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RESEARCH ARTICLE

Combined effects of goethite nanoparticles with metallic contaminants and an organophosphorus pesticide on *Eisenia andrei*

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Abstract

The effects of mixtures of nanoparticles (NPs) and other chemicals have been poorly studied in terrestrial invertebrates. In this study, we investigated the effects of binary mixtures of goethite (α-FeOOH) NPs and metallic (Cd and Pb) or organic (chlorpyrifos, CPF) contaminants in *Eisenia andrei* earthworms. We used the filter paper contact test to evaluate (i) the uptake of NPs in organisms exposed to the mixtures of NPs+Metals and NPs+CPF and (ii) the potential effects of the mixture of NPs+CPF on the CPF-induced inhibition of the biomarker enzymes acetylcholinesterase (AChE) and carboxylesterases (CES). We used the artificial soil test to deepen the study on joint effects of NPs+CPF. All compounds were applied separately and in binary mixtures. In the single exposure treatment, Fe levels decreased significantly in organisms exposed to NPs on filter paper, suggesting systemic effects aimed at eliminating Fe incorporated through NPs. Conversely, earthworms exposed to binary mixtures showed Fe levels similar (NPs+Metals) to or higher (NPs+CPF) than controls. The earthworms single exposed to NPs presented no changes in AChE and CES activities. In the artificial soil test, the only treatment that showed AChE inhibition after 72 h was single CPF exposure, while no significant changes were observed in CES activity. However, after 7-day exposure in artificial soil or 72-h exposure on filter paper, the mixture of NPs+CPF induced a similar degree of AChE and CES inhibition as single CPF exposure. All these suggested that NPs did not produce neurotoxic effects, and that the inhibition of the enzymes' activities in all cases was due to the presence of the pesticide. On the other hand, the differences in the pattern of Fe accumulation in the earthworms indicate that the presence of other contaminants in the exposure media can modify the uptake and/or the excretion of Fe and evidence the interactions that may be found in binary mixtures of metal oxide NPs and other pre-existing contaminants in the soil ecosystem.

Keywords *Eisenia andrei* · Goethite · Nanoparticles · Cadmium · Lead · Chlorpyrifos

Introduction

Nanotechnology has recently grown to become a multi-billion dollar industry with over 3000 registered consumer products containing nanoparticles (NPs) (DTU Environment 2018). The remarkable versatility of these particles is reflected, among others, in their use in the development of self-cleaning glasses, antimicrobial patches, and therapeutic agents for the treatment of various pathologies (Dowling 2004; Sanvicens and Marco 2008; Missaoui et al. 2018). Moreover, potential applications of NPs for remediation processes of contaminated water and soil are being investigated (Wu et al. 2019). In particular, iron oxides and oxyhydroxides engineered NPs, such as goethite, are a promising material, for example, in catalysis, sensors, and also in remediation processes (Cross et al. 2010; Chernyshova et al. 2011; Parveen et al. 2012; Hjorth et al. 2017; Nanorem 2017).

NPs can enter the environment not only through production or application processes but also through waste management

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disposal and outdoor aging products. For example, paints containing nano-Ag that were exposed to ambient outdoor weather for 1 year showed strong leaching of NPs during initial runoff events (Kaegi et al. 2010). Upon release and emission, they can interact with numerous pre-existing contaminants, such as pesticides and heavy metals. These interactions are enhanced by the high surface area of NPs and can alter the bioavailability and potential toxic effects of contaminants (Pachapur et al. 2016).

Unlike other contaminants, metals cannot be degraded. They concentrate in water, soil, and sediments and/or accumulate in living organisms (Vijver et al. 2003). Due to intense industrialization and urbanization, the release of contaminants such as heavy metals continues to rise. Among them, cadmium (Cd) and lead (Pb) have become widespread in the environment because of human activities (Alloway 2013). Cd has the ability to bioaccumulate in tissues at high concentrations and can be biomagnified through the trophic chain. Anthropogenic Cd sources include, for example, Ni-Cd batteries, plastics, and also from the application of soil fertilizers based on phosphates that are contaminated with this metal (Roberts 2014; Wu et al. 2016). Anthropogenic Pb comes mostly from mining, smelting of ore, fossil fuels, manufacture, and use of Pb-containing products (ATSDR 2019).

Chlorpyrifos (CPF) is a broad-spectrum organophosphorus (OP) non-systemic insecticide and acaricide, used to control crop pests worldwide (Jänsch et al. 2006). The mode of action of CPF is through acetylcholinesterase (AChE) inhibition at the synaptic junction. This leads to alterations in the normal functioning of the nervous system and eventually death. In addition, carboxylesterases (CES) are a family of enzymes that catalyze the hydrolysis of carboxylic esters by the addition of water (Wheelock et al. 2005). Like cholinesterases (ChEs), CES are B-esterases because they are inhibited by OPs (Aldridge 1953); hence, they are also used in environmental studies of exposure to OP pesticides. The combined monitoring of CES and AChE activities can provide more useful information on exposure to OPs than the measurement of AChE alone in many aquatic and terrestrial species (Wheelock et al. 2005; Cacciatore et al. 2013).

