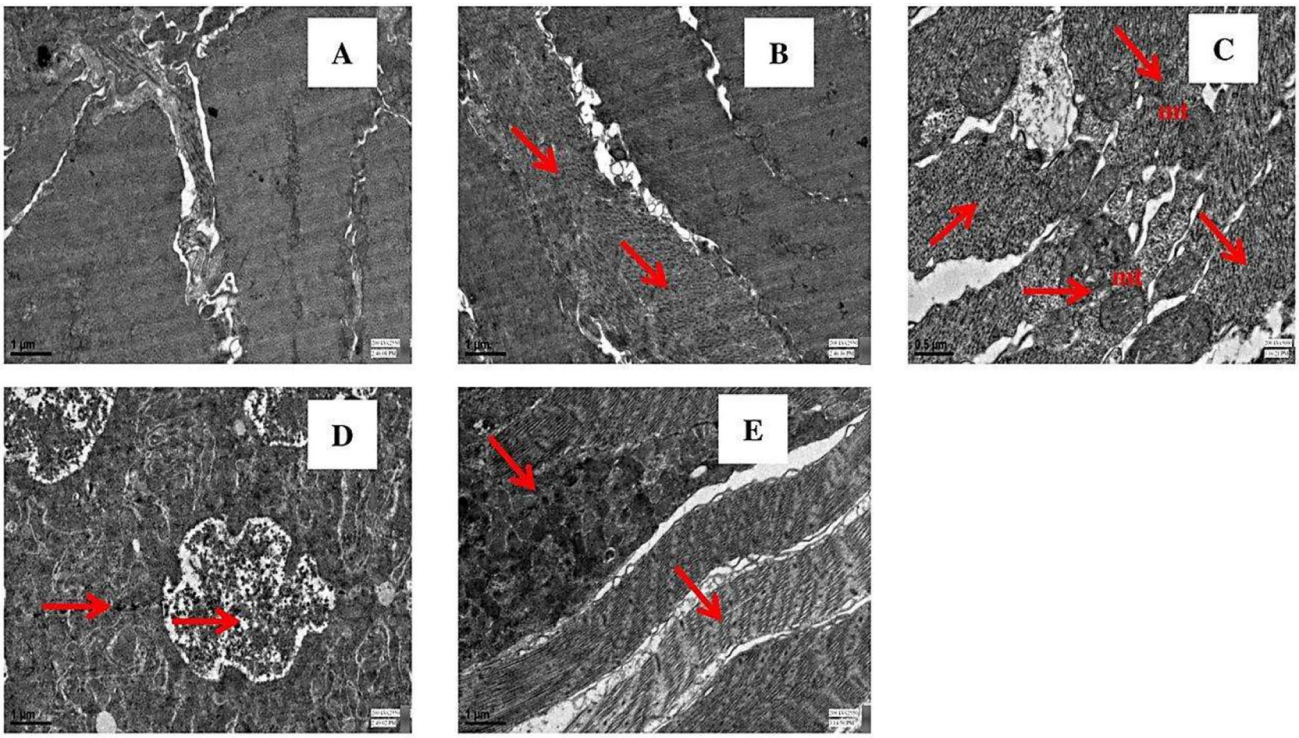

Fig. 8 Transmission electron microscopic (TEM) images of the gut wall of *E. eugeniae* after the exposure of ZnO-NPs and $ZnCl_2$ at 28 days showing uptake and accumulation (**A**) Control; (**B**) ZnO-NPs exposure at 100 mg kg^{-1}; (**C**) ZnO-NPs exposure at 750 mg kg^{-1} and showing abnormal mitochondria (mt); (**D**) $ZnCl_2$ exposure at 100 mg kg^{-1}; (E) $ZnCl_2$ exposure at 750 mg kg^{-1}, showing accumulation in the integumentary system of earthworm

