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Research Paper

Nano versus bulk: Evaluating the toxicity of lanthanum, yttrium, and cerium oxides on *Enchytraeus crypticus*

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ABSTRACT

Considering the increase in demand for rare earth elements (REEs) and their accumulation in soil ecosystems, it is crucial to understand their toxicity. However, the impact of lanthanum, yttrium and cerium oxides (La_2O_3, Y_2O_3 and CeO_2, respectively) on soil organisms remains insufficiently studied. This study aims to unravel the effects of La_2O_3, Y_2O_3 and CeO_2 nanoparticles (NPs) and their corresponding bulk forms (0, 156, 313, 625, 1250 and 2500 mg/kg) on the terrestrial species *Enchytraeus crypticus*. The effects on survival, reproduction (21 days (d)), avoidance behavior (2 d) and DNA integrity (2 and 7 d) of *E. crypticus* were evaluated. No significant effects on survival were observed. For La_2O_3, the bulk form affected more endpoints than the NPs, inducing avoidance behavior (1250 mg/kg) and DNA damage (1250 mg/kg - 2 d; 2500 mg/kg - 7 d). The Y_2O_3 NPs demonstrated higher toxicity than the bulk form: decreased reproduction ($\geq$ 1250 mg/kg); induced avoidance behavior ($\geq$ 625 mg/kg) and DNA damage ($\geq$ 156 mg/kg - 2 d; 2500 mg/kg - 7 d). For CeO_2, both forms exhibited similar toxicity, decreasing reproduction (625 mg/kg for bulk and 2500 mg/kg for NPs) and inducing DNA damage at all tested concentrations for both forms. REEs oxides toxicity was influenced by the REEs type and concentration, exposure time and material form, suggesting different modes of action. This study highlights the distinct responses of *E. crypticus* after exposure to REEs oxides and shows that REEs exposure may differently affect soil organisms, emphasizing the importance of risk assessment.

1. Introduction

Rare earth elements (REEs) based nanoparticles (NPs) are increasingly used in high-technology industry due to their catalytic, magnetic and electronic properties (Adeel et al., 2021a; Herrmann et al., 2016; Balusamy et al., 2015). For example, lanthanum oxide (La_2O_3) NPs have applications in sensors, electronics, fuel cells, magnetic data storage, antimicrobials, catalysis, automobiles, water treatment, phosphate removal and biomedicine (Balusamy et al., 2015). Yttrium oxide (Y_2O_3) NPs are gaining attention for their anticancer and biomedical applications (Emad et al., 2023; Rajakumar et al., 2021). Cerium oxide (CeO_2) NPs are used in physicochemical polishing agents, coatings, glass additives and catalytic additive in diesel fuel, resulting in a component of diesel exhaust particulate matter (Hu et al., 2018; Dahle and Arai, 2015; Sager and Wiche, 2024). According to Dahle and Arai (2015) approximately 95 % of NPs accumulate in sludge during wastewater treatment, with the application of wastewater biosolids to agricultural soils being an important terrestrial exposure pathway for NPs (Dahle and Arai, 2015; De la Torre Roche et al., 2015). Recently, CeO_2 NPs are also being studied to apply as a slow-release phosphate fertilizer since these NPs can efficiently capture and interact with dissolved phosphate in water treatment applications (Qu et al., 2024). In addition, the use of CeO_2 NPs in pest control, crop disease suppression, and growth/yield enhancement have been increasing (De la Torre Roche et al., 2015). Typically, NPs (such as those composed of silver, silicon dioxide and titanium dioxide) exhibit higher toxicities than their bulk counterparts, due to their smaller sizes and increased reactivity across biological systems (Dahle and Arai, 2015).

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The intensification of REEs mining, coupled with the use of REEs enriched fertilizers, has raised concerns about their environmental impact on soil ecosystems (Sager and Wiche, 2024; Otero et al., 2005; Sysolyatina and Olkova, 2023). Reported global concentrations of REEs in agricultural soils range from 5.5 to 72 mg/kg of yttrium (Y), 2.3–184 mg/kg of lanthanum (La) and 1.3–454 mg/kg of cerium (Ce) (Sager and Wiche, 2024). In soils near to REEs mines, concentrations as high as 6900 mg/kg for La and 12,000 mg/kg for Ce have been detected (Tang et al., 2022). Limited data regarding biological exposure have demonstrated that REEs inhibited photosynthesis and growth and disrupted metabolism on plants, inhibited growth and induced cytogenetic effects and organ-specific toxicity on animals and induced pneumoconiosis and carcinogenesis in humans (Yun et al., 2024). Given the existing evidence of their adverse effects on organisms, it is extremely important to study the ecotoxicity of REEs. Regarding soil organisms, Li et al. (2018) found that the 50 % lethal concentration (LC_{50}) for La ($La(NO_3)_3.6H_2O$) exposure was 1650 mg/kg for *Enchytraeus crypticus,* 1850 mg/kg for *Eisenia andrei*, 1690 mg/kg for *Folsomia candida* and 960 mg/kg for *Porcelio scaber (*Li et al., 2018*),* suggesting that lethality towards soil organisms may only occur in areas with severe REEs pollution (Sager and Wiche, 2024). However, reproduction seems to be more sensitive to REEs exposure, with 50 % effect concentration (EC_{50}) values for La ($La(NO_3)_3.6H_2O$) exposure reported as 1010 mg/kg for *E. crypticus,* 529 mg/kg for *E. andrei*, 1220 mg/kg for *F. candida* and 312 mg/kg for *P. scaber (*Li et al., 2018*)*. La_2O_3 NPs and bulk (25–1000 mg/kg) were reported to increase mortality and abnormalities in internal organelles while reducing reproduction and digestive and cast enzymes (Adeel et al., 2021b). For Y_2O_3 there is not any study with terrestrial organisms for both NPs and bulk forms. CeO_2 NPs did not affected mortality or reproduction of *Eisenia fetida* and there was minimal effects on lipid peroxidation but a decrease in the moisture content and histological changes was observed (Servin et al., 2018; Lahive et al., 2014). Affected feeding rates (increase for low concentration and decrease for high concentrations) and increased lipid peroxidation was also reported for CeO_2 NPs exposed through feeding on the soil organism *P. scaber* (Malev et al., 2017). Given the scarcity of studies on the toxicity of REEs in soil organisms (Egler et al., 2022), and even fewer studies on the effect of REEs based NPs, it is imperative to investigate their potential ecotoxicity to understand the risk they pose to terrestrial ecosystems.

