



Effects of cobalt oxide nanomaterial on plants and soil invertebrates at different levels of biological organization

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Received: 26 August 2018 / Accepted: 17 February 2019 / Published online: 4 March 2019
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Abstract

Purpose Metallic nanomaterials (MNM) like cobalt oxide (*nano*-Co₃O₄) are currently attracting enormous interest owing to their unique size and shape-dependent properties and potential applications in various sectors. The aims of this study were to assess the toxicity of *nano*-Co₃O₄ and to propose a risk limit through the estimation of a Predicted No Effect Concentration (PNEC) for this MNM to soil biota.

Materials and methods For this purpose, a battery of sub-lethal ecotoxicological tests was performed to assess the influence of this MNM on four plant species (endpoints: germination and growth) and two invertebrate species (endpoints: avoidance and reproduction) following standard protocols. Further, biochemical endpoints (acetylcholinesterase [AChE], catalase [CAT], glutathione-S-transferase [GST] activity, and lipid peroxidation [LPO]) were also assessed in *Eisenia andrei*, one of the invertebrate species tested, in order to contribute for refining the PNEC value.

Results and discussion The recorded data showed a significant inhibition in the germination of *L. lycopersicum* and in the growth of *Z. mays*, even at the lowest concentration tested (269.3 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄). Concerning the soil invertebrates, the results showed only significant avoidance ($p < 0.05$) by *E. andrei* in the soil contaminated with the highest concentration tested (1000 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄), while no significant ecotoxicological effect on reproductive outputs of both species was recorded. However, the data reported for AChE, CAT, GST, and LPO showed significant effects at the range of concentrations tested in *E. andrei*. Thus, we recorded, the occurrence of oxidative stress and the enhancement of lipid peroxidation, on this invertebrate species.

Conclusions The data obtained in this study supports the proposal of a PNEC value of 9.1 mg kg⁻¹ soil_{dw} for *nano*-Co₃O₄ in soil. The integration of data from biochemical endpoints allowed the refinement of the PNEC value and to obtain a more protective threshold.

Keywords Avoidance · *Eisenia andrei* · *Folsomia candida* · Oxidative stress biomarkers · Plant growth · Reproduction

Responsible editor: Dong-Mei Zhou

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1 Introduction

Metallic nanomaterials (MNM) are widely manufactured and used in different fields of application (Tjong and Chen 2004; Singh and Nalwa 2011; Zhou et al. 2011; Wu et al. 2012b; Trujillo-Reyes et al. 2014; Wahid et al. 2014; Laurent et al. 2018; Lu and Astruc 2018). Hence, the progressive production and application of MNM will conduct to relevant releases into the environment (Lowry et al. 2012; Nowack et al. 2012), with consequences that remain unknown. Accordingly, the emergence of these contaminants in the natural environment may occur at any stage of its life cycle, and the possible mechanisms of release, as well as the environmental compartments affected (water, air, and soil), depend on nanotechnology products and applications, uses, and properties, as described by Nowack et al. (2015) and on their interaction with biotic and abiotic

environment identified as nano–bio–eco interactions by He et al. (2018). The interaction with the biotic components of ecosystems is to be expected (Lowry et al. 2012; Nowack et al. 2012; Maurer-Jones et al. 2013), what makes of utmost importance the investigation on the persistence, fate, hazards, and risks of nanomaterials (NM) in general, and of MNM in particular (Ray et al. 2009; Lachance et al. 2013). Most of the studies on MNM toxicity are focused on aquatic ecosystems. Considerable work has been performed with aquatic species and several data and reviews are already published (e.g., Griffitt et al. 2008; Kahru et al. 2008; Ziccardi et al. 2008; Wang and Wang 2014; Chen et al. 2015). However, there is still a gap of knowledge concerning the impacts and the possible risk of NM to terrestrial ecosystem (Gardea-Torresdey et al. 2014; Pulicharla et al. 2015; Gomes et al. 2017). Only a limited number of MNM were studied for their soil ecotoxicity and main attention was given on the phytotoxicity of MNM such as CeO₂, TiO₂, ZnO, CuO, TiSiO₄, Ag, and Au (Dietz and Herth 2011; Wu et al. 2012a; Gardea-Torresdey et al. 2014; Bouguerra et al. 2016).

Among MNM, the magnetic ones, essentially iron, nickel, and cobalt, received a great attention in medicine, biotechnology, pharmaceutical industry and cancer treatment (Rutnakornpituk et al. 2002; Pankhurst et al. 2003; Ito et al. 2005; Wang et al. 2005; Pandey et al. 2015), medical imaging (Kim et al. 2001; Ito et al. 2005; Laconte et al. 2005; Parkes et al. 2008), and on groundwater and wastewater treatment approaches (Cundy et al. 2008; Calcagnile et al. 2012). Cobalt NM in particular have been widely studied for their application in electrodes for lithium batteries, fabrication of gas sensors, solar energy absorption, and as effective catalysts in environmental cleanliness and chemical engineering (Wang et al. 2002; Liu et al. 2005; Man et al. 2011; Jamal et al. 2012; Karami 2013). Furthermore, it was proved that assembling nano-sized cobalt with other NM such as gold and carbon nanotubes can improve the performance of highly sensitive sensors and provide an enhanced quality to electronic devices (Ando et al. 1997; Fu et al. 2005; Yang et al. 2006).

Despite the toxicological information for human and mouse cells (Colognato et al. 2008; Kwon et al. 2009; Papis et al. 2009; Ponti et al. 2009; Jiang et al. 2012), to date studies investigating the toxicity of Co NM to biota are restricted. Even for aquatic ecosystem, when compared to other NM, there are few studies (Chen et al. 2015). For terrestrial ecosystems, the phytotoxic effect of cobalt oxide NM (*nano*-Co₃O₄, 10–30 nm) was investigated by assessing the impact on germination and root elongation of lettuce, radish, and cucumber seeds in Petri dish assays (Wu et al. 2012a). For a wide range of concentrations varying between 1 and 5 g L⁻¹, these authors observed no significant effects in all endpoints measured (Wu et al. 2012a). Ghodake et al. (2011) demonstrated that *nano*-Co₃O₄ (50 nm) tested at concentrations up to 20 mg L⁻¹ caused a significant reduction on root length of *Allium cepa* after 1, 2, and 3 days of exposure in glass vessels. More recently, the cytogenetic effects of pure cobalt and cobalt

oxide NM colloidal suspensions (5–80 nm) on *Sesbania cannabina* species were assessed (Pandey et al. 2015; Srivastava 2015). Authors observed various mitotic perturbations and anomalies in meristematic cells after 3 h of exposure of rooted seeds to both cobalt NM (Pandey et al. 2015; Srivastava 2015). Regarding soil invertebrates, the bioavailability of cobalt core-shell nanoparticles (3.9 nm) to the earthworm *Eisenia fetida* was investigated (Coutris et al. 2012). Authors showed a significant accumulation of cobalt, released from 1.25 µg of cobalt nanoparticles (NP) per gram of supplied food, in blood vessels, pseudo-hearts, and digestive organs of worms after 28 days of exposure in artificial OECD soil (Coutris et al. 2012). Recently, Antisari et al. (2014) also observed a significant accumulation of cobalt in the tissues of the earthworm *Lumbricus rubellus* obtained from food contaminated with (20–60 nm) cobalt NP (2.5 mg of Co-NP in 10 g of food) and supplied at the surface of the artificial OECD soil (once a week for 4 weeks). Besides the bioaccumulation, the study also showed reduction in the degree of unsaturated fatty acids in the tissues of exposed worms (Antisari et al. 2014). In the same study, the authors used the soils contaminated with the supplied food (20 µg of Co-NP g⁻¹ soil) for assessing the effects on soil microbial biomass after extra incubation for more 28 days and they recorded significant reduction on this endpoint measured by the fumigation-extraction method. These results reflected the possible impact on soil microbial communities of cobalt NM (Antisari et al. 2014).

According to published data, concerning cobalt NM toxicity to soil biota, the effects are still poorly investigated, and insufficient for risk assessment purposes. Thus, the main aims of this work were (i) to assess the phytotoxicity of cobalt oxide NM on seed's germination and seedling's growth using four plant species (two dicotyledonous and two monocotyledonous); (ii) to determine the effects of cobalt oxide NM on the behavior and the reproductive output of soil earthworms *E. andrei* and soil arthropods *Folsomia candida*; (iii) to evaluate the effects on neurologic and oxidative stress biomarkers (acetylcholinesterase [AChE], catalase [CAT], glutathione-S-transferase [GST] activity, and lipid peroxidation [LPO]) of the exposed earthworms *E. andrei*; and (iv) to use the ecotoxicological data obtained to propose a risk limit for this MNM, integrating data from different species and endpoints at different levels of biological organization.

