Mixture toxicity study of metal oxide nanoparticles and chlorpyrifos on earthworms at low concentrations: a short-term exposure approach

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Abstract

Co-exposure soil studies of pollutants are necessary for an appropriate ecological risk assessment. Here, we examined the effects of two-component mixtures of metal oxide nanoparticles (NPs, ZnO or goethite) with chlorpyrifos (CPF) under laboratory conditions in short-term artificial soil assays using *Eisenia andrei* earthworms. We evaluated effects on metal accumulation, oxidative stress enzymes, and neurotoxicity related biomarkers in single and combined toxicity assays.

Exposure to ZnO NPs increased Zn levels compared to control in single and combined exposure (ZnO NPs + CPF) at 72 h and 7 days, respectively. In contrast, there was no indication of Fe increase in organisms exposed to goethite NPs. One of the most notable effects on oxidative stress biomarkers was produced by single exposure to goethite NPs. This shows that the worms were more sensitive to goethite NPs than to ZnO NPs, regardless of nano-structure. Statistically significant differences between single and combined exposure were found for catalase and superoxide dismutase (goethite NPs) and for glutathione S-transferase (ZnO NPs) activities, mostly at 72 h. Acetylcholinesterase and carboxylesterase activities indicated that ZnO NPs were not neurotoxic to earthworms and similar degrees of inhibition were observed after single CPF and ZnO NPs + CPF exposure. These findings suggest a necessity to evaluate mixtures of NPs with co-existing contaminants in soil, and that the nature of metal oxide NPs and exposure time are relevant factors to be considered when assessing combined toxicity, as it may have an impact on ecotoxicological risk assessment.

1. Introduction

The successful synthesis of nanoparticles (NPs) showing fascinating physicochemical properties has facilitated the golden age of these industries. As a result, this has brought NPs into many products and applications (Rizwan et al. 2017). Among them, metal oxide nanoparticles (MO-NPs) are widely used because of their distinct electronic, mechanical, magnetic and photocatalytic properties (Khan et al. 2017). For example, engineered iron and iron oxides NPs have become important in various areas as they have broad industrial applications, like manufacturing, materials science, and remediation (Waychunas et al. 2005; Baragaño et al. 2020). In addition, zinc oxide nanoparticles (ZnO NPs) are among those used in cosmetics products for personal use. They also are employed in pharmaceutical products, industrial coatings, plastics, wood, pigments, sensors or electronic and optical devices such as solar cells, (Mu and Sprando 2010; Ma et al. 2013; Brunetti et al. 2015) among other uses. As a consequence of their extensive applications, they can reach terrestrial compartments through deliberate or unintentional release. Therefore, soils are not only sources of naturally occurring NPs (Nowack & Bucheli, 2007; Waychunas et al. 2005), but also one of the major sinks of engineered NPs (Klaine et al. 2008; Rajput et al. 2018). Once in the soil, there is a possibility that engineered NPs accumulate in non-target organisms and exert harmful effects on them (Martínez et al. 2021).

The most accepted toxic mechanism of MO-NPs is the occurrence of oxidative stress induced by reactive oxygen species (ROS) (Wu et al. 2014; Dayem et al. 2017). The unique properties of MO-NPs, particularly
the reactive surface area, are correlated with their potential to generate ROS that are associated with toxic effects. In cells, protection against ROS is achieved by non-enzymatic and enzymatic antioxidant defense systems (e.g. superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), among others) (Bernard et al. 2015). SOD catalyzes the disproportionation of superoxide anion to oxygen and hydrogen peroxide, and CAT breaks down hydrogen peroxide into water and oxygen. GST, a phase II detoxification enzyme, conjugates electrophilic compounds with GSH (Bernard et al., 2015; Sanchez-Hernandez, 2006; Stephensen et al., 2002). When ROS production exceeds antioxidant defenses, a redox imbalance is produced within cells leading to macromolecular oxidative damage (Dayem et al. 2017). As such, the determination of oxidative stress related to MO-NPs may help address concerns about the impact of biological exposure to these type of nanoparticles.

Due the complexity of the soil ecosystem and strong interactions with human activities, MO-NPs may probably interact with other components naturally present in the soil environment, or related to industrial and agricultural activities, among others. Additionally, pollutants such as metals and pesticides, can reach soils forming complex mixtures of chemicals. Therefore, soil organisms can be affected by mixture effects due to co-exposition to different pollutants (Kabir et al. 2018). Besides, NPs may function as chemicals carriers, enabling the uptake of substances into cells (Naasz et al 2018).