Enchytraeids are often selected in ecotoxicity tests due to their importance in soil ecosystems and sensitivity to environmental stressors (Huang et al., 2020). *E. crypticus* is a suitable candidate for evaluating REEs toxicity (Huang et al., 2020). To our knowledge, no study has yet investigated the potential toxic effects of Y_2O_3, La_2O_3 and CeO_2 NPs on *E. crypticus*, highlighting a critical research gap. Thus, this work aims to unravel the impact of the REEs based materials, specifically Y_2O_3, La_2O_3 and CeO_2 NPs and their corresponding bulk forms, on the soil species *E. crypticus.* Individual endpoints such as survival, reproduction, and avoidance behavior were evaluated. Additionally, REEs genotoxicity was assessed using the comet assay to better understand the toxicity mechanisms of these materials in both forms. The assessment of the relative toxicity of Y_2O_3, La_2O_3 and CeO_2 in their nano and bulk forms is essential to understand *E. crypticus* sensitivity to REEs exposure and to contribute to REEs risk assessment in terrestrial ecosystems.

2. Material and methods

2.1. Test species

The species used for the experiment was *E. crypticus*. *E. crypticus* was selected as an ecologically important terrestrial model organism since they play an important role in soil organic matter decomposition and are widespread in various soil types, being sensitive to inorganic and organic chemicals (Castro-Ferreira et al., 2012).Their cultures were kept in laboratory, on agar plates made of Oxoid bacteriological agar and a sterilized mixture of salt solutions containing 2 mM $CaCl_2.2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$. The organisms were fed ground autoclaved oatmeal twice a week (ad libitum) and maintained under controlled conditions (20 ± 1 °C; 16:8 h (h) light:dark photoperiod). Only adults with a visible clitellum and similar size were selected for the experiments.

2.2. Test medium

The Standard Landwirtschaftliche Untersuchungs-und Forschungs-Anstalt (LUFA) 2.2 natural soil (Speyer, Germany) was used for the experiments, with the following main characteristics, according to the supplier: pH (0.01 M $CaCl_2$) = 5.8; organic carbon = 1.71 %; cation exchange capacity = 9.2 meq/100 g; maximum water-holding capacity (WHC) = 44.1 %; and grain size distribution of 7.2 % clay, 8 % silt, and 77.5 % sand. The soil was dried (48 h; 60 °C) before use.

2.3. Test materials and spiking

All three materials, La_2O_3 (CAS: 1312-81-8), Y_2O_3 (CAS: 1314-36-9) and CeO_2 (CAS: 1306-38-3), in both bulk and NPs forms were obtained in powder form from Sigma-Aldrich. According to the manufacturer, the NPs sizes are below 50 nm for Y_2O_3 NPs and CeO_2 NPs, and below 100 nm for La_2O_3 NPs. The morphology and size of the NPs were examined through scanning electron microscopy (SEM) and transmission electron microscopy (TEM), using a STEM HD2700 electron microscope operating at 300 kV. SEM micrographs of the bulk materials were captured using a Hitachi SU-70 instrument operating at 15 kV. For STEM analysis, samples were prepared by evaporating diluted suspensions of the NPs onto a copper grid coated with an amorphous carbon film. For SEM analysis, samples of bulk materials were prepared by placing a small volume of a dilute suspension in ethanol onto a glass slide glued to the sample holder using double-sided carbon tape, followed by carbon sputter coating. The quantification of the materials (nano and bulk) in the experimental media was performed by inductively coupled plasma mass spectrometry (ICP-MS) using an iCAP™ Q ICP-MS equipment, at the beginning of the exposure.

The nominal concentrations selected for the reproduction tests were 0, 156, 313, 625, 1250 and 2500 mg of La_2O_3, Y_2O_3 and CeO_2 nano or bulk/kg soil dry weight (DW). Powder of the test materials was incorporated into DW soil, mixed and then moistened to 50 % of the maximum WHC. Spiking was done individually, replicate by replicate, following standard guidelines (OECD- Organisation for Economic Co-operation and Development, 2012). For the control, a batch was prepared, homogenized to 50 % of the maximum WHC, and divided equally among the replicates. The soil was allowed to equilibrate for one day before the test start.

2.4. Reproduction tests

The standard OECD guidelines (OECD, 2016) was followed for the enchytraeid reproduction test. Concisely, 10 organisms (adults with similar size and visible clitellum) were introduced in test containers with 20 g of moist soil and food (25 mg autoclaved oats). The tests ran over 21 days (d) under controlled conditions: 20 °C and a 16 h: 8 h light: dark photoperiod. Food and water were replenished weekly, being the water content adjusted based on the weight loss of the test vessel. Four replicates (n = 4) per experimental condition were used, with an additional one without organisms for pH measurement. At the end of the test, the organisms were fixated with 96 % ethanol and Bengal rose (1 % in ethanol). The replicates were sieved through three meshes (0.6, 0.2, and 0.1 mm) to separate individuals from the soil and to facilitate counting using a stereo microscope. Survival (number of adults) and reproduction (number of juveniles) were assessed.

2.5. Avoidance tests

The avoidance test was performed following the earthworm avoidance test guidelines (International Organization for Standardization (ISO), 2008). Plastic containers measuring 2.5 × 6.5 cm in diameter, each equipped with a removable divider and covered with lids, were used. Five biological replicates ($n = 5$) with pools of organisms per treatment were done. Each replicate consisted of 50 g of soil: 25 g of control soil and 25 g of spiked soil. Per replicate, 10 selected enchytraeids (adults with a visible clitellum and similar sizes) were placed along the contact line between the control and spiked soil. As a test validation, a dual control test was performed with both compartments filled with control soil. The containers were kept for 48 h under controlled conditions: 20 ± 1 °C and a 16:8 h light:dark photoperiod. At the end of the test, each side of the container was independently searched for worms. Percentage of avoidance (A) per treatment was calculated according to the followed expression: $= \frac{C-T}{N} \times 100$, where C is the number of organisms in the control soil, T in the spiked soil and N is the total of organisms in the replicate.