2 Materials and methods

2.1 Test substrate, test substance, and experimental design

The standard artificial OECD soil (OECD 1984) composed by a mixture of quartz sand, kaolin, and sphagnum peat (5% organic matter and pH 6.0 ± 0.5) was used in this study. For all the tests, the OECD soil was spiked with suspensions of

cobalt oxide (II, III) NM (herein mentioned as *nano*-Co₃O₄) (particle size < 50 nm, 99.5% of purity, supplied by Sigma-Aldrich™ as powder) prepared with Milli-Q water in order to obtain the range of concentrations tested in the soil (0, 269.3, 350.1, 455.2, 591.7, 769.2, and 1000 mg *nano*-Co₃O₄ kg⁻¹ soil_{dw}). The maximum concentration tested was 1000 mg *nano*-Co₃O₄ kg⁻¹ soil_{dw}. This concentration was selected because there is little ecotoxicological information available for this NM, and this is the maximum concentration of a substance that should be tested according to standard protocols, when no ecotoxicological data exists. In the present study, we assumed that magnetic stirring of suspensions was a more environmentally realistic option to disperse NMs, as they are expected to be aggregated and in polydisperse in the environment. For this purpose, the *nano*-Co₃O₄ powder was suspended in Milli-Q water and magnetic stirred for 30 min in the appropriate mass (to obtain the range of concentrations above described) in the volume of water required to adjust the water holding capacity (WHC) of the test soil to 45% ± 5% of its maximum value, and immediately added to the soil.

2.2 Characterization of *nano*-Co₃O₄ powder and aqueous suspension and chemical analysis of spiked soil

In order to analyze how the morphological properties and the stability of *nano*-Co₃O₄ varied when suspended in water, in a concentration gradient, a range of concentrations varying between 1.0, 10.0, 100.0, and 1000.0 mg L⁻¹ of *nano*-Co₃O₄ was prepared and stirred during 30 min in Milli-Q water before analysis. The suspensions were then characterized for the hydrodynamic size (*Z* average) and number distribution, and for surface charge in suspension (zeta potential ζ). The characterization was done by dynamic light scattering (DLS) and by electrophoretic light scattering (ELS), with a Zeta Sizer Nano ZS, Zen 3500, with a 532-nm laser (Malvern ZetaSizer 2013). All the measurements were made at pH (pH 6.0 ± 0.5) and 20 °C, thus keeping the same conditions defined for soil incubation during the ecotoxicological tests. The zeta potential (ζ) parameter was calculated by the Zetasizer Nano Software, version 6.01, using the Smoluchowsky equation (Malvern ZetaSizer 2013) and was reported in this study. The magnitude of the zeta potential gives an indication of the stability of the dispersed NM in the suspension, which is greater for values equal or above |± 30| mV (Malvern ZetaSizer 2013). All the measurements were made at backward scattering angle (173°). The distribution of particle sizes was determined by measuring the polydispersity index (Pdl). Further, considering that when Pdl is greater than 0.3, the *Z*-average diameter values should be used, the number distribution mean was reported, whenever the multimodal mean number distribution data was consistent run to run, and when > 90% of the “% by number” was in peak 1, (NanoComposix 2015).

In addition, transmission electron microscopy (TEM) was used to obtain images at a higher resolution of this nanomaterial, in order to better characterize the form and size of nanoparticles. For this purpose, a Hitachi H8100 with a LaB6 filament operated at an acceleration voltage of 200 kV was used. The images were acquired by an Olympus KeenView digital Camera with the iTEM software. The sample in powder was suspended in ethanol and a drop was allowed to dry on a Cu grid with a formvar film, before observation.

The quantification of cobalt nanomaterial (effective concentrations) in soil was assessed in soil samples collected from three random selected replicates at the end of one short-term exposure (avoidance test with earthworm) and two chronic, i.e., long-term exposure tests (plant growth and earthworm reproduction tests). For each replicate, 0.2 g of soil was digested by using *aqua regia* solution (HCl:HNO₃) at 95 °C, for 60 min, and Co element analyzed with a Thermo X Series ICP-MS equipment. A detection limit of 0.1 mg kg⁻¹ for the cobalt element was obtained. Average and standard deviation values of three measurements made for all analyzed replicates are presented. The gathered values are reported as mg kg⁻¹ soil_{dw} of cobalt. Accuracy of the analysis was checked through calculation of recovery percentages of soil samples spiked with a suspension of *nano*-Co₃O₄, which showed to be between 93.0 and 100.7%.

2.3 Ecotoxicological assessment

2.3.1 Tests with higher plants: germination and growth

In order to assess the phytotoxic effects of *nano*-Co₃O₄ on terrestrial plants, two monocotyledonous plant species [*Zea mays* (corn) and *Avena sativa* (oat)], and two dicotyledonous plant species [*Lycopersicon lycopersicum* (tomato) and *Brassica oleracea* (cabbage)] were used for seed germination and seedling growth tests. Tests with plants were performed according to the ISO 11269-2 standard (ISO 2012a). For this purpose, 200 g_{dw} of soil spiked with the concentrations described above (Section 2.1) were added to each replicate, and then the soil was saturated with additional water. The soil was placed in plastic pots (11.7 cm diameter, 6.2 cm height) and 20 seeds were added to each pot and slightly covered with the contaminated soil. The soil moisture was assured and controlled during all the period of experiment to guarantee the necessary conditions for seed germination and growth. Four replicates were prepared for both the control replicates, with uncontaminated OECD soil and, for each tested concentration of the NM. At the beginning of the test, a solution of nutrients (Substral® - Fertilizer NPK: 6-3-6; nitrogen (N): 6%; phosphate (P₂O₅): 3%; potassium (K₂O): 6%; iron (Fe): 0.03%; trace elements: Cu, Mn, Mo, and Zn) 10% diluted according to the recommendations of the vendor were added to all pots in a similar volume. Pots were maintained at the constant conditions of light and temperature as defined by the standard

protocol. The endpoints seed germination, fresh and dry biomass were reported for each species after 14 days of exposure, starting after test validation.

2.3.2 Terrestrial invertebrates: culture conditions, avoidance, and reproduction tests

The terrestrial organisms selected for this work were the standard soil earthworm *Eisenia andrei* (Oligochaeta: Lumbricidae) and the soil arthropod *Folsomia candida* (Collembola: Isotomidae). The test organisms were selected from laboratorial cultures maintained at controlled conditions (temperature: 20 ± 2 °C; pH: 6.0–7.0; photoperiod: 16hL: 8hD). The earthworms (*E. andrei*) are cultured in plastic boxes (10 to 50 L) containing a substrate composed by peat, dry and defaunated horse manure. The organisms are fed every 2 weeks with six table spoons of oatmeal previously hydrated with deionized water and cooked for 5 min. Test organisms were collected from synchronized cultures with a homogeneous age structure (between 2 months and 1 year old), from those with a fully developed clitellum and an individual fresh weight between 300 and 600 mg.

The arthropods (*F. candida*) are maintained in plastic containers with culture medium composed by moistened plaster of Paris mixed with activated charcoal in a 8:1 (w:w) proportion. The cultures are fed with 5 mg of granulated dry yeast, twice a week to avoid spoilage by fungi. Before starting the experiment, cultures are synchronized to obtain juveniles 9–12 days old.

The avoidance tests with *E. andrei* were carried out in rectangular (1370.7 cm³) plastic boxes (four replicates per test concentration) according to the ISO 17512-1 standard (ISO 2008). The boxes were divided into two equal compartments with a plastic divider. To each compartment, 200 g of dry soil was added. In one side of the boxes, the soil was spiked with a suspension of the NM to obtain the above described concentrations of *nano*-Co₃O₄, while in the other side, the same mass of soil was added only moistened with deionized water. The plastic card was removed and 10 adult earthworms weighing between 250 and 600 mg were placed on the line between both soils, in each test container, giving them the ability to freely move between the both sides of the boxes. Thereafter, the lids of the boxes were perforated for air circulation. Dual-control test containers (five replicates) were prepared with uncontaminated soil moistened with deionized water in both sides. After 48 h of exposure, the plastic card was placed again in the middle line and the number of earthworms in each side of the test containers was counted, and the avoidance percentage was calculated, through the following formula:

$$\text{Avoidance (\%)} = ((C-T)/N) \times 100$$

- C number of organisms observed in the control soil (spiked only with distilled water);
- T number of organisms observed in the test soil per concentration (spiked with *nano*-Co₃O₄ suspension);
- N total number of organisms per replicate.

A positive (+) response indicated avoidance and a negative (−) response indicated a non-response or a preference by the test soil.