CPF is an organophosphate (OP) insecticide, acaricide and miticide, and as other OPs exerts its acute toxicity through inhibition of the activity of acetylcholinesterase (AChE), an enzyme that hydrolyzes the neurotransmitter acetylcholine (Thompson and Richardson 2004). Several authors have concluded that the other enzymes that could be altered by OP exposure and that can be used to monitor OP exposition are carboxylesterases (CES) (Jansen et al. 2009; Cacciatore et al. 2013; Sanchez-Hernandez et al. 2015).

Recent long-term studies (28 or 56 days) have reported joint effects of co-exposure to CPF and ZnO NPs on earthworms (García-Gómez et al. 2019; Lončarić et al. 2020). Regarding goethite, we have already demonstrated, using the paper contact test, the combined effects of these NPs plus different heavy metals or CPF on the pattern of Fe accumulation (Cáceres-Wenzel et al. 2020). Moreover, short (72 h) co-exposure to goethite NPs + CPF in artificial soil reduced OP-induced AChE inhibition

Altogether, these results show the need to perform co-exposure studies for a correct evaluation of the ecological risk associated to the increase presence of engineered NPs in soils. Therefore, the purpose of this investigation was to deepen the knowledge of the extent of adverse effects that the interaction between MO-NPs and OPs produce towards earthworms. To this end, we comparatively investigated the possible differences in single and combined toxicity to goethite NPs, ZnO NPs and a mixture of each NP with an organic contaminant (CPF) on Eisenia andrei earthworms in short-term artificial soil assays. We evaluated effects on metal accumulation, oxidative stress enzymes (CAT, GST and SOD) and neurotoxicity related biomarkers (AChE and CES). These findings will help to better understand the potential risk that these NPs and CPF may produce to the terrestrial ecosystem.

2. Materials And Methods
2.1. **Chemicals**

Water suspensions of ZnO NPs (45% w/w, 30 nm) and goethite NPs (20% w/w, 100 nm) were from Sigma-Aldrich (Argentina). The morphology and average diameter was examined using a NTS-Supra 40 Carl Zeiss scanning electron microscope. Prior to the bioassays, the stock and working NPs dispersion were sonicated for 30 minutes.

A commercial formulation of chlorpyrifos (Terminator Ciagro®, Ciagro S.A., Argentina) was used in the assays. Acetylthiocholine iodide, bovine serum albumin (BSA), chlorodinitrobenzene (CDNB), 5,5′ dithiobis-2 nitrobenzoic acid (DTNB), glutathione (GSH), hydrogen peroxide (H₂O₂), methionine, p-nitrophenyl butyrate, nitro blue tetrazolium (NBT), riboflavin, were from Sigma–Aldrich (Argentina). In all assays, otherwise detailed, analytical grade chemicals were used.

2.2. **Earthworms**

As previously described (Cáceres-Wenzel et al. 2020), adult *E. andrei* (Oligochaeta, Lumbricidae) earthworms were from our own laboratory culture stocks. Earthworms with well-developed clitellum were left to purge their gut content on damp filter paper for 24 h (in the dark at 22°C). Afterwards, they were washed with tap water, dried and weighed (0.30–0.50 g).

2.3. **Artificial Soil Test**

The test was carried out with OECD soil (OECD 1984) with two exposure periods, 72 h and 7 days. The soil was constituted of 10% ground sphagnum peat (organic matter), 20% kaolinite clay, and 70% fine sand. Calcium carbonate was added to adjust the pH to 6.0 ± 0.5.

Soil was spiked with CPF to a final concentration of 18 mg/kg soil dry weight (DW). ZnO NPs or goethite NPs were added as water suspensions (to 75% of the soil water holding capacity, WHC) to obtain a concentration of 100 mg NPs/kg soil (DW). Soil for co-exposure assays, was treated with CPF and then was mixed with NP suspensions as previously stated. Distilled water was added to control and CPF treatments to attain the same water holding capacity. The spiking procedure as well as the chosen CPF concentration was described in a previous study (Cáceres-Wenzel et al. 2020). Control and other soil treatments were placed in 120-mm diameter/70-mm height containers (replicated 3 times). Six earthworms were added in each container protected with perforated plastic film. The assays were performed in a room at 20 ± 2°C under 16h L-8h D photoperiod. After 72 h and 7 days, earthworms were removed, rinsed with distilled water, transferred to plastic Petri dishes with moist paper filter to void their gut content, weighed and finally stored at -80°C until use.