2.6. Comet assays

The comet assay with *E. crypticus* was performed according to the optimization of Maria et al. (2018). Per replicate, 15 adult organisms with visible clitellum were used for each nano or bulk form of La_2O_3, Y_2O_3 and CeO_2 experiment. Organisms were introduced in test vessels containing 20 g of moist soil and food supply (25 mg autoclaved oats). Test conditions were 20 ± 1 °C and 16 h: 8 h photoperiod. Five replicates per treatment were used ($n = 5$) and sampling was done at 2 and 7 d. All the procedures during the comet assay were performed under dimmed light conditions to prevent additional DNA damage. During the sampling procedure, organisms were collected, rinsed in ISO water, maintained for 30 min, transferred to cold phosphate-buffered saline (PBS) and chopped with scissors (10 to 15 times), being 40 μL of cold cell suspension collected at the end. The cell suspension was mixed with 140 μL of 1 % low melting point agarose (LMPA), at 37 °C, and immediately distributed in a pre-coated glass microscope slide with 1 % normal melting point agarose (NMPA), and a glass coverslip was placed. The slides were kept at 4 °C until solidification. Positive controls were made with 75 μM hydrogen peroxide (H_2O_2). The slides were immersed in a lysis solution (at 4 °C for 24 h) and placed in a tank with electrophoresis buffer for alkaline treatment (20 min). The electrophoresis was performed for 15 min, 25 V and 300 mA (4 °C). The slides were neutralized with cold PBS for 10 min at 4 °C and washed in cold distillate water (10 min at 4 °C) before being placed to air dry and stained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization on a fluorescence microscopy. Per slide, 100 no overlapping nucleoids were counted and each one was evaluated with a visual classification according to 5 classes of damage from 0 (no tail) to 4 (almost all DNA in tail) (Collins, 2004) (Fig. 1). Then the genetic index damage (GDI), varying from 0 to 400, was calculated for each slide by multiplying the number of nucleoids in each class by the corresponding factor. Results were expressed as arbitrary units (au). GDI = (number of nucleoids class 0 × 0) + (number of nucleoids class 1 × 1) + (number of nucleoids class 2 × 2) + (number of nucleoids class 3 × 3) + (number of nucleoids class 4 × 4).

2.7. Data analysis

Statistical analysis and graphics were done with SigmaPlot Version 12.5 software. Tests for normality (Shapiro-Wilk test) and homoscedasticity (Levene test) were done. After one way analysis of variance (ANOVA), Dunnett's post hoc test was used for control versus treatments comparisons. For non-parametric data the Kruskal-Wallis test was done. Two-way ANOVA was performed to compare the two forms of each material, NPs versus bulk. Significant differences were considered for a significance level (p) < 0.05.

3. Results and discussion

3.1. Materials characterization and quantification

The average NPs sizes, assessed by electron microscopy, were as follow: 14.4 ± 3.6 nm for Y_2O_3 NPs, 37.4 ± 11.9 nm for La_2O_3 NPs and 18.6 ± 7.9 nm for CeO_2 NPs. These sizes are consistent with the ranges specified by the manufacturers (< 50 nm for Y_2O_3 and CeO_2 NPs, and < 100 nm for La_2O_3 NPs). The three nanomaterials exhibited irregular shape and pronounced aggregation/agglomeration (Fig. 2).

The three bulk materials also exhibited diverse morphologies. Bulk Y_2O_3 displayed irregular or cuboid structures and La_2O_3 bulk material showed irregular, fractured structures while bulk CeO_2 displayed pillar or irregular, fractured structures. The averages lengths for these bulk materials were 9.63 ± 4.09 μm for Y_2O_3, 1.54 ± 0.84 μm for La_2O_3 and 2.03 ± 0.47 μm for CeO_2.

At 0 h, the concentrations of Y_2O_3, La_2O_3 and CeO_2 measured in the soil generally agreed with the nominal concentrations for both NPs and bulk forms (Table 1).

3.2. Reproduction tests

Y_2O_3, La_2O_3 and CeO_2 NPs and bulk forms did not significantly affect the survival of *E. crypticus* ($p > 0.05$; Fig. 3 A, C and E) across all tested concentrations (156 to 2500 mg/kg) after 21 d of exposure. Li et al. (2018) exposed *E. crypticus* to various concentrations of La ($La(NO_3)_3.6H_2O$; 0–10,000 mg/kg) and reported an LC_{50} of 1650 mg/kg after 21 d of exposure (Li et al., 2018). The discrepancy in results may be due to the different materials used as La source and exposure method. Specifically, Li et al. (2018) spiked the soil with aqueous solutions of $La(NO_3)_3.6H_2O$, which is a highly water soluble salt and let the spiked soil to equilibrate for 2 weeks, before starting exposures. In contrast, in the present study, water insoluble La_2O_3 powdered forms were mixed with the soil that was let to equilibrate during 1 d before the start of the assay. This most likely resulted in different bioavailability of La ions, thereby influencing lethality towards *E. crypticus*. Gong et al. (2021) reported LC_{50} values of 277 mg/L at 14 d of exposure for La and 326 mg/L at 14 d of exposure for Ce for *E. crypticus* organisms (Gong et al., 2021). While, Huang et al. (2020) estimated ultimate *E. crypticus* LC_{50} values of 279 mg/L for Ce and 358 mg/L for La (Huang et al., 2020). However, the exposure conditions in these studies differed significantly from the present study. The authors used a simulated soil solution-quartz sand system as the exposure medium to avoid the binding of REEs to test matrix and the impact of complex soil processes on REEs bioavailability (Gong et al., 2021). This exposure design allows for greater

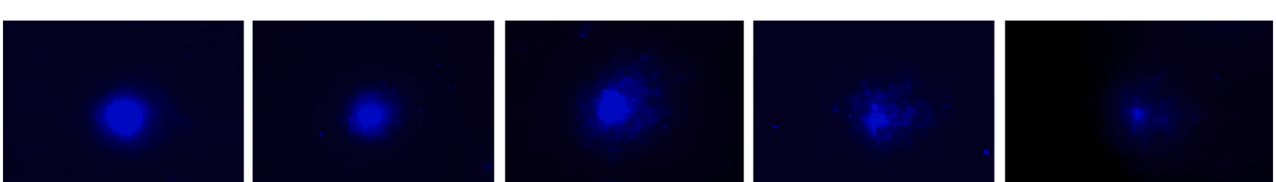

Fig. 1. DNA damage classes from *E. crypticus* (x 400 magnification): class 0, class 1, class 2, class 3 and class 4, from left to right.