For the arthropods (*F. candida*), the test run in plastic circular boxes also divided into two equal compartments by a plastic card, following similar experimental design for the earthworms and according to ISO 17512-2 standard (ISO 2011). The amount of soil in each section of the test container was 30 g soil_{dw} moistened with deionized water. Five replicates were prepared for each *nano*-Co₃O₄ concentration and dual control replicates (in a total of 5) were also prepared. For this test, 20 organisms were placed in the line between both soils. Thereafter, boxes were closed with a perforated lid. At the end of the exposure period (48 h), carried out at the same conditions described for culture maintenance, the plastic card was placed again in the line between both soils and tap water was added to both sides simultaneously, to visualize floating organisms in the surface of water. Some dark ink drops were added to the water to facilitate identification and counting of organisms. The missed animals were considered dead and the avoidance percentage was calculated by using the formula described above.

The reproduction tests with *E. andrei* and *F. candida* were carried out according to ISO 11268-2 (ISO 2012b) and ISO 11267 (ISO 2014) standard protocols, respectively. For the test with *E. andrei*, exposure was achieved in plastic test containers (11.7 cm diameter and 13 cm height). All replicates contained 500 g of soil (spiked with the suspensions of the NM to attain the concentrations described above) and 10 worms with a fully developed clitellum and individual fresh weight between 250 and 600 mg were added to each replicate. Four replicates per concentration and five replicates for the control were prepared for the reproduction test with *E. andrei*. Adult earthworms were removed from the test containers after 28 days of exposure leaving produced cocoons in the soil until 56 days of exposure have been completed. During the test, organisms were fed once a week, with 5 g per box of defaunated horse manure, and the soil moisture content was weekly monitored and adjusted whenever necessary. At the end of the test, juveniles from each test container were counted.

For the tests with arthropods, 10 synchronized organisms of 10–12 days old were added to each replicate with 30 g soil_{dw} (four replicates per concentration and per control). In addition, a fifth replicate without organisms was prepared for each treatment (control and spiked soils) for soil moisture and pH control at the end of the experiment. The reproduction tests

with *F. candida* took 28 days to be completed. During the exposure period, the collembolans were fed weekly with granulated dry yeast added to the soil surface. The test containers were maintained at 20 ± 2 °C and a photoperiod of 16h^L: 8h^D. At the end of the test, each test container was filled with tap water. The mixture was stirred carefully to let all the animals float to the surface and the juveniles were counted after the addition of few dark ink drops. For this purpose, photos for the dark surface of all containers were taken using a digital camera and the digitalized pictures were used for counting the floating organisms with the support of the ImageJ software (<http://imagej.nih.gov/ij/>).

The validity criteria of each standard test above described were checked at the end of the corresponding exposure periods.

2.4 Biomarker's assays

Adults of *E. andrei* removed from the test containers of the reproduction test, after 28 days of exposure were used for the assessment of the extent of oxidative stress induced by *nano*-Co₃O₄. For this purpose, five selected worms from each container were immediately sacrificed by snap-freezing in liquid nitrogen, and then stored at -80 °C until further analysis. For the biomarker assays, stored worms were then thawed in ice and were homogenized in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% Triton X-100) with ultrasounds (T 10 basic ULTRA-TURRAX®) at a temperature of 4 °C. After homogenization, the samples were centrifuged at 4000 rpm for 5 min using a refrigerated centrifuge (4 °C). The supernatants were divided into several aliquots of equal volume (500 µl) in Eppendorf tubes and stored at -80 °C until enzymatic analysis. Protein content, lipid peroxidation (LPO) and acetylcholinesterase (AChE) and glutathione-S-transferase (GST) activity were measured using a spectrophotometer with a microplate reader (Thermo Scientific, MULTISKAN GO). Catalase activity (CAT) determinations were performed using a spectrophotometer (VWR UV-3100PC). Triplicate measurements were performed for a total of four replicates per each tested concentration.

The total amount of protein of each replicate was determined spectrophotometrically (at 595 nm) according to Bradford (1976), using bovine gamma globulin as standard, in order to express enzyme activities per milligram of protein.

The AChE activity was determined following the photometric methodology described by Ellman et al. (1961) with some modifications. The Ellman procedure is based on the hydrolysis of acetylthiocholine by AChE present in samples. During the enzymatic reaction thiocholine is released and reacts with 5,5'-dithio-bis-nitrobenzoic acid (DTNB) and 3-carboxy-4-nitrothiolate anion (TNB anion) revealing yellow coloration. This anion has a strong absorption at 412 nm. Results were expressed as millimole of the reaction product per minute per milligram of protein.

CAT activity was measured spectrophotometrically at 240 nm, following the protocols adapted by Aebi (1984). The technical procedure was based on the quantification of the decrease in the substrate (hydrogen peroxide) concentration, which is proportional to the enzymatic activity in the reaction medium. Results were expressed as millimole of the consumed substrate per minute per milligram of protein (Aebi 1984).

The GST activity was evaluated according to the protocol described by Habig et al. (1974). Basically, the isoenzymes GST are responsible to catalyze the conjugation reaction between 2,4-dinitrochlorobenzene (CDNB) and reduced glutathione (GSH) allowing the formation of thioether. This compound can be quantified by the increase in absorbance at 340 nm. GST activity was expressed in terms of micromole of the reaction product per minute per milligram of protein (Habig et al. 1974).

LPO was evaluated following the technical procedure described by Buege and Aust (1978) based on the quantification of thiobarbituric acid reactive substances (TBARS). The TBARS concentration was measured spectrophotometrically at 535 nm after the reaction of malondialdehyde (MDA: by-products of the peroxidation of membrane lipids) with 2-thiobarbituric acid (TBA). Data expressed in picomole of MDA equivalents per mg of protein (Buege and Aust 1978).

2.5 Data analysis

The seeds germination and the growth of seedlings (fresh and dry mass produced), the reproduction of soil invertebrates, and the biochemical endpoints data were analyzed by one-way ANOVAs followed by Dunnett tests to assess the effect of the *nano*-Co₃O₄ concentrations on the responses of test organisms, using the software SPSS (version 18.0). NOEC (non-observed effect concentration) and LOEC (lowest observed effect concentration) values were obtained from ANOVA analysis. Prior to ANOVA analysis, the assumptions of normality and homoscedasticity were checked through Kolmogorov–Smirnov and Levene's tests (for $p \leq 0.05$).

With the same purpose, but now for the avoidance behavior of earthworms and arthropods, the Fisher's exact test was performed using GraphPad software (<http://graphpad.com/quickcalcs/contingency1.cfm>). A one-tailed test was performed for each nanomaterial exposure concentration, to test the null hypothesis of no avoidance of the contaminated soil by the test organisms. For the analysis of the dual controls, a two-tailed test was used, assuming an equal distribution of the individuals on both sides of the test chamber. For the growth inhibition tests with plants, EC₂₀ values and the corresponding 95% confidence limits were calculated using the nonlinear least squares regression model procedure supplied by the Statistica software, (version 10). All graphs were made with the software OriginPro 8.5.1.

3 Results

3.1 Characterization of *nano-Co₃O₄* in aqueous suspension and metal release in spiked soils

TEM images (Fig. 1) demonstrated that *nano-Co₃O₄* were able to form aggregates with spherical shape and size ranging from 100 to 500 nm. However, it is highly possible that the aggregation observed on TEM images resulted from the procedure described for sample preparation. The results gathered from the DLS and ELS analysis for *nano-Co₃O₄* aqueous suspensions at the range of concentrations (1–1000 mg L⁻¹) were presented in Table 1. The main aim of DLS analysis was to perceive the behavior of the particles in suspension since the NM was mixed in water before spiking the soil. The Z-average hydrodynamic diameter of *nano-Co₃O₄* suspended in Milli-Q water was very high for all tested concentrations (Table 1). In addition, the PDI value (>0.3) reported for all suspensions suggested to consider the average diameter by number distribution due to the important level of polydispersity and the presence of large aggregates in suspensions. According to the particle size distribution (number in percentage) more than 90% of *nano-Co₃O₄* formed aggregates sizes above 500 nm in the highest concentrations tested (100 and 1000 mg L⁻¹). The data demonstrated that *nano-Co₃O₄* was able to form aggregates 10 and 16 times bigger than the manufactured size (primary size 50 nm) at 100 and 1000 mg L⁻¹, respectively, when suspended in water. Further, as evidenced by the negative and low zeta potential values (close to -30 mV) recorded for the tested suspensions, such aggregates were stable only for concentrations between 1 and 100 mg L⁻¹ of *nano-Co₃O₄* (Table 1). In opposition, at the highest concentration (1000 mg L⁻¹) of *nano-Co₃O₄*, the

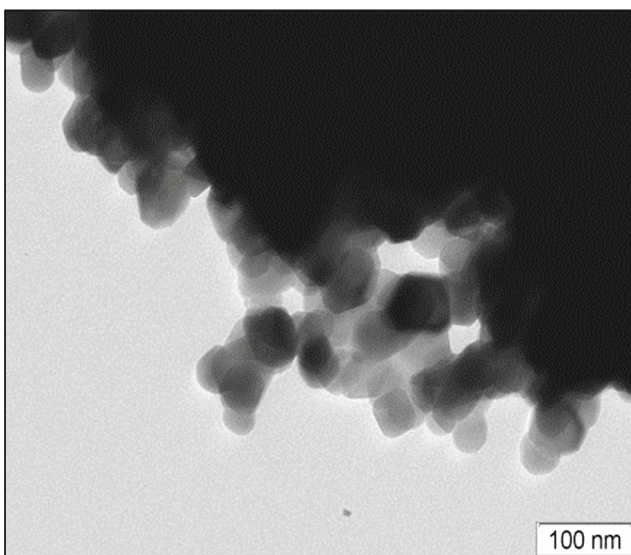


Fig. 1 Transmission electron microscopy (TEM) image of *nano-Co₃O₄*. Scale bar corresponds to 100 nm

aggregates were not stable regarding the high zeta potential values registered. Thus, the registered data showed that as much as concentration of *nano-Co₃O₄* in water suspension increases, bigger aggregates could be formed, and the aggregation instability increases.