2.4. **Zn and Fe levels**

The Zn and Fe total concentrations were quantified by flame atomic absorption spectroscopy (575 AA Varian; Springvale, Australia) after a digestion process with ultrapure concentrated nitric acid performed in an Anton Parr Multiwave GO plus microwave digestor (Graz, Austria).
2.5. **Biochemical analysis**

Exposed individuals were homogenized (1:3 w/v) with addition of ice cold 100 mM Tris buffer (pH 7.4) by a mechanical homogenizer. Then, the homogenates were centrifuged for 30 min at 9000×g at 4°C to obtain the post-mitochondrial fraction (supernatant), which was stored at -80°C for future assays of CAT, GST, SOD, AChE and CES activity. The protein content was determined by the Lowry assay (Lowry et al., 1951).

2.5.1. **CAT**

CAT activity was determined by the method of Saint-Denis et al. (1998). The H$_2$O$_2$ dismutation was monitored over a 2-min period at 240 nm and 25°C. The reaction mix contained H$_2$O$_2$ 9.2 mM in Tris 100 mM pH 7.5 buffer and supernatant. An extinction coefficient ($\varepsilon$) of 0.04 mM$^{-1}$ cm$^{-1}$ was used to calculate H$_2$O$_2$ concentration. The results were expressed as µmol of degraded H$_2$O$_2$ min$^{-1}$ mg of protein$^{-1}$.

2.5.2. **GST**

GST activity was determined using CDNB and GSH as substrates and monitoring the appearance of the conjugated complex (GS-CDNB, $\varepsilon$ = 9.6 mM$^{-1}$ cm$^{-1}$) at 340 nm (Habig and Jakoby 1981). The reaction mix contained phosphate 100 mM pH 6.5 buffer, 50 mM GSH and 50 mM CDNB. The results were expressed as nmol of conjugate min$^{-1}$ mg of protein$^{-1}$.

2.5.3. **SOD**

SOD activity was measured according to the method of Giannopolitis and Ries (1977) in 96 wells microplates (Causin et al. 2020). The reaction mix contained an O$_2$·$^-$/ generating solution (14.3 mM methionine, 82.5 µM NBT, 2.5 µM riboflavin), 50 mM potassium phosphate pH 7.0 buffer + 0.1 mM Na$_2$EDTA, and supernatant. An MIR-153 Incubator (SANYO, Gunma, Japan) was used to illuminate microplates for 4, 8 and 12 min at a controlled temperature of 20 ± 1°C. After incubation, absorbance readings were taken at 570 nm in an RT-2100C microplate reader (Rayto, Shenzhen, Germany). One unit of SOD was defined as the amount of sample that inhibits the photoreduction of NBT by 50%.

2.5.4. **AChE**

AChE activity was measure following Ellman et al. (1961). The reaction mix contained 50 mM Tris buffer pH 8, 0.25 mM DTNB, and 12 mM acetylthiocholine iodide as substrate. Absorbance was read continuously at 412 nm and was amended for spontaneous hydrolysis of acetylthiocholine iodide. An $\varepsilon$ of 13.6 mM$^{-1}$ cm$^{-1}$ was used for the calculations. AChE activity was expressed as nmol of acetylthiocholine hydrolyzed min$^{-1}$ mg of protein$^{-1}$.

2.5.5. **CES**
CES activity was measured following the method of Cacciatore et al., (2013) using p-nitrophenyl butyrate as substrate and recording continuously the absorbance at 400 nm. The reaction mix contained 100 mM phosphate buffer pH 8.0 + 5% acetone, 1 mM p-nitrophenyl butyrate, and sample. CES activity was quantified using an \( \varepsilon \) of 18.6 mM\(^{-1}\) cm\(^{-1}\) (p-nitrophenol). The activity was expressed as nmol of product min\(^{-1}\) mg of protein\(^{-1}\).

### 2.6. Statistical Analysis

Results were expressed as mean ± SD and analysed using the Instat Graph Pad software (version 3.01, GraphPad Software, La Jolla, CA, USA, [www.graphpad.com](http://www.graphpad.com)). One-way ANOVA analyses were carried out and inter-group comparisons were made using the Tukey test. Prior to the analysis, data were tested for normality and variance homoscedasticity. In all cases, the level of significance was 0.05.