a wide spectrum of zinc concentrations can manifest in the environment. Metals have varying degrees of influence on soil organisms, according to Edwards and Arancon et al. (2022). The dosages were established according to the LC50 value (662 mg kg^{-1} soil) of Zn nanoparticles for *Eisenia fetida* over a 28-day exposure period following Panda et al. (1999). It aligns with the findings of previous studies conducted by Lebedev et al. (2015), Yausheva et al. (2016), and Zhang et al. (2022). The study administered multiple doses of zinc, specifically 0, 100, 250, 500, and 750 mg kg^{-1}, to replicate the typical levels of zinc concentration found in natural soil with precision. Lebedev et al. (2015) employed a soil sample with an LC50 range of 828 to 995 mg kg^{-1} in their study. Thus, in alignment with the aforementioned prior studies, we have selected diverse dosage concentrations, specifically the minimum (100 mg kg^{-1}), intermediate (250–500 mg kg^{-1}), and maximum (750 mg kg^{-1}). The investigation assessed the impact of extended exposure to ZnO-NP/$ZnCl_2$ on earthworms in relation to their viability, reproductive abilities, behavior, bioaccumulation, and neurotoxicity. The application of the highest concentrations of Zn in nanoparticles, specifically 750 mg kg^{-1}, resulted in a survival rate of 63.3%. In contrast, the survivability rate was found 53.3% after a 28-day exposure period when the same concentrations of Zn were administered in ionic form. The lethality rate was observed to be 30% upon exposure to ZnO-NPs, whereas treatment with

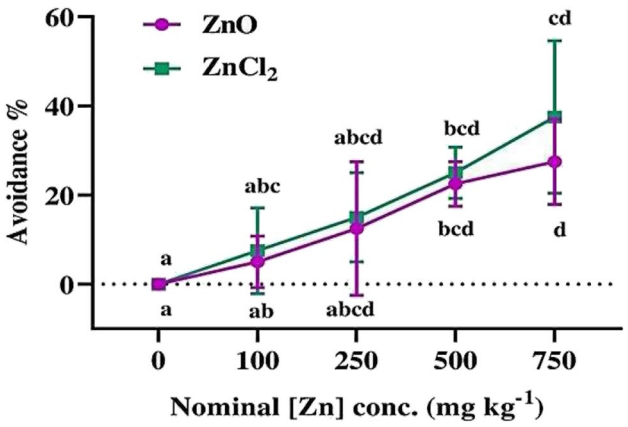

Fig. 9 Avoidance behavior of *E. eugeniae* when exposed in a control versus treated soil with ZnO-NPs and $ZnCl_2$ (100, 250, 500, and 750 mg kg^{-1}) separately after 48 h. The values are presented as the percent ± SD; $n = 4$. Different alphabetical letters represent significant differences between different concentrations in both treatment groups: $p < 0.05$. SD standard deviation

$ZnCl_2$ at a dosage of 750 mg kg^{-1} resulted in a lethality rate of 43.3% on the 21st day, specifically during the third week. The findings of the research are consistent with previous studies that have posited the higher toxicity of ions in comparison to nanoparticles (García-Gómez et al. 2020; Hu et al. 2020; Filipiak and Bednarska 2021). In a study by Bicho et al. (2017), *Enchytraeus crypticus* was subjected to $CuCl_2$ and CuO nanoparticles at concentrations of 400 and 800 mg Cu kg^{-1}, respectively, in an experimental setting. The entire population did not survive in LUFA 2.2 soil, whereas no fatalities were detected in the presence of nanomaterials at concentrations of 1600 and 3200 mg Cu kg^{-1} soil. Earthworms were exposed to various concentrations of necessary elements, such as Co and Cu, in a study carried out by Irizar et al. (2015). They were established that in under-regulated settings, earthworms' body weight, growth, production of cocoons, and reproductive development were decreased.