In Table 2, the effective concentrations of cobalt in spiked soils corresponding to each nominal tested concentrations of cobalt oxide NM were reported. The measurements of total metal concentration in soil were carried out for all plant's tests after 15 days of exposure, and for a short and a long-term exposure (48 h and 56 days) respectively, for the earthworm's tests.

The data obtained for spiked soils at the end of the tests showed no remarkable differences in the concentrations of cobalt for the same nominal concentrations in soil for the earthworm's test and the real concentration of cobalt ranged from ≈ 190 to ≈ 730 mg kg⁻¹ soil_{dw} (Table 2). Similar values were obtained in soil samples collected from the plant tests (Table 2).

3.2 Effect of cobalt oxide nanomaterial on plant germination and growth

All the tests with plant species fulfilled the validity criteria described by the standard guidelines (ISO 2012a). The data gathered in this study showed significant adverse effects on the fresh and the dry weight of *Z. mays* (germination: $F = 0.82$, $d.f_1 = 28$, $d.f_2 = 34$, $p = 0.564$; fresh weight: $F = 3.132$, $d.f_1 = 28$, $d.f_2 = 34$, $p = 0.018$; dry weight: $F = 4.023$, $d.f_1 = 28$, $d.f_2 = 34$, $p = 0.005$). In opposition, no significant effects were recorded for these endpoints in the other monocotyledonous species *A. sativa* (germination: $F = 1.792$, $d.f_1 = 21$, $d.f_2 = 27$, $p = 0.15$; fresh weight: $F = 2.558$, $d.f_1 = 21$, $d.f_2 = 27$, $p = 0.051$; dry weight: $F = 2.089$, $d.f_1 = 21$, $d.f_2 = 27$, $p = 0.098$). Regarding the dicotyledonous species, *L. lycopersicum* (germination: $F = 2.601$, $d.f_1 = 22$, $d.f_2 = 28$, $p = 0.047$; fresh weight: $F = 2.964$, $d.f_1 = 28$, $d.f_2 = 34$, $p = 0.023$; dry weight: $F = 0.818$, $d.f_1 = 28$, $d.f_2 = 34$, $p = 0.565$) displayed significant inhibition in the percentage of germinated seeds at the highest concentration tested (1000 mg *nano-Co₃O₄* kg⁻¹ soil_{dw}), in opposition no significant effects were observed for *B. oleracea* (germination: $F = 1.568$, $d.f_1 = 21$, $d.f_2 = 27$, $p = 0.206$; fresh weight: $F = 1.739$, $d.f_1 = 22$, $d.f_2 = 28$, $p = 0.159$; dry weight: $F = 1.356$, $d.f_1 = 22$, $d.f_2 = 28$, $p = 0.275$). Additionally, and even though the difference between the tested concentrations and the control was not significant (at the highest tested concentration 1000 mg *nano-Co₃O₄* kg⁻¹ soil_{dw}), *B. oleracea* showed slight reduction in all the measured endpoints (Fig. 2). Nevertheless, looking to all data (Fig. 2), it was possible to perceive that *Z. mays* and *L. lycopersicum* were the most sensitive species to the cobalt oxide NM.

Table 3 summarizes the ecotoxicological data obtained for *nano-Co₃O₄* for all tested organisms. The *Z. mays* was

Table 1 DLS and ELS data for *nano*-Co₃O₄ suspensions in Milli-Q water

<i>Nano</i> -Co ₃ O ₄ (mg L ⁻¹)	Primary size (nm)	Peak 1 (nm)	Number % in peak 1	Average diameter by number distribution (nm)	PdI	Zeta potential (mV)
1000	< 50	505.1 ± 180	90.7 ± 13.2	506.5 ± 181	0.8 ± 0.4	18.3 ± 1
100		805 ± 187.4	91.3 ± 9	804.8 ± 185.7	0.77 ± 0.2	-32.7 ± 2.2
10		309.6 ± 75.8	100.0 ± 0	309.8 ± 77.1	1 ± 0	-32 ± 1.4
1		273.8 ± 103	93.2 ± 0.3	268.9 ± 100.6	0.87 ± 0	-25.9 ± 1.1

reported to have an EC₂₀ of 291.1 and 440.5 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄ for fresh and dry biomass respectively, and NOEC values < 269.3 and LOEC values ≤ 269.3 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄ for fresh and dry mass, respectively (Table 3). While the *L. lycopersicum* displayed only a NOEC and a LOEC (769.2 and 1000 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄, respectively) values for the seed's emergence (Table 3).

3.3 Effect of cobalt oxide nanomaterials on soil earthworms and arthropods

All the tests were valid according to the criteria described in standard protocols. No mortality was recorded at the different NM exposure for earthworm's and arthropods. No significant avoidance was observed for *F. candida* (Fisher's exact test: $p > 0.05$) for all the concentrations tested. However, *E. andrei* significantly avoided the soils spiked with the intermediate and the highest tested concentrations of *nano*-Co₃O₄ (591.7 and 1000 mg kg⁻¹ soil_{dw}) (Fisher's exact test: $p = 0.018$) (Fig. 3). Nevertheless, only a maximum avoidance percentage of 50% was recorded for these concentrations, which was below the limit of 80% of avoidance necessary to consider that the habitat function of soils is compromised (Hund-Rinke and Wiechering 2001). The NOEC and the LOEC

values for the avoidance tests were obtained based on the Fisher exact test analysis for *E. andrei* (Table 3). Regarding reproduction tests, this endpoint was not significantly affected for both tested organisms exposed to the different concentrations of *nano*-Co₃O₄ (*F. candida*: $F = 0.901$, d.f.₁ = 21, d.f.₂ = 27, $p = 0.513$; *E. andrei*: $F = 0.293$, d.f.₁ = 22, d.f.₂ = 28, $p = 0.934$) (Fig. 4).

