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EFFECTS OF AGEING AND SOIL PROPERTIES ON ZINC OXIDE NANOPARTICLE  
AVAILABILITY AND ITS ECOTOXICOLOGICAL EFFECTS TO THE EARTHWORM

*EISENIA ANDREI*

Running title: Bioavailability of ZnO nanoparticles

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**Abstract:** To assess the influence of soil properties and ageing on the availability and toxicity of Zn applied as nanoparticles (ZnO NPs) or as Zn<sup>2+</sup> ions (ZnCl<sub>2</sub>), three natural soils were individually spiked with either ZnO NPs or ZnCl<sub>2</sub> and incubated for up to 6 months. Available Zn concentrations in soil were measured by pore water extraction (ZnPW), while exposures of earthworms (*Eisenia andrei*) were performed to study Zn bioavailability. ZnPW was lower when Zn was applied as nanoparticles than as ionic form, and decreased with increasing soil pH. ZnPW for both Zn forms were affected by ageing, but varied among the tested soils, highlighting the influence of soil properties. Internal Zn concentration in the earthworms (ZnE) was highest for the soil with high organic carbon content (5.4%) and basic pH (7.6) spiked with ZnO NPs, but the same soil spiked with ZnCl<sub>2</sub> showed the lowest increase in ZnE compared to the control. Survival, weight change, and reproduction of the earthworms were affected by both Zn forms, but differences in toxicity could not be explained by soil properties or ageing. This shows that ZnO NPs and ZnCl<sub>2</sub> behave differently in soils depending on soil properties and ageing processes, but differences in earthworm toxicity remain unexplained. This article is protected by copyright. All rights reserved

**Keywords:** Ageing, Availability, Ecotoxicity, Nanoparticles, Zinc

## INTRODUCTION

The use of nanoparticles (NPs) in a variety of applications has exponentially increased over the last 30 years [1]. As a consequence, manufactured NPs are increasingly entering the environment [2], but only limited data is available on their potential hazard [3]. The high production of NPs as well as their potential for release into the environment, and subsequent effects on ecosystem health are becoming a growing concern. A thorough knowledge of the behavior and effects of NPs in environmental media is essential for risk assessment [4].

Zinc oxide (ZnO) is one of the most commonly used types of metal-based NPs, having the third highest annual production volume [5]. ZnO NPs are used in electronics applications, solar panel devices, medicine, cosmetics, sunscreens (UV-filters), and applied as antibacterial agents [6]. ZnO NPs can enter the environment via waste water at industrial sites or through domestic sewage; and by the application of sewage sludge in agriculture they may also end up in soil [7].

The distribution, mobility, and bioavailability of Zn in soils are controlled by a range of physicochemical characteristics, including the nature and heterogeneity of the soil constituents, the surface charge of soil colloids, and variations in soil pH and redox status [8]. The properties of nanoscale materials may differ substantially from those of the respective bulk materials [9]. Under the influence of soil properties, such as pH and organic matter content, ZnO NPs show a high variability in bioavailability and toxicity [10-12]. For assessing their hazard and potential risk in soil, it is essential to determine under which conditions and how ZnO NPs exert their ecotoxicological effects. Unfortunately, the ecotoxicity studies available show a significant lack of characterization of the exposure of soil organisms to ZnO NPs. Once in the soil, complex processes can affect NPs, which can act as colloids. NPs may form aggregates or agglomerates, which can lead to sedimentation, or may be prone to dissolution and release of free metal ions [13]. Upon contact with water, ZnO NPs partially dissolve to Zn ions ( $Zn^{2+}$ ), so their effects may partly be due to the soluble forms of Zn [14]. This process may also occur in soil, and it therefore is essential to

substantiate whether Zn toxicity is produced by the NP forms, as well as the free ions released in soil.

It has been observed that long time periods are required for ionic Zn to reach equilibrium in spiked soils, a process called ageing [15]. In soils spiked with ZnO NPs the same processes occur [16], while further dissolution to ionic Zn forms means that it may take even longer time to reach apparent equilibrium compared to soils directly spiked with ionic Zn. These long-term processes have shown to decrease bulk Zn toxicity in soil over time, while ageing also seems to reduce ZnO NP toxicity (and bioavailability) in soil, as was shown for springtails by Waalewijn-Kool et al. [17]. Hence future ecotoxicity tests with ZnO NPs should focus on their long-term effects in relation to their fate and bioavailability [6]. The difference in Zn bioavailability between freshly spiked and aged soils may also be explained by major soil properties [18].

To assess their potential ecotoxicological risk, metal bioaccumulation and effects in soil organisms have been studied [19-20]. Earthworms are common in a wide range of soils and have largely been used in bioassays for evaluating hazardous chemicals in soils [21]. Earthworms are more susceptible to metal pollution than many other soil invertebrates, and have a number of characteristics (large size, behaviour, and high biomass) that make them highly suitable for use as bioindicator organisms for determining the toxicity of chemicals in soil [22]. As so-called eco-engineers, they play an important role in decomposition and soil-forming processes, but they also can easily accumulate chemicals from soil and subsequently, as important prey, introduce them into the food chain [19]. Consequently, earthworms have been adopted as standard organisms for ecotoxicological testing [23].

The purpose of the present study was to determine the influence of ageing on the availability and toxicity of ZnO NPs to earthworms (*Eisenia andrei*) in soils with contrasting properties. Three natural soils with different physicochemical soil properties were selected to assess the fate and effects of ZnO NPs. In addition, an ionic Zn ( $ZnCl_2$ ) treatment was included for comparison. Pore water extraction was applied to assess changes in the availability of the two studied Zn forms over a

6-month ageing period. Bioavailability and toxicity were measured by exposing *E. andrei* to ZnO NPs or ZnCl<sub>2</sub> aged in soils for different periods of time. Internal Zn concentrations in the animals, survival, weight change, and reproduction were measured to evaluate Zn bioavailability and toxicity.

## MATERIALS AND METHODS

### *Soils*

Three uncontaminated soils with contrasting properties were selected from different countries. Two soils were collected from the surface horizon of fields in Spain (SPCA; forestland in Granada) and the Netherlands (NLGA; a garden in Bilthoven), homogenized, 5 mm sieved and air dried. The third soil was the LUFA 2.2 natural standard soil (LUFA Speyer, Germany). Before the start of the tests, the following physicochemical properties were determined: soil pH in 1 M potassium chloride (soil:KCl, ratio 1:2.5 [w:v]) and pH in pore water; electric conductivity (EC); calcium carbonate content (CaCO<sub>3</sub>), organic carbon (OC) content, particle-size distribution, and cation exchange capacity (CEC), according to Romero-Freire et al. [24] and water holding capacity (WHC) [25] (Table 1).

Soils were spiked in the laboratory with ZnO NPs (Nanosun Zinc Oxide P99/30) with a primary particle diameter size of 20-40 nm (Figure SI-1). To study the effect of dissolved (ionic) Zn, one treatment with the soluble salt ZnCl<sub>2</sub> (Merck, zinc chloride pure) was included. Test concentrations were based on toxicity data obtained in an earlier study with the same type of ZnO NPs [11]. The spiking concentrations correspond with EC10 and EC50 for the effects of ZnO NPs, and EC50 for effects of ZnCl<sub>2</sub> on earthworm reproduction. The selected dosing levels were adjusted to take into account the influence of soil pH and CEC on the effect concentrations. Test concentrations of ZnCl<sub>2</sub> were 500 mg Zn kg<sup>-1</sup> in LUFA and NLGA, and 1250 mg Zn kg<sup>-1</sup> in SPCA; ZnO NP concentrations tested were 500 and 1000 mg Zn kg<sup>-1</sup> in LUFA and NLGA, and 1250 and 2500 mg Zn kg<sup>-1</sup> in SPCA. Uncontaminated controls were also included.

The ZnO NPs were mixed into the soils as a powder in order to avoid dissolution of the particles prior to addition to the soil, while ZnCl<sub>2</sub> was introduced as an aqueous solution. Soils were intensively mixed with a spoon to avoid modifying the NPs, to achieve as homogenous distribution of the Zn as possible. After spiking, soils were moistened to 50% of their WHC. Soils were dosed as a single batch which was then split into separate aliquots for each time point and replicate. Soils were incubated in a climate room (Weiss Technik Benelux B.V., Rotterdam, The Netherlands) at 20°C and 75% relative air humidity, with a light/dark cycle of 12/12 h. Soil moisture content was checked weekly by weighing the test containers and if needed readjusted.

#### *Extraction procedure to assess Zn availability*

To assess available Zn concentrations, two methods were applied: pore water extraction and extraction with a diluted Cu(NO<sub>3</sub>)<sub>2</sub> solution. We expected that the latter method would allow discrimination between particulate and freely available Zn forms. Since this method did not seem to work as expected, we will not discuss this further; we included a brief description in the Supporting Information.

At 1, 3, 56, and 168 d after spiking the soils, pore water extractions were performed. For that purpose, 50 g soil samples were placed in Teflon containers and moistened to 100% WHC, mixed and equilibrated for 7 d at room temperature. Soils were then centrifuged for 45 min at 2000 × g at 10 °C, through a 0.45 µm membrane filter (Whatman NC45, cellulose nitrate diameter 47 mm) placed between two circular filters (Whatman filter paper cat. n° 10001-047, diameter 47 mm) [26]. The pH of the extracted pore water was measured with an inoLab pH 7110 pH meter (WTW Wissenschaftlich-Technische Werkstätten GmbH, Germany), and electric conductivity was measured by an EC Multiline P4 with a cell TetraCon 325 (WTW, Germany). Samples were acidified with a drop of concentrated nitric acid and refrigerated for further analysis. Pore water extraction was performed in duplicate for each treatment, including two controls with water only.

#### *Earthworm tests*

Earthworm tests were performed using soils incubated for 1, 56, and 140 d after spiking.

Earthworms (*E. andrei*) were obtained from a laboratory culture at the Department of Ecological Science of the Vrije Universiteit in Amsterdam. The earthworms were fed with horse manure free of pharmaceuticals and incubated at 20 °C. The tests used adult earthworms with well-developed clitellum, which were acclimatized for 24 h in the respective control soils before starting the exposures.

The earthworm tests followed OECD guideline 222 [27], including 28 d exposure of adult animals followed by another 28 d incubation of cocoons to enable the assessment of juvenile production. Four replicate test containers were used for each Zn concentration and control, containing approx. 500 g soil (dry weight equivalent) moistened to 50% WHC. Ten adult earthworms were added to each test container after being gently cleaned on moistened paper towels and weighed. Furthermore, 10 g (wet weight) of horse manure:distilled water (1:2 ratio) were added to each container to feed the earthworms. The containers were maintained under the same conditions as mentioned above for soil incubation. Container weights were monitored weekly to maintain soil moisture content and additional food was added if required.

After 28 d, test containers were emptied into a tray and surviving adults were collected by hand sorting and weighed. Loss in earthworm weight after 28 d was calculated ( $W_L$ ) relative to the initial weight and expressed as the percentage reduction. Surviving earthworms were incubated on moist filter paper in Petri dishes for approx. 24 h to void their gut contents. Subsequently, they were frozen, freeze-dried and stored for analysis. Soils containing cocoons were returned to the respective containers and incubated for another 28 d. After this period the number of juveniles was determined by placing the containers in a water bath at 60 °C forcing juveniles to emerge to the surface, where they were counted.

#### *Metal analysis*

To check spiked concentrations, approx. 0.1 g oven-dried soil samples were digested in 2 mL of a 4:1 mixture of nitric acid (65% pro analysis; Riedel-de Haen) and hydrochloric acid (37%



pro analysis, Baker) in tightly closed Teflon containers, which were heated in an oven at 140 °C for 7 h. Measured Zn concentrations were used in all data analyses. To determine the Zn concentration in earthworms, one freeze-dried individual earthworm of each replicate test container was digested using the same acid mixture and procedure as described for soil samples (n=4). Total Zn concentrations in soils (ZnT), earthworms (ZnE), and porewater Zn concentrations (ZnPW) were measured by flame atomic absorption spectrometry (AAS; Perkin Elmer AAnalyst 100). Instrumental drift was monitored by regularly running standard element solutions between samples. All Zn analyses included procedural blanks. Certified reference materials were also measured for ZnT (ISE sample 989, River Clay from Wageningen, The Netherlands) and for ZnE (Dogfish Liver DOLT-4, National Research Council Canada). Procedural blanks for estimating the detection limits (n=20) were  $<0.003 \text{ mg L}^{-1}$  for Zn. Digested blanks contained Zn concentrations below the limit of detection. Recovery of Zn (mean $\pm$ SD) from the reference soil was  $95\pm 6.9 \%$  and from the DOLT-4 reference material was  $97\pm 2.4\%$  (both n =3).

#### *Data analyses*

Normal distribution of the data was verified using a Kolmogorov-Smirnov test. Significant differences were determined by ANOVA and multiple comparisons were performed with Tukey's test ( $p < 0.05$ ). Partition coefficients ( $K_{d_{PW}}$ ) were calculated as ZnT ( $\text{mg kg}^{-1}$ ) divided by ZnPW ( $\text{mg L}^{-1}$ ). To compare soils and treatments with different Zn concentrations, bioaccumulation factors (BAF) for the accumulation of Zn in the earthworms were calculated by dividing ZnE by ZnT. To determine the influence of soil properties and ageing on Zn availability and earthworm responses for the three test soils, principal component analyses (PCA) were performed using the 'CANOCO for Windows' program v4.02. To study the effect of ageing by relating soil properties and earthworm behaviour, we assumed that soils, which were incubated under controlled conditions, had reached equilibrium well before day 140. The PCA analyses were done with the data of the earthworm exposures that started on day 140 and the chemical analysis data from day 168, the latter date coinciding with the end of the 28 day exposure of the earthworms. Ordination

diagrams were explained with soils shown as points and most of the studied variables ( $Kd_{PW}$ ,  $pH_{PW}$ ,  $BAF$ ,  $ZnE$  and  $W_L$ ) as arrows, according to González-Alcaraz et al. [28].

## RESULTS

### *Available Zn*

Measured concentrations (mean $\pm$ SD) in the test soils on average were  $97 \pm 6\%$  ( $n=48$ ) of the nominal (background and added) Zn concentrations, and variation among replicate samples was less than 18.5% for all treatments (Table 2).  $Zn_{PW}$  was lower in ZnO NP treatments than in soils spiked with  $ZnCl_2$ , with the difference being a factor of 4.4-32 in LUFA and NLGA soils spiked at  $500 \text{ mg kg}^{-1}$  and a factor of 1.5-15 at  $1000 \text{ mg kg}^{-1}$ . In the SPCA soil spiked the difference was a factor of 1.9-7.5 and 1.2-3.3 when dosing at  $1250 \text{ mg kg}^{-1}$  and  $2500 \text{ mg kg}^{-1}$  respectively (Table 2). For  $ZnCl_2$  treatments, average  $Zn_{PW}$  corresponded with 5.04-11.4% of the total Zn in LUFA, 1.71-2.57% in NLGA and 0.08-0.34% in SPCA soil. In the ZnO NP treatments,  $Zn_{PW}$  was  $\leq 2.53\%$  of  $Zn_T$  in LUFA and  $\leq 0.61\%$  in the other two soils, the fraction of Zn found in the pore water was lowest in SPCA soil (Table 2). In LUFA and NLGA,  $Zn_{PW}$  tended to increase with time for all treatments, while in SPCA it remained constant or slightly decreased with time.

Partition coefficients calculated from  $Zn_{PW}$  ( $Kd_{PW}$ ) were significantly affected by ageing in LUFA for both Zn forms ( $ZnCl_2$  and ZnO NPs). In NLGA  $Kd_{PW}$  values showed only a significant decrease with ageing for ZnO NPs (Tukey,  $p < 0.05$ ), while in SPCA they did not show significant changes for ZnO NPs but significantly increased with time for  $ZnCl_2$  (Tukey,  $p < 0.05$ ) (Figure 1).

### *Toxicity and bioaccumulation of Zn in earthworms*

Earthworm responses to the different test soils and treatments at different times of ageing are shown in Table 3. Survival was higher than 78% for all treatments and sampling times, except for SPCA spiked with  $ZnCl_2$  after 56 d of ageing where survival was only 25%. Earthworm weight loss ( $W_L$ ), after 28 d of exposure, increased significantly with ageing in all test soils (Tukey,  $p < 0.05$ ) except for NLGA spiked with ZnO NPs ( $500 \text{ mg kg}^{-1}$ ). The number of juveniles produced per earthworm during the 28 d exposure period in the controls was 1.8 in NLGA, 3.1 in LUFA and 3.6

in SPCA for exposures starting after 1 d ageing (data not shown). Compared to the control, earthworm reproduction was most affected by  $\text{ZnCl}_2$ , with complete inhibition in LUFA and SPCA at all ageing times and with  $\geq 82\%$  reduction in NLGA (Table 3). LUFA soil also showed a significant decrease in earthworm reproduction after 140 d ageing for both ZnO NP treatments (Tukey,  $p < 0.05$ ). In NLGA, no significant reduction ( $p > 0.05$ ) in reproduction with time was seen for the ZnO NP treatments. In SPCA, earthworm reproduction with time was not affected by the ZnO NP treatment of  $1250 \text{ mg Zn kg}^{-1}$  soil while at  $2500 \text{ mg kg}^{-1}$  almost no reduction was seen compared to the control after 56 d.

ZnE differed within each soil (Table 4). Earthworms from the control soils had ZnE concentrations ranging from  $105$  to  $143 \text{ } \mu\text{g g}^{-1}$ , increasing in the order: LUFA < NLGA < SPCA. ZnE differed from the controls for both  $\text{ZnCl}_2$  and ZnO NP treatments, except for earthworms kept in SPCA soil spiked with  $\text{ZnCl}_2$  which showed similar ZnE concentrations than the corresponding controls. In general, soils spiked with ZnO NPs showed a trend of increasing ZnE with increasing ZnT. The highest ZnE was found for the highest treatments with ZnO NPs, and amounted to  $284$  and  $387 \text{ } \mu\text{g Zn g}^{-1}$  earthworm in LUFA and NLGA, respectively, spiked with  $1000 \text{ mg Zn kg}^{-1}$  soil. The highest ZnE recorded was  $408 \text{ } \mu\text{g Zn g}^{-1}$  in earthworms kept in SPCA soil spiked with  $2500 \text{ mg kg}^{-1}$  Zn. In earthworms exposed to LUFA soil, ZnE showed a decrease with ageing for the  $\text{ZnCl}_2$  treatment, while for the ZnO NP treatments ZnE showed an increase after 56 d of ageing and a decrease after 140 d. In earthworms exposed to NLGA, ZnE was highest after 56 d of ageing and significantly lower after 140 d (Tukey,  $p < 0.05$ ). Upon exposure to SPCA, ZnE did not show remarkable changes upon ageing (Table 4).

Bioaccumulation factors calculated for the controls with the Zn background differed among the soils with the following pattern: LUFA > NLGA > SPCA. In the treatments with Zn, BAF was also lowest in the SPCA soil while NLGA and LUFA had similar BAF values. The SPCA soil had the highest BAF in the lowest treatment with ZnO NPs ( $1250 \text{ mg Zn kg}^{-1}$  soil). At the same concentration added as  $\text{ZnCl}_2$ , BAFs were similar to those in the ZnO NP treatment of  $2500 \text{ mg Zn}$

kg<sup>-1</sup> soil. It should be noted that earthworm survival was only 25% in SPCA soil spiked with ZnCl<sub>2</sub> for exposures started after 56 d, making the BAF estimate less reliable. Except for the highest ZnO NP treatment, BAF values in SPCA decreased with ageing. In LUFA and NLGA, BAFs were higher in the ZnCl<sub>2</sub> treatments and did not show clear trends with ageing, while for the ZnO NP treatments they dose-relatedly decreased with increasing ZnT.

#### *Influence of soil properties and ageing on Zn bioavailability*

The pH measured in pore water differed among treatments and incubation times (Table S1), and was higher in soils spiked with ZnO NPs than in the control soils. In soils spiked with ionic Zn, pore water pH decreased or remained similar compared to the control. With ageing, in general, pH decreased for all treatments; this decrease was most pronounced in LUFA and NLGA soils spiked with ZnO NPs.

To study the influence of soil properties, a principal component analysis (PCA) was performed using ZnE, BAF, W<sub>L</sub>, K<sub>d</sub><sub>PW</sub>, and pH of the pore water for the three test soils and the different treatments (ZnCl<sub>2</sub> and ZnO NP) as well as two different ageing periods (1 and 168 d).

Because earthworm reproduction in ZnCl<sub>2</sub> treatments was very low in NLGA and zero in the other two soils (Table 3), this variable was not taken into account in this analysis. On day 1, the alkaline Spanish soil (SPCA) grouped together with the variables pH<sub>PW</sub>, K<sub>d</sub><sub>PW</sub>, and ZnE on the positive side of the main gradient (X-axis) (Figure 2a). Treatments with Zn applied as ZnCl<sub>2</sub> in this soil showed more separation from the ZnO NP treatments and variables for time 1 d. NLGA spiked with ZnO NPs appeared in the centre of the gradient, and shifted to the negative side of the X-axis when it was spiked with ZnCl<sub>2</sub>. Pore water pH and K<sub>d</sub><sub>PW</sub> were negatively related, and BAF was positively related to ZnCl<sub>2</sub> treatment in NLGA soil. Results for LUFA soil were similar to those for NLGA but with less clear differences between treatments. In the secondary gradient (Y-axis), W<sub>L</sub> was negatively correlated with SPCA soil spiked with ZnCl<sub>2</sub> (negative side). The remaining soils were not segregated well in this gradient. The results obtained 168 d after spiking the soils (Figure 2b)

showed similar aggregation patterns of the variables as after 1 d, but the soils were better segregated with higher explained variance (85.3%).

## **DISCUSSION**

### *Zinc oxide availability*

Our results showed that at the same concentration of Zn added, soils spiked with ZnO NPs had lower ZnPW concentrations than those spiked with ZnCl<sub>2</sub>. This was also found by Waalewijn-Kool et al. [12] and indicates that ZnO NPs behaved differently compared to ionic Zn. The lower pore water Zn concentrations obtained for the ZnO NPs compared with ZnCl<sub>2</sub> might suggest that a considerable proportion of the ZnO NPs remained in the particulate form, probably as agglomerates [7]. It cannot be excluded however, that part of the ZnO NPs present in the pore water did pass the 0.45 µm filter used when collecting pore water. Although we did not see large differences between Zn concentrations in pore water before and after ultrafiltration using a 3 kDa filter in a previous study [12], it is possible that our pore-water collection method was not fully adequate for separating NPs from dissolved Zn. This element requires further studies.

Soil pH was affected by the addition of Zn, with an increase in pH in soils spiked with the ZnO NPs and a decrease in pH in soils treated with ionic Zn. Difference in Zn solubility can also be related to soil properties, such as pH. Franklin et al. [29] found that dissolved Zn concentrations in pore water were higher in soils with lower pH, which matches with the results obtained in the present study. Low Zn availability at higher pH has been explained by stronger sorption to the solid phase in basic soils [30], as we observed in our study in the carbonate-rich SPCA soil. Moreover, in the SPCA soil, less Zn was available when spiked with ZnO NPs than with ionic Zn. It has been demonstrated that water solubility of ZnO is highly pH-dependent [29]. In addition, it was also reported that soils with low pH and low organic matter content have a higher availability of Zn [31], which is in agreement with our results, where LUFA soil (with the lowest OC content and low pH)

showed the highest ZnPW for both the ZnO NP and ZnCl<sub>2</sub> treatments. The NLGA soil had a pH similar to that of LUFA but a higher OC content; this soil showed lower ZnPW compared to LUFA for the ZnCl<sub>2</sub> treatment but similar ZnPW in the treatments with ZnO NPs. Natural organic matter can modify the surface charge of NPs, affecting their aggregation [29], and Li et al. [32] found that Zn ions could have a high affinity for binding to or complexation with dissolved organic carbon. In a study with ZnO NPs, Waalewijn-Kool et al. [12] found the highest ZnPW in the most organic soil (with 15% of organic matter), although under acidic pH (pH(CaCl<sub>2</sub>)=5). In the present study, the SPCA soil had the highest OC content but alkaline pH, so it seems that soil pH could determine Zn availability in soils spiked with ZnO NPs better than organic matter content, for this Zn form. *K<sub>d</sub>* can be used to express the adsorption of Zn [33]. Our *K<sub>d</sub>* values for the three tested soils decreased in the order: SPCA>NLGA>LUFA, regardless of the applied Zn form. This indicates the highest adsorption and therefore lowest availability of Zn in SPCA soil and the highest Zn availability in LUFA soil. The *K<sub>d</sub>* values decreased with decreasing soil pH, which agrees with literature data [34].

Upon ageing, Zn availability increased in the LUFA and NLGA soils for all treatments, while in SPCA soil no significant differences were found for ZnO NPs and a decrease was observed for ZnCl<sub>2</sub>. Our results for the times 1 to 3 d, which is relatively short-term, showed a greater decrease in Zn availability in the LUFA soil (with the lowest CEC), although it increased again after 6 months of incubation. Lock and Janssen [18] also observed a faster rate of adsorption in soils with low CEC. Hence ageing or equilibration of contaminated soil might provide a more realistic insight into ZnO NP behaviour and therefore its potential toxicity under natural conditions. The equilibrium processes of metals between pore water and the soil solid phase are rather complex and NP solubility could be continuously changing with time [29], so the influence of soil components on Zn solubility cannot be ignored [12].

*Zinc toxicity and Zn bioaccumulation by earthworms*

Nanoparticles are expected to be less toxic than ionic forms. Notter et al. [35] derived a nanofactor of 2 to indicate the difference in toxicity of both metal species. Zinc concentrations for the treatments used in the present study were based on effective concentrations (EC50) for effects on earthworm reproduction of ZnO NPs and ZnCl<sub>2</sub> [11], therefore no effects on survival were expected. This indeed was the case for almost all soils and treatments (>78% of survival) except for the SPCA soil after 56 d of ageing (Table 3).

After 28 d of exposure, earthworm body weights showed a decrease in all soils and varied between treatments and soils, which suggests that the observed differences can be related to soil type. According to Janssen et al. [36], W<sub>L</sub> variation could be caused primarily by soil factors.

Hooper et al. [7] observed greater W<sub>L</sub> of the earthworm *Eisenia veneta* exposed to 750 mg Zn kg<sup>-1</sup> soil as ZnO NPs compared to treatments with ionic Zn. This disagrees with our finding of greater or the same W<sub>L</sub> in the ZnCl<sub>2</sub> treatments compared to the ZnO NPs in all test soils. Heggelund et al. [11] found a dose-related increase in W<sub>L</sub> of the earthworm *Eisenia fetida* in ZnCl<sub>2</sub> treated soils, but not in soils spiked with ZnO NPs at concentrations of 238 to 2500 mg Zn kg<sup>-1</sup> d.w. soil. In our ZnO NP treatments, earthworms in LUFA and SPCA showed similar results at the two tested ZnO NP concentrations, while in NLGA there was a small decrease with increasing ZnT. In addition, ageing effects were observed in all soils, with an increase in W<sub>L</sub> with time in all treatments. The higher W<sub>L</sub> was observed for the LUFA soil for both ZnCl<sub>2</sub> and ZnO NP treatments. The observed earthworm weight changes in our test soils suggest that, upon exposure to ZnO NPs, W<sub>L</sub> was not dose-related and it is probably influenced by soil properties. It also suggests that there is an ageing effect with an increase in W<sub>L</sub> with time, influenced by soil properties.

Reproduction of *E. andrei* is, in general, more sensitive and more ecologically relevant than the other earthworm toxicity endpoints [11, 37]. Earthworm reproduction is known to be influenced by soil properties [38], and this indeed was observed from the difference in juvenile numbers in the controls. Earthworm reproduction was more affected by ZnCl<sub>2</sub> than by ZnO NPs, with almost total inhibition of reproduction in soils spiked with ionic Zn. A dose-related effect on reproduction was

seen for the ZnO NP treatments in our test soils. It is remarkable that in SPCA soil, spiked with higher Zn concentrations, reproduction was similar to that in the other two soils spiked at lower total Zn concentrations. This could be explained by the low ZnPW concentration in this soil (Table 2). However, it has to be noted that in SPCA, inhibition of earthworm reproduction was not always dose-related, and in some cases inhibition was stronger at lower available Zn levels (e.g., after 56 and 140 d of ageing).

Studies using artificially spiked soils should be considered with care as metal solubility and toxicity may change with time (ageing). Therefore results of ecotoxicity tests with Zn in freshly spiked soils could differ from those with field-contaminated soils [39]. In the present study, an increase of earthworm  $W_L$  was seen in all test soils and treatments with ageing, while only a decrease in earthworm reproduction with time was observed in the case of LUFA treated with ZnO NPs. In a study with enchytraeids, no effect of ageing on Zn toxicity was detected, which was explained by the high adsorption capacity of soil components (clay and organic matter content) [40]. In the present study, the changes in the reproduction toxicity of Zn with ageing could mainly be attributed to the higher Zn availability (ZnPW) in LUFA soil that showed an increase with ageing (Table 2). Therefore, further studies on ZnO NP toxicity with ageing are needed using different soil types and exposure levels.

Earthworms are able to sequester and retain, as well as autoregulate internal Zn concentration for essential functions, therefore their ZnE can remain constant regardless of the concentrations of total and available Zn in soil [38]. Heggelund et al. [11] found that *E. fetida* kept in control soils with different pH (4.5-7.2) had average Zn internal concentration of 123-132  $\mu\text{g Zn g}^{-1}$  earthworm (n=300), which is within the range of ZnE values in our controls ranging between 105 and 143  $\mu\text{g Zn g}^{-1}$  earthworm. Heggelund et al. [11] also observed that in soils spiked with ZnO NPs and  $\text{ZnCl}_2$ , the earthworms showed higher ZnE in the NP treatments, which agrees with our findings. We found somewhat higher ZnE in the NLGA and SPCA soils spiked with the highest concentrations of ZnO NPs ( $387 \pm 65$  and  $408 \pm 158 \mu\text{g Zn g}^{-1}$  earthworm, respectively). The



observed ZnE in our test soils spiked with different concentrations and forms of Zn highlights the need for further studies on the influence of soil type on Zn bioavailability, as well as their potential role in the capability of earthworms to regulate their Zn body concentrations [31].

The bioaccumulation factor (BAF) is a good indicator to compare among soils, taking into account the difference in the applied concentrations, but BAF value alone does not provide enough information because of metal auto-regulation mechanisms in earthworms. In addition, after 24 h of depuration some 5% of the gut content may still remain in earthworms, and the gut loading of soil can vary based on the properties of the soil and soil moisture content [41], adding more bias to the use of BAF values. Nevertheless, BAF is a good measure to compare tissue concentrations of earthworms exposed in different soils, taking into account the differences in the applied concentrations. In the present study, BAFs were lowest for SPCA compared to the other test soils, for both Zn forms. In general, BAFs in soils spiked with ZnO NPs showed a dose-dependent pattern (opposite to  $Kd_{PW}$  in the PCAs, Figure 2) with the lowest values at the highest soil concentrations. This behaviour had been observed earlier for other essential elements, such as molybdenum, for which internal concentrations in exposed earthworms may be regulated to fairly constant levels [19].

According to the PCA analysis, the BAF was inversely related with  $pH_{PW}$ , which suggests that along with the available Zn, the pH could have an important role in earthworm Zn bioaccumulation. This agrees with Spurgeon et al. [42], who indicated that Zn uptake in earthworms can be dependent on soil pH, making it hard to predict Zn uptake by earthworms from available Zn concentrations [31]. BAFs were inversely related with pH, independent of the Zn type studied [11]. The only exception was SPCA soil, in which the lowest BAFs were found and which were significantly lower for  $ZnCl_2$  compared to ZnO NPs. The BAF or ZnE did not explain the  $W_L$  (Figure 2), with the earthworms having the lowest ZnE showing the highest  $W_L$ . The lowest  $W_L$  was found in SPCA soil, which suggests again an effect of soil properties rather than the ZnE on  $W_L$ . These results suggest that in this soil, earthworms may be capable of sequestering Zn, leading

to higher body concentrations than expected when Zn was applied as ZnO NPs. Additional studies are needed to unravel the complex mechanisms of Zn bioaccumulation in earthworms exposed to nano-particulate Zn and the role of soil properties.

## CONCLUSIONS

The present study introduces new data on the effect of long-term incubation on the fate and effects of ZnO NPs in different soils, which may help improving the risk assessment of chronic ZnO NP exposures. We compared the effect of Zn applied as nanoparticles (ZnO NPs) and as Zn<sup>2+</sup> ions (ZnCl<sub>2</sub>) on Zn availability and bioavailability to the earthworm *E. andrei* at different incubation times after spiking in three natural soils with contrasting properties. Zinc concentrations in pore water were lower in soils spiked with ZnO NPs compared with ZnCl<sub>2</sub>. Zinc availability was lowest in the soil with alkaline pH and with high organic carbon content. For treatments with ZnO NPs, soil pH best explained the difference in Zn availability, while organic carbon explained Zn availability in soils spiked with ZnCl<sub>2</sub>. The effect of ageing on the availability of Zn showed differences without regular trends among soils as well as between treatments (ZnCl<sub>2</sub> and ZnO NPs). Earthworms showed varying internal Zn concentrations among soils, which were highest in the soil with the highest OC content and basic pH, following exposure to Zn applied as ZnO NPs, even though this was the soil which showed lowest ZnPW.

Toxicity of Zn to earthworm reproduction was highest for ZnCl<sub>2</sub> treatments, with almost complete reproduction inhibition, but there were no clear differences in survival and weight loss (W<sub>L</sub>) between treatments. An effect of soil ageing on Zn toxicity to the earthworms was only observed for weight loss, which increased with time. No differences were seen for the other variables, so no significant effects of ageing were detected that could explain differences in earthworm toxicity.

More research is necessary to understand ZnO NP interactions with different soil constituents and how soil properties control Zn availability. It is also essential to deepen the knowledge on the importance of long-term processes for Zn availability for a proper risk assessment of ZnO NPs as well as Zn-polluted soils.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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*Data Availability*—The Supporting Information shows characterization of the ZnO NPs, pH of the test soils at different incubation times, and results of the Cu(NO<sub>3</sub>)<sub>2</sub> extraction method; further data are available upon request from the corresponding author (anaromerof@ugr.es).

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Figure 1. Average partition coefficients ( $Kd_{PW}$ , in  $\text{mL g}^{-1}$ ), expressed as the total Zn concentration in soil (ZnT) divided by pore water Zn concentration (ZnPW) ( $n=2$ ) at 1, 3, 56, and 168 d after spiking the three test soils with  $\text{ZnCl}_2$  (panel a) or ZnO NPs (panel b) (numerical data are shown in Table 2). Nominal zinc concentration in LUFA and NLGA soils was  $500 \text{ mg kg}^{-1}$ , and in SPCA soil  $1250 \text{ mg kg}^{-1}$ . Letters indicate significant differences between  $Kd_{PW}$  for different periods of ageing (Tukey  $p<0.05$ ).

Figure 2. Principal Component Analysis (PCA) for the relationship between earthworm toxicity and soil properties in three test soils spiked with ZnO NPs and  $\text{ZnCl}_2$ . Parameters included in the PCA are the partitioning coefficient calculated using pore water Zn concentrations ( $Kd_{PW}$ ), pH of the pore water ( $\text{pH}_{PW}$ ), bioaccumulation factor (BAF), Zn concentration in earthworm (ZnE), and earthworm weight loss ( $W_L$ ). PCA were run for two periods of ageing of the spiked soils, 1 and 168 days. Figure 2a shows the results for time 1 day; variance explained by the two first components is 69.3 % (X-axis: 45.7 %; Y-axis: 23.6 %). Figure 2b shows the results for time 168 days; variance explained by the two first components is 85.3 % (X-axis: 58 %; Y-axis: 27.3 %).

**Table 1.** Physicochemical properties (mean±SD) of the soils used to assess the effects of ageing and the influence of soil properties on the (bio)availability of ZnO nanoparticles (ZnO NPs) and ionic Zn (applied as ZnCl<sub>2</sub>).

Soil	Country	pH (KCl)	pH (PW)	EC (mS cm <sup>-1</sup> )	CaCO <sub>3</sub> (%)	OC (%)	Clay (%)	CEC (cmol <sup>+</sup> kg <sup>-1</sup> )	WHC (%)	Background Zn (mg kg <sup>-1</sup> )
<b>LUFA2.2</b>	Germany	5.6±0.07	6.7±0.04	0.05±0.02	<1	1.55±0.15	8.27±0.78	8.19±1.96	45	15.2±0.85
<b>NLGA</b>	Netherlands	5.9±0.03	6.9±0.07	0.03±0.01	<1	3.44±0.19	4.80±0.59	18.8±1.18	51	92.5±0.77
<b>SPCA</b>	Spain	7.6±0.03	8.0±0.06	0.08±0.04	37±0.44	5.43±0.38	23.6±0.90	21.4±2.00	62	154±1.77

pH measured in potassium chloride extractions (KCl) and pore water extractions (PW). EC: electric conductivity; OC: organic carbon content; CEC: Cation Exchange Capacity; WHC: water holding capacity.

**Table 2.** Effect of ageing on the availability of Zn in soil. Shown are the nominal and average measured total Zn concentrations found immediately after spiking soils ( $\pm$ SD; n=3) (ZnT; in mg Zn kg<sup>-1</sup> dry soil) in soils spiked with ionic Zn (ZnCl<sub>2</sub>) or ZnO nanoparticles (ZnO NPs), and pore water Zn concentrations (ZnPW; n=2; mg Zn L<sup>-1</sup>), obtained at different points in time after spiking the soil. Zn recoveries are shown as the percentage of ZnT extracted in the ZnPW (mean $\pm$ SD). Also included are  $Kd_{PW}$  values (mean $\pm$ SD), derived as the ratio of ZnT and ZnPW concentrations, and indicating the strength of Zn binding to the different soils.

Soil	Treatment	Nominal Zn mg kg <sup>-1</sup>	Measured ZnT mg kg <sup>-1</sup>	Ageing Days	ZnPW $\pm$ SD mg L <sup>-1</sup>	Recovery $\pm$ SD %	$Kd_{PW}\pm$ SD L kg <sup>-1</sup>	
LUFA	ZnCl <sub>2</sub>	500	487 ( $\pm$ 87.4)	1	84.1 $\pm$ 4.50 a	7.78 $\pm$ 0.41	6 $\pm$ 0.31	
				3	60.5 $\pm$ 4.67 a	5.59 $\pm$ 0.43	8 $\pm$ 0.62	
				56	54.4 $\pm$ 4.31 a	5.04 $\pm$ 0.40	9 $\pm$ 0.71	
				168	124 $\pm$ 14.7 b	11.4 $\pm$ 1.36	4 $\pm$ 0.47	
	ZnO NP	500	497 ( $\pm$ 36.7)	1	2.64 $\pm$ 0.42 a	0.24 $\pm$ 0.04	191 $\pm$ 30.4	
				3	3.26 $\pm$ 0.08 a	0.30 $\pm$ 0.01	153 $\pm$ 3.64	
				56	12.3 $\pm$ 2.11 b	1.12 $\pm$ 0.19	41 $\pm$ 7.04	
				168	25.4 $\pm$ 1.52 c	2.30 $\pm$ 0.14	20 $\pm$ 1.18	
	ZnO NP	1000	873 ( $\pm$ 38.6)	1	5.51 $\pm$ 0.30 a	0.28 $\pm$ 0.02	159 $\pm$ 8.51	
				3	5.46 $\pm$ 0.85 a	0.28 $\pm$ 0.04	162 $\pm$ 25.25	
				56	37.4 $\pm$ 0.42 b	1.93 $\pm$ 0.02	23 $\pm$ 0.26	
				168	49.0 $\pm$ 1.91 c	2.53 $\pm$ 0.10	18 $\pm$ 0.69	
	NLGA	ZnCl <sub>2</sub>	500	495 ( $\pm$ 55.3)	1	16.5 $\pm$ 2.77 a	1.71 $\pm$ 0.29	30 $\pm$ 5.10
					3	19.1 $\pm$ 0.89 ab	1.98 $\pm$ 0.09	26 $\pm$ 1.21
					56	23.6 $\pm$ 0.71 b	2.45 $\pm$ 0.07	21 $\pm$ 0.63
					168	24.8 $\pm$ 1.53 b	2.57 $\pm$ 0.16	20 $\pm$ 1.23
ZnO NP		500	490 ( $\pm$ 63.9)	1	2.48 $\pm$ 0.18 a	0.26 $\pm$ 0.02	198 $\pm$ 14.7	
				3	2.12 $\pm$ 0.13 a	0.22 $\pm$ 0.01	231 $\pm$ 13.9	
				56	2.04 $\pm$ 1.00 a	0.22 $\pm$ 0.11	273 $\pm$ 134	
				168	5.68 $\pm$ 0.25 b	0.60 $\pm$ 0.03	86 $\pm$ 3.87	
ZnO NP		1000	858 ( $\pm$ 49.0)	1	2.95 $\pm$ 0.06 a	0.18 $\pm$ 0.004	291 $\pm$ 6.27	
				3	3.06 $\pm$ 0.39 a	0.18 $\pm$ 0.02	282 $\pm$ 28.4	
				56	2.67 $\pm$ 0.14 a	0.16 $\pm$ 0.01	322 $\pm$ 17.4	
				168	10.2 $\pm$ 1.68 b	0.61 $\pm$ 0.10	85 $\pm$ 14.0	
SPCA		ZnCl <sub>2</sub>	1250	1259 ( $\pm$ 230)	1	6.84 $\pm$ 0.54 c	0.34 $\pm$ 0.03	185 $\pm$ 14.5
					3	4.95 $\pm$ 1.48 bc	0.24 $\pm$ 0.07	266 $\pm$ 79.6
					56	1.57 $\pm$ 0.08 a	0.08 $\pm$ 0.004	801 $\pm$ 39.6
					168	2.13 $\pm$ 0.06 ab	0.10 $\pm$ 0.003	591 $\pm$ 15.7
	ZnO NP	1250	1287 ( $\pm$ 134)	1	0.91 $\pm$ 0.20	0.04 $\pm$ 0.01	1451 $\pm$ 304	
				3	0.73 $\pm$ 0.02	0.04 $\pm$ 0.001	1753 $\pm$ 38.8	
				56	0.81 $\pm$ 0.06	0.04 $\pm$ 0.003	1592 $\pm$ 125	
				168	0.83 $\pm$ 0.20	0.04 $\pm$ 0.01	1598 $\pm$ 379	
	ZnO NP	2500	2514 ( $\pm$ 258)	1	2.05 $\pm$ 0.20	0.05 $\pm$ 0.005	1233 $\pm$ 118	
				3	1.43 $\pm$ 0.52	0.04 $\pm$ 0.01	1888 $\pm$ 691	
				56	1.33 $\pm$ 0.07	0.03 $\pm$ 0.002	1894 $\pm$ 95	
				168	1.46 $\pm$ 0.21	0.04 $\pm$ 0.01	1736 $\pm$ 252	

Lowercase letters represent significance difference between sampling days for each treatment (Tukey HSD test.  $p < 0.05$ ).

**Table 3.** Effect of ageing on the toxicity of ZnO nanoparticles (ZnO NPs) and ionic Zn (applied as ZnCl<sub>2</sub>) to the earthworm *Eisenia andrei* exposed to three different natural soils. Shown are percentage average of survival (% ± SD) (n=40) and weight loss (W<sub>L</sub>, % ± SD) (n=40) after 4 weeks, and reduction of the number of juveniles produced per earthworm after 8 weeks in percentage compared to the corresponding controls (reproduction reduction) (% ± SD). Earthworm assays were performed after different periods of ageing of the spiked soils.

Soil	Treatment	Ageing days	Survival±SD %	W <sub>L</sub> ±SD %	Reproduction Reduction±SD %	
LUFA	ZnCl <sub>2</sub> 500 mg kg <sup>-1</sup>	1	90 ± 14	11 ± 8 a	100 (nsd)	
		56	98 ± 5	12 ± 4 a	100 (nsd)	
		140	95 ± 10	54 ± 1 b	100 (nsd)	
	ZnO NP 500 mg kg <sup>-1</sup>	1	98 ± 5	4 ± 8 a	31 ± 24	a
		56	100 (nsd)	8 ± 4 a	89 ± 13	b
		140	93 ± 10	31 ± 1 b	100 (nsd)	b
	ZnO NP 1000 mg kg <sup>-1</sup>	1	98 ± 5	4 ± 6 a	90 ± 8	a
		56	78 ± 19	9 ± 6 a	96 ± 5	ab
		140	85±13	49 ± 7 b	100 (nsd)	b
NLGA	ZnCl <sub>2</sub> 500 mg kg <sup>-1</sup>	1	100 (nsd)	15 ± 8 a	98 ± 3	
		56	95 ± 6	29 ± 4 b	94 ± 11	
		140	95 ± 10	30 ± 6 b	82 ± 18	
	ZnO NP 500 mg kg <sup>-1</sup>	1	98 ± 5	26 ± 8	41 ± 41	
		56	93 ± 15	26 ± 5	-5 ± 38	
		140	95 ± 6	25 ± 4	22 ± 21	
	ZnO NP 1000 mg kg <sup>-1</sup>	1	95 ± 10	17 ± 2 a	74 ± 12	
		56	98 ± 5	27 ± 2 b	79 ± 17	
		140	100 (nsd)	21 ± 7 ab	61 ± 25	
SPCA	ZnCl <sub>2</sub> 1250 mg kg <sup>-1</sup>	1	98 ± 5 b	21 ± 5 a	100 (nsd)	
		56	25 ± 13 a	32 ± 4 b	100 (nsd)	
		140	83 ± 35 b	41 ± 5 c	100 (nsd)	
	ZnO NP 1250 mg kg <sup>-1</sup>	1	100 (nsd) b	14 ± 1 a	45 ± 32	
		56	88 ± 10 a	18 ± 7 ab	41 ± 31	
		140	100 (nsd) b	26 ± 2 b	75 ± 16	
	ZnO NP 2500 mg kg <sup>-1</sup>	1	100 (nsd)	9 ± 1 a	83 ± 17	c
		56	95 ± 6	16 ± 2 b	1 ± 17	a
		140	100 (nsd)	27 ± 3 c	45 ± 12	b

Lowercase letters represent significance difference between treatments (Tukey HSD test.  $p < 0.05$ ); nsd (no standard deviation): all earthworms survived after 4 weeks (n=40) or no reproduction was observed in the samples after 8 weeks (n=4).

**Table 4.** Average Zn concentrations in earthworms *Eisenia andrei* with corresponding standard deviation (ZnE±SD; n=4) after 4 weeks exposure to ZnCl<sub>2</sub> or ZnO NPs in three different soils aged for different periods of time. Also given are bioaccumulation factors (BAF) calculated as Zn concentrations in the earthworms divided by measured total Zn concentrations in the soils (see Table 2).

Soil	Treatment	Nominal Zn mg kg <sup>-1</sup>	Ageing days	ZnE±SD µg g <sup>-1</sup>	BAF±SD
LUFA	Control	0	1	108 ± 4 a	7.06 ± 0.25
			56	105 ± 11 a	6.90 ± 0.70
			140	117 ± 8 ab	7.66 ± 0.50
	ZnCl <sub>2</sub>	500	1	214 ± 33 de	0.44 ± 0.07 b
			56	204 ± 24 cde	0.42 ± 0.05 b
			140	161 ± 7 bc	0.33 ± 0.02 b
	ZnO NP	500	1	188 ± 21 cde	0.38 ± 0.04 b
			56	195 ± 32 cde	0.39 ± 0.06 ab
			140	182 ± 26 cd	0.37 ± 0.05 b
	ZnO NP	1000	1	229 ± 33 e	0.26 ± 0.04 a
			56	284 ± 49 f	0.33 ± 0.06 a
			140	190 ± 37 cde	0.22 ± 0.04 a
NLGA	Control	0	1	125 ± 8 a	1.35 ± 0.10
			56	118 ± 6 a	1.28 ± 0.07
			140	128 ± 10 a	1.39 ± 0.12
	ZnCl <sub>2</sub>	500	1	213 ± 20 bc	0.43 ± 0.04 b
			56	330 ± 59 ef	0.67 ± 0.11 b
			140	208 ± 26 bc	0.42 ± 0.06 b
	ZnO NP	500	1	203 ± 34 bc	0.42 ± 0.07 b
			56	302 ± 91 de	0.62 ± 0.20 ab
			140	189 ± 21 ab	0.39 ± 0.04 b
	ZnO NP	1000	1	249 ± 44 bcd	0.29 ± 0.06 a
			56	387 ± 65 f	0.45 ± 0.07 a
			140	268 ± 46 cde	0.31 ± 0.05 a
SPCA	Control	0	1	143 ± 8 a	0.93 ± 0.05
			56	139 ± 7 a	0.90 ± 0.04
			140	132 ± 10 a	0.86 ± 0.07
	ZnCl <sub>2</sub>	1250	1	192 ± 37 ab	0.15 ± 0.03 b
			56	175 ± 15 ab	0.14 ± 0.01 a
			140	168 ± 22 ab	0.13 ± 0.02 a
	ZnO NP	1250	1	286 ± 38 bc	0.22 ± 0.03 c
			56	239 ± 38 ab	0.19 ± 0.03 b
			140	243 ± 50 ab	0.19 ± 0.04 b
	ZnO NP	2500	1	292 ± 55 bc	0.12 ± 0.02 a
			56	408 ± 158 c	0.15 ± 0.05 ab
			140	336 ± 89 c	0.13 ± 0.04 a

Lowercase letters in ZnE indicate significant differences in each soil sample. Lowercase letters in BAF represent significant differences between treatment for each sampling day (both with Tukey HSD test.  $p < 0.05$ ).

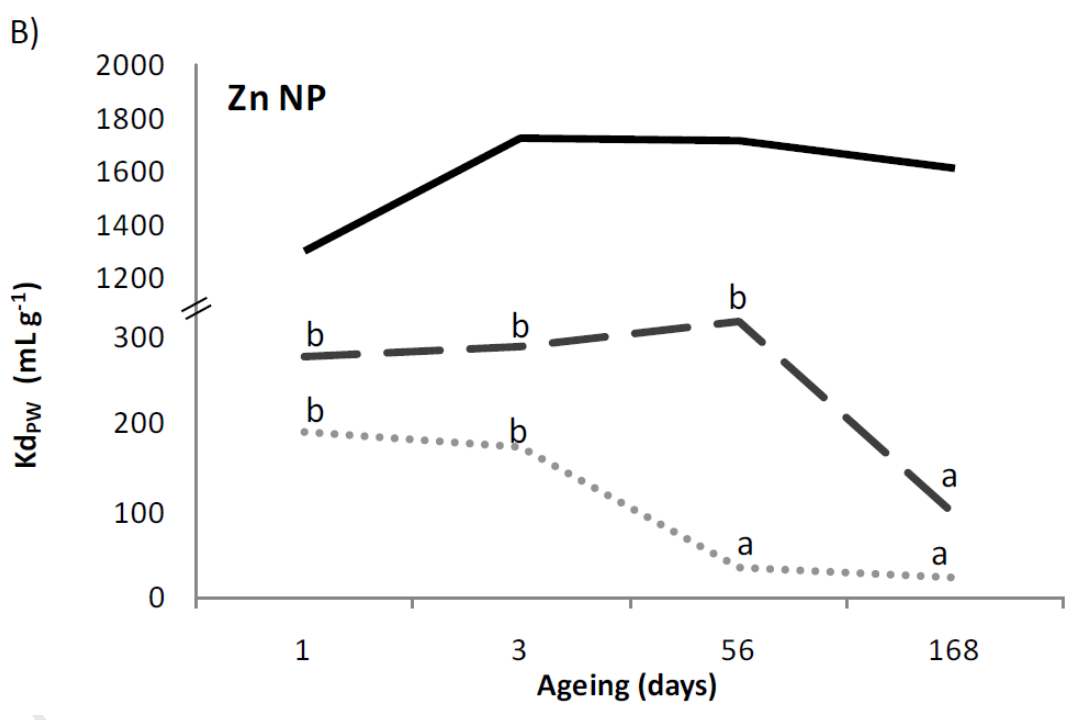
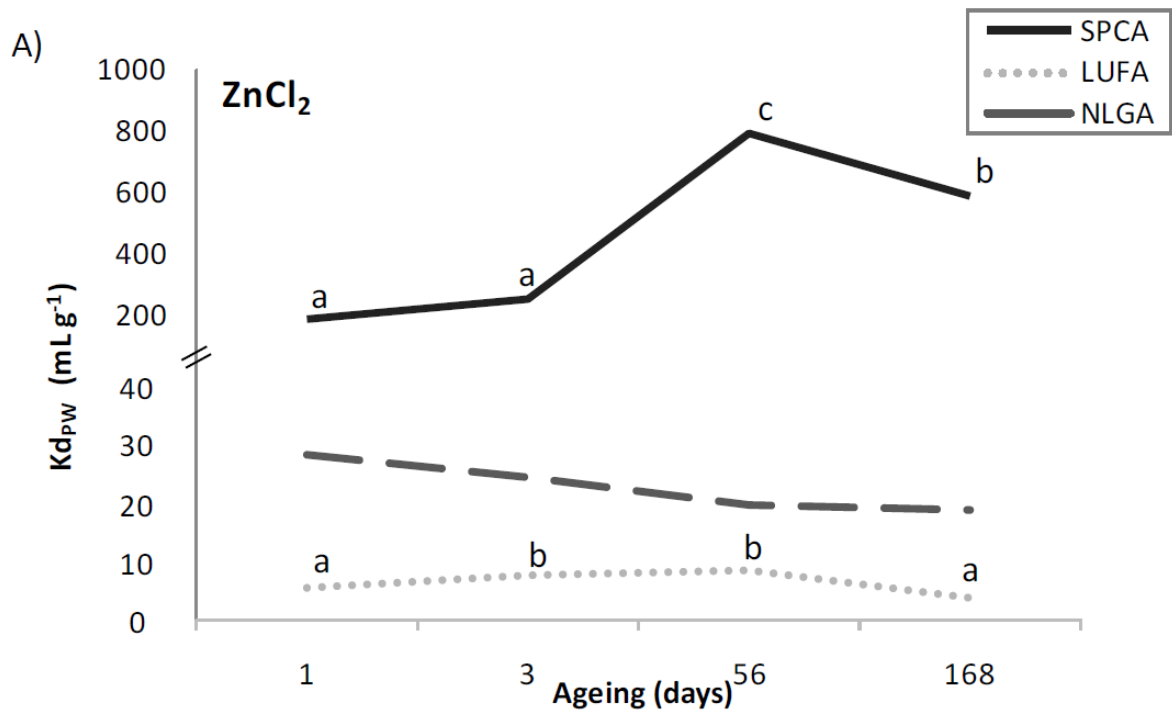


Figure 1

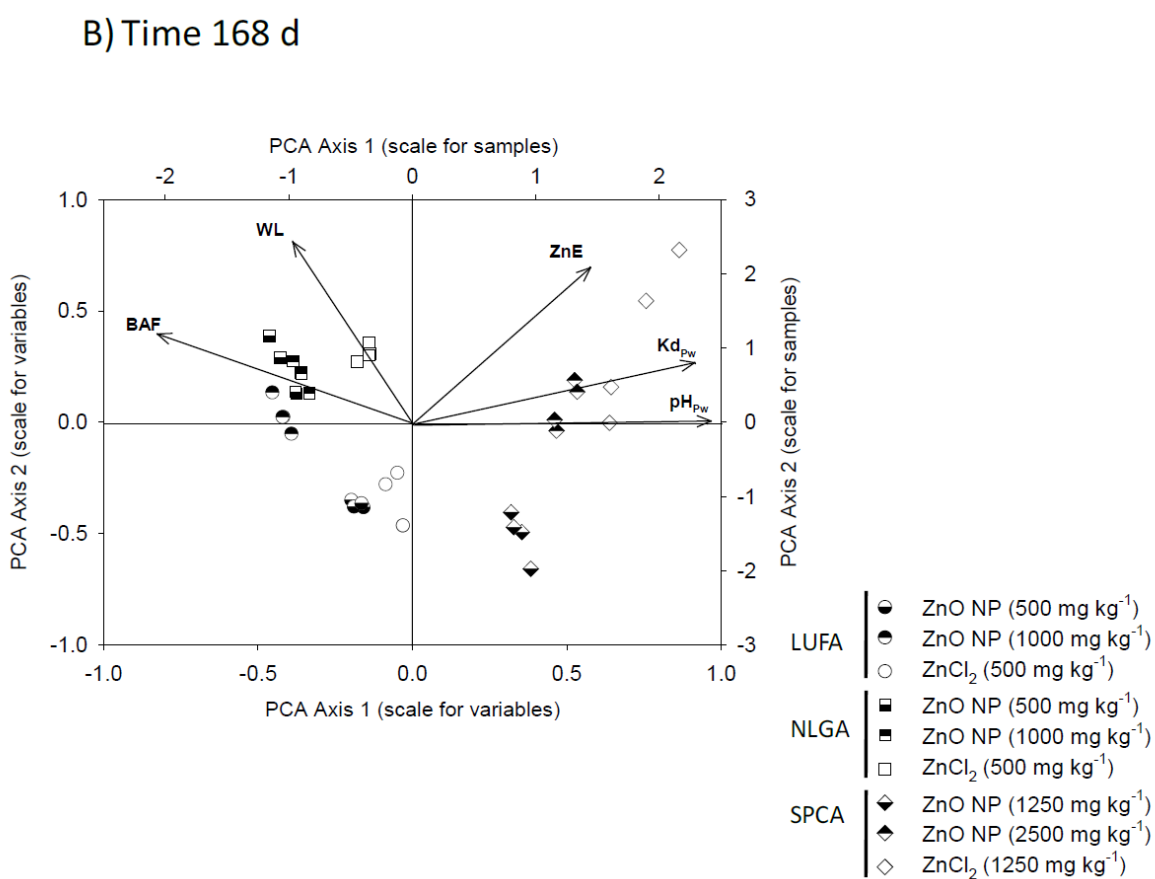
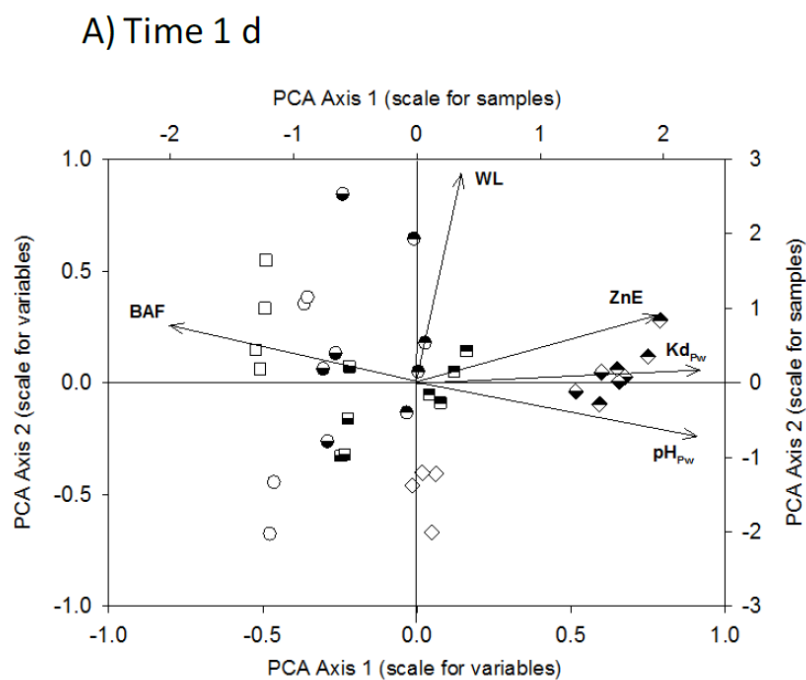


Figure 2