The study found that the EC50 value of $ZnCl_2$ was 268.6 mg kg^{-1} for reproduction, indicating a negative impact on the production of juvenile worms. In contrast, the EC50 value for the NP form was 533.4 mg kg^{-1} in the experimental setting. The reproduction inhibition percentage was 22.22% in ZnO-NPs and 31.42% in $ZnCl_2$ exposure at 100 mg kg^{-1} with respect to control worm. Similarly, at the higher concentration (750 mg kg^{-1}), reproduction inhibition was 61.11% (ZnO-NPs) and 74.28% ($ZnCl_2$) with respect to control. At 56 days of exposure, the juvenile worms were decreased in both treatments in a concentration-dependent manner. The fertility rate appeared $46.67 \pm 1.15\%$ and $30.00 \pm 1.73\%$ in $ZnCl_2$ and ZnO-NPs exposure, respectively. It was found that the fertility rate was lower in $ZnCl_2$ rather than ZnO-NPs treatment, confirming that the exposure of Zn ion had a greater impact on reproduction. The highest concentration of Zn accumulation occurred in the tissues of worms exposed to 500 and 750 mg kg^{-1} of ZnO-NPs at the end of the experiment. $ZnCl_2$ significantly reduces earthworm reproduction rate despite the increased accumulation in ZnO-NPs treatment. This trend was particularly prominent at the highest concentration of ZnO-NPs (750 mg kg^{-1}). It was notably elevated throughout the gut of the exposed worms, as depicted in Fig. 8. Additionally, the presence of nanoparticles within the cytoplasm of the worm was observed in both dispersed and clustered arrangements (Fig. 8). In general, the findings indicate that the accumulation of Zn follows a concentration-dependent pattern. The determination of metal bioaccumulation is contingent upon various factors, such as size and concentration (Alves et al. 2019). The possibility of a relation between the bioaccumulation characteristic and the accumulation of 20 nm Au-NPs at high concentrations was previously investigated by Bocca et al. (2020). He et al. (2020) found that plants can absorb Au ion ($Au^{1\pm}$ & $Au^{3\pm}$) from soil and their tissues conduct a process of sequential reduction, transforming from one metal ion into another (Gardea-Torresdey et al. 2005). The study also suggests the potential transformation of ZnO into Zn metal by earthworms. Alhussan et al. (2021) reported that endocytosis was the mechanism through which 50 nm NP was internalized by HeLa cells. The consequences of exposure to Au-NPs through drinking water were examined in a recent mice study. In the range of 10–58 nm, it was also found that uptake tended to decline as particle size increased. According to Sani et al. (2021), improved approaches must be developed further in order to study the aggregation state at extremely low concentrations and determine the mechanisms influencing nanoparticle uptake in soil and tissues.

In the context of enzymatic studies, it has been observed that living organisms possess specific enzymes that are capable of binding zinc within body tissues through the aid of metallothionein proteins, thereby facilitating the rapid elimination of Zn (Slobodian et al. 2021). As demonstrated in a prior investigation conducted by Singh et al. (2022), the application of ZnO-NPs and $ZnCl_2$ has the potential to significantly increase the activity of SOD, CAT, and LPO, as well as the total GSH content, following a 28-day exposure period ($p \leq 0.05$). Except AChE, we have previously examined other antioxidant enzymes (Singh et al. 2022). The AChE enzyme, a neuro-biomarker, is a delicate enzyme that is present in the neural systems of both vertebrates and invertebrates and functions as an efficient neurological enzyme (Wong-Guerra et al. 2021 and Jankowska et al. 2023). The study revealed that the highest dose of ZnO-NPs and $ZnCl_2$ resulted in elevated levels of the AChE enzyme. After a 28-day period of exposure, the activity of AChE enzymes was shown to be more activated in the treatment with $ZnCl_2$ (536.32 ± 11 mol min^{-1}) compared to ZnO-NPs (497.7 ± 59 mol min^{-1}) at a higher dose of 750 mg kg^{-1}, when compared to the control (145.88 ± 28 to 149.41 ± 23 mol min^{-1}). The activation of AChE enzyme correlated with the behavior of earthworm, since AChE enzyme is a neurotoxic indicator to assess the effects of nanoparticles on neuro-membrane of earthworms (Romani et al. 2003). The behavior of *E. eugeniae* was altered upon ZnO-NPs and $ZnCl_2$ exposure that correlated with the activation of AChE enzyme activity. Specifically, a dosage of 750 mg kg^{-1} was found to be associated with this increase. After being exposed to $ZnCl_2$, the enzyme showed increased levels of activation; however, the extent of activation depended on the concentration of the aforementioned Zn compounds. It has been observed that on the 28th day, the activation of AChE in earthworms gets boosted by metals, thereby elevating its catalytic efficacy. Excessive activation of the enzyme may pose a potential hazard towards the organism. Researches have demonstrated that being exposed to organophosphate and carbamate pesticides can result in the