Among soil organisms, earthworms are highly appropriate terrestrial model organisms for toxicity tests, as they play a vital role in decomposition, nutrient cycles, and the development and maintenance of soil structure. Particularly, *Eisenia andrei* is widely used as a standard organism to evaluate soil quality because of its low cost, easy handling, and the standardization of acute and subchronic toxicological tests (OECD 1984; Gong and Perkins 2016).

Recently, we have shown that dermal exposure of *E. andrei* to mixtures of goethite (α-FeOOH) NPs and Cd or Pb reduced the bioavailability of these metals (Cáceres Wenzel et al. 2016). Therefore, testing for joint effects of mixtures is crucial as it may show different toxicity patterns compared with the single chemical, and in the environment, pesticides and metals are likely to co-exist in most agricultural soil ecosystems (Wang et al. 2015). In this study, we wanted to investigate the effects of binary mixtures of goethite NPs with metallic (Cd and Pb) or organic (CPF) contaminants on Fe accumulation in *E. andrei*. We then evaluated the potential effects of the binary mixture of NPs and CPF on the OP-induced inhibition of AChE and CES.

Materials and methods

Chemicals

Goethite NPs (product number 720704, Sigma–Aldrich, Argentina) were provided as a water suspension (20% w/w). According to the manufacturer, nominal range of particle size was less than 100 nm. NP suspensions for bioassays were prepared by sonicating the stock suspension for 30 min followed by dilution in distilled water.

The following compounds: Chlorpyrifos (CPF, PESTANAL® 99.9% purity), 5,5′ dithiobis-2 nitrobenzoic acid (DTNB), acetylthiocholine iodide (AcSChI), p-nitrophenyl butyrate, and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Argentina). $CdCl_2 \cdot 2\frac{1}{2} H_2O$ was purchased from M&B Laboratory Chemicals and $Pb(NO_3)_2$ from Mallinckrodt. All other reagents were of analytical grade.

Goethite NP characterization

The morphology of goethite NPs was analyzed by scanning electron microscopy (SEM) using a Carl Zeiss model NTS-Supra 40, Germany (Centro de Microscopía Avanzada, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires). SEM specimens were prepared by drop-casting one or two drops of NPs solution (10 mg/L) onto carbon-coated copper grids and allowing to dry at room temperature overnight. The SEM technique was combined with Energy Dispersive Spectroscopy (EDS) to obtain composition measurements.

Organisms selected

E. andrei (Oligochaeta, Lumbricidae) were obtained from the culture stocks maintained in our laboratory since 1997. Long-term use of isolated laboratory cultures may lead to production of individuals adapted to laboratory conditions and/or inbreeding (Lowe and Butt 2007). To increase genetic variation in our culture stocks, we periodically introduced individuals coming from other research institutions. In addition, our population was kept in 10–12 40-L plastic containers, but individuals from each of them were regularly exchanged to promote

random reproduction. Prior to the bioassays, earthworms were randomly removed from these containers, rinsed with tap water, and stored on damp filter paper for 24 h (in the dark at 22 °C) to void the gut contents. The earthworms used in the assays were all adults with well-developed clitellae (0.30–0.50 g after voiding the gut content).

Bioassays using the filter paper contact toxicity method

Earthworms were either single exposed (72 h) to filter papers (Whatman No. 1, surface = 60 cm^2) impregnated with NPs, Cd, Pb, and CPF, or to the mixture of NPs+Cd, NPs+Pb, and NPs+CPF following the OECD guidelines (OECD 1984) with modifications (the filter papers were dried outside the vial instead of inside).

Preliminary experiments were carried out in order to choose the concentration of Cd, Pb, and CPF to be used in the assays, so as to allow an appropriate evaluation of the effects of the mixtures. Thus, prior to the binary mixture assays, earthworms were exposed to different Cd concentrations (0–16.6 μg Cd/cm^2) for 72 h. One hundred percent mortality was observed at the highest concentration tested, while at the other concentrations, no mortality was recorded. An appreciable accumulation of this metal was observed at the lowest concentration (0.4 μg Cd/cm^2), so it was selected for further studies. For comparative purposes, an equimolar Pb concentration (0.214 mM, equivalent to 0.74 μg Pb/cm^2) was used. At this level of Pb, no mortality was observed.