3.4 *E. andrei* biomarker parameters

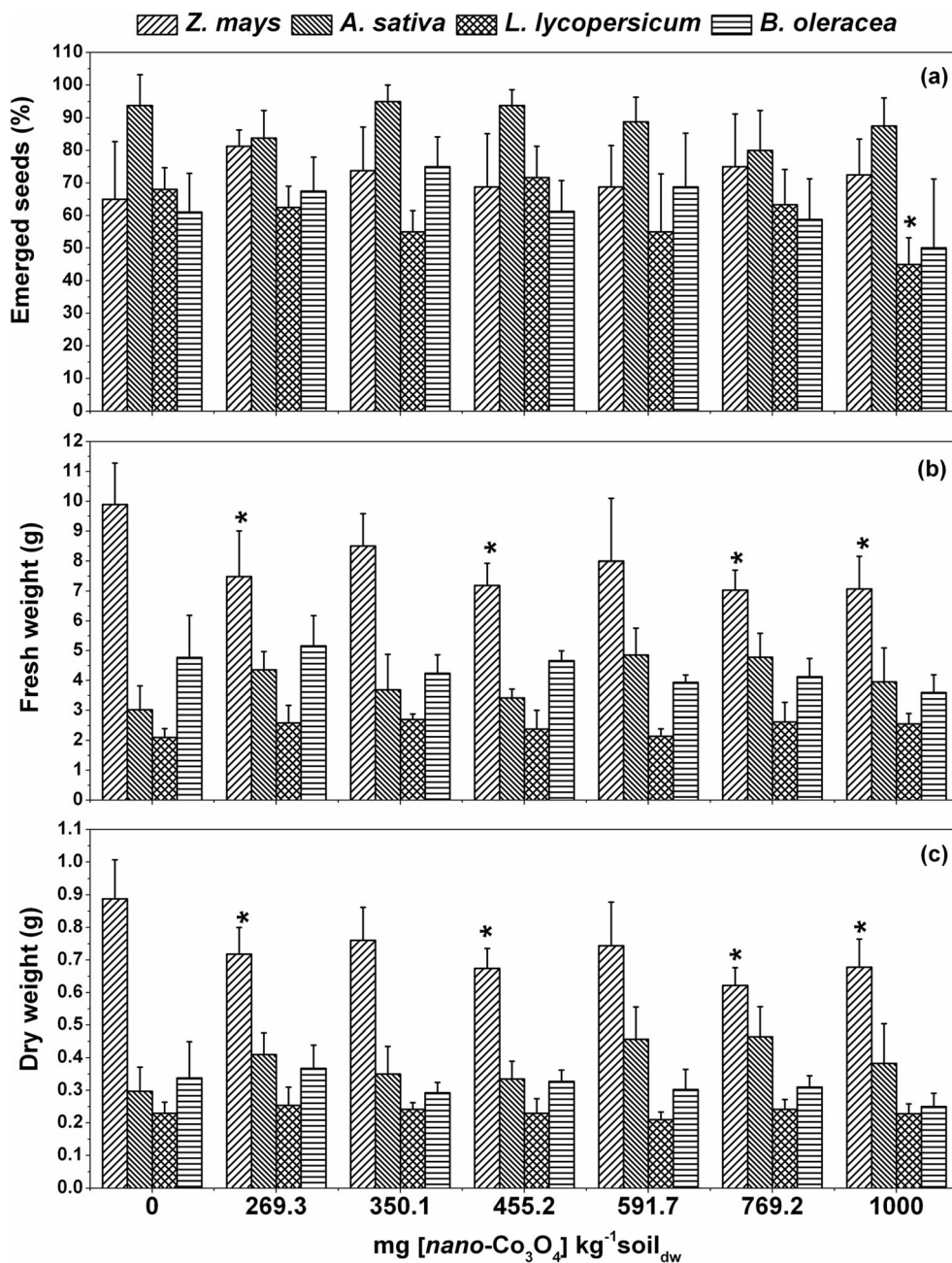
The data recorded for biochemical analysis of exposed *E. andrei* are presented in Fig. 5. A significant stimulation ($F = 5.279$, d.f.₁ = 78, d.f.₂ = 84, $p < 0.01$) of AChE activity was observed for the two lowest concentrations tested (269.3 and 350.1 mg kg⁻¹ soil_{dw}), while no significant effects were observed for the remaining concentrations. In terms of CAT activity, the worms collected from soils contaminated with 350.1, 455.2, and 769.2 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄ displayed significant highest values for this biomarker ($F = 3.659$, d.f.₁ = 80, d.f.₂ = 86, $p = 0.003$). The soil-dwelling earthworms taken from soils treated with the highest concentration of *nano*-Co₃O₄ (1000 mg kg⁻¹ soil_{dw}) have a significantly lower GST activity ($F = 5.416$, d.f.₁ = 74, d.f.₂ = 80, $p < 0.01$). The analysis of the LPO activity exhibited significant differences ($F = 3.410$, d.f.₁ = 80, d.f.₂ = 86, $p = 0.005$), being the

Table 2 Total content of Co (mg kg⁻¹ soil_{dw}) in soils for each nominal tested concentration in mg of *nano*-Co₃O₄ kg⁻¹ soil_{dw}, at the end of earthworm's avoidance and reproduction tests and at the end of terrestrial plant tests, respectively

Nominal concentrations of mg [<i>nano</i> -Co ₃ O ₄] kg ⁻¹ soil _{dw}	Earthworms (<i>E. andrei</i>)		Plants (15 days)			
	Avoidance (48 days)	Reproduction (56 days)	<i>Z. mays</i>	<i>A. sativa</i>	<i>L. lycopersicum</i>	<i>B. oleracea</i>
0	< DL	< DL	< DL	< DL	< DL	< LD
269.3	194.4 ± 0.3	191.2 ± 0.3	184.2 ± 0.4	189.8 ± 0.4	186.8 ± 0.3	188.4 ± 0.1
350.1	255.6 ± 0.3	249.3 ± 0.4	240.7 ± 0.4	247.9 ± 0.4	244.0 ± 0.2	246.0 ± 0.4
455.2	330.9 ± 0.5	336.7 ± 0.5	325.4 ± 0.4	335.1 ± 0.4	329.9 ± 0.5	332.6 ± 0.9
591.7	432.2 ± 0.2	425.2 ± 0.3	411.5 ± 0.3	423.9 ± 0.3	417.2 ± 0.6	420.7 ± 0.6
769.2	563.2 ± 0.5	550.8 ± 0.5	533.2 ± 0.4	549.2 ± 0.4	540.6 ± 0.6	545.1 ± 1.1
1000	730.1 ± 0.6	724.6 ± 0.7	702.2 ± 0.8	723.2 ± 0.8	711.9 ± 0.6	717.8 ± 0.3

DL stands for detection limit

Fig. 2 Average values of **a** emerged seeds percentage, **b** average above soil fresh weight, and **c** average above soil dry weight in monocotyledonous (*Z. mays* and *A. sativa*), and dicotyledonous species (*L. lycopersicum* and *B. oleracea*) grown in OECD soil spiked with *nano*-Co₃O₄ suspensions. Error bars represent the standard error. * stands for significant differences from the control (Dunnett test: $p < 0.05$)



MDA content significantly higher only in worms collected at the lowest concentration of the *nano*-Co₃O₄.

4 Discussion

Seed germination and seedling growth tests for evaluating the impact of NM on soils are imperative in ecotoxicological studies e.g. (Lin and Xing 2007; Castiglione et al. 2011; Hatami et al. 2014; Andersen et al. 2016; Bouguerra et al. 2016; Gavina et al. 2016; De la Rosa et al. 2017; Baskar et al. 2018; Rajput et al. 2018a, b). They are relevant to identify for the most sensitive

species, the risks of impaired soil biomass production function. In our study, we found that *nano*-Co₃O₄ had an effect on germination of tomato seeds (*L. lycopersicum*) but only for the highest concentrations tested (1000 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄) (Fig. 2a). Nevertheless, no significant effect on seeds germination was observed for the other tested species (*Z. mays*, *A. sativa*, *B. oleracea*). The great majority of published data reported no effect of MNM on seed germination for different species and at concentration ranges usually ≤ 1000 mg kg⁻¹ or mg L⁻¹ (sometimes higher) in soil or under hydroponic conditions, respectively. For example, *nano*-TiSiO₄ (< 50 nm) showed no significant effects (0–1000 mg kg⁻¹ soil_{dw}) on *Z. mays*, *A. sativa*, *L.*

Table 3 Toxicity data for invertebrates and plants with effect concentrations expressed as mg nano-Co₃O₄ kg⁻¹ soil_{dw}, and corresponding 95% confidence intervals in the same units (when available between brackets)

Biota	Endpoint	NOEC	LOEC	EC ₂₀
Invertebrates				
<i>E. andrei</i>	Avoidance (48 h)	455.2	591.7	n.d.
	Reproduction (56 days)	≥ 1000	> 1000	n.d.
	Biomarkers (28 days)			
	AChE	≤ 269.3	≤ 269.3	n.d.
	CAT	269.3	350.1	n.d.
	GST	769.2	1000	n.d.
	LPO	≤ 269.3	≤ 269.3	n.d.
<i>F. candida</i>	Avoidance (48 h)	≥ 1000	> 1000	n.d.
	Reproduction (56 days)	≥ 1000	> 1000	n.d.
Plants				
<i>Z. mays</i>	Germination	≥ 1000	> 1000	n.d.
<i>A. sativa</i>		≥ 1000	> 1000	n.d.
<i>L. lycopersicum</i>		769.2	1000	n.d.
<i>B. oleracea</i>		≥ 1000	> 1000	n.d.
<i>Z. mays</i>	Fresh weight	≤ 269.3	≤ 269.3	291.1 (– 327.2 to 909.4)
<i>A. sativa</i>		≥ 1000	> 1000	n.d.
<i>L. lycopersicum</i>		≥ 1000	> 1000	n.d.
<i>B. oleracea</i>		≥ 1000	> 1000	n.d.
<i>Z. mays</i>	Dry weight	≤ 269.3	≤ 269.3	440.5 (– 183.2 to 1064.2)
<i>A. sativa</i>		≥ 1000	> 1000	n.d.
<i>L. lycopersicum</i>		≥ 1000	> 1000	n.d.
<i>B. oleracea</i>		≥ 1000	> 1000	n.d.

