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Romero-Freire, A.; Lofts, S.; Martin Peinado, F.J.; van Gestel, C.A.M.

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EFFECTS OF AGING AND SOIL PROPERTIES ON ZINC OXIDE NANOPARTICLE AVAILABILITY AND ITS ECOTOXICOLOGICAL EFFECTS TO THE EARTHWORM *EISENIA ANDREI*

ANA ROMERO-FREIRE,*† STEPHEN LOFTS,‡ FRANCISCO J. MARTÍN PEINADO,† and CORNELIS A.M. VAN GESTEL§
†Department of Soil Science, University of Granada, Granada, Spain
‡NERC Centre for Ecology and Hydrology, Lancaster Environment Centre, Bailrigg, Lancaster, United Kingdom
§Department of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit, Amsterdam, The Netherlands

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Abstract: To assess the influence of soil properties and aging on the availability and toxicity of zinc (Zn) applied as nanoparticles (Zn oxide [ZnO]-NPs) or as Zn^{2+} ions (Zn chloride [$ZnCl_2$]), 3 natural soils were individually spiked with either ZnO-NPs or $ZnCl_2$ and incubated for up to 6 mo. Available Zn concentrations in soil were measured by porewater extraction (ZnPW), whereas earthworms (*Eisenia andrei*) were exposed to study Zn bioavailability. Porewater extraction concentrations were lower when Zn was applied as NPs compared to the ionic form and decreased with increasing soil pH. For both Zn forms and Zn-PW values were affected by aging, but they 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 Zn-NPs, but the same soil spiked with $ZnCl_2$ 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 aging. This shows that ZnO-NPs and $ZnCl_2$ behave differently in soils depending on soil properties and aging processes, but differences in earthworm toxicity remain unexplained. *Environ Toxicol Chem* 2017;36:137–146. © 2016 SETAC

Keywords: Aging Availability Ecotoxicity Nanoparticle Zinc

INTRODUCTION

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

Zinc (Zn) oxide (ZnO) is 1 of the most commonly used types of metal-based NPs, with the third highest annual production volume [5]. Zinc oxide NPs (ZnO-NPs) are used in electronics applications, solar panel devices, medicine, cosmetics, and sunscreens (ultraviolet light filters) and applied as antibacterial agents [6]. Zinc oxide NPs can enter the environment via wastewater 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 nano-scale 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 what 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. Nanoparticles may form aggregates or agglomerates, which can lead to sedimentation, or may be prone to dissolution and release of free metal ions [13]. On contact with water, ZnO-NPs partially dissolve to Zn ions (Zn^{2+}), so their effects may partly be caused by 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 aging [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 to reach apparent equilibrium compared to soils directly spiked with ionic Zn. These long-term processes have been shown to decrease bulk Zn toxicity in soil over time, whereas aging 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

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* Address correspondence to anaromerof@ugr.es
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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, behavior, 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 aging 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 (Zn chloride [$ZnCl_2$]) treatment was included for comparison. Porewater extraction was applied to assess changes in the availability of the 2 studied Zn forms over a 6-mo aging period. Bioavailability and toxicity were measured by exposing *E. andrei* to ZnO-NPs or $ZnCl_2$ 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). 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 porewater (pH_{PW}), electric conductivity, calcium carbonate content ($CaCO_3$), organic carbon content, particle-size distribution, and cation exchange capacity (CEC), according to Romero-Freire et al. [24], as well as water holding capacity [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 nm to 40 nm (Supplemental Data, Figure S1). To study the effect of dissolved (ionic) Zn, 1 treatment with the soluble salt $ZnCl_2$ (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 10% and 50% effective concentrations for the effects of ZnO-NPs and the 50% effective concentration for effects of $ZnCl_2$ 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 $ZnCl_2$ were 500 mg Zn kg^{-1} in LUFA and NLGA and 1250 mg Zn kg^{-1} in SPCA; ZnO-NP concentrations were 500 mg Zn kg^{-1} and 1000 mg Zn kg^{-1} in LUFA and NLGA and 1250 mg Zn kg^{-1} and 2500 mg Zn kg^{-1} in SPCA. Uncontaminated controls were also included.

The ZnO-NPs were mixed into the soils as a powder to avoid dissolution of the particles prior to addition to the soil, while $ZnCl_2$ was introduced as an aqueous solution. Soils were intensively mixed with a spoon to avoid modifying the NPs, to achieve as homogenous a distribution of the Zn as possible. After spiking, soils were moistened to 50% of their water holding capacity. 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-controlled room (Weiss Technik Benelux) at 20 °C and 75% relative air humidity, with a 12:12-h light: dark cycle. 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, 2 methods were applied: porewater extraction and extraction with a diluted $Cu(NO_3)_2$ solution. We expected that the latter method would allow discrimination between particulate and freely available Zn forms. Because this method did not seem to work as expected, we will not discuss this further; we included a brief description in the Supplemental Data.

At 1 d, 3 d, 56 d, and 168 d after spiking the soils, porewater extractions were performed. For that purpose, 50-g soil samples were placed in Teflon containers and moistened to 100% water holding capacity, 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 2 circular filters (Whatman filter paper 10001-047, diameter 47 mm) [26]. The pH of the extracted porewater was measured with an inoLab pH 7110 pH meter (Wissenschaftlich-Technische Werkstätten), and electrical conductivity was measured by a Multiline P4 with a cell TetraCon 325 (Wissenschaftlich-Technische Werkstätten). Samples were acidified with a drop of concentrated nitric acid and refrigerated for further analysis. Porewater extraction was performed in duplicate for each treatment, including 2 controls with water only.

Earthworm tests

Earthworm tests were performed using soils incubated for 1 d, 56 d, and 140 d after spiking. Earthworms (*E. andrei*) were obtained from a laboratory culture at the Department of Ecological Science, Vrije Universiteit, Amsterdam, The

Table 1. Physicochemical properties (mean ± standard deviation) of the soils used to assess the effects of aging and the influence of soil properties on the (bio) availability of zinc oxide nanoparticles and ionic zinc (applied as $ZnCl_2$)

Soil	Country	pH[a] (KCl)	pH[a] (PW)	EC (mS cm^{-1})	$CaCO_3$ (%)	OC (%)	Clay (%)	CEC ($cmol^{+}kg^{-1}$)	WHC (%)	Background Zn (mg kg^{-1})
LUFA2.2	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
NLGA	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
SPCA	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

[a]The pH was measured in potassium chloride extractions and porewater extractions.
EC = electric conductivity; KCl = potassium chloride; OC = organic carbon content; PW = porewater; CEC = cation exchange capacity; WHC = water holding capacity; Zn = zinc.

Netherlands. Earthworms were fed with horse manure free of pharmaceuticals and incubated at 20 °C. The tests used adult earthworms with a well-developed clitellum, which were acclimatized for 24 h in the respective control soils before starting the exposures.

The earthworm tests followed Organisation for Economic Co-operation and Development 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 approximately 500 g soil (dry wt equivalent) moistened to 50% water holding capacity. Ten adult earthworms were added to each test container after being gently cleaned on moistened paper towels and weighed. Furthermore, 10 g (wet wt) 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 (in *Soils*) for soil incubation. Container weights were monitored weekly to maintain soil moisture content, and food was added if required.

After 28 d, test containers were emptied into a tray and surviving adults collected by hand sorting and weighed. Loss in earthworm weight after 28 d was calculated relative to the initial weight and expressed as the percentage reduction. Surviving earthworms were incubated on moist filter paper in Petri dishes for approximately 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, approximately 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, 1 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 (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 $<0.003\ mg\ L^{-1}$ for Zn. Digested blanks contained Zn concentrations below the limit of detection. Recovery of Zn (mean ± standard deviation [SD]) from the reference soil was $95 \pm 6.9\%$ and that 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 analysis of variance, and multiple comparisons were performed with Tukey's test ($p < 0.05$). Partition coefficients (K_{dPW}) were calculated as ZnT (milligrams per kilogram) divided by ZnPW (milligrams per kilogram). To compare soils and treatments with different Zn concentrations, bioaccumulation factors (BAFs) for the accumulation of Zn in the earthworms were calculated by dividing ZnE by ZnT. To determine the influence of soil properties and aging on Zn availability and earthworm responses for the 3 test soils, principal component analyses were performed using the CANOCO for Windows program, Ver 4.02. To study the effect of aging by relating soil properties and earthworm behavior, we assumed that soils, which were incubated under controlled conditions, had reached equilibrium well before day 140. The principal component 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-d exposure of the earthworms. Ordination diagrams were explained with soils shown as points and most of the studied variables (K_{dPW}, pH_{PW}, BAF, ZnE, and weight loss) as arrows, according to González-Alcaraz et al. [28].

RESULTS

Available Zn

Measured concentrations (mean ± 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 <18.5% for all treatments (Table 2). Porewater Zn concentration values were lower in ZnO-NP treatments than in soils spiked with $ZnCl_2$, with the difference being a factor of 4.4 to 32 in LUFA and NLGA soils spiked at $500\ mg\ kg^{-1}$ and a factor of 1.5 to 15 at $1000\ mg\ kg^{-1}$. In the spiked SPCA soil the difference was a factor of 1.9 to 7.5 and 1.2 to 3.3 when dosing at $1250\ mg\ kg^{-1}$ and $2500\ mg\ kg^{-1}$, respectively (Table 2). For $ZnCl_2$ treatments, average ZnPW corresponded with 5.04% to 11.4% of the total Zn in LUFA, 1.71% to 2.57% in NLGA, and 0.08% to 0.34% in SPCA soil. In the ZnO-NP treatments, ZnPW was ≤2.53% of ZnT in LUFA and ≤0.61% in the other 2 soils, and the fraction of Zn found in the porewater was lowest in SPCA soil (Table 2). In LUFA and NLGA, ZnPW tended to increase with time for all treatments, whereas in SPCA it remained constant or slightly decreased with time.

Partition coefficients calculated from ZnPW (K_{dPW}) were significantly affected by aging in LUFA for both Zn forms ($ZnCl_2$ and ZnO-NPs). In NLGA K_{dPW} values showed only a significant decrease with aging for ZnO-NPs (Tukey, $p < 0.05$), whereas 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 aging are shown in Table 3. Survival was >78% for all treatments and sampling times, except for SPCA spiked with $ZnCl_2$ after 56 d of aging where survival was only 25%. Earthworm weight loss, after 28 d of exposure, increased significantly with aging in all test soils (Tukey, $p < 0.05$) except for NLGA spiked with ZnO-NPs ($500\ mg\ kg^{-1}$). 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 aging (data not shown). Compared to the control, earthworm reproduction was most affected by $ZnCl_2$, with complete inhibition in LUFA and SPCA at all aging times and with ≥82% reduction in NLGA (Table 3). The LUFA soil also showed a

Table 2. Effect of aging on the availability of zinc in soil[a]

Soil	Treatment	Nominal Zn (mg kg^{-1})	Measured ZnT (mg kg^{-1} dry soil)	Aging (d)	ZnPW ± SD[b] (mg L^{-1})		Recovery ± SD (%)	K_{dPW} ± SD (L kg^{-1})
LUFA	$ZnCl_2$	500	487 (±87.4)	1	84.1 ± 4.50	a	7.78 ± 0.41	6 ± 0.31
				3	60.5 ± 4.67	a	5.59 ± 0.43	8 ± 0.62
				56	54.4 ± 4.31	a	5.04 ± 0.40	9 ± 0.71
				168	124 ± 14.7	b	11.4 ± 1.36	4 ± 0.47
	ZnO NP	500	497 (±36.7)	1	2.64 ± 0.42	a	0.24 ± 0.04	191 ± 30.4
				3	3.26 ± 0.08	a	0.30 ± 0.01	153 ± 3.64
				56	12.3 ± 2.11	b	1.12 ± 0.19	41 ± 7.04
				168	25.4 ± 1.52	c	2.30 ± 0.14	20 ± 1.18
	ZnO NP	1000	873 (±38.6)	1	5.51 ± 0.30	a	0.28 ± 0.02	159 ± 8.51
				3	5.46 ± 0.85	a	0.28 ± 0.04	162 ± 25.3
				56	37.4 ± 0.42	b	1.93 ± 0.02	23 ± 0.26
				168	49.0 ± 1.91	c	2.53 ± 0.10	18 ± 0.69
NLGA	$ZnCl_2$	500	495 (±55.3)	1	16.5 ± 2.77	a	1.71 ± 0.29	30 ± 5.10
				3	19.1 ± 0.89	ab	1.98 ± 0.09	26 ± 1.21
				56	23.6 ± 0.71	b	2.45 ± 0.07	21 ± 0.63
				168	24.8 ± 1.53	b	2.57 ± 0.16	20 ± 1.23
	ZnO NP	500	490 (±63.9)	1	2.48 ± 0.18	a	0.26 ± 0.02	198 ± 14.7
				3	2.12 ± 0.13	a	0.22 ± 0.01	231 ± 13.9
				56	2.04 ± 1.00	a	0.22 ± 0.11	273 ± 134
				168	5.68 ± 0.25	b	0.60 ± 0.03	86 ± 3.87
	ZnO NP	1000	858 (±49.0)	1	2.95 ± 0.06	a	0.18 ± 0.004	291 ± 6.27
				3	3.06 ± 0.39	a	0.18 ± 0.02	282 ± 28.4
				56	2.67 ± 0.14	a	0.16 ± 0.01	322 ± 17.4
				168	10.2 ± 1.68	b	0.61 ± 0.10	85 ± 14.0
SPCA	$ZnCl_2$	1250	1259 (±230)	1	6.84 ± 0.54	c	0.34 ± 0.03	185 ± 14.5
				3	4.95 ± 1.48	bc	0.24 ± 0.07	266 ± 79.6
				56	1.57 ± 0.08	a	0.08 ± 0.004	801 ± 39.6
				168	2.13 ± 0.06	ab	0.10 ± 0.003	591 ± 15.7
	ZnO NP	1250	1287 (±134)	1	0.91 ± 0.20		0.04 ± 0.01	1451 ± 304
				3	0.73 ± 0.02		0.04 ± 0.001	1753 ± 38.8
				56	0.81 ± 0.06		0.04 ± 0.003	1592 ± 125
				168	0.83 ± 0.20		0.04 ± 0.01	1598 ± 379
	ZnO NP	2500	2514 (±258)	1	2.05 ± 0.20		0.05 ± 0.005	1233 ± 118
				3	1.43 ± 0.52		0.04 ± 0.01	1888 ± 691
				56	1.33 ± 0.07		0.03 ± 0.002	1894 ± 95.0
				168	1.46 ± 0.21		0.04 ± 0.01	1736 ± 252

[a]Shown are the nominal and average measured total zinc concentrations (ZnT) found immediately after spiking soils (±standard deviation [SD], $n = 3$) in soils spiked with ionic Zn ($ZnCl_2$) or Zn oxide nanoparticles and porewater Zn concentrations (ZnPW; $n = 2$), obtained at different points in time after spiking the soil. Recovery is shown as the percentage of ZnT extracted in the ZnPW. Also included are porewater partition coefficient values, derived as the ratio of ZnT and ZnPW concentrations and indicating the strength of Zn binding to the different soils.
[b]Lowercase letters represent significance difference between sampling days for each treatment (Tukey's honestly significant difference test, $p < 0.05$).
K_{dPW} = porewater partition coefficient; $ZnCl_2$ = zinc chloride; ZnO NP = zinc oxide nanoparticle.

significant decrease in earthworm reproduction after 140-d aging 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 mg Zn kg^{-1} soil, whereas at 2500 mg kg^{-1} almost no reduction was seen compared to the control after 56 d.

Zinc concentration values in the earthworms differed within each soil (Table 4). Earthworms from the control soils had ZnE concentrations ranging from 105 μg g^{-1} to 143 μg g^{-1}, increasing in the order LUFA < NLGA < SPCA. Zinc concentration values in the earthworms differed from the controls for both $ZnCl_2$ and ZnO-NP treatments, except for earthworms kept in SPCA soil spiked with $ZnCl_2$, 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 μg Zn g^{-1} and 387 μg Zn g^{-1} earthworm in LUFA and NLGA, respectively, spiked with 1000 mg Zn kg^{-1} soil. The highest ZnE recorded was 408 μg Zn g^{-1} in earthworms kept in SPCA soil spiked with 2500 mg kg^{-1} Zn. In earthworms exposed to LUFA soil, ZnE showed a decrease with aging for the $ZnCl_2$ treatment, whereas for the ZnO-NP treatments ZnE showed an increase after 56 d of aging and a decrease after 140 d. In earthworms exposed to NLGA, ZnE was highest after 56 d of aging and significantly lower after 140 d (Tukey, $p < 0.05$). On exposure to SPCA, ZnE did not show remarkable changes on aging (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, whereas NLGA and LUFA had similar BAF values. The SPCA soil had the highest BAF in the lowest treatment with ZnO-NPs (1250 mg Zn kg^{-1} soil). At the same concentration added as $ZnCl_2$, BAFs were similar to those in the ZnO-NP treatment of 2500 mg Zn kg^{-1} soil. It should be noted that earthworm survival was only 25% in SPCA soil spiked with $ZnCl_2$ 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 aging. In LUFA and NLGA, BAFs were higher in the $ZnCl_2$ treatments and did not show clear trends with aging, whereas for the

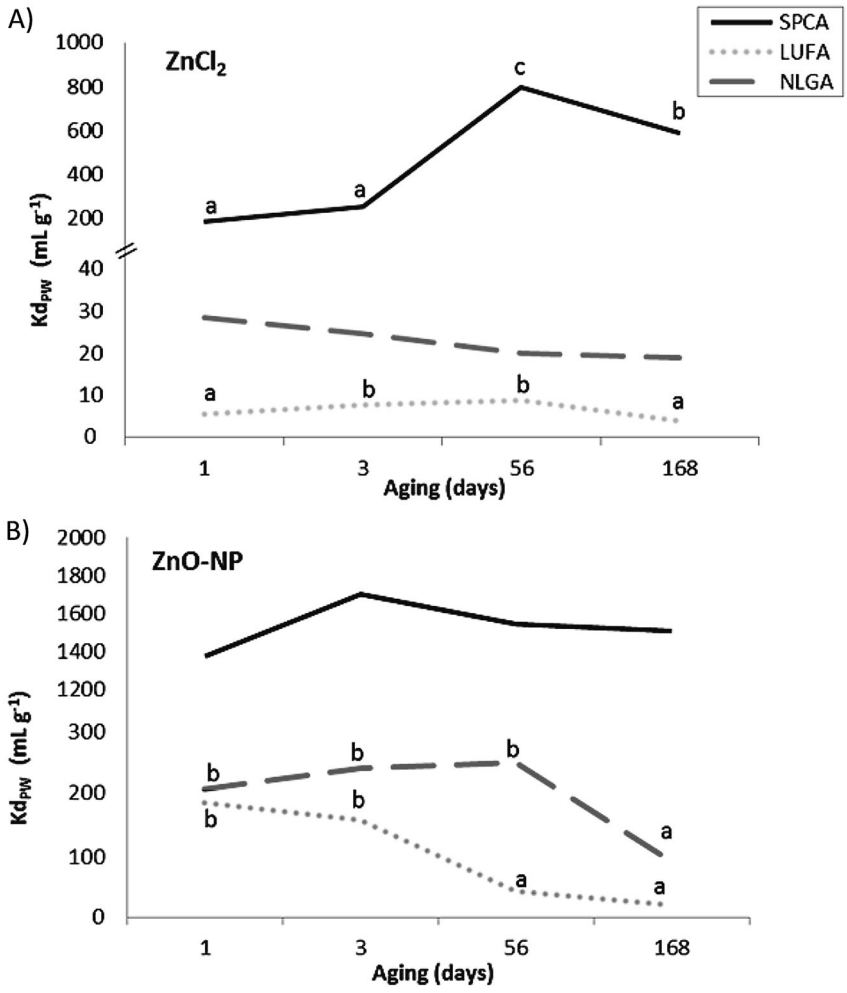

Figure 1. Average partition coefficients (K_{dPW}), expressed as the total zinc (Zn) concentration in soil divided by the porewater Zn concentration ($n = 2$) at 1 d, 3 d, 56 d, and 168 d after spiking the 3 test soils with Zn chloride (**A**) or Zn oxide nanoparticles (**B**) (numerical data are shown in Table 2). Nominal Zn concentration in LUFA and a garden in Bilthoven, The Netherlands (NLGA) soils was 500 mg kg^{-1}, and that in forestland in Granada, Spain (SPCA) soil was 1250 mg kg^{-1}. Letters indicate significant differences between K_{dPW} for different periods of aging (Tukey $p < 0.05$). $ZnCl_2$ = Zn chloride; ZnONP = Zn oxide nanoparticle.

ZnO-NP treatments they dose-relatedly decreased with increasing ZnT.

Influence of soil properties and aging on Zn bioavailability

The pH measured in porewater differed among treatments and incubation times (Supplemental Data, Table S1) and was higher in soils spiked with ZnO-NPs than in the control soils. In soils spiked with ionic Zn, porewater pH decreased or remained similar compared to the control. With aging, 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 was performed using ZnE, BAF, weight loss, K_{dPW}, and pH of the porewater for the 3 test soils and the different treatments ($ZnCl_2$ and ZnO-NP) as well as 2 different aging periods (1 d and 168 d). Because earthworm reproduction in $ZnCl_2$ treatments was very low in NLGA and 0 in the other 2 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_{PW}, K_{dPW}, and ZnE on the positive side of the main gradient (x axis; Figure 2A). Treatments with Zn applied as $ZnCl_2$ in this soil showed more separation from the ZnO-NP treatments and variables for time 1 d. The NLGA soil spiked with ZnO-NPs appeared in the center of the gradient and shifted to the negative side of the x axis when it was spiked with $ZnCl_2$. Porewater pH and K_{dPW} were negatively related and BAF was positively related to $ZnCl_2$ 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), weight loss was negatively correlated with SPCA soil spiked with $ZnCl_2$ (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

ZnO availability

The present results showed that at the same concentration of Zn added, soils spiked with ZnO-NPs had lower ZnPW concentrations than those spiked with $ZnCl_2$. This was also found by Waalewijn-Kool et al. [12] and indicates that ZnO-NPs behaved differently compared to ionic Zn. The lower porewater Zn concentrations obtained for the ZnO-NPs

Table 3. Effect of aging on the toxicity of zinc oxide nanoparticles and ionic zinc (applied as $ZnCl_2$) to the earthworm *Eisenia andrei* exposed to 3 different natural soils[a]

Soil	Treatment		Aging (d)	Survival ± SD (%)	W_L ± SD[b] (%)	Reproduction reduction ± SD[b] (%)
LUFA			1	90 ± 14	11 ± 8 a	100 (nsd)
	$ZnCl_2$	500 mg kg^{-1}	56	98 ± 5	12 ± 4 a	100 (nsd)
			140	95 ± 10	54 ± 1 b	100 (nsd)
			1	98 ± 5	4 ± 8 a	31 ± 24 a
	ZnO NP	500 mg kg^{-1}	56	100 (nsd)	8 ± 4 a	89 ± 13 b
			140	93 ± 10	31 ± 1 b	100 (nsd) b
			1	98 ± 5	4 ± 6 a	90 ± 8 a
	ZnO NP	1000 mg kg^{-1}	56	78 ± 19	9 ± 6 a	96 ± 5 ab
			140	85 ± 13	49 ± 7 b	100 (nsd) b
NLGA			1	100 (nsd)	15 ± 8 a	98 ± 3
	$ZnCl_2$	500 mg kg^{-1}	56	95 ± 6	29 ± 4 b	94 ± 11
			140	95 ± 10	30 ± 6 b	82 ± 18
			1	98 ± 5	26 ± 8	41 ± 41
	ZnO NP	500 mg kg^{-1}	56	93 ± 15	26 ± 5	–5 ± 38
			140	95 ± 6	25 ± 4	22 ± 21
			1	95 ± 10	17 ± 2 a	74 ± 12
	ZnO NP	1000 mg kg^{-1}	56	98 ± 5	27 ± 2 b	79 ± 17
			140	100 (nsd)	21 ± 7 ab	61 ± 25
SPCA			1	98 ± 5 b	21 ± 5 a	100 (nsd)
	$ZnCl_2$	1250 mg kg^{-1}	56	25 ± 13 a	32 ± 4 b	100 (nsd)
			140	83 ± 35 b	41 ± 5 c	100 (nsd)
			1	100 (nsd) b	14 ± 1 a	45 ± 32
	ZnO NP	1250 mg kg^{-1}	56	88 ± 10 a	18 ± 7 ab	41 ± 31
			140	100 (nsd) b	26 ± 2 b	75 ± 16
			1	100 (nsd)	9 ± 1 a	83 ± 17 c
	ZnO NP	2500 mg kg^{-1}	56	95 ± 6	16 ± 2 b	1 ± 17 a
			140	100 (nsd)	27 ± 3 c	45 ± 12 b

[a]Shown are percentage average of survival ($n = 40$) and weight loss ($n = 40$) after 4 wk and reduction of the number of juveniles produced per earthworm after 8 wk in percentage compared to the corresponding controls (reproduction reduction). Earthworm assays were performed after different periods of aging of the spiked soils.

[b]Lowercase letters represent significance difference between treatments (Tukey's honestly significant difference test, $p < 0.05$); all earthworms survived after 4 wk ($n = 40$) or no reproduction was observed in the samples after 8 wk ($n = 4$).

nsd = no standard deviation; SD = standard deviation; W_L = weight loss; $ZnCl_2$ = zinc chloride; ZnO NP = zinc oxide nanoparticle.

compared with $ZnCl_2$ 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 some of the ZnO-NPs present in the porewater did pass the 0.45-μm filter used when collecting porewater. Although we did not see large differences between Zn concentrations in porewater before and after ultrafiltration using a 3-kDa filter in a previous study [12], it is possible that our porewater collection method was not fully adequate for separating NPs from dissolved Zn. This element requires further study.

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. Differences in Zn solubility can also be related to soil properties, such as pH. Franklin et al. [29] found that dissolved Zn concentrations in porewater were higher in soils with lower pH, which matches 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 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 reported that soils with low pH and low organic matter content have a higher availability of Zn [31], which is in agreement with the present results, where LUFA soil (with the lowest organic carbon content and low pH) showed the highest ZnPW for both the ZnO-NP and $ZnCl_2$ treatments. The NLGA soil had a pH similar to that of LUFA but a higher organic carbon content; this soil showed lower ZnPW compared to LUFA for the $ZnCl_2$ 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[$CaCl_2$] 5). In the present study, the SPCA soil had the highest organic carbon 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. The partition coefficient K_d can be used to express the adsorption of Zn [33]. Our K_d values for the 3 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_d values decreased with decreasing soil pH, which agrees with literature data [34].

On aging, Zn availability increased in the LUFA and NLGA soils for all treatments, whereas in SPCA soil no significant differences were found for ZnO-NPs and a decrease was observed for $ZnCl_2$. Results for the times 1 d 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 mo of incubation. Lock and Janssen [18] also observed a faster rate of adsorption in soils with low CEC. Hence, aging or equilibration of contaminated soil might provide a more realistic insight into ZnO-NP

Table 4. Average zinc (Zn) concentrations in the earthworm *Eisenia andrei* with corresponding standard deviation ($n = 4$) after 4-wk exposure to Zn chloride or Zn oxide nanoparticles in 3 different soils aged for different periods of time: Also given are bioaccumulation factors calculated as Zn concentrations in the earthworms divided by measured total Zn concentrations in the soils (see Table 2)

Soil	Treatment	Nominal Zn ($mg\,kg^{-1}$)	Aging (d)	ZnE ± SD[a] ($\mu g\,g^{-1}$)	BAF ± SD[a]
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
	$ZnCl_2$	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
	$ZnCl_2$	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
	$ZnCl_2$	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

[a]Lowercase letters in Zn concentration in the earthworm *Eisenia andrei* indicate significant differences in each soil sample. Lowercase letters in bioaccumulation factor represent significant differences between treatment for each sampling day (both with Tukey's honestly significant difference test, $p < 0.05$).
BAF = bioaccumulation factor; SD = standard deviation; ZnE = Zn concentration in the earthworm *Eisenia andrei*; $ZnCl_2$ = zinc chloride; ZnO NP = zinc oxide nanoparticle.

behavior and, therefore, its potential toxicity under natural conditions. The equilibrium processes of metals between porewater 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].

Zn toxicity and 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 (50%) for effects on earthworm reproduction of ZnO-NPs and $ZnCl_2$ [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 aging (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], weight loss variation could be caused primarily by soil factors. Hooper et al. [7] observed greater weight loss of the earthworm *Eisenia veneta* exposed to $750\,mg\,Zn\,kg^{-1}$ soil as ZnO-NPs compared to treatments with ionic Zn. This disagrees with our finding of greater or the same weight loss in the $ZnCl_2$ treatments compared to the ZnO-NPs in all test soils. Heggelund et al. [11] found a dose-related increase in weight loss of the earthworm *Eisenia fetida* in $ZnCl_2$-treated soils but not in soils spiked with ZnO-NPs at concentrations of $238\,mg\,Zn\,kg^{-1}$ to $2500\,mg\,Zn\,kg^{-1}$ dry weight soil. In our ZnO-NP treatments, earthworms in LUFA and SPCA showed similar results at the 2 tested ZnO-NP concentrations, whereas in NLGA there was a small decrease with increasing ZnT. In addition, aging effects were observed in all soils, with an increase in weight loss with time in all treatments. The higher weight loss was observed for the LUFA soil for both $ZnCl_2$ and ZnO-NP treatments. The observed earthworm weight changes in our test soils suggest that, on exposure to ZnO-NPs, weight loss was not dose-related and is probably influenced by soil properties. It also suggests that there is an aging effect with an increase in weight loss 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

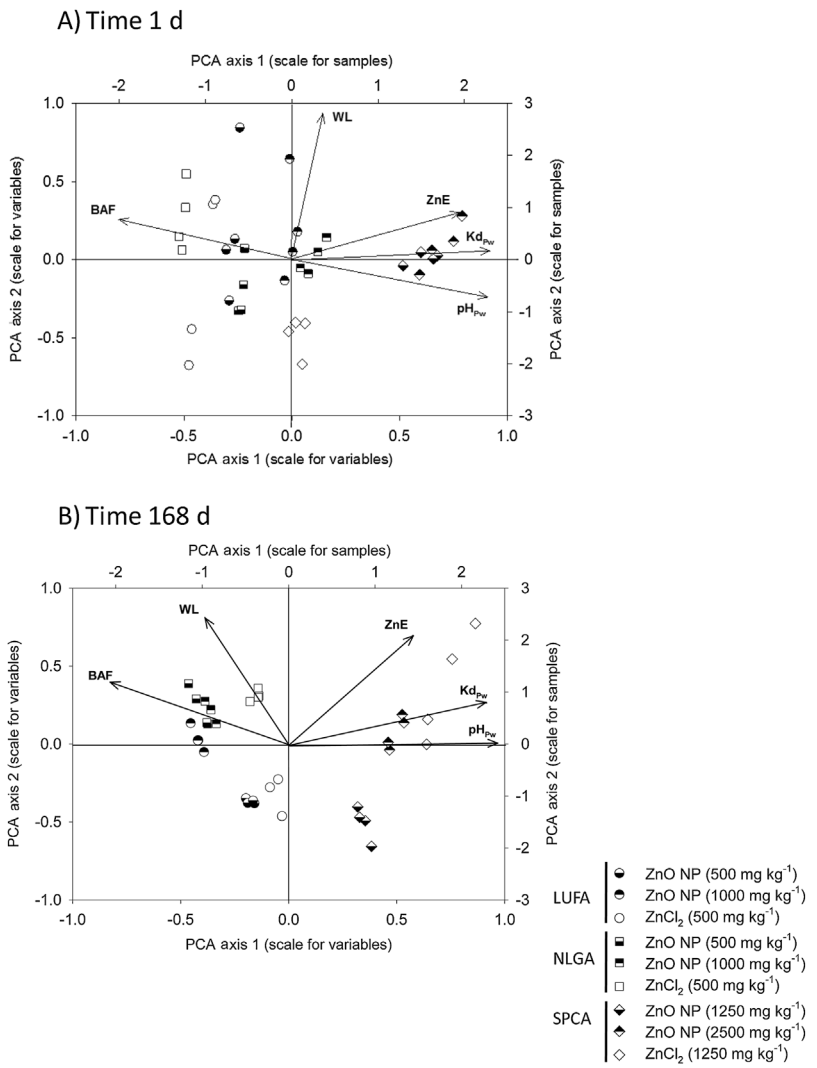

Figure 2. Principal component analysis for the relationship between earthworm toxicity and soil properties in 3 test soils spiked with zinc (Zn) oxide nanoparticles and Zn chloride. Parameters included in the principal component analysis are the partitioning coefficient calculated using porewater Zn concentrations, the pH of the porewater, the bioaccumulation factor, the Zn concentration in earthworm (ZnE), and earthworm weight loss. Principal component analyses were run for 2 periods of aging of the spiked soils, 1 d and 168 d. (**A**) Results for time 1 d; variance explained by the 2 first components is 69.3 % (*x* axis 45.7%, *y* axis 23.6%). (**B**) Results for time 168 d; variance explained by the 2 first components is 85.3% (*x* axis 58 %, *y* axis 27.3%). PCA = principal component analysis; $ZnCl_2$ = Zn chloride; ZnO-NP = Zn oxide nanoparticle.

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 $ZnCl_2$ 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 the present test soils. It is remarkable that in SPCA soil spiked with higher Zn concentrations reproduction was similar to that in the other 2 soils spiked at lower total Zn concentrations. This could be explained by the low Zn-PW 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 that, in some cases, inhibition was stronger at lower available Zn levels (e.g., after 56 d and 140 d of aging).

Studies using artificially spiked soils should be considered with care because metal solubility and toxicity may change with time (aging). 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 weight loss was seen in all test soils and treatments with aging, whereas 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 aging 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 aging could mainly be attributed to the higher Zn availability (ZnPW) in LUFA soil that showed an increase with aging (Table 2). Therefore, further studies on ZnO-NP toxicity with aging 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 concentrations of 123 μg Zn g^{-1} to 132 μg Zn g^{-1} earthworm ($n = 300$), which is within the range of ZnE values in our controls, between

105 μg Zn g^{-1} and 143 μg Zn g^{-1} earthworm. Heggelund et al. [11] also observed that in soils spiked with ZnO-NPs and $ZnCl_2$, 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 ± 65 μg Zn g^{-1} and 408 ± 158 μg Zn g^{-1} earthworm, respectively). The observed ZnE in the present 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 BAF is a good indicator to compare among soils, taking into account the difference in the applied concentrations; but a BAF value alone does not provide enough information because of metal autoregulation mechanisms in earthworms. In addition, after 24 h of depuration some 5% of the gut content may 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, the 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 K_{dPW} in the principal component analysis; Figure 2), with the lowest values at the highest soil concentrations. This behavior 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 principal component 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]. The 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 weight loss (Figure 2), with the earthworms having the lowest ZnE showing the highest weight loss. The lowest weight loss was found in SPCA soil, which suggests again an effect of soil properties rather than the ZnE on weight loss. 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 nanoparticulate 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 to improve the risk assessment of chronic ZnO-NP exposures. We compared the effect of Zn applied as NPs and as Zn^{2+} ions ($ZnCl_2$) on the Zn availability and bioavailability to the earthworm *E. andrei* at different incubation times after spiking in 3 natural soils with contrasting properties. Zinc concentrations in porewater were lower in soils spiked with ZnO-NPs compared with $ZnCl_2$. 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, whereas organic carbon explained Zn availability in soils spiked with $ZnCl_2$. The effect of aging on the availability of Zn showed differences without regular trends among soils as well as between treatments ($ZnCl_2$ and ZnO-NPs). Earthworms showed varying internal Zn concentrations among soils, which were highest in the soil with the highest organic carbon content and basic pH, following exposure to Zn applied as ZnO-NPs, even though this was the soil which showed the lowest ZnPW.

Toxicity of Zn to earthworm reproduction was highest for $ZnCl_2$ treatments, with almost complete reproduction inhibition; but there were no clear differences in survival and weight loss between treatments. An effect of soil aging 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 aging 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.3512.

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Data availability—The Supplemental Data show characterization of the ZnO-NPs, pH of the test soils at different incubation times, and results of the $Cu(NO_3)_2$ extraction method; further data are available on request from the corresponding author (anaromerof@ugr.es).

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