In the case of CPF, earthworms were first exposed to varying concentrations of the pesticide dissolved in acetone (0–1 μg/cm^2) for 72 h in order to choose a concentration that would inhibit 40–50% the activity of AChE enzyme. A half-maximal effective concentration (EC_{50}) of 0.81 ± 0.02 μg/cm^2 was determined with OriginPro 9.0 software (OriginLab, Northampton, MA). Therefore, a CPF concentration of 0.75 μg/cm^2 was selected for further studies.

For the assays involving NPs, filter papers were impregnated with 1 mL of an aqueous suspension of 3 g NPs/L (final concentration, 50 μg NPs/cm^2). This is in line with concentrations recently reported for remediation processes (5 g/L) (Nanorem 2017). In the case of the assays involving mixtures of NPs and metals, after drying the NP-impregnated papers in a fume hood, flat-bottom vials were lined with the papers and 1 mL of equimolar solutions of Cd or Pb was pipetted into each vial while rotating it horizontally to impregnate the papers in a homogeneous way (final metal concentrations, 0.40 μg Cd/cm^2 or 0.74 μg Pb/cm^2). Solvent control vials contained filter papers impregnated only with water.

In the case of the studies involving mixtures of NPs and CPF (50 μg NPs/cm^2 + 0.75 μg CPF/cm^2), filter papers were first impregnated with 1 mL of aqueous medium (water or NPs suspension in distilled water). Then, 1 mL of organic solvents (acetone or CPF solution prepared in acetone) was added according to the different treatments. Papers were dried in the fume hood, and then 1 mL of distilled water was added to each vial to moisten the filter paper. Solvent controls followed the same treatment, i.e., papers were first impregnated with 1 mL of distilled water, then with 1 mL of acetone. They were later dried and moistened with 1 mL of distilled water.

In all cases, one worm per vial was added, and 6 earthworms for each treatment were prepared for all experiments. After addition of earthworms, each vial was covered with a piece of nylon stockings and closed with a cap with a ventilation hole (cap diameter 25 mm/hole diameter 15 mm) and placed in the dark at 22 ± 2 °C. At the end of the exposure period, earthworms were rinsed with distilled water. Those destined to atomic absorption spectroscopy (AAS) determinations were used immediately as described below. Organisms used for enzyme determinations were stored at − 80 °C until use.

Bioassays using artificial soil

Earthworms were exposed to artificial soil spiked with NPs, CPF, and the mixture of NPs and CPF following the OECD guidelines (OECD 1984) with slight modifications (the test duration was modified to 72 h and 7 days instead of 7 and 14 days). The artificial soil used consisted of 10% ground sphagnum peat (< 0.5 mm), 20% kaolinite clay (> 50% kaolinite), and 70% fine sand with more than 50% of the particles between 50 and 200 μm. A small amount of calcium carbonate was added to adjust the pH to 6.0 ± 0.5. The NP application concentration was based on our previous studies (Cáceres Wenzel et al. 2016). CPF concentration was selected after exposing earthworms to varying concentrations (0–87 mg/kg) of the pesticide in soil for 7 days in order to choose a concentration that would inhibit 50% (EC_{50}) activity of AChE enzyme. This study was carried out using either pure CPF (99.9%) or a commercial formulation of CPF (TERMINATOR CIAGRO®, Ciagro S.A., Argentina). No significant differences were found between Pure-CPF or Formulated-CPF (results not shown). Therefore, and to simulate crop field conditions, the commercial formulation of CPF was used for soil studies. The concentration selected was 18 mg/kg, based on the EC_{50} value for AChE inhibition.

The soil for the CPF treatments (single and mixture exposure) was prepared 24 h before starting the experiment. CPF was added to artificial dry soil as an acetone solution to obtain a final concentration of 18 mg/kg of dry soil, gently mixed and left for 24 h in a fume hood to let acetone evaporate. For the control treatment, the same amount of acetone as in CPF single treatment was added. For NP single exposure, an appropriate amount of an aqueous suspension of goethite NPs (to 75% of the soil water holding capacity, WHC) was mixed with

soil to obtain a concentration of 100 mg NPs/kg of dry soil. The soil destined to the binary mixture exposure, which was previously treated with CPF, was mixed with the same NP suspension as mentioned above. Distilled water was added to CPF and control treatments to reach the same WHC. Each treated soil was divided into three equal parts and placed inside plastic containers (120-mm diameter/70-mm height). Six worms were placed in each container covered with perforated plastic film to prevent moisture loss and allow exchange of air. The containers were stored at 20 ± 2 °C under photoperiod of 16-h light/8-h dark. Single earthworms were removed from soil after 72 h and 7 days of treatment and transferred to individually marked plastic Petri dishes with moist paper filter to void the content of their guts (24 h, in the dark) and later stored at − 80 °C until use in enzymatic determinations.

Fe accumulation in earthworms

To determine the uptake of Fe, the total metal concentration of sampled individuals from the filter paper contact test was quantified by AAS after a digestion process (Cáceres Wenzel et al. 2016). Optimal digestion conditions for the quantification of Fe levels in tissues were determined using untreated organisms spiked with a known concentration of NPs. The digestion process was performed with ultrapure concentrated nitric acid at $T = 100$ °C for 8 h. The results showed that the treatment was effective since the recovery of Fe was 98 ± 12%.

Samples were measured in a 575 AA Varian atomic absorption spectrophotometer applying the method of direct flame atomization in an air-acetylene flame with background correction. All the glassware was prewashed with a mixture containing 5% nitric acid plus 5% hydrochloric acid, thoroughly rinsed with double-distilled water and dried. Blank values were negligible. Detection limit was 0.10 mg Fe/L. Values of metal concentration were expressed as micrograms of metal per gram wet tissue.

Enzyme activities

Earthworms were homogenized on ice in 100 mM Tris buffer (pH 7.4), at 1:3 tissue:buffer ratio (w:v), using a mechanical homogenizer. The homogenates were centrifuged at 4 °C for 30 min at 9000×g. The supernatant (post-mitochondrial fraction) and the pellet from each sample were used for the determination of AChE activity. Although most authors study AChE activity in supernatants, others have shown that in many invertebrate species, AChE activity can be found both in the supernatant and in the pellet (Basack et al. 1998; Kristoff et al. 2006). To our knowledge, there is no preliminary data on *E. andrei*. Therefore, we decided to study the effects of the different treatments on AChE activity in both fractions in order to analyze whether they display differential sensitivity in this species. CES activity was only measured in the supernatants. Proteins were determined according to the method of Lowry et al. (1951), using BSA as standard.

AChE activity

AChE activity was determined according to the method of Ellman et al. (1961) using a Shimadzu UV-1603 spectrophotometer. Kinetic measurements were performed in 50 mM Tris buffer pH 8, 0.25 mM DTNB, and 12 mM AcSChI as substrate. Activity was recorded continuously at 412 nm and was corrected for spontaneous hydrolysis of the substrate. The mean readings of two replicates per sample were taken for calculations using the absorption coefficient of 13.6 mM^{-1} cm^{-1}. Specific activity was expressed as nmol of acetylthiocholine hydrolyzed per min per mg protein.

CES activity

CES activity was evaluated using p-nitrophenyl butyrate as substrate (Cacciatore et al. 2013). Activity was measured, in duplicate, in 100 mM phosphate buffer pH 8.0 containing 5% acetone and 1 mM p-nitrophenyl butyrate. The activity was monitored at 400 nm and the specific activity was calculated using the molar extinction coefficient of p-nitrophenol (18.6 mM^{-1} cm^{-1}). The results were expressed as nmol of product per min per mg of protein.

Statistical analysis

Results were expressed as mean ± S.D. One-way ANOVA analyses were performed and comparisons between groups were made by Tukey's test. In all cases, data were tested prior to analysis for normality (Kolmogornov–Smirnoff test) and variance homoscedasticity (Bartlett's test). The level of significance used was 0.05. The statistical software Instat Graph Pad version 3.01 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and R (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) were used.

Results

Goethite NP characterization

Goethite NPs exhibited an acicular shape, with a long axis length of 100.0 ± 5.7 nm (Fig. 1). EDS technique confirmed their composition, which was consistent with goethite (Table 1).

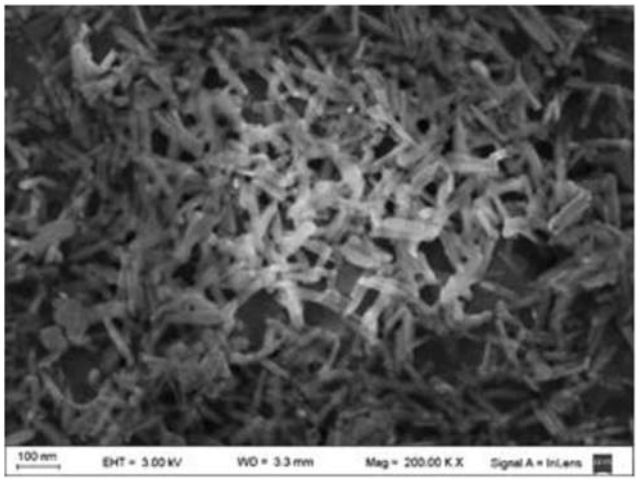

Fig. 1 SEM image of goethite nanoparticle (NPs) suspension with a magnification of × 200,000

Fe accumulation using the filter paper contact test

A significant decrease in Fe levels was observed in organisms after single exposure to NPs and Cd, ($p < 0.05$) (Fig. 2). In contrast, no significant differences ($p > 0.05$) were observed in the Fe levels of individuals exposed either to Pb or to CPF alone, or to the mixtures of NPs+Metals. However, there was a significant increase (65%, $p < 0.05$) in organisms exposed to the mixture of NPs+CPF. Acetone did not affect Fe levels; thus, only control organisms exposed to distilled water are shown.

Enzyme activities

Filter paper contact test

The inhibition of AChE activity in supernatant and pellet was determined after 72 h of filter paper exposure to goethite NPs, CPF, and to the mixture of NPs+CPF (Fig. 3). CPF and the binary mixture significantly inhibited ($p < 0.05$) AChE activity in both fractions. When the activity was measured in the supernatant, inhibitions of 41% and 35% were obtained for CPF and the binary mixture, respectively, while in the pellets, inhibitions of 29% and 36% were obtained for CPF and the binary mixture, respectively. Single exposure to goethite NPs did not affect the activity of AChE, in both the supernatant and pellet ($p > 0.05$).

CES activity in the supernatants followed the same trend as AChE activity (Fig. 4), since only CPF and the binary mixture of NPs+CPF significantly inhibited the activity of the enzyme (49% and 53% respectively).

Artificial soil test

The inhibition of AChE and CES activities was determined in the supernatants of 9000×*g* after 72-h and 7-day exposure to soil spiked with goethite NPs, CPF, and with the mixture of NPs+CPF (Figs. 5 and 6).

At 72 h, only CPF caused a marked inhibition ($p < 0.05$) of AChE activity. No significant differences were found in earthworms single exposed to goethite NPs or to the binary mixture ($p > 0.05$) compared with control (Fig. 5). However, after 7 days, CPF and the mixture significantly inhibited ($p < 0.05$) AChE activity. At this time point, inhibitions of 72% and 60% were obtained for CPF and the binary mixture, respectively, whereas goethite NPs did not inhibit AChE activity.

Neither of the treatments altered the activity of CES of earthworms exposed for 72 h ($p > 0.05$) (Fig. 6). Nevertheless, after 7-day exposure, CPF and the mixture treatments showed significant inhibition of CES activity (59% and 60% respectively) ($p < 0.05$), while no significant differences were found in earthworms single exposed to goethite NPs ($p > 0.05$) compared with control organisms.

Table 1 Elemental analysis by EDS of goethite NPs shown in Fig. 1

Element	Weight (%)	Atomic (%)
C	3.32	7.17
O	41.26	66.86
Si	0.26	0.24
Fe	54.82	25.45
S	0.34	0.28

EDS, energy dispersive spectroscopy; *NPs*, nanoparticles

Discussion

To date, few studies have investigated the co-exposure of NPs and other chemicals in soil invertebrates. The present study is, to our knowledge, the first assessment of the effects of binary mixtures of goethite NPs and Cd, Pb or CPF in the earthworm *E. andrei*, especially on Fe uptake and modulation of CPF toxicity upon exposure.

Fe uptake

Terrestrial invertebrates are able to regulate, to a certain degree, the accumulation of essential metals such as Cu, Zn, and Fe (Dallinger 1993; van Gestel et al. 1993). Our results using the filter paper contact test offer some evidence for this, as single exposure for 72 h to goethite NPs caused a decrease in Fe levels. Similarly, we have previously reported decreased levels of Fe in *E. andrei* exposed to goethite NPs in artificial soil (Cáceres Wenzel et al. 2016). We have also reported in that previous study that during the first 48 h of exposure to goethite NPs using the filter paper contact test, earthworms showed increased Fe levels. Consequently, the early absorption of goethite NPs may be triggering a series of systemic

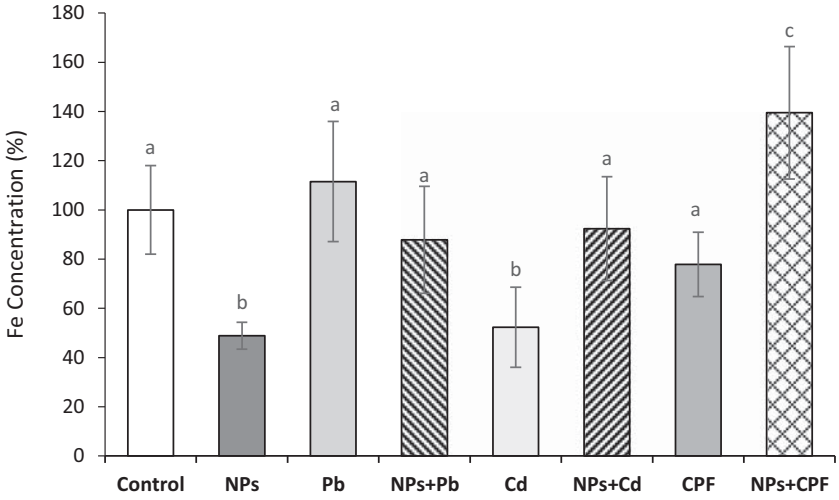

Fig. 2 Fe levels of earthworms exposed on filter paper. The results are expressed as percentage of Fe compared with control and represent the mean ± the standard deviation. Different letters indicate significant differences ($p < 0.05$). Acetone did not affect Fe levels; thus, only control organisms exposed to distilled water are shown. Control value, 54 ± 9 μg Fe/g tissue (wet weight). NPs, nanoparticles (50 μg/cm^2); CPF, chlorpyrifos (0.75 μg/cm^2); Cd (0.40 μg/cm^2); Pb (0.74 μg/cm^2)

effects that would later lead to the excretion of part of the Fe basal levels. Coincidentally, single exposure to Cd seemed to trigger an analogous systemic effect. According to the literature, non-essential metals like Cd and Pb tend to accumulate mostly in the posterior section of the earthworm, particularly in the digestive tract and in the chloragogen tissue (Morgan et al. 1989). This tissue contains granules called chloragosomes. Over time, chloragogen cells increase in size, separate from the epithelium, and release the chloragosomes to the coelomic fluid, from where the latter are eliminated through the nephridia (Karaca et al. 2010). It has been postulated that chloragosomes may be essential to the endogenous ion, acid-base, and electron balance, and in this way, they are involved in the homeostatic mechanisms (Fischer and Horvith 1977). There are different types of chloragosomes. One type consists of complexes rich in phosphates that bind metals such as Pb. A second type, named cadmosomes, contains ligands rich in thiol groups that bind Cd, mainly low molecular weight proteins such as metallothioneins (Karaca et al. 2010). It has recently been reported that dermal exposure of *E. fetida* to Cd induced the dose-dependent expression of metallothionein mRNA with a maximum induction factor of 11, whereas in the case of those exposed to Pb, the induction was much smaller, with a factor close to 2 (Homa et al. 2015). These differences in the behavior of Cd and Pb in the worms' tissues could be related to the differential effect of both metals on the regulation of Fe levels. However, further research is needed to confirm this hypothesis.

Unlike earthworms exposed for 72 h to NPs alone, those exposed to binary mixtures showed Fe levels similar (NPs+Metals) to or higher (NPs+CPF) than controls. These results indicate that the presence of other contaminants in the exposure media can modify the uptake and/or the excretion of Fe. However, it has to be taken into account that the experiments were done using a methodology that only involves dermal contact with the chemicals (filter paper contact test). Interestingly, in a recent study performed in soil (a methodology that evaluates toxicity through two routes: by direct contact with the contaminant and by ingestion), García-Gómez et al. (2019) also reported an increase in metal concentration in earthworms exposed to mixtures of ZnO NPs and CPF compared with controls and with those exposed to ZnO NPs alone. Similarly, other

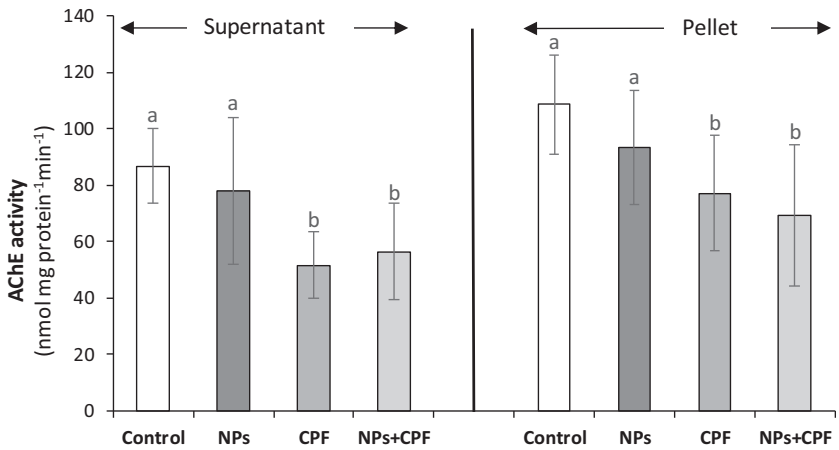

Fig. 3 AChE activity in earthworms exposed on filter paper. The activity was measured in the supernatant, and the pellet of 9000×*g* centrifugation. Solvent controls were performed by impregnating the filter papers with water and acetone as it is described in the "Materials and methods" section. The results represent the mean ± the standard deviation. Different letters indicate significant differences ($p < 0.05$). NPs, nanoparticles (50 μg/cm^2); CPF, chlorpyrifos (0.75 μg/cm^2)

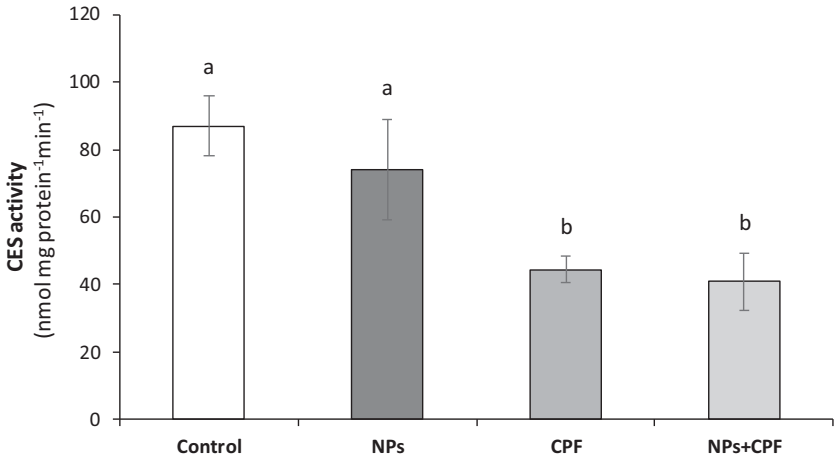

Fig. 4 CES activity in earthworms exposed on paper filter. Solvent control was performed by impregnating the filter paper with water and acetone as it is described in the "Materials and methods" section. The results represent the mean ± the standard deviation. Different letters indicate significant differences ($p < 0.05$). NPs, nanoparticles (50 μg/cm^2); CPF, chlorpyrifos (0.75 μg/cm^2)

studies have shown that the presence of organic compounds increased metal accumulation in animal and plant species exposed to metallic NPs (Fang et al. 2016; Ma et al. 2017).

AChE and CES activities

Determining the inhibition of ChEs is an important sublethal endpoint in the evaluation of exposure and effect by OP and carbamate pesticides. Caselli et al. (2006) characterized the activity of these enzymes in *E. andrei* and found that they are mainly represented by AChE. There are numerous studies on the effects of OP pesticides on earthworms, and most of them are focused on the study of individual pesticides or on binary mixtures of OPs (Booth and O'Halloran 2001; Collange et al. 2010; Velki and Hackenberger 2013a, b; Stepić et al. 2013; Chen et al. 2014). On the other hand, there is little information about the effects of NPs on ChEs of soil organisms.

Earthworms exposed to CPF showed a reduction in AChE activity. This inhibition was observed in pellet-AChE as well as in supernatant-AChE, suggesting that both enzyme sources have similar sensitivity to the OP, and using both toxicity methods. AChE activity was not affected in earthworms exposed to goethite NPs in any of the experiments. Similarly, García-Gómez et al. (2019) reported that ZnO NPs did not cause significant changes in AChE activity in *E. andrei* exposed (28 days) in soil at exposure concentrations even higher than the one used in the present study. In contrast, other authors have reported induction of this activity in *Enchytraeus albidus* earthworms exposed in artificial soil to high concentrations (750 mg/kg and 1000 mg/kg) of ZnO NPs (Hackenberger et al. 2019).

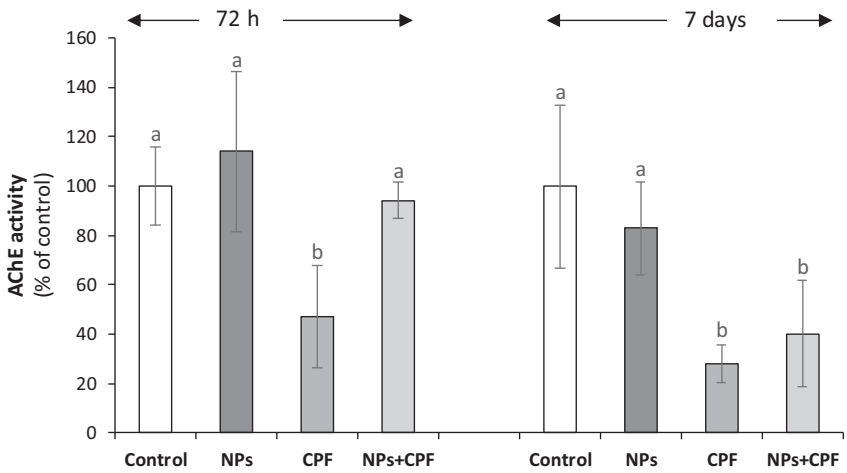

Fig. 5 AChE activity in earthworms exposed in artificial soil. Solvent control was performed by adding acetone and water as it is described in the "Materials and methods" section. The results are expressed as percentage of control and represent the mean ± the standard deviation. Seventy-two-hour control value, 41.9 ± 6.6 nmol mg protein^{-1} min^{-1}. Seven-day control value, 75.3 ± 24.8 nmol mg protein^{-1} min^{-1}. Means noted with different letters within each treatment duration are significantly different ($p < 0.05$). NPs, nanoparticles (100 mg/kg); CPF, chlorpyrifos (20 mg/kg)

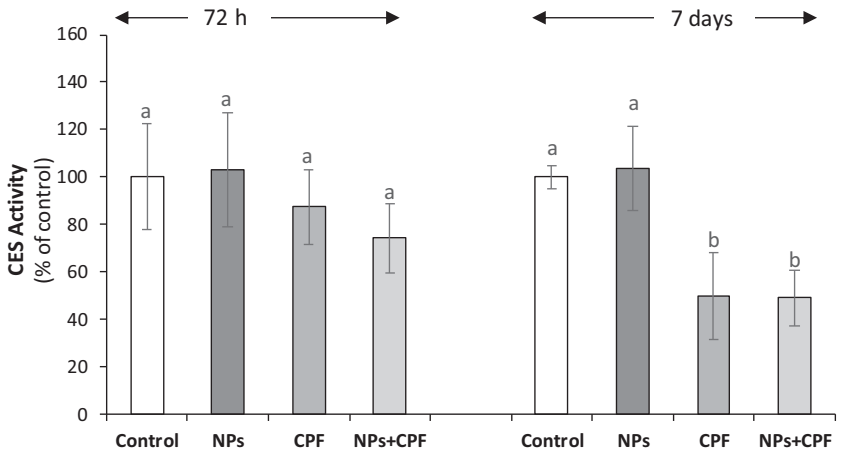

Fig. 6 CES activity in earthworms exposed in artificial soil. Solvent control was performed by adding acetone and water as it is described in the "Materials and methods" section. The results are expressed as percentage of control and represent the mean ± the standard deviation. Seventy-two-hour control value, 61.3 ± 13.7 nmol mg protein^{-1} min^{-1}. Seven-day control value, 98.1 ± 4.9 nmol mg protein^{-1} min^{-1}. Means noted with different letters within each treatment duration are significantly different ($p < 0.05$). NPs, nanoparticles (100 mg/kg); CPF, chlorpyrifos (20 mg/kg)

In regard to joint effects, the co-exposure with goethite NPs diminished the effect of the OP on AChE activity at a short exposure time in artificial soil (72 h). However, this behavior was not observed at 72 h using the filter paper contact test or at 7 days using the artificial soil test. Instead, in these last two assays, exposure to the mixture of NPs+CPF induced a similar degree of inhibition than single CPF exposure. Similarly, García-Gómez et al. (2019) reported that AChE activity in *E. andrei* earthworms exposed (28 days) to natural soil spiked with the mixture of ZnO NPs and CPF significantly diminished compared with control, and that this inhibition was similar to the one observed in organisms single exposed to CPF.

CES comprise a group of enzymes with low substrate specificity that has been frequently reported as more sensitive to OPs than ChEs in invertebrates (Wheelock et al. 2008; Cacciatore et al. 2013). To the best of our knowledge, the effects of metal oxide NPs on *E. andrei* CES activity have not been studied before. Present results using both toxicity tests followed the same trend as those for AChE. Earthworms exposed to the mixture showed similar inhibition of CES activity than the earthworms exposed only to CPF. On the contrary, goethite NPs did not inhibit the activity of CES. Several isoenzymes with CES activity are present in total tissue homogenates; thus, there are different substrates to measure their activity (Sanchez-Hernandez and Wheelock 2009). Our results using p-nitrophenyl butyrate as substrate are in agreement with the observation of Oneto et al. (2005) that CES activity in earthworms measured with phenylthioacetate as substrate would be equally sensitive to OP inhibition as ChEs activity. However, this would be valid if the test is performed using the artificial soil test at exposure times not less than 7 days, or the filter paper contact test.

Conclusions

Our study shows the importance of evaluating the uptake of NPs in co-exposure studies with other contaminants. In this regard, the use of the contact filter paper test proved to be a quick and easy alternative for assessing combined effects of mixtures in earthworms.

Fe levels in the earthworms exposed only to goethite NPs were lower than basal values, suggesting systemic effects. On the contrary, earthworms exposed to binary mixtures showed Fe levels similar (NPs+Metals) to or higher (NPs+CPF) than controls. These differences in the pattern of Fe accumulation indicate that the presence of other contaminants in the exposure media can modify the uptake and/or the excretion of Fe and evidence the interactions that may be found in binary mixtures of metal oxide NPs and other pre-existing contaminants in the soil ecosystem.

E. andrei exposed to the binary mixture of NPs+CPF showed similar inhibitions in AChE and CES activities to organisms exposed only to CPF both in the paper filter contact test and in the artificial soil test (7-day exposure). Furthermore, the enzymatic activities in the individuals exposed only to NPs were comparable with those registered in control organisms. This suggests that NPs did not produce neurotoxic effects, and that the inhibition of the enzymes' activities in all cases was due to the presence of the pesticide.

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Author contributions The experiments on artificial soil were performed by JSF, MICW, and FNB; MICW, JSF, and ACC wrote the manuscript.

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