n.d., not determined; NOEC, no observed effect concentration; LOEC, low observed effect concentration; EC₂₀, effect concentration that causes a reduction of 20% in the parameter under evaluation

Fig. 3 Behavioral response of *F. candida* and *E. andrei*, exposed to the OECD soil spiked with suspensions of nano-Co₃O₄ at different concentrations. Values correspond to the average percentage of avoidance of spiked soil ± standard deviation. * stands for significant avoidance of the spiked soil when the observed distribution of test organisms between soils is compared with the expected distribution for no avoidance response (Fisher's exact test: $p < 0.05$)

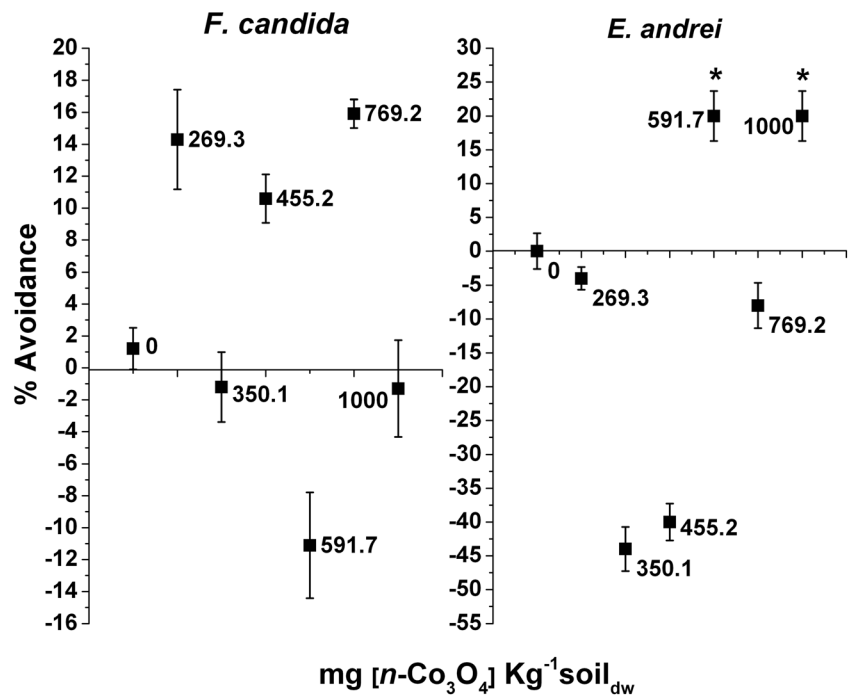
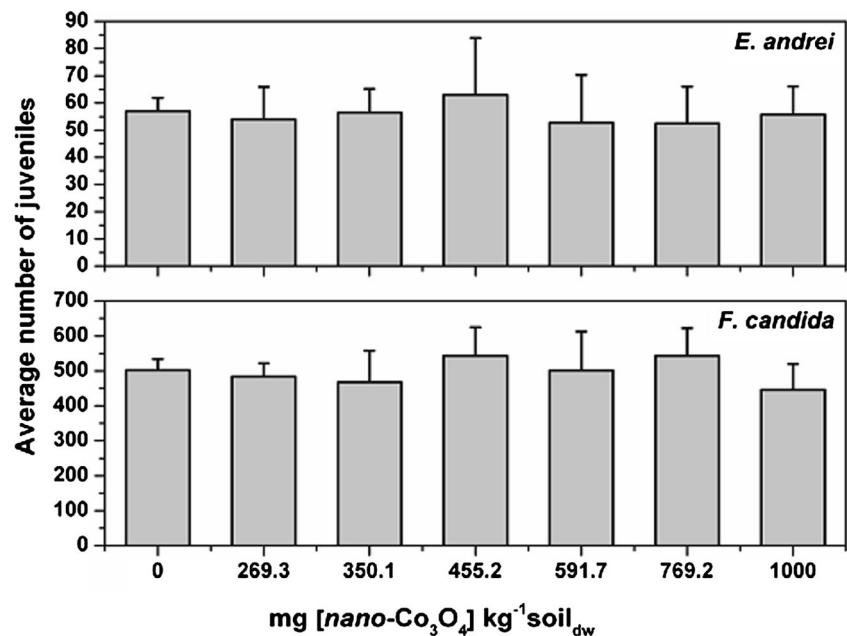


Fig. 4 Reproductive output of *F. candida* and *E. andrei* per treatment, exposed to the OECD soil spiked with different concentrations of *nano*-Co₃O₄. Error bars represent standard deviation from the mean

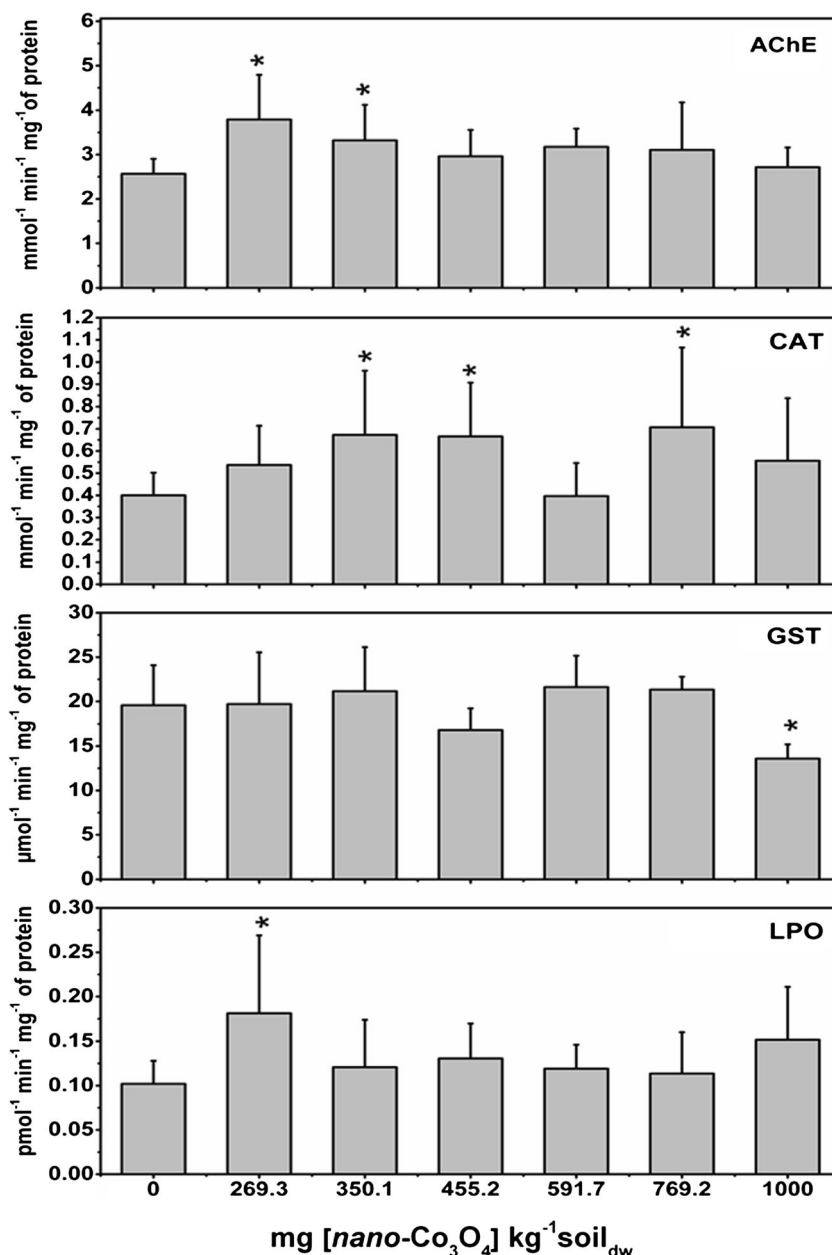


lycopersicum, and *L. sativa* (Bouguerra et al. 2016). The *nano*-CuO (< 50 nm) slightly impaired the germination rate of rice (*Oryza sativa*) in Hoagland's solution at 1000 mg L⁻¹ (da Costa et al. 2016). Yang et al. (2015) showed no effect on germination of rice (*Oryza sativa* L.) and maize (*Zea mays* L.) exposed to 2000 mg L⁻¹ of *nano* (CeO₂; CuO; TiO₂; ZnO; Fe₂O₃; Al₂O₃) dispersed in deionized water. In a recent study, Jahan et al. (2018) observed significant enhanced germination of the red bean (*Vigna angularis*) seeds, particularly at concentrations 20 to 100 μg mL⁻¹, on exposure to both metal oxides ZnO and TiO₂ NMs in hydroponic conditions. The magnetic MNM of Fe₂O₃ (50–100 nm) showed no significant effect on *Arabidopsis thaliana* (Lee et al. 2010) at concentrations of 400, 2000, and 4000 mg L⁻¹ dispersed in a nutritive medium. Likewise, Wu et al. (2012a) observed no significant inhibition induced by the MNM Fe₂O₃ and Co₃O₄ (20–40 and 10–30 nm, respectively) in the germination of *lettuce*, *cucumber* and *radish* (1000–5000 mg L⁻¹ in filter paper). Nevertheless, the opposite was recorded, for example, for magnetic *nano*-NiO (30 nm; EC₅₀ = 28 mg L⁻¹ and 401 mg L⁻¹ for lettuce and radish, respectively) (Wu et al. 2012a). The MNM effects on seed germination in different culture media, exposure conditions and for various MNM sizes and concentrations were reviewed by Du et al. (2016) and Rizwan et al. (2017). Seed germination was considered as an important physiological response of plant against stressful condition. Nonetheless, most of researchers related the poor sensitivity of the endpoint to the impermeability of seed coats to toxic elements and its ability to protect plant embryos during the early stage of seedling emergence (Wierzbička and Obidzińska 1998; Liu et al. 2007; Ma et al. 2010a). However, such coats could be sensitive in several cases depending on species, concentrations and bioavailability, and physical-