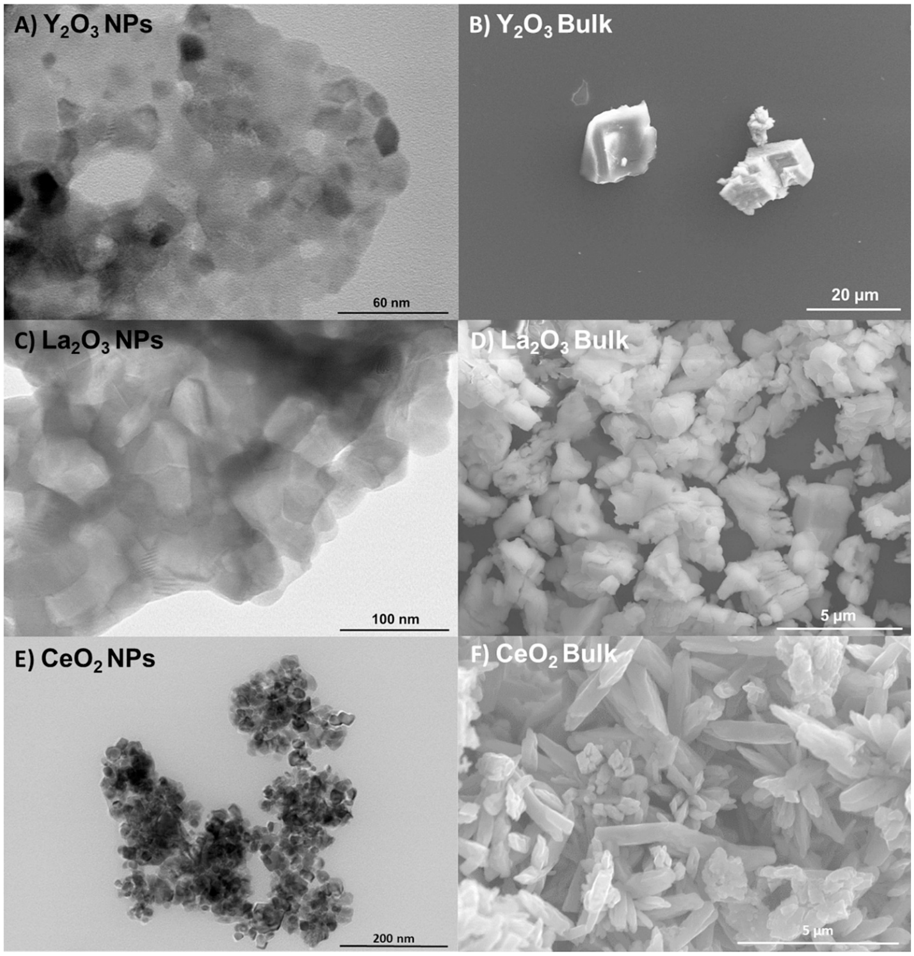

Fig. 2. Transmission electron microscopy (TEM) images of Y_2O_3 (**A**), La_2O_3 (**C**), and CeO_2 (**E**) nanoparticles (NPs) and scanning electron microscopy (SEM) images of Y_2O_3 (**B**), La_2O_3 (**D**), and CeO_2 bulk forms (**F**).

Table 1
Determined concentrations of Y_2O_3, La_2O_3, and CeO_2 nanoparticles (NPs) and bulk in the exposure media (LUFA 2.2 soil) at the beginning of the exposure tests (day 0). Results are expressed as average value ± standard deviation ($n = 3$).

Nominal concentrations (mg/kg)	Measured concentrations (mg/kg)					
	La_2O_3 NPs	La_2O_3 Bulk	Y_2O_3 NPs	Y_2O_3 Bulk	CeO_2 NPs	CeO_2 Bulk
0	5.05 ± 0.09		4.01 ± 0.16			
156	158 ± 2.9	153 ± 4.7	159 ± 11.5	153 ± 5.9	160 ± 12.2	149 ± 3.8
313	320 ± 6.5	322 ± 12.4	317 ± 14.8	318 ± 5.7	309 ± 5.2	301 ± 7.1
625	629 ± 15.3	626 ± 11.6	623 ± 20.5	617 ± 21.3	634 ± 9.8	604 ± 10.7
1250	1248 ± 30.5	1148 ± 50.3	1230 ± 36.2	1312 ± 41.6	1272 ± 39.3	1175 ± 35.2
2500	2478.12 ± 96.2	2378 ± 46.8	2446 ± 74.5	2545 ± 74.6	2485 ± 111	2277 ± 91.3

bioavailability of REEs to *E. crypticus*, resulting in more evident toxic effects at lower concentrations, but is less environmentally relevant. Experiment designs must be formulated with the factors likely to influence bioavailability in mind, therefore, tests should be conducted in media that are as close to realistic conditions as possible to have relevance (Tourinho et al., 2012). It is well know, that sorption on clay minerals, phosphates, hydroxides and organic matter decrease the solubility and mobility of REEs in soils (Sager and Wiche, 2024). Besides, their mobility and bioavailability are influenced by pH, temperature and concentration/type of organic and inorganic ligands (Gonzalez et al., 2014). REEs may accumulate on the soil surface due to their sorption onto soil colloids leading to low bioavailability (Ramos et al., 2016). Under these circumstances, the nominal concentrations quantified in the spiked soil of the present study may not represent the real risk to the organisms, as the quantified values may not be bioavailable. Future quantification of the materials on the organisms' tissues could more precisely shows the real risk of these REEs by indicating how much was bioavailable for *E. crypticus* internalization. Previous studies concluded that low bioavailability levels of Ce substantially reduce the contribution of Ce exposure values via direct soil ingestion (Ramos et al., 2016). Despite using the same materials and very similar exposure conditions, contradictory results were observed when comparing La and Ce lethality in *E. crypticus*: Gong et al. (2021) found La to be more toxic than Ce (Gong et al., 2021), whereas Huang et al. (2020) reported that Ce was the most lethal to *E. crypticus*, followed by Gadolinium (Gd) and La (Huang et al., 2020). These discrepancies highlight the importance of a multi-endpoint approach in REEs risk assessment, as in the present study, with more endpoints assessed, CeO_2 forms were evidently more toxic than La_2O_3.

In the present study, La_2O_3 did not affect the reproduction of *E. crypticus* ($p > 0.05$; Fig. 3 D). Li et al. (2018) exposed *E. crypticus* to a range of La ($La(NO_3)_3.6H_2O$) concentrations (12.4–10,000 mg/kg) and reported an EC_{50} of 1010 mg/kg after 21 d of exposure (Li et al., 2018).