### 3. Results

#### 3.1. Metal accumulation

Single CPF exposure did not affect Zn or Fe levels of earthworms exposed for 72 h or 7 days (Fig. 1). On the other hand, Zn increased significantly in earthworm tissue (37%, \( p < 0.05 \)) compared to control in single ZnO NPs exposure for 72 h. However, there was no evidence of Zn uptake in organisms exposed to the mixture (\( p > 0.05 \)) (Fig. 1A). At day 7 of single exposure to ZnO NPs, there were no changes in the Zn levels compared to control, but a statistically significant slight increase of this metal (15%) was found in organisms exposed to the binary mixture (Fig. 1B). On the contrary, Fe uptake was not registered in organisms exposed to goethite NPs and the mixture for 72 h or 7 days (\( p > 0.05 \); Fig. 1C and D, respectively).

#### 3.2. Oxidative stress enzyme activities

**3.2.1. Single CPF exposure**

CPF exposure impacted CAT activity at 72 h, as an increase of 58% was registered in organisms exposed to the pesticide compared to controls (Fig. 2A and D). Nevertheless, no changes were observed in CAT activity after 7 day-exposure (\( p > 0.05 \)) (Fig. 3A and D). Likewise, CPF had no effect on GST or SOD activity at any exposure time (Fig. 2 and Fig. 3).

**3.2.2. Effects of MO-NPs and MO-NPs + CPF mixtures**

As presented in Fig. 2A, B and C, single exposure to ZnO NPs for 72 h had no effect on CAT, GST or SOD activities compared to controls. Only a considerable increase (78%; \( p < 0.05 \)) in GST activity was observed after 72 h exposure to ZnO NPs + CPF (Fig. 2B).
After 72 h, earthworms single exposed to goethite NPs showed a significant increase in CAT (64%, Fig. 2D) and also in GST activity (63%, Fig. 2E) compared to untreated worms (p < 0.05). SOD activity was not modified (Fig. 2F). The mixture of goethite NPs + CPF did not alter CAT and GST (p > 0.05), but an inhibition of 63% of SOD activity was observed (p < 0.05) (Fig. 2D, E and F).

Exposure to MO-NPs or to MO-NPs + CPF for 7 days (Fig. 3) did not modify CAT, GST and SOD activities, with only one exception: worms single treated with goethite NPs showed a significant decrease in SOD activity (p < 0.05) (Fig. 3F).

### 3.3. AChE and CES activities

Regarding AChE and CES activities (Figs. 4 and 5, respectively), earthworms exposed to CPF showed statistically significant inhibitions of 53% and 72% in AChE activity compared to controls after 72 h and 7 days, respectively, and a decrease of 59% in CES at day 7 (p < 0.05). Earthworms treated with ZnO NPs + CPF showed a significant AChE inhibition (47% and 76%, respectively compared to control) after 72 h and 7 days, and an inhibition of 61% in CES activity only at day 7 (p < 0.05). Single ZnO NPs treatment did not exert significant differences in the activity of these enzymes compared to controls (p > 0.05).

### 4. Discussion

In this study, we focused on possible differences in the uptake of metals by single and combined exposure to two MO-NPs with the pesticide CPF on *E. andrei* earthworms. We also studied the effects of these pollutants on oxidative stress and neurotoxicity related biomarkers.