chemical properties of pollutants. Ma et al. (2010b) and Nair and Chung (2014) reported that La₂O₃ (22 nm; 2000 mg L⁻¹) and CuO (30 nm; 0.5–100 mg L⁻¹) MNM inhibited root growth and consequently germination of *Cucumis sativus* and *Arabidopsis thaliana*, respectively, due to the release of toxic metal ions or surface transformation in exposure medium or in plant tissues, respectively. Whereas, Wu et al. (2012a) demonstrated that radish (*Raphanus sativus* L.) and cucumber (*Cucumis sativus*) germination was enhanced by the adhesion of *nano*-Co₃O₄ (10–30 nm) on seed surface and by the release of free metal ions (Wu et al. 2012a). However, the authors reported also that the toxicity of the MNM varies with crops and seed size (i.e., lettuce > cucumber > radish). Furthermore, it was proved that the seed germination are less affected in soil exposures than in hydroponic exposures when evaluating the effects of zero-valent iron (0–5000 mg L⁻¹) and Ag (0–100 mg L⁻¹) NM in ryegrass (*Lolium perenne* L.), barley (*Hordeum vulgare* L.), and flax (*Linum usitatissimum* L.) in both media (El-Temsah and Joner 2010); besides, authors demonstrated that the soil quality should not be neglected in such studies since seed germination was less sensitive in a clayey soil than in a sandy soil. All previous data and studies support the results gathered in our study regarding the germination of tested species in OECD soil spiked with *nano*-Co₃O₄. The inhibition of tomato seed germination at the highest concentration tested (1000 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄) was likely related to the seed size or/and coat permeability.

The shoot's weight is also known as an important endpoint for assessing plant growth and morphology and accordingly, plant's health. Several studies reported the effects of NM and in particular MNM on aerial biomass of plants e.g. (Deng et al. 2014; Nair and Chung 2014; Bouguerra et al. 2016; De la

Fig. 5 Acetylcholinesterase (AChE), catalase (CAT), glutathione-S-transferase (GST), and lipid peroxidation (LPO) expressed as mean values \pm standard deviation ($\bar{A}v \pm STDEV$) for *E. andrei* exposed to the OECD soil spiked with different concentrations of *nano-Co₃O₄*, for 28 days. Error bars represent standard deviation. *stands for significant differences from the control (0 mg kg⁻¹ soil_{dw})



Rosa et al. 2017; Jesmer et al. 2017; Rizwan et al. 2017) for different species. However, to the best of our knowledge, there were no previous studies assessing the effect of *nano-Co₃O₄* on terrestrial plant species exposed in soil-based systems. In our study, only corn (*Z. mays*) displayed a significant toxic response to *nano-Co₃O₄* in all tested concentrations in terms of both fresh and dry biomass above soil ($EC_{20} = 291.1$ and 440.5 mg *nano-Co₃O₄* kg⁻¹ soil_{dw}, respectively) (Table 3; Fig. 2b, c). However, no significant effects were observed for oat, cabbage and tomato plants. Cobalt is known as a phytotoxic metal. For instance, Chaney (1983) demonstrated that plants accumulating 50 to 100 mg kg⁻¹ soil_{dw} of Co displayed severe toxic symptoms. Keeling et al. (2003) also observed that Co, at concentrations above 111 μg g⁻¹ supplied

in the growing substrate, caused a decrease in biomass production and promoted the yellowing and necrosis of foliage in *Berkheya coddii*. Chatterjee and Chatterjee (2000) proved that 0.5 mM of cobalt sulfate (supplied with the basal nutrient solution to pots) decreased the dry weight of root, stem and leaves of *Brassica oleracea* L. Authors also observed effects on plant morphology after 8 days of exposure and they reported chlorosis in young leaves and chlorotic mottling in newly emerged leaves (Chatterjee and Chatterjee 2000). The main aim of the study carried out by Bakkaus et al. (2005) was to understand the effect and the pathway of Co accumulation in a monocotyledonous species (wheat: *Triticum aestivum*) and in one dicotyledonous species (tomato: *Lycopersicon esculentum*). The authors demonstrated that

cobalt had a different distribution in plant leaves. In wheat, Co followed the same pathway of potassium and calcium in parallel veins; the same was not observed in tomato leaves. In tomato leaves, Co needs first to be confirmed and then segregated from other elements. Authors suggested that this may be the reason for the tolerance of dicotyledonous species to cobalt (Bakkaus et al. 2005). In our study, the tolerance of both dicotyledonous species tested (*L. lycopersicum* and *B. oleracea*) to *nano*-Co₃O₄ was once again registered. Li et al. (2009) also reported different degrees of tomato (*Lycopersicon esculentum* L.), barley (*Hordeum vulgare* L.), and oilseed rape (*Brassica napus* L.) sensitivity, two dicots and one monocot species, exposed in seven different natural European soils spiked with CoCl₂ to obtain a range of concentrations of Co between 160 and 1600 mg kg⁻¹. These authors demonstrated that soil properties interfere in Co phytotoxicity and tomato was the most sensitive species (EC₅₀ values varying from 7 to 736 mg kg⁻¹ of Co depending on soil properties) to Co. Further, when looking for the Co concentration (ranging from 180 to 723 of Co mg kg⁻¹ soil_{dw}) in our treated soil (Table 2), we expected important toxicity response for all species, since lowest concentrations, as discussed previously, showed to cause several problems to plant growth and morphology. However, it seems that nano-sized cobalt oxide is less phytotoxic than ionic cobalt at least for three out of four species tested in this study. The lower sensitivity was likely related with the high aggregation level of this NM that has likely reduced the metal bioavailability and uptake by plants; this may be also related to the complex structure of plant cell walls formed by three layers, each one with a specific structure and function, assembled with cellulose microfibrils, cross-linked with hemicellulose and pectin (Serag et al. 2013). Besides, the nanometric cells size pores with diameters estimated between 5 and 20 nm (Carpita et al. 1979; Tepfer and Taylor 1981). This is also in agreement with some previous studies, proving that, at least in some conditions, the formation of aggregates with micrometric size may decrease the toxic effect of MNM (Yang and Watts 2005; Lin and Xing 2007; Jahan et al. 2018). All these aspects could explain the low sensitivity of tomato, cabbage and oat to *nano*-Co₃O₄ tested in this study. However, although uptake and translocation of the NM could have been prevented by aggregation, the MNM could broadly adsorb on root surface causing several structural anomalies. Wild and Jones (2009) observed that carbon nanotube may alter root tissues, facilitating the transport of NM into the cell's cytoplasm. Further, some authors hypothesized that aggregates may also damage hydraulic movement between soil and roots promoting symptoms of water scarcity. Asli and Neumann (2009) exposed *Z. mays* roots to 300 and 1000 mg L⁻¹ of *nano*-TiO₂ (P25) under hydroponic conditions. These authors observed that the highest concentration inhibited root hydraulic conductivity and subsequently plant growth and transpiration (Asli and Neumann 2009). All these

previous hypothesis and data may explain the highest sensitivity of *Z. mays* to *nano*-Co₃O₄. In addition, we could not neglect the effect of small nano-aggregates that could cross root epidermal pores since we registered a high polydispersity level (PdI > 0.5; Table 1) in the *nano*-Co₃O₄ suspension suggesting the possible presence of small aggregates or even of isolated nanoparticles (although in a low percentage by number < 10%) that could cross cell pores. The release of ions from the surface of NM aggregates is also another possibility for more detrimental effects.