suppression of AChE activity in both controlled experiments and factual conditions (Zheng et al. 2013; Wu et al. 2020). Romani et al. (2003) reported an increase in AChE activity (Vm/Km) in *Sparus auratus* following exposure to sublethal doses of copper. The assessment of the impact of metallic substances on AChE as an environmental biomarker is of utmost importance, despite its intricate nature (Vieira and Nunes 2021). This holds particularly true in settings that are polluted with diverse chemical substances. Metal ions have been found to have an impact on the enzyme acetylcholinesterase (AChE); however, the conclusions of these studies have been inconsistent (Romani et al. 2003; Wong-Guerra et al. 2021; Zatta et al. 2003).

The study established a relationship between the behavior of earthworm and AChE enzyme after the exposure of Zn-contaminated soil. The avoidance response was found to persist during a 48-h exposure to ZnO-NPs and $ZnCl_2$, even when regulated. The avoidance tests are quick, reasonable, and simple to do as compared to other common sublethal experiments, including reproduction. The exposure to $ZnCl_2$ resulted in higher levels of avoidance behavior in comparison to ZnO-NPs at all concentrations. The avoidance behavior can be ascribed to the immediate effect of unbound zinc ions on sensory perception, rather than being indicative of endogenous zinc toxicity. The avoidance behavior of earthworms changed at a concentration-dependent manner upon the exposure to ZnO-NPs and $ZnCl_2$. Nevertheless, the behavioral response is dependent on the species of earthworms tested, the contaminants in which they are exposed, and other soil-specific variables (Hermes et al. 2020). Chaudhuri et al. (2021) reported that chemoreceptive organs located in the outer area of the prostomium and proximal segments of earthworms demonstrated a responsive behavior towards metal ions. The statement mentioned pertains to the free ion activity model (FIAM), which proposes that the reaction of unbound metallic ions with the substrate's membrane leads to metal reactions in biological systems (Wu et al. 2019).

Interaction between the endpoints induced by ZnO-NPs/$ZnCl_2$ in *E. eugeniae*

In general, the manifestation of sublethal reproduction impairment and avoidance behavior indicated that zinc exposure had a greater impact on these endpoints compared to Zn accumulation in the exposed tissues. $ZnCl_2$ was found more toxic than ZnO-NPs, instead of the higher accumulation of ZnO-NPs; it may raise concerns about ZnO-NPs' effects on earthworms. According to the postulated finding, the act of avoiding a certain behavior is a rapid reaction to a sensory effect that is actively attributed to free Zn, rather than the magnitude of internal zinc toxicity. The behavior of earthworms may be disrupted by changes in the activity of the AChE enzyme. This was seen in this study because exposure to $ZnCl_2$ can maximize avoidance behavior due to increased activation of AChE enzymes. The exposure to ZnO-NPs did not exhibit adverse effect in relation to the high bioaccumulation of Zn contents. The worms exposed to ZnO-NPs exhibited the highest accumulation of Zn, while those exposed to $ZnCl_2$ demonstrated a significant reduction in survivability and the subsequent impact on reproductive efficiency. The study implies that zinc has the ability to produce significant internal and external thresholds for avoidance behavior. In addition, the possible impact of zinc on reproductive mechanisms may offer a powerful approach for assessing the environmental risks associated with soils contaminated by this specific element.