Our results concerning single exposition to ZnO NPs suggest that earthworms might be able to regulate intracellular Zn by excreting the remaining fraction and that this effect may take time. In a previous report, Świątek et al. (2017) studied the toxicokinetics of Zn in *E. andrei* up to 21 days and found significant uptake of Zn in earthworms treated with 500 and 1000 mg/kg of ZnO NPs in the first days. The highest average internal Zn levels were attained at day 2 and 4, respectively. Then, they observed a decrease in metal concentration similar to basal levels, concluding that earthworms could regulate Zn efficiently. Our results are in agreement with this as we observed a significant increase in Zn concentration after 3 days and a later decrease in internal metal concentration in earthworms exposed to 100 mg/kg of ZnO NPs. In contrast, present results show that after 72 h oligochaetes exposed to the mixture showed basal Zn levels, while an increase in metal concentration was detected only after 7 days. García-Gómez et al. (2019) observed a similar trend in earthworms exposed to different mixture ratios of ZnO NPs and CPF for 21 days. Li et al. (2020) reported that the presence of bifenthrin, a pyrethroid insecticide, augmented the accumulation of ZnO and CuO NPs in *E. fetida* earthworms after 7, 14 and 21 days. The cause of these findings is still not well known and requires more understanding about the effects of the mixture on the earthworm body system and the relevance of physicochemical interactions between the pollutants that could alter the metal bioavailability.
It is well known that terrestrial invertebrates are able to regulate until some extent the accumulation of Fe (van Gestel et al. 1993; Świątek et al. 2017). In previous investigations involving artificial soil, we reported a decrease in Fe levels after 10-day exposure to 10, 100 and 1000 mg/kg of goethite NPs (Cáceres Wenzel et al. 2016) which suggested that the exposure of earthworms of the species *E. andrei* to these NPs can promote a decrease in basal Fe levels over time. Present results indicate that at shorter times (72 h and 7 days), earthworms exposed to goethite NPs were able to regulate Fe levels so as to maintain them similar to control levels. Interestingly, in studies using the paper contact test method we observed an increase in Fe levels after 24 and 48 h treatment with goethite NPs and a later decrease below basal levels after 72 h (Cáceres Wenzel et al. 2016; Cáceres-Wenzel et al. 2020). One of the reasons of the discrepancies with the present results might be associated with the methodological approach employed since the physicochemical properties of NPs and their bioavailability rely on the surrounding matrix properties (Peijnenburg et al. 2016). Similarly, present results showed no differences in Fe levels between single and combined exposures using artificial soil but, in studies using the paper contact test method, combined exposure to goethite NPs + CPF increased metal uptake after 72 h (Cáceres Wenzel et al. 2016; Cáceres-Wenzel et al. 2020).

Antioxidant enzymes have commonly been employed as biomarkers to determine pollutants' effects. Regarding single CPF exposure, our results indicate that CPF can affect oxidative stress biomarkers, as we found an increase in CAT activity after 72 h. This is in agreement with Torabi Farsani et al. (2021), as they registered an elevated CAT activity in *E. fetida* treated with different concentrations of CPF. On the other hand, our results show that ZnO NPs did not alter *E. andrei*’s enzymatic antioxidant defense at any time of exposure. In contrast, single exposure to goethite NPs affected several oxidative stress biomarkers. Regarding combined exposures, we observed a rise in GST activity and a decrease in SOD activity in earthworms 72h exposed to ZnO NPs + CPF and goethite NPs + CPF, respectively. In both cases these effects reversed after 7 days, suggesting that they were transient. To our knowledge, short-term toxicity approaches for these mixtures, have not been previously documented. Recently, García-Gómez et al. (2020) showed that earthworms exposed to natural soils treated with ZnO NPs and its mixture with CPF for 28 days did not cause significant biochemical alterations in CAT and GST activity.

In a previous study, we did not register alterations on AChE activity in earthworms after goethite NPs exposure. (Cáceres-Wenzel et al. 2020). In agreement with the latter, our present results show that single exposure to ZnO NPs did not alter the activity of AChE. Regarding the mixture of goethite NPs + CPF we have previously reported a diminution of the inhibition of CPF on AChE in earthworms exposed for a short time (72 h), and an enzyme inhibition similar to single CPF exposure in organisms exposed for a longer period (7 days) (Cáceres-Wenzel et al. 2020). However, present results show that the mixture of ZnO NPs + CPF did not follow this pattern. AChE activity showed an inhibition similar to single CPF exposure at both times of exposure. A possible explanation for this observation is that at shorter time exposures goethite NPs, but not ZnO NPs, might be exerting a potential enzyme protective effect against CPF-induced neurotoxicity. This would be related to the adsorption of CPF onto goethite NPs, consequently preventing it from inducing its effect on AChE.
Regarding CES activity, our prior study (Cáceres-Wenzel et al. 2020) showed that goethite NPs + CPF mixture produced the same degree of inhibition as the one seen in earthworms treated with CPF alone. Goethite NPs, on the other hand, had no effect on CES activity. Present results are in agreement with this, as single exposure of ZnO NPs did not alter CES activity; meanwhile the mixture showed an inhibition similar to the one observed in earthworms single exposed to CPF after 7 days. As tissue homogenates contain several CES isoenzymes, measuring their activity requires a variety of substrates (Sanchez-Hernandez et al., 2009). In our case, CES activity measured with p-nitrophenylbutirate as substrate resulted equally sensitive to inhibition by CPF and binary mixtures of MO-NPs + CPF.

5. Conclusions

The results obtained in this co-contaminant study have shown that both investigated MO-NPs and their mixtures with the OP CPF affect *E. andrei* earthworms at different levels. The concentrations of the pollutants tested in these experiments are lower than the ones previously reported in the literature and thus, more “environmentally realistic”.