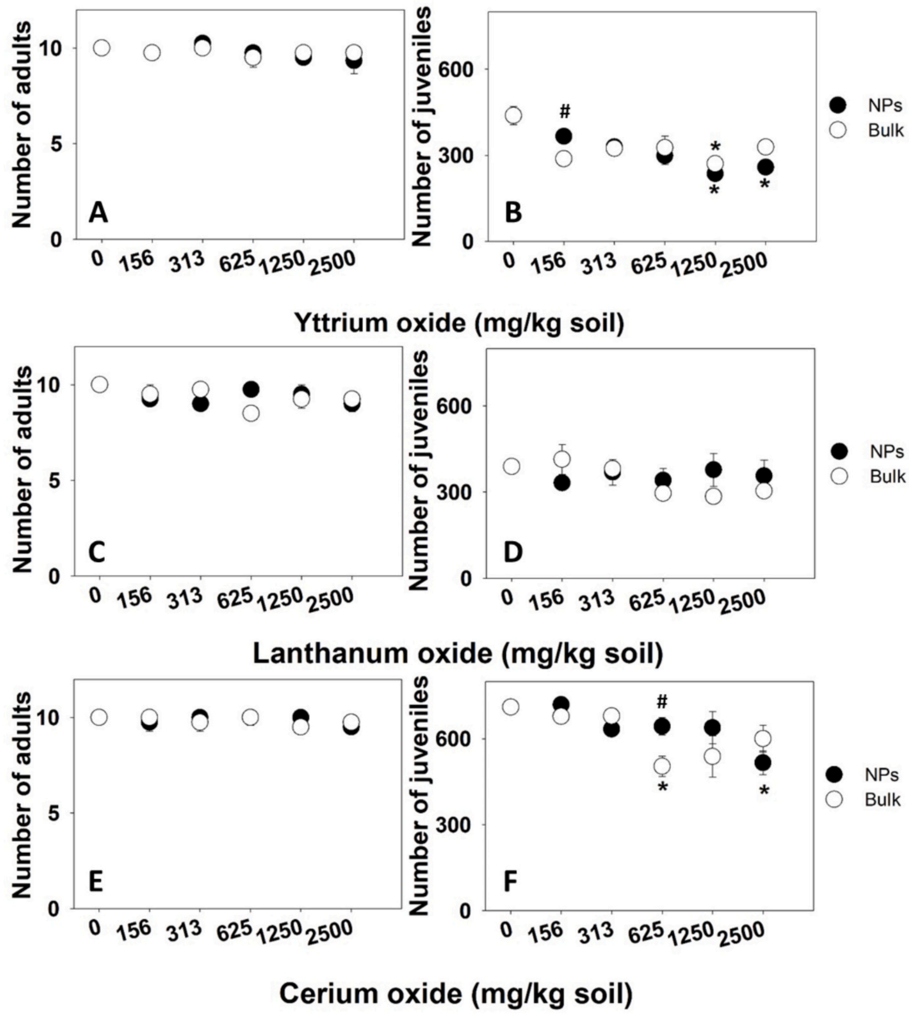

Fig. 3. Effects on survival (number of adults) and reproduction (number of juveniles) of *Enchytraeus crypticus* after 21 days to yttrium oxide (Y_2O_3) (**A**, **B**), lanthanum oxide (La_2O_3) (**C**, **D**), and cerium oxide (CeO_2) (**E**, **F**) nanoparticles (NPs) and bulk in LUFA 2.2 soil. Data are expressed as average value ± standard error. * Significant differences with control group - 0 mg/kg ($p < 0.05$). # Significant differences between the two forms (NPs versus bulk) within the same concentration ($p < 0.05$).

As mentioned previously for survival, this difference in results may be attributed to different material used ($La(NO_3)_3.6H_2O$, a highly water soluble salt) and on different exposure methods. Additionally, some authors have reported less dissolved La^{3+} ions from La_2O_3 NPs and bulk due to La^{3+} precipitation with phosphates in the soil medium (De la Torre Roche et al., 2015), which may explain the reduced toxicity towards *E. crypticus*. It is also noteworthy that the measured concentration for the highest level of La_2O_3 bulk was lower than expected (2027 mg/kg instead of 2500 mg/kg) which may have influenced the observed lack of effect on reproduction. In contrast, a significant decrease in the reproductive outcome of *E. crypticus* was observed for Y_2O_3 (1250 mg/kg bulk; 1250 and 2500 mg/kg NPs) and CeO_2 (625 mg/kg bulk; 2500 mg/kg NPs) NPs and bulk exposure ($p < 0.05$; Fig. 3 B and F). CeO_2 NPs have been shown to induce size-dependent toxicity effects on the survival and fertility of *Caenorhabditis elegans* (Dahle and Arai, 2015). The lanthanides (including La and Ce) have similar chemical properties, resulting in similar predicted toxicity, but some studies show lanthanides' toxicity increases with increasing atomic mass. Previous studies have reported that Ce was more toxic than La, attributed to the higher charge density of Ce that allows it easier to adsorb (Kotelnikova et al., 2022). De la Torre Roche et al. (2015) reported that CeO_2 and La_2O_3 exhibit different mechanisms of uptake and transport in plants, with soil phosphates negatively influencing the uptake and transport of both La_2O_3 bulk and NPs by lettuce (De la Torre Roche et al., 2015). This finding may relate to the results of the present study, where the toxicity of CeO_2 exposure was higher than La_2O_3 exposure to *E. crypticus*. To the best of our knowledge, no studies have assessed the effects of Y_2O_3 on animals, making this study the first to contribute to the risk assessment of this type of REEs oxide. Yttrium nitrate was found to have no effect on the reproduction of rats at a dose of 90 mg/kg (Yan et al., 2023). Reproduction was found to be more sensitive than survival, making this individual endpoint more suitable for risk assessment than lethality (Li et al., 2018).

3.3. Avoidance test

Avoidance behavior is an important protective mechanism, as organisms may evade contaminated soil (Barreto et al., 2023). To our knowledge, there are no previous studies reporting the potential avoidance responses of *E. crypticus* (or any other soil organism) to REEs-based materials exposure. In the present study, *E. crypticus* exhibited a stronger avoidance behavior after 2 d of exposure to Y_2O_3 NPs compared to La_2O_3 and CeO_2, with increasing avoidance at concentrations ≥625 mg/kg ($p < 0.05$; Fig. 4A). Conversely, Y_2O_3 bulk did not induce an avoidance reaction in the organisms ($p > 0.05$; Fig. 4C). It seems that the chemoreceptors of *E. crypticus* detected the presence of Y_2O_3 NPs on the