The potential benefits of *E. eugeniae* to eliminate zinc contaminants from soil environments

The earthworm *E. eugeniae* showed a varied impact on Zn bioaccumulation in soil amended with different Zn species (ZnO-NPs and $ZnCl_2$) at various concentrations. As a result, earthworms are a conceivably significant organism for the elimination and bioremediation of components in soil ecosystems, since they have tendency to uptake and accumulate the existing materials in soil. It is crucial to remember that different Zn species respond differently to earthworms; thus, remediation procedures for various components should be validated before employing earthworms to remediate Zn contamination in soil. Additionally, when earthworms reduce the bioavailability of NPs, they can be utilized as ecological engineers to remediate soil NP pollution directly; however, if the earthworms enhance the bioavailability of NPs, then the NP pollution remediation using earthworms should be combined with other methods like additional biological remediation and cleaning the soil. Earthworm *E. eugeniae* affects soil-based zinc bioavailability and induced toxicity on behavior and physiological attributes. Additional research is necessary before employing earthworms as an effective means of reducing NP pollution, including the potential impact of soil types, earthworm species, and NPs on the interaction between earthworms and NPs.

Conclusion

The present study examined the comparative effects of Zn ion ($ZnCl_2$) and nano ZnO on the earthworm *E. eugeniae* in garden soil. The experiment was conducted at five distinct concentrations for both ZnO-NPs and $ZnCl_2$, including 0 (control), 100 mg kg^{-1}, 250 mg kg^{-1}, 500 mg kg^{-1}, and 750 mg kg^{-1}, following a chronic exposure period of 56

days. According to the study, the accumulation of ZnO-NPs was significantly higher in the gut of worms exposed to ZnO-NPs compared to those exposed to $ZnCl_2$, particularly at concentrations exceeding 750 mg kg^{-1}. The potential hazards associated with $ZnCl_2$ could exceed those of nano ZnO due to factors such as increased dissolution and surface reactivity. Zinc chloride ($ZnCl_2$) was found to have a more detrimental impact on acetylcholinesterase (AChE) activity, reproduction, and avoidance behavior. Overall, the study demonstrates that earthworms exposed to ZnO-NPs exhibited higher Zn accumulation in comparison to those exposed to an equivalent concentration of $ZnCl_2$. The study concluded that the toxicity observed in the worms is primarily attributed to Zn ion uptake from the soil substrate, which varies depending on different concentration forms. The results obtained provide a significant contribution towards the progress of a scientific approach for ecotoxicological evaluation and explanation of the fundamental mechanism of toxicity of ZnO-NPs in soil. It indicates the significance of conducting a comprehensive analysis of the correlation between uptake and toxicity in future nanotoxicological assessments. Additionally, the study provides evidence that contradicts the finding that ZnO-NPs have harmful impacts on the organisms that inhabit the soil. Nevertheless, the toxicity of $ZnCl_2$ was shown to be larger than that of ZnO-NPs. It is possible that the ionic forms of zinc, rather than the size of zinc itself, are responsible for this differential in toxicity. Further, additional research is necessary to clarify the underlying mechanisms of their toxicity, as this could potentially affect the persistence and trophic mobility of zinc in terrestrial ecosystems.

Author contribution KS wrote the first draft of the manuscript, designed the work, set the experiment, recorded, and analyzed. MMA helped in the analysis of the data. AK reviewed and edited the MS. SY conceptualized, edited, and approved the version to be published.

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Data availability The data sets used and analyzed during the present study are available from the corresponding author (kmshweta@gmail.com) on reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors have given consent to publish.

Competing interests The authors declare no competing interests.

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