Earthworms exposed to ZnO and goethite NPs effectively regulated Zn and Fe levels in tissues and only the binary mixture of ZnO NPs + CPF enhanced metal accumulation after 7 days. This suggests that the occurrence of other contaminants in soil may affect the toxicokinetics of Zn.

Among the biomarkers evaluated, oxidative stress enzymes were affected by goethite NPs, while fewer alterations were observed in goethite NPs + CPF, ZnO NPs, and ZnO NPs + CPF groups. This indicates that earthworms were more sensitive to goethite NPs than to ZnO NPs exposure regardless of nano-structure. However, the effects were time-dependent and mostly transient.

ZnO NPs did not produce neurotoxic effects, as AChE and CES activities in single NPs exposure were similar to controls. In addition, we have already reported comparable results for goethite NPs which suggests a poor or null ability of these MO NPs to exert neurotoxic effects in *E. andrei*. The comparison of the inhibitions induced by CPF alone and ZnO NPs + CPF indicate that they are due to the presence of CPF. This differs from results previously reported that suggest a potential protective effect of goethite NPs against CPF inhibition at short times of exposure (Cáceres-Wenzel et al. 2020).

Altogether, our findings demonstrate how important is the assessments of mixtures of NPs with co-existing contaminants in soil, as this may have an impact on the ecological risk assessment. Factors such as the nature of MO-NPs and exposure time should be considered when assessing MO-NPs + CPF mixtures.

**Abbreviations**

AChE: acetylcholinesterase, BSA: bovine serum albumin, CES: carboxylesterase, CAT: catalase, CDNB: chlorodinitrobenzene, CPF: chlorpyrifos, DTNB: 5,5′ dithiobis-2 nitrobenzoic acid, GST: glutathione-S-

**Declarations**

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**Author Contributions**

**Marcela I. Cáceres-Wenzel:** Methodology, Investigation, Formal analysis, Writing- Original draft preparation, Writing- Reviewing & Editing; **Florencia N. Bernassani:** Investigation, Formal analysis, Writing- Original draft preparation, **Julio S. Fuchs:** Methodology, Conceptualization, Supervision; **Eduardo Cortón:** Original draft preparation, Resources, Funding acquisition, **Adriana C. Cochón:** Conceptualization, Resources, Writing- Reviewing & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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**Declarations**

**Ethics approval**

Not applicable

**Consent to participate and publish**

All coauthors (Marcela I. Cáceres-Wenzel, Florencia N. Bernassani, Julio S. Fuchs, Eduardo Cortón and Adriana C. Cochón) have approved the manuscript and agreed with the submission.

**Competing interests**

The authors have no relevant financial or non-financial interests to disclose.

**Data Availability**

This article has all the data that were generated and processed during this study. On reasonable request, additional datasets can be supplied by the corresponding author.

**References**


Figures
Figure 1

Zn and Fe levels of *E. andrei* earthworms exposed to ZnO NPs, goethite NPs and their binary mixtures with CPF for 72 h and 7 days. The concentration of Zn or Fe relative to the control is stated as a percentage (mean ± SD). Significant differences are indicated by different letters (p<0.05). NPs (100 mg/kg soil); CPF (18 mg/kg soil)
Figure 2

CAT, GST and SOD activities of *E. andrei* earthworms exposed to ZnO NPs, goethite NPs and their binary mixtures with CPF for 72 h. The data are presented as a percentage of control (mean ± SD). Significant differences are indicated by different letters (p<0.05). NPs (100 mg/kg soil); CPF (18 mg/kg soil)
Figure 3

CAT, GST and SOD activity of *E. andrei* earthworms exposed to ZnO NPs, goethite NPs and their binary mixtures with CPF for 7 days. The data are presented as a percentage of control (mean ± SD). Significant differences are indicated by different letters (p<0.05). NPs (100 mg/kg soil); CPF (18 mg/kg soil)
Figure 4

AChE activity of *E. andrei* earthworms exposed to ZnO NPs and the binary mixture with CPF for 72 h and 7 days. The data are presented as a percentage of control (mean ±SD). Significant differences are indicated by different letters (p<0.05). NPs (100 mg/kg soil); CPF (18 mg/kg soil)

Figure 5
CES activity of *E. andrei* earthworms exposed to ZnO NPs and the binary mixture with CPF for 72 h and 7 days. The data are presented as a percentage of control (mean ± SD). Significant differences are indicated by different letters (p<0.05). NPs (100 mg/kg soil); CPF (18 mg/kg soil).