Toxicity of ZnO Nanoparticles, ZnO Bulk, and $ZnCl_2$ on Earthworms in a Spiked Natural Soil and Toxicological Effects of Leachates on Aquatic Organisms

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Abstract The present study assessed the uptake and toxicity of ZnO nanoparticles (NPs), ZnO bulk, and $ZnCl_2$ salt in earthworms in spiked agricultural soils. In addition, the toxicity of aqueous extracts to *Daphnia magna* and *Chlorella vulgaris* was analyzed to determine the risk of these soils to the aquatic compartment. We then investigated the distribution of Zn in soil fractions to interpret the nature of toxicity. Neither mortality nor differences in earthworm body weight were observed compared with the control. The most sensitive end point was reproduction. $ZnCl_2$ was notably toxic in eliminating the production of cocoons. The effects induced by ZnO-NPs and bulk ZnO on fecundity were similar and lower than those of the salt. In contrast to ZnO bulk, ZnO-NPs adversely affected fertility. The internal concentrations of Zn in earthworms in the NP group were greater than those in the salt and bulk groups, although bioconcentration factors were consistently <1. No relationship was found between toxicity and internal Zn amounts in earthworms. The results from the sequential extraction of soil showed that $ZnCl_2$ displayed the highest availability compared with both ZnO. Zn distribution was consistent with the greatest toxicity showed by the salt but not with Zn body concentrations. The soil extracts from both ZnO-NPs and bulk ZnO did not show effects on aquatic organisms (Daphnia and algae) after short-term exposure. However, $ZnCl_2$ extracts (total and 0.45-μm filtered) were toxic to Daphnia.

Zinc oxide (ZnO) nanoparticles (NPs) are widespread and are increasingly applied in various commercial products, such as personal care products, pharmaceuticals, medicine, as well as other industrial applications, including coating and paints, leading to concerns about their environmental fate and potential toxicity (Chang et al. 2012; Peralta-Videa et al. 2011). The main differences between NPs and their bulk counterparts relate to their high surface-to-volume ratio and the consequent changes in physicochemical, optical, reactive, and electrical properties. In addition, the environment in which NPs are present determines their behavior, reactivity, and potential toxicity (Baalousha et al. 2008; Bian et al. 2011). Information regarding the impact of ZnO-NPs on human health as the result of occupational or public exposure is already available, but data concerning the potential impact of these NPs in the environment is still scarce despite the rapid increase of peer-reviewed articles (Maurer-Jones et al. 2013; Ma et al. 2013; Kahru and Dubourguier 2010). Of note, data on the ecotoxicity of metal oxide NPs remain limited compared with those of other nanostructures, such as carbon-based, silver, and silica NPs. Among the metal oxides, nano-TiO_2 has been the most studied (Bigorgne et al. 2011).

The interaction between NPs and the soil matrix can greatly modify their availability due to aggregation, release

of the metal ion, oxidation, and sorption to soil components, among other processes; in turn, they can modify NP toxicity (Pan and Xing 2012). Despite this, most studies have been performed in artificial media. Therefore, experiments performed with natural soils are important for the study of NPs toxicity because these more realistically resemble environmental conditions (Zhao et al. 2012). Regarding organisms, more information is available about freshwater receptors compared with organisms that live in the soil (Kahru and Dubourguier 2010). In general, this information focuses on tests of acute toxicity (Tourinho et al. 2012; Li et al. 2011), thus leaving a gap (with some exceptions) in the knowledge about potential long-term effects (Kool et al. 2011; Manzo et al. 2011).

In this study, earthworms (*Eisenia fetida*) were exposed to ZnO-NPs in natural soil to evaluate the potential acute and long-term effects of exposure. Natural soil was used to increase the relevance of the study to represent natural conditions. The results were compared with the effects of $ZnCl_2$ and bulk ZnO. A concentration of 1,000 mg Zn kg^{-1} of soil dry weight (dw) was tested. Earthworms were selected based on their environmental relevance. Moreover, earthworms ingest large amounts of soil and they are continuously exposed to contaminants by direct dermal contact with chemicals in soil solution and soil atmosphere. To help to interpret the toxicity found, we studied the distribution of metal in the most labile soil fractions. We wanted to determine whether special characteristics of NPs can affect their mobility or availability and, consequently, their toxicity and accumulation in earthworm tissues. In addition, leachates from treated and control soils were obtained to assess the risks of transferring contaminants to adjacent surface or groundwater. The effects on two aquatic organisms belonging to different taxa (daphnia and algae) were determined.

The objectives of this study were to (1) compare the toxicity to earthworms of ZnO-NPs with the effects of bulk ZnO and soluble salt $ZnCl_2$, which were used as reference compounds for size-dependent and solubility effects; (2) assess the influence of the chemical form on the Zn distribution in soil fractions as well as on its incorporation in worms and toxicity; (3) relate toxic effects to the internal concentrations of Zn in earthworms; and (4) study the impact of leachates from treated soils on aquatic organisms (*Daphnia magna* and C*hlorella vulgaris*).

The results will draw attention to the effects of ZnO-NPs compared with ZnO bulk and $ZnCl_2$ on such environmentally relevant species as earthworms, Daphnia, and algae. Reliable ecotoxicological information regarding the effects of ZnO-NPs added to a natural soil is desirable to decrease the uncertainty of environmental risk assessments associated with the use of these compounds.

Materials and Methods

Materials and Organisms

Uncoated ZnO-NP powder (advertised particle size <100-nm diameter) and the bulk form of ZnO were purchased from Sigma-Aldrich (Germany), and $ZnCl_2$ salt was purchased from Panreac (Spain). The size and shape of the NPs, pristine material, was determined previously by the investigators with a transmission electron microscope (Fernandez et al. 2013). The mean size and SD were calculated by observing 200 ZnO-NPs in random view fields. The particle size distribution appeared to be approximately log-normal with 75 % of the particles (by number) having diameters from 20 to 80 nm. The mean ± SD of the NPs was 58.40 ± 30.13 nm.

The soil for ecotoxicity testing was collected from the top soil layer (0- to 20-cm soil depth excluding the vegetal cover) of a field located near Madrid (Spain) at GPS coordinates N40°27′18″, W03°44′55″. The soil was air-dried and sieved (2-mm mesh). It was used as control and to prepare the Zn treatments. The main physicochemical characteristics of this soil were as follows: clay 7.8 %; silt 18.8 %; sand 73.4 % (pH 6.8), and organic matter (OM) 1.9 %. OM, pH, and electrical conductivity (EC) were determined following the protocols of the Spanish Ministry of Agriculture (1994). OM was determined by dichromate-oxidation, and pH was measured in a 1:2.5 (w:v) soil-water suspension and EC in a 1:5 (w:v) soil-water suspension using a conductivity meter. The pH and EC tests were performed at the beginning of the experiment and after cocoons were harvested (35 days).

Eisenia fetida (Oligochaeta:Lumbricidae) were obtained from our own laboratory cultures. Clitellated adults (300 to 500 mg/individual) were kept on moist filter paper for 24 h to void the contents of their guts; then they were washed, dried, and weighed before being placed in test units. *D. magna* <24 h old and *C. vulgaris* in the exponential growth phase, both species from our own laboratory cultures, were used to measure immobilization and growth, respectively, in the soil aqueous extracts.

Soil Treatments

Soil was contaminated with one of the following chemicals: ZnO-NPs, ZnO bulk, or $ZnCl_2$ salt to generate the three treatments in this study. All of them were prepared at 1,000 mg (Zn-based) kg^{-1} soil oven dw.

ZnO-NPs and ZnO bulk were directly added to dried soil and hand blended according to previous studies (Franklin et al. 2007; Waalewijn-Kool et al. 2012) showing that the distribution of ZnO-NPs in the soil was not influenced by whether the NPs were added as a dry powder or as a

suspension. The treated soils were 2-mm sieved three times to ensure homogenization of the samples. $ZnCl_2$ salt was added to the soil as an aqueous solution to assess the toxicity of the ionic metal. The concentration was conditioned by the milliliters of water needed to reach the 50 % water-holding capacity (WHC) of soil. A nontreated soil was used as a control. Soils were wetted until 50 % WHC and left in the dark at 20 °C for 24 h before use to allow initial stabilization of mixtures before the worms were added.

Experimental Procedure

Twelve glass test containers (170 Ø × 90 mm height) were filled with 750 g (dw) of the treated soil or control soil with three replicates per group. MilliQ water was added to the containers until 80 % WHC. Ten adult earthworms per container were placed on the soil surface. The test units were covered with perforated plastic film and kept for 28 days under a continuous light source of 400–800 lux at 20 ± 2 °C. To avoid the introduction of additional organic material into the system, which could have effects on Zn forms behavior over time, and to increase the oral intake of soil particles as a consequence of hunger stress, the earthworms were not fed during this period.

The test of acute toxicity on the earthworms was based on Organisation for Economic Co-operation and Development (OECD 1984) recommendations. Mortality was assessed at 7 and 14 days by carefully emptying the test units. After the worms were counted, the surviving animals and soil were replaced back into the test units. Any behavioral or pathological symptoms were noted.

On day 28, the adults were removed from the units. The test units were left under the same conditions for 1 additional week (35 days from the start of the experiment). Then, cocoons from the same units were isolated by hand and placed in a moistened petri dish to hatch (20 °C, dark); the number of cocoons per unit was recorded. Hatching was monitored daily for 1 month to obtain the total number of hatchlings and calculate the rate of hatching success (Jensen et al. 2007). Fecundity was considered as the number of cocoons laid per adult per month and fertility as the number of offspring per cocoon.

Surviving earthworms were counted on day 28, rinsed with distilled water, kept for 24 h on moist filter paper, and weighed. Then earthworms were purged for an additional period of 24 h to decrease their gut contents as much as possible, frozen at −20 °C for 24 h, lyophilized (Telstar Cryodos), and analyzed for total Zn content as described in Chemical Analysis. The earthworms' bioconcentration factor (BCFworm) was calculated as the Zn concentration in the body (dw) divided by total Zn in soil (dw). Toxicity to aquatic organisms (*D. magna* and *C. vulgaris)* was determined for the aqueous extract samples (obtained as described in chemical analysis section: DIN method) according to OECD (2004, 2006) protocols.

Chemical Analysis

Lyophilized earthworms were ground to a fine powder with an agate mortar and pestle. All earthworms from each of the three replicates of each treatment were treated and analyzed together. Total Zn concentrations in whole worms (dw-material basis) were determined by wet acid digestion (10 mL of HNO_3 + 10 mL of HCl + 10 mL of double-deionized water) in Teflon bombs in a microwave oven (Mars; CEM, Matthews, NC, USA). The process included a heating ramp ≤170 psi in 20 min followed by a 20-min plateau at 1,200 watts. The extract was filtered (no. 42 filter paper; Whatman) and diluted with water to 50 mL.

Chemical analyses for Zn were performed in soil treatments before and after the toxicity assays and in earthworms after exposure. The soil samples were digested in a microwave oven equipped with a rotating tray using an acid mixture (10 mL of HNO_3 + 10 mL of HF + 10 mL of double-deionized water) following the previously described process but prolonging the plateau ≤100 min. A certified reference soil provided by the Institute for Reference Materials and Measurements of the European Commission (ERM-CC141) was used to identify the quality of the results of total Zn content.

Zn distribution in the different soil fractions was determined by sequential extraction. The most active fractions (reactive pools) were sequentially determined in three steps according to the methodology used by Pietrzak and McPhail (2004): F1 = water soluble (WS-Zn) with double-deionized water for 2 h ratio 1:10); and F3 = sorbed (SORB-Zn) with 1 % NaCaHEDTA in 1 M NH_4OAc for 2 h (using a soil-to-extractant ratio of 1:20). The difference between the total and the reactive pools is considered to be nonreactive or inert (Römkens et al. 2009). After each successive extraction, the soil suspension was centrifuged (4,500 rpm, 15 min), and the supernatant obtained was filtered through 0.45-μm cellulose acetate paper and acidified with HNO_3. Further studies to determine the nature of Zn (Zn ion, ZnO, or ZnO-NPs) in each fraction were not performed.

The aqueous extracts (leachates) were obtained following the DIN 38414-S4 (1984) method. In brief, 50 g of soil was mixed with 500 mL of water and continuously agitated for 24 h. The liquid phase extract was separated by centrifugation (4,000 rpm, 7 min). The extraction was performed in triplicate at the end of the toxicity soil test. Every extract was divided into three parts. The first one was kept as such; the second was filtered through 0.45-μm pores; and the third was acidified with HNO_3 and analyzed for Zn

Table 1 pH and EC of soil used for the earthworm exposures at start and the end of the assay

Treatment	pH (1:2.5)		EC (1:5) (μS cm^{-1})	
	Day 0	Day 35	Day 0	Day 35
Control	6.80 ± 0.01	6.73 ± 0.01[a]	284 ± 12	314 ± 21[a]
ZnO-NP	7.42 ± 0.02	6.97 ± 0.03[b]	216 ± 15	283 ± 18a
ZnO bulk	7.70 ± 0.02	7.22 ± 0.01c	216 ± 13	293 ± 16[a]
$ZnCl_2$	6.06 ± 0.02	5.93 ± 0.01[d]	906 ± 24	761 ± 30[b]

The ratio of soil to water (w:v) used in the measurements is included in parentheses. All values are mean ± SD ($n = 3$). Different superscript letters indicate significant statistical differences within columns ($p < 0.05$)

content. The first and the second parts were tested for toxicity to the aquatic organisms. This single extraction was performed to evaluate potential Zn losses by leaching from contaminated soils.

The soil samples were extracted and analyzed in triplicate using each of the procedures. PerkinElmer Pure standard checks were used for the quality-assurance system (certified by NIST-SRM). Standard solutions of Zn were prepared for each extraction in a background solution of the extracting agents. The Zn concentrations in all of the extracts obtained were determined using flame/graphite furnace atomic absorption spectrometry depending on the Zn concentration range (Analyst 700; PerkinElmer).

Statistical Analysis

The data were analyzed statistically using STATGRAPHICS software (version 5.0). Statistically significant differences between individual means for chemical and toxicological data were identify by analysis of variance with Fisher's least significant difference procedure (LSD; $p < 0.05$).

Results and Discussion

Physicochemical Soil Properties

pH and EC of the control and spiked natural soils are listed in Table 1. Addition of ZnO-NPs and ZnO bulk increased soil pH by 0.6 and 0.8, respectively, in agreement with other investigators (Kool et al. 2011; Zhao et al. 2013; Heggelund et al. 2014), although the differences decreased with time. This decrease could be associated with the slight increase of Zn^{2+} in solution released from the ZnO-NP or bulk because this ion is a strong Lewis acid (Zhao et al. 2013). For $ZnCl_2$ salt treatment, the decrease in pH value compared with that of the control soil observed at the beginning of the assay could be explained by the excess Zn ions causing a release of protons from sorption sites on the soil (Kool et al. 2011). The addition of both ZnO-NPs and ZnO bulk decreased EC values compared with the control. In contrast, the addition of $ZnCl_2$ strongly increased the EC due to the incorporation of the dissolved salt. During the assay, EC values in the oxide treatments increased as a result of their solubilization in the medium. Earthworm activities also contributed to the increasing levels of soluble salt in the soils (Chaudhuri et al. 2012, Sizmur et al. 2011). However, the conductivity of the salt-treated soil decreased with time, most likely due to the movement of ions to less labile fractions of the soil over time (Trelo-ges et al. 2002).

Zn Concentrations in Soil and Extracts

The test concentration of 1,000 mg Zn kg^{-1} soil was selected based on a previous study performed in our laboratory with the same soil contaminated with ZnO-NPs at four different doses ranging from 125 to 1,000 mg Zn kg^{-1} (dw). In that work, no significant differences were observed in survival and growth rates of the adult earthworms, and only negative effects on reproduction were statistically significant at the highest concentration. In addition, this concentration not only is high enough to provoke undesirable effects on earthworms, it is also well above the solubility of the ZnO oxides (both NPs and bulk); hence, the proportion of the corresponding ions in the soil will be low compared the ZnO molecule as such.

At the beginning of the assay, the total Zn concentration was 52.60 mg kg^{-1} dw in the control soil (background level) and 972–1,023 mg kg^{-1} dw in the Zn treatments, which is very close to the nominal concentration of 1,000 mg Zn kg^{-1} soil dw. A good distribution of applied Zn in the soil was obtained, and there was low variation among repetitions (<10 %).

It is known that Zn added to soils may undergo sorption to soil components (mineral and organic) among others processes. Moreover, Bystrzejewska-Piotrowska et al. (2012) showed that ZnO-NP extractability with water decreased significantly after 10 days. In our assay, the availability and distribution of Zn in the natural soil of the control as well as in the treatments was studied after 35 days of incubation using a sequential chemical-extraction technique (Table 2). Sequential extraction has been used extensively to assess the partitioning of trace elements in environmental samples. However, literature involving the application of this technique to NPs is scarce (Coutris et al. 2012; Zhao et al. 2012). In this work, only fractions involved in displacement processes were obtained because they are related to the labile Zn portion and more easily accessible to soil-inhabiting organisms (earthworms in this work). The WS-Zn and EX-Zn fractions in soils are the

Table 2 Total Zn concentration measured in control and spiked soils (nominal 1,000 mg Zn kg^{-1} soil) at the start (day 0) and in soil extracts obtained by DIN and sequential-extraction procedure at the end (day 35) of the earthworm assay

Treatment	Zn (mg kg^{-1} soil dw)						
	Total content	Single extraction (DIN)		Sequential-extraction procedure			
		Non filtered	Filtered (0.45-μm)	WS (H_2O)	EX ($MgCl_2$)	SORB (NH_4Ac/NaCaHEDTA)	Nonreactive pool
Control	52.60 ± 0.44	1.20 ± 0.35[a]	0.9 ± 0.28[a]	0.53 ± 0.06[a]	3.68 ± 0.59[a]	13.6 ± 2.0[a]	34.79 ± 11.07[a]
ZnO-NP	972.9 ± 40	18.40 ± 0.63[b]	6.20 ± 0.07[b]	7.29 ± 0.37[b]	386.5 ± 7.1[b]	285.1 ± 12.5[b]	320.94 ± 18.3[c]
ZnO Bulk	1,023.2 ± 61	22.90 ± 0.35[c]	8.40 ± 0.14[c]	8.17 ± 0.80[b]	372 ± 30.5[b]	360.8 ± 33[c]	258.71 ± 62.4[b,c]
$ZnCl_2$	982.1 ± 91	23.4 ± 0.7[c]	21.0 ± 1 d	19.61 ± 1.36[c]	491.0 ± 12.6[c]	259.1 ± 5.7[b]	230.21 ± 18.8[b]

All values are mean ± SD ($n = 3$). Different superscript letters indicate significant statistical differences between treatments and control for the same column

Table 3 Effects on reproduction, BCFworm, and internal Zn concentrations on *E. fetida* after 28-day exposure

Treatment	Cocoons/worm/month (fecundity)	Offspring/cocoon (fertility)	offspring/worm	BCF worm	Internal Zn (mg kg^{-1} dw)
Control soil	2.63 ± 0.36[a]	2.06[b]	5.4 ± 1.1[b]	2.31 ± 0.04[a]	121 ± 2[a]
ZnO-NP	1.08 ± 0.32[b]	1.42[a]	1.5 ± 0.4[a]	0.21 ± 0.03[b]	204.9 ± 34.7[c]
ZnO-Bulk	1.50 ± 0.44[b]	2.81[c]	4.2 ± 1.1[b]	0.15 ± 0.01[c]	158.9 ± 11.7[b]
Cl_2Zn	0.03 ± 0.05[c]	NM	NM	0.13 ± 0.01[c]	126.8 ± 5.3[ab]

All values are mean ± SD ($n = 3$). Different superscript letters indicate significant statistical differences between treatments and control for the same column

NM not measured because only one cocoon was found in this group

most easily mobilized and are considered the bioaccessible fraction (directly available pool). The ethylene diamine tetraacetic acid (EDTA) extractable fraction (SORB-Zn) represents sorbed metal reacting with binding sites located on the surfaces of clay, amorphous metal oxides, and soil OM. Different patterns of partitioning of Zn were observed for the salt and the oxides (Table 2). The maximum WS-Zn concentration corresponded to the $ZnCl_2$-spiked soil treatment (2 % of total). The percentage of WS-Zn in soil treated with ZnO bulk was 0.85 and 0.75 % for ZnO-NPs. Considering EX-Zn, $ZnCl_2$ salt again showed the highest portion of the total (49 %), and Zn oxides achieved similar values (39 and 37 % of the total for NPs and bulk, respectively). In the control soil, >66 % of total Zn was found in a nonreactive pool. Nevertheless, the added Zn was mainly directly available and on the surfaces of soil particles as reactive forms (WS, EX, and SORB-Zn fractions): 67.9, 74.1, and 77.0 % of applied Zn for ZnO-NPs, ZnO bulk, and $ZnCl_2$, respectively. The lower WS-Zn and EX-Zn size fractions suggested that the amount of bioavailable Zn was lower in the soils treated with ZnO-NPs or ZnO bulk than with $ZnCl_2$ salt. This is consistent with the highest toxicity shown by the salt, but it contrasts with the Zn body concentrations as listed in Table 3. ZnO-NPs were the Zn species with the highest content in the nonreactive pool indicating that they were associated with less accessible forms according with Zhao et al. (2012). However, these data were obtained during a short time period (35 days), and the accessible Zn fraction might increase over time due to Zn release from ZnO-NPs (Coutris et al. 2012).

DIN extracts were obtained with the purpose of evaluating the potential Zn leaching from soils contaminated with ZnO-NPs, ZnO bulk, and $ZnCl_2$ salt to aquatic bodies. These data are listed in Table 2. Zn-extractable concentrations from soil were determined as such and filtered through 0.45-μm pores. The nonfiltered extracts from salt and ZnO bulk soils contained similar concentrations of Zn, but they were significantly greater than the metal amount measured in leachates from ZnO-NPs. However, in the filtered samples, Zn concentrations from both ZnO-NP and ZnO bulk decreased, suggesting that the dissolved molecules from ZnO-bulk or NPs were joined mainly to soil particles or to organic dissolved matter, that their size exceeded the filter size, or a mix of these possibilities. As expected, the Zn concentration in the filtered samples from $ZnCl_2$-spiked soil did not differ from the nonfiltered extracts, signifying that the most Zn was present in the ionic form.

Toxic Effects on Earthworms

No mortality was detected in any of Zn treatments or the control after 28 days. This result was expected based on results from previous studies (Heggelund et al. 2014; Lock and Janssen 2003; Hooper et al. 2011) where *Eisenia sp*. adults were exposed to Zn (in artificial soil from ZnO-NPs, ZnO bulk, and $ZnCl_2$ salt) in a concentration range from 250 to 10,000 mg kg^{-1} without lethal effects. Earthworms in all groups (both treatments and control) lost weight (weighed 10–15 % less than initial weight) during the test period, likely because of the absence of food. Comparing final values, the mean body weight of worms exposed to Zn treatments was not statistically different from that of the control group.

The effects on reproduction are listed in Table 3. The fecundity of worms was decreased in all Zn-treated soils, especially in the $ZnCl_2$ salt group, where inhibition of cocoon production compared with the control was >98 %. The highest toxicity shown by the salt is consistent with the highest available fractions (WS-Zn and EX-Zn) obtained in the sequential extraction (Table 2). The fecundity in both oxide groups was statistically similar, and the decrease was not as dramatic as that observed with the salt (59 and 43 % inhibition for NPs and bulk, respectively). Moreover, some cocoons appeared to be malformed, with unfinished cocoon shells, and some juveniles appeared to be paler and less healthy. This fact seems to indicate that the size and high specific surface of the NPs did not increase toxicity with respect to bulk material and that the toxicity may be due to the release of Zn ions at a rate lower than that of the salt. In this study, the fecundity of *E. fetida* in the ZnO-NP group was similar to the results by Heggelund et al. (2014) and Hooper et al. (2011). Moreover, these investigators found that $ZnCl_2$ salt caused much more inhibition of fecundity than did NPs.

In contrast, notable differences were found between the fertility of earthworms in the ZnO-NP and ZnO-treated soils and the control (Table 3). Relative to the control, ZnO-NPs decreased fertility by 72 %, whereas ZnO bulk increased the final number of offspring per cocoon by 36 %. The high fertility observed in cocoons from the ZnO bulk samples was unexpected, but similar results have been found by other investigators for different metal oxides NPs (Heckmann et al. 2011). No fertility data could be obtained from the $ZnCl_2$ salt group due to the near absence of cocoons.

Although no significant differences in fecundity were found between ZnO-NPs and ZnO bulk, differences in fertility caused a significant decrease in the number of juveniles in the NP group (Table 3). This effect is of major importance because the exposure of earthworms to ZnO-NPs could lead to a serious depletion of the earthworm population over time. The increased negative effects on fertility associated with ZnO-NPs (compared with bulk) may be explained by differing capacities in penetrating biological membranes and different mechanism of action between the two forms of Zn (Ma et al. 2011, 2013).

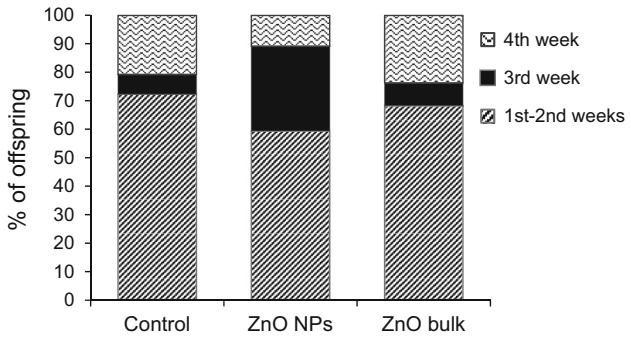

Fig. 1 Emergence of hatchlings over time. Percentage of total juveniles within each treatment after 1, 2, 4, and 4 weeks

Differences were also observed in hatch time between treatments. The time taken for the emergence of juveniles within each group is shown in Fig. 1. The timing of emergence of hatchlings in the ZnO bulk treatment was similar to that of the control; however, cocoons from the ZnO-NP treatment took longer to hatch.

Toxic Effects on Aquatic Organisms

The toxicity of soil leachates to aquatic organisms depends both on the nature of the contaminants and on the transfer of pollutants from the soil to leachates. Therefore, it is important to perform ecotoxicological analysis with leachates because they allow for studding the integration of chemical and toxicological effects.

Aqueous extracts (DIN method) were tested for toxicity in *D. magna* and *C. vulgaris*. No mortality was observed for the aquatic invertebrate *D. magna* exposed to ZnO-NPs or to ZnO bulk extracts. This is explained because total Zn concentration in extracts (Table 2) were lower than the LC_{50} (*D. magna*) values in the range 2.6–3.2 mg Zn L^{-1} (equivalent to 26–32 mg Zn kg^{-1} soil) for ZnO-NPs and bulk reported in the literature (Blinova et al. 2010; Wiench et al. 2009; Heinlaan et al. 2008). In contrast, extracts from $ZnCl_2$-treated soil induced 75 and 63 % mortality relative to the control for original and 0.45-μm filtrated samples, respectively. These results also agree with bibliographic LC_{50} for Zn ion between 0.35 and 3.3 mg Zn L^{-1} depending on physicochemical characteristics of the spiked natural freshwater (De Schamphelaere et al. 2005).

Zn concentration in the DIN nonfiltered extracts was similar in the three Zn species (Table 2) but was greatly

decreased in the 0.45-μm filtrates of the ZnO (both forms), which could explain differences of toxicity observed compared with the salt. Moreover, the toxicity of ZnO-NPs could be due to dissolved Zn ions (Ma et al. 2013). Then the extracts from ZnO-NPs were then filtered by 0.02 μm to determine the concentration of ion Zn in these samples. This concentration was very low (0.32 ± 0.07 mg Zn L^{-1}) and can explain the lack of toxicity observed (De Schamphelaere et al. 2005).

No toxic effects were identified on *C. vulgaris* exposed to filtered and nonfiltered extracts from any of the Zn-treated soils. This lack of effects differs from other published results where the EC_{50} values for dissolved Zn have been reported to be as low as 0.04 (Muyssen and Janssen 2001) or 0.153 mg Zn L^{-1} (Sbihi et al. 2012).

The nontoxic nature of leachates from soils contaminated at 1,000 mg kg^{-1} with ZnO-NPs to aquatic organisms suggests that the risk of soils contaminated with ZnO-NPs due to the transference of metal from soil to surface and groundwaters is low.

Internal Earthworm Zn Concentration and Bioconcentration

Data regarding the internal Zn contents in worms are listed in Table 3. Levels of Zn in earthworms exposed to the control and to the soil treated with $ZnCl_2$ were statistically similar ($p < 0.05$), but they differed from the amounts of Zn in worms living in ZnO-NP soil. It is remarkable that earthworms exposed to $ZnCl_2$ salt at an external concentration 20 times greater than the control soil reached an internal Zn concentration similar to that in the worms in the control group. These data coincide with the value of nearby 120 mg kg^{-1} body dw established by Lock and Janssen (2001) and by Smith et al. (2010) for the regulation of this essential metal by *E. fetida* and *E. andrei*, respectively, regardless of $ZnCl_2$ exposure concentration in soils contaminated ≤1,000 mg kg^{-1} soil dw. The Zn body burden from ZnO-NP treatment (205 mg Zn kg^{-1} dw) was greater than that from ZnO bulk and $ZnCl_2$ salt, which is in agreement with Hooper et al. (2011). The internal overload reached in worms exposed to ZnO-NPs suggests that novel entry pathways and novel mechanisms may be involved in Zn autoregulation when it is added in NP form.

A correlation between body residues and toxicity was not found in this study. Zn concentration in worms followed the order control ≈ $ZnCl_2$ salt < ZnO bulk < ZnO-NPs. However, the highest effects on fecundity were observed for $ZnCl_2$ salt. One hypothesis is that animals assimilated Zn more quickly when it was added as $ZnCl_2$ salt than as oxides due to presumed greater bioavailability of the salt, and the adverse effects were most likely caused by increased intake rate rather than internal concentration (Lock and Janssen 2001; Van-Straalen et al. 2005). The highest intake rate correlates with the metal in solution, and it is associated to direct uptake by way of the skin. Direct uptake by way of the skin in worms inhabiting in ZnO-NP or ZnO bulk soil is of low importance (Li et al. 2011). Consequently, in the absence of lethal effects, earthworms can incorporate Zn at a lower speed by oral ingestion, and thus they can achieve greater final NP