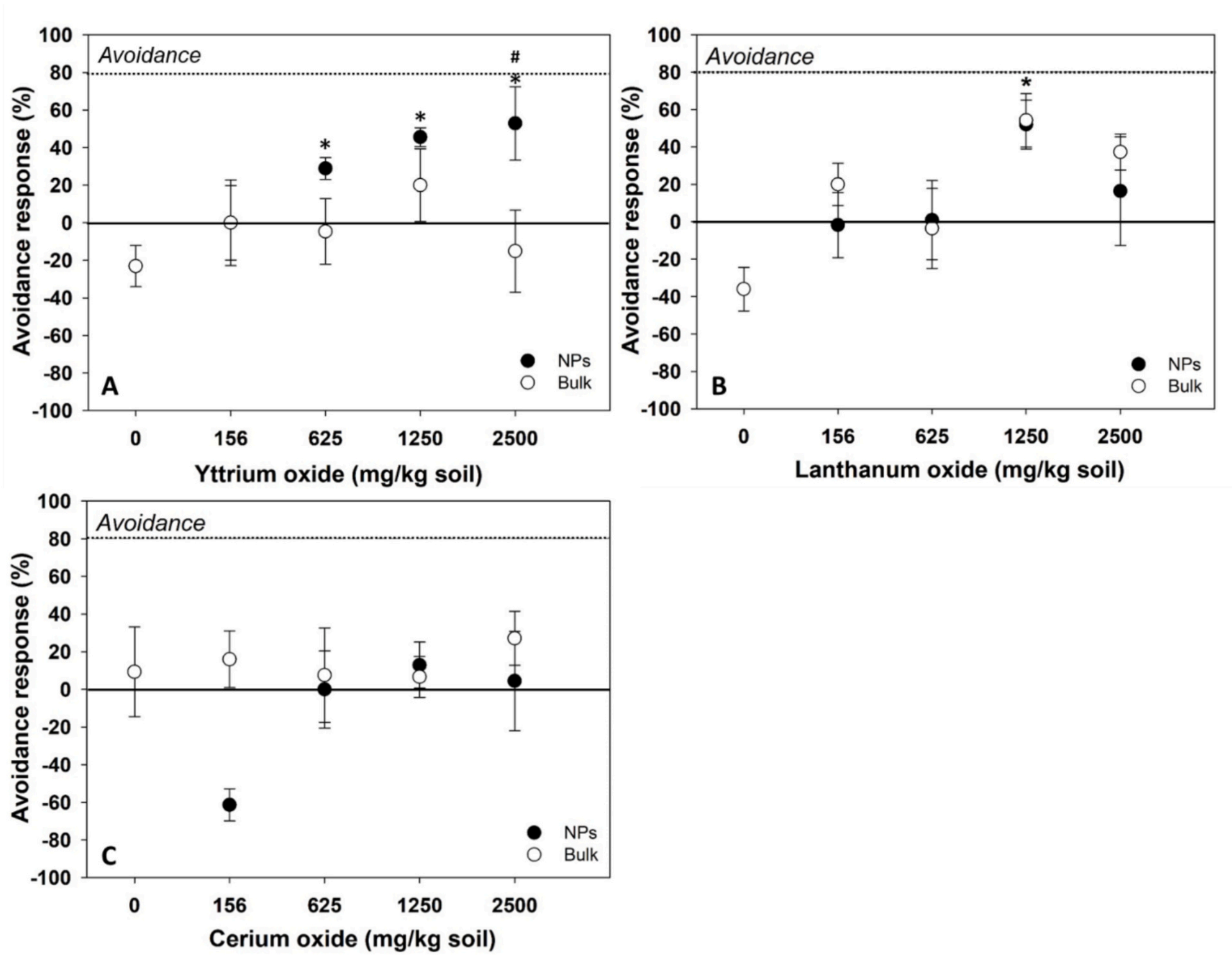

Fig. 4. Avoidance responses (%) of *Enchytraeus crypticus* after 2 days of exposure to yttrium oxide (Y_2O_3) (**A**), lanthanum oxide (La_2O_3) (**B**), and cerium oxide (CeO_2) (**C**) nanoparticles (NPs) and bulk in LUFA 2.2 soil. Data are expressed as average value ± standard error. * Significant differences with control group - 0 mg/kg ($p < 0.05$). # Significant differences between the two forms (NPs versus bulk) within the same concentration ($p < 0.05$).

soil, processed this information via the nervous system and initiate a response of avoidance behavior, however the same was not detect for Y_2O_3 bulk at the same concentrations. Exposure to high concentrations of REEs has been linked to neurotoxic effects, characterized by decreased AChE activity and blocking of K-type Ca^{2+} channels in invertebrates (Sager and Wiche, 2024). Y_2O_3 bulk neurotoxic effects may be the cause for the no avoidance response in the organisms in the present study. After 2 d of exposure, both CeO_2 NPs and bulk did not elicit a significant avoidance response in *E. crypticus* ($p > 0.05$; Fig. 4C). For La_2O_3, the form of the material influenced the outcome; while La_2O_3 NPs had no effect, a significant avoidance response was observed at 1250 mg/kg of La_2O_3 bulk ($p < 0.05$; Fig. 4B). Previous studies have reported that neurotoxic effects, such as inhibition of the neurotransmitter acetylcholinesterase (AChE), induced by the exposure to the contaminated soil can result in the absence of avoidance behavior in organisms, as they become incapable of avoiding the contaminated soil (Barreto et al., 2023; Capitão et al., 2022). Thus, it is plausible that the observed lack of avoidance in certain conditions in the present study may have been caused by AChE inhibition. Therefore, assessing the *E. crypticus* AChE activity after REEs-based materials exposure is essential to validate this hypothesis. Adell et al. (2021) observed a dose-dependent inhibition of AChE activity in *E. fetida* exposed to La_2O_3 NPs and bulk (≥ 100 mg/kg), with the effect of La_2O_3 NPs being greater than that of the bulk form (Adeel et al., 2021a). The authors suggested that inhibition of AChE could be due to NPs adsorption or physical interaction with the enzyme structure, or the release of ions from both, NPs and bulk forms (Adeel et al., 2021a). Guo et al. (2020) reported that ZnO NPs suppressed AChE activity, due to the interaction of Zn^{2+} released from the NPs with AChE altering its secondary structure or via a transcriptional down-regulation mechanism (Guo et al., 2020). Wang et al. (2009) also concluded that AChE inhibition by metallic nanoparticles was primarily caused by adsorption or interaction with AChE, however, metal ions released from nanoparticles suspensions could also reduce AChE activity (Wang et al., 2009). It is possible that REEs NPs have a similar mode of action regarding AChE inhibition. Given the fundamental roles of AChE, the mechanisms involved in suppressing AChE activity by REEs exposure are needed to further explore.

3.4. Comet assay

Molecular-level effects, such as genotoxicity, are increasingly used in ecotoxicology, due to their high sensitivity and mechanistic values for contaminant risk assessment (Gonzalez et al., 2014). In the present study, while the effects on reproduction and avoidance occur at higher concentrations, DNA damage was observed at much lower concentrations, comparable to those found in agricultural soils. This finding demonstrates the potential toxicity of these compounds. All REEs-based materials, in both NPs and bulk forms, induced some level of DNA damage. However, different response patterns were observed for different materials and forms: 1) CeO_2 exposure was the most severe to the DNA integrity of *E. crypticus*, as all tested concentrations, at 2 and 7 d, in both forms, induced similar DNA damage ($p < 0.05$; Fig. 5C); 2) For

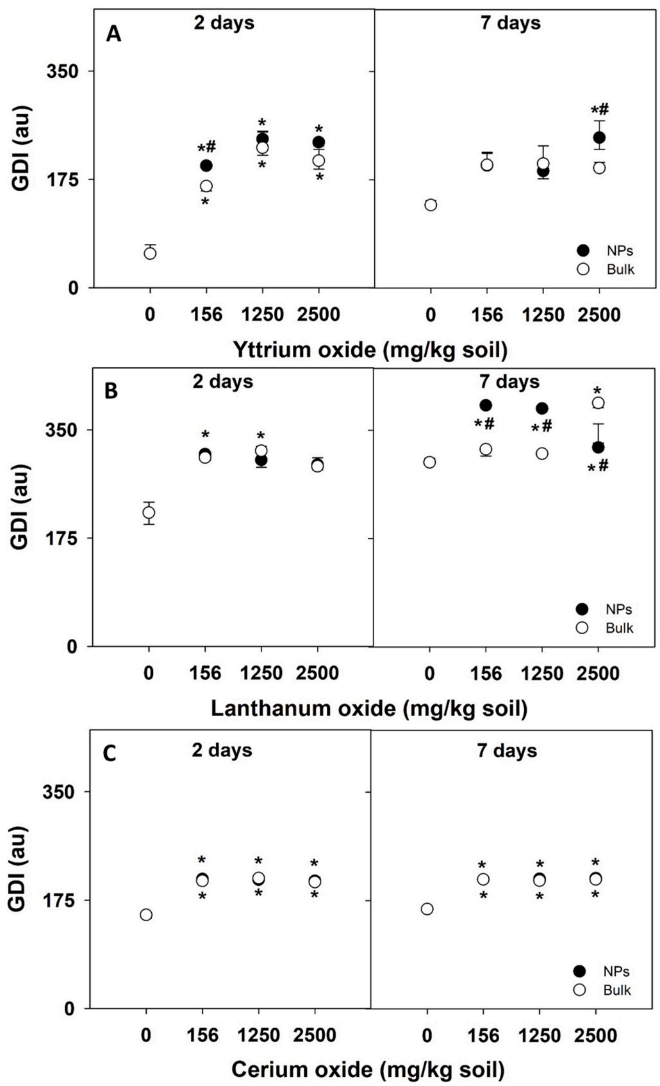

Fig. 5. The DNA damage measured as genetic damage indicator (GDI, in arbitrary units (au)) of *Enchytraeus crypticus* after 2 and 7 days of exposure to yttrium oxide (Y_2O_3) (**A**), lanthanum oxide (La_2O_3) (**B**), and cerium oxide (CeO_2) (**C**) nanoparticles (NPs) and bulk in LUFA 2.2 soil. Data are expressed as average value ± standard error. * Significant differences with the corresponding control group - 0 mg/kg ($p < 0.05$). # Significant differences between the two forms (NPs versus bulk) within the same concentration ($p < 0.05$).

Y_2O_3, both forms induced DNA damage at all tested concentrations, after 2 d of exposure. However, after 7 d the organisms seemed to recover with a significant effect only detected at 2500 mg/kg of Y_2O_3 NPs ($p < 0.05$; Fig. 5A); 3) For La_2O_3 NPs, a different response pattern was observed, with less damage at 2 d of exposure (only at 156 mg/kg) and DNA damage at all tested concentrations after 7 d. While for La_2O_3 bulk, few effects were detected (1250 mg/kg at 2 d and 2500 mg/kg at 7 d) ($p < 0.05$; Fig. 5B). A study with crickets (*Acheta domesticus)* reported a slower elimination or higher retention of La from NPs exposure compared to its bulk form after 7 d of depuration (De la Torre Roche et al., 2015). This may explain why DNA damage persisted across all concentrations for NPs exposure, while for La_2O_3 bulk, DNA damage was observed only at the highest concentration after 7 d. Y_2O_3 NPs and microparticles 28 d oral exposure in Wistar rats (30, 120 and 480 mg/kg body weight per day) also induced genotoxicity detected by significant differences on the comet and micronucleus assays (Panyala et al., 2019). Studies associate the primary molecular mechanism of REEs exposure in organisms with oxidative stress due to increased reactive oxygen species (ROS) formation (Sysolyatina and Olkova, 2023). Excess ROS production induces lipid peroxidation and damage to macromolecules (such as DNA), leading to cell death (Kotelnikova et al., 2021). Sager et al. (2024) reported that REEs affect redox mechanisms like ROS formation, lipid peroxidation and increase activity of enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferases (Sager and Wiche, 2024). Additionally, lipid peroxidation was observed on *E. fetida* after exposure to La and Ce, indicating that these REEs can cause oxidative damage to earthworms (Tang et al., 2022). Based on this knowledge, it is possible that the DNA damage observed in *E. crypticus* in the present study is triggered by REEs-induced ROS production, leading to DNA oxidation and depletion of antioxidant defence. However, since different REEs materials induced DNA damage, at varying levels, the production of ROS was not uniform across the three REEs oxides, suggesting that the type of REEs may be responsible for this variation. Moreover, for the REEs in NPs form, a direct effect on DNA, through direct binding to DNA or DNA repair enzymes, may promote DNA instability (Abegoda-Liyanage and Pathiratne, 2023). These different mechanistic hypotheses may explain the varying response patterns observed for different forms (nano versus bulk) of the same material. It is noted that, with exception of CeO_2 material, REEs NPs are more toxic concerning DNA damage than their bulk counterparts. For both, Y_2O_3 NPs and La_2O_3 NPs, higher impact on DNA is noted after 7 d of exposure when comparing to the bulk forms. This may be due to the direct interaction of NPs with DNA after entrance into the nucleus, in combination with indirect mechanisms such as oxidative stress induced by generation of ROS, or as a result of inflammatory responses, as suggested previously for TiO_2 NPs (Abegoda-Liyanage and Pathiratne, 2023). Further molecular studies are needed to unravel the mechanism of action regarding REEs DNA damage.

It appears that the impact of REEs-based materials on the evaluated endpoints of *E. crypticus* depends on the concentration, exposure time and type/form of the material, as the tested REEs materials displayed different modes of action. The differences in toxicity of individual REEs may be due to variations in paramagnetic properties (slight differences in charge density) influencing physiological processes and bioavailability based on their mobility on soil or transport properties within the organism (Sager and Wiche, 2024). Among all the REEs types and forms tested, La_2O_3 NPs were the least toxic to *E. crypticus*, resulting only in DNA damage, while Y_2O_3 NPs were the most toxic, decreasing reproduction, inducing avoidance behavior and causing DNA damage. The distinct toxicity between forms (nano versus bulk) of the same material can also be attributed to the variations in bioavailability due to different uptake processes or basic physiological processes of the organism, such as detoxification mechanisms (Huang et al., 2020). Earthworms, polychaetas like *E. crypticus*, are reported to respire through skin and ingest soil, so it is possible that REEs materials may enter through skin and/or gastrointestinal tract (Chouhan and Tripathi, 2020). Studying the uptake of REEs NPs and bulk form on *E. crypticus* could help explaining the different toxicity mechanisms reported in the present study for different forms of the same material. He et al. 2019, did an important work comparing the uptake of ZnO nanoparticles and ionic Zn in *E. crypticus* and conclude that ionic zinc had higher uptake and elimination rates than nanoparticulate zinc (He et al., 2019). A similar research could be developed in the future for REEs nanoparticles and bulk forms.

Although NPs are generally described as more toxic than their bulk counterparts, this conclusion was not consistently observed for the three REEs materials in the present study. For La_2O_3, the bulk form affected more endpoints than the NPs form, inducing avoidance and DNA damage. De la Torre Roche et al. (2015) reported La_2O_3 NPs accumulation in lettuce (*Lactuca sativa* grown in 350 or 1200 g of La_2O_3 bulk/NPs amended soil) was equivalent to or significantly less than that of La_2O_3 bulk, and that La_2O_3 NPs aggregated to sizes approximating their bulk counterparts (De la Torre Roche et al., 2015). This may help to explain our results, as aggregation/agglomeration was also observed in the NPs characterization process. However, further investigation is needed to confirm this hypothesis. For Y_2O_3, the NPs form was evidently more toxic than the bulk form, affecting all the tested endpoints. A previous study documented the critical role of particle size in induced toxicity: smaller sized Ag NPs (20 nm) had greater ability to cross cell membranes of *E. fetida* via membrane proteins (porins), resulting in more effects on metabolic pathways and cellular damage compared to larger Ag NPs (80 nm) at the same concentration (500 mg/kg) (Hu et al., 2012). Addel et al. (2021) suggested that REEs NPs induce more histopathological changes and increase ROS production, leading to an imbalance in the antioxidant enzyme system, which may be the reason for the higher toxicity of REEs NPs versus bulk (Adeel et al., 2021a). For CeO_2, very similar toxicity was detected, with both forms affecting reproduction and DNA integrity of *E. crypticus*. This similarity may be due to CeO_2 NPs toxicity arising from the dissolution and release of ions similarly to the bulk form and not from the particle itself, resulting in a similar pattern of toxicity to its bulk counterpart. However, further research at the biochemical and molecular levels is needed to a more comprehensive assessment of the diverse physiological aspects influencing CeO_2 NPs and bulk toxicity patterns.

Recently, REEs have been classified as contaminants of environmental concern (CEC) because they are not regulated or routinely monitored in the environment and their mechanisms of environmental and human toxicity are poorly understood (Egler et al., 2022; Gwenzi et al., 2018). Thus, information on the toxic effects of REEs on terrestrial organisms is crucial to the risk assessment (Egler et al., 2022). Considering the maximum concentrations of REEs reported in agricultural soils (72 mg/kg for Y, 184 mg/kg for La and 454 mg/kg for Ce (Sager and Wiche, 2024)) and the effect concentrations in the present study (156 mg/kg of all tested REEs types, except La_2O_3 bulk, induced DNA damage), it seems that REEs NPs and bulk forms can pose some risk to terrestrial organisms. As enchytraeids are ecologically relevant soil-dwelling annelids, that play an important role in organic matter decomposition and soil bioturbation, (Castro-Ferreira et al., 2012) this level of REEs contamination in agricultural soils may negatively impact these ecosystems. In mining zones the risk is greater since higher concentrations of REEs have been detected (6900 mg/kg of La and 12,000 mg/kg of Ce (Tang et al., 2022)). This study provides a valuable foundation for exploring the effects of REEs NPs and bulk on *E. crypticus*, aiming to narrow the critical knowledge gap in REEs toxicity in terrestrial ecosystems.

4. Conclusions

The toxicity of the three tested REEs-based oxides (La_2O_3, Y_2O_3 and CeO_2) varied with the form (bulk vs nano) significantly influencing the resulting toxicity towards *E. crypticus*. No effects on survival of *E. crypticus* were detected for any REEs materials in either form, even at concentrations as high as 2500 mg/kg of REEs. DNA integrity was the most sensitive endpoint, with DNA damage being detected, to a degree, for all tested REEs types (La_2O_3, Y_2O_3 and CeO_2) and forms (NPs versus bulk). *E. crypticus* decreased reproduction and avoidance behavior was also found after exposure to some REEs types (Y_2O_3 NPs, Y_2O_3 bulk, CeO_2 NPs, CeO_2 bulk and La_2O_3 bulk). This work highlights the importance of a multi-endpoint approach, at individual and molecular levels, for REEs risk assessment, since it facilitated the toxicity comparison between the different REEs types and forms towards *E. crypticus*. Notably, when comparing different forms of the same material (bulk versus nano) different results were obtained depending on the type of REEs: for La_2O_3, the bulk form affected more endpoints than the NPs; in contrast, for Y_2O_3, the NPs form was evidently more toxic than the bulk, affecting all tested endpoints; and, for CeO_2, both forms exhibited similar toxicity. In conclusion, REEs-based oxides toxicity was influenced by the REEs type/concentration, time of exposure (for DNA damage, 2 versus 7 d) and the form of the material (nano versus bulk), suggesting different modes of action. Based on the effect in the *E. crypticus* DNA integrity observed at a low concentration (156 mg/kg) and considering the REEs concentrations on agricultural soils, it appears that both NPs and bulk forms of REEs can pose risks to these terrestrial ecosystems. This study reinforces the importance of REEs risk assessment and elucidates the varied responses of *E. crypticus* to different REEs oxides (NPs and bulk forms), thereby increasing the knowledge about REEs toxicity in terrestrial ecosystems.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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