Several studies with terrestrial organisms such as soil earthworms and springtails have been performed to assess the toxicity of MNM. These studies have mainly focused the evaluation of short-term behavioral responses, reproductive output and biochemical performance e.g. (Hu et al. 2010; Cañas et al. 2011; Heckmann et al. 2011; Kool et al. 2011; Gomes et al. 2015a, b; Bouguerra et al. 2016; Brami et al. 2017; Garcia-Velasco et al. 2017; Jesmer et al. 2017; Liang et al. 2017). *Nano*-Co₃O₄ showed no or low toxicity for both tested invertebrates' species regarding their behavioral ability to avoid the MNM and their reproductive function, at least for the range of concentrations tested in the present study. A significant avoidance response of *E. andrei* was only recorded at 591.7 and 1000 mg kg⁻¹ soil_{dw} of *nano*-Co₃O₄, reflecting the ability of earthworms to detect *nano*-Co₃O₄ in soil. This effect was likely related to the MNM concentration in soil (Fig. 3) and occurred despite their aggregation. To the best of our knowledge, there are no studies describing the impact of cobalt NM on avoidance response of earthworms and collembolans. Nevertheless, this finding was in accordance with our previous study (Bouguerra et al. 2016) for *nano*-TiSiO₄ at the same range of concentrations tested and for the same species. Only OECD soil contaminated with 1000 mg kg⁻¹ soil_{dw} of TiSiO₄ caused a significant avoidance of earthworms. Also, titanium dioxide NM (100% and 83% anatase) with different sizes (5 and 21 nm) were weakly avoided at concentrations up to 1000 mg kg⁻¹ in artificial and natural soil by both *E. andrei* and *E. fetida* earthworms (McShane et al. 2012). Similarly, *E. fetida* only avoided a natural soil spiked with Al₂O₃ NM at the highest concentrations tested 5000 and 10,000 mg kg⁻¹ (Coleman et al. 2010). As far as the reproduction was assessed, no significant effect was recorded in the reproduction output of both *E. andrei* and *F. candida*. Lock et al. (2004) recorded an EC₅₀ value of 1480 mg kg⁻¹ soil_{dw} of ionic Co (added to the artificial OECD soil) for *F. candida* reproduction. In our study, the highest effective concentration of Co in soil was 730 mg kg⁻¹ soil_{dw}, what explains the absence of reproduction effects at least for this species. Nevertheless, we think that concentrations above these become truly environmental unrealistic. At the nano-sized scale, a higher toxicity of Co was expected as a high ability for cell internalization was also expected; however, this was not observed probably due to nanoparticles aggregation, or due to interaction with

soil components. Furthermore, Coutris et al. (2012) observed that earthworms (*E. fetida*) fed with horse manure (0.5 g/worm) spiked with $1.20 \pm 0.45 \mu\text{g g}^{-1}$ of cobalt NM (Co core/ Co_3O_4 -CoO shell; 3.9 nm) once a week for a period of 4 months had negligible bioaccumulation factor of cobalt in spermathecae when compared to other organs. Such results may explain the absence of effects in the reproductive function of earthworms in our study. Even though worms may accumulate the cobalt NM, it will not directly affect the reproductive organs and consequently their reproduction activity.

Although effects at the individual level were negligible, significant differences were recorded in the adult earthworm's enzyme activities after 28 days of exposure. Our results showed a significant stimulation in CAT activity, as well as a significant increase in LPO at the lowest and intermediate concentrations tested. In earthworms exposed to $1000 \text{ mg kg}^{-1} \text{ soil}_{\text{dw}}$ of *nano*- Co_3O_4 , a significant decrease in GST activity was observed. The oxidative stress observed especially at the lowest concentration tested suggested the uptake and the possible bioaccumulation of Co by *E. andrei*. Coutris et al. (2012) demonstrated that Co ions released from Co nanoparticles were mostly accumulated in pseudo-hearts and digestive organs (esophagus, crop, gizzard and gut) when supplied in food. In addition, the strict contact of earthworms with contaminated soils increased the possible internalization of released Co ion into biological membrane and consequently inside cell tissues. The release of Co ions may also happen inside the gut. The internalization of *nano*- Co_3O_4 has been previously observed in human cells by Colognato et al. (2008) and Papis et al. (2009) and evidences of oxidative stress were also shown. Nonetheless, the LPO levels, after 4 weeks of exposure, indicated that worms suffered oxidative damage induced by the exposure to *nano*- Co_3O_4 . These levels were more significantly pronounced at lowest concentration. The high CAT activity in parallel with the highest aggregation of the MNM in the intermediate concentrations may have contributed to reduce cell membrane's damages caused by the oxidative stress. The increase in CAT activity upon *nano*-cobalt exposure was previously observed in primary T cell (selected from human peripheral blood mononuclear cells) after 4 h of exposure, at $6 \mu\text{M}$ of Co nanoparticles (30–70 nm; median size 50 nm) (Jiang et al. 2012). Conversely to cellular damage caused by oxidative stress, our results showed the low ability of *nano*- Co_3O_4 to alter the earthworm's neuronal functions as shown by the non-inhibition of AChE activity. Rault et al. (2007) pointed out that AChE is the main cholinesterase present in the whole body of six different earthworms; therefore, authors suggested that it could be a powerful marker of neuronal activity besides the use of earthworm's whole body for measuring its activity. To the best of our knowledge, there were no studies reporting the effect of cobalt NM on AChE activity in soil organisms. Furthermore, little work was done to investigate the effect of NM and in particular MNM on AChE activity, except the study from Wang et al. (2009). These authors, through

an in vitro study, pointed out for some data suggesting the interaction between AChE activity and different types of NM including metals, metal oxides, and carbon nanotubes (SiO_2 , TiO_2 , Al_2O_3 , carbon-coated copper, multi-walled carbon nanotubes, and single-walled carbon nanotubes). The study demonstrated that metals and metal oxide NM had a low ability to inhibit AChE activity, when compared with multi-walled carbon nanotubes and single-walled carbon nanotubes (Wang et al. 2009).

Therefore, the data reported in the present study and the ecotoxicological data reported in Table 3 allowed us to propose a PNEC (Predicted No Effect Concentration) of $9.1 \text{ mg kg}^{-1} \text{ soil}_{\text{dw}}$ of *nano*- Co_3O_4 , by applying an assessment factor of 50 to the lowest NOEC obtained for standard endpoints (for earthworm's avoidance). This assessment factor was selected because only data for two trophic levels are available (EC 2003). We did not use the NOEC value for the most sensitive species, since for example for *Z. mays* it was not possible to determine a NOEC with the range of concentrations tested. Nevertheless, the NOEC used for the calculation of the PNEC value was similar to the EC20 recorded for *Z. mays* dry mass production. The integration of biochemical endpoints allows increasing the degree of protection, while decreasing uncertainty. Taking into account these endpoints at the biochemical level, the PNEC value will be refined to $5.3 \text{ mg kg}^{-1} \text{ soil}_{\text{dw}}$ of Co_3O_4 , NM.

5 Conclusions

In summary, *nano*- Co_3O_4 can significantly affect the emergence and growth of some plant species. Soil invertebrates like *E. andrei* can notice their presence in soil and start avoiding it. Moreover, long-term exposure can affect the metabolic activity of invertebrate species, although without compromise their reproductive activity, at least for the exposure period tested. The registered effect for this organism could be mainly related to the direct uptake of *nano*- Co_3O_4 aggregates. However, more data are needed to evaluate the effects of *nano*- Co_3O_4 for other soil organisms in order to reduce the uncertainty of the PNEC values estimated. Biochemical endpoints could be extremely important in the first tier of environmental risk assessment, when few data are available, to guarantee an adequate level of protection. For instance, the possible effects of this NM in the activity of soil microbial community must be assessed considering its relevant role in soil functions. Special attention should be given for the effect of this metal oxide NM in agriculture soils, since the use of biosolids for fertilization is one of the most relevant pathways for the entrance of these emergent contaminants in this environmental compartment. The relatively high PNEC value recorded reflects the low toxicity of this MNM, which in turn may result from its aggregation and lower bioavailability. However, what is expected is that NMs are in the soil

fundamentally aggregated or complexed with matrix components. Disaggregation may occur progressively with soil aging, but the slow release of nanoparticles will certainly help to maintain their toxicity low.

Funding This research is part of the project REALISE (PTDC/AAC-AMB/120697/2010) funded by the Portuguese Government (Program Ciência – Inovação 2010) and by the European Social Fund – COMPETE. Sirine Bouguerra was supported by an investigator fellowship (Ref. PTDC/AAC-AMB/120697/2010) from the project. Ana Gavina was also supported by a Ph.D. Grant (Ref. SFRH/BD/94902/2013) from FCT – Foundation for Science and Technology. This research was also partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020.

Compliance with ethical standards

Research involving human participants and/or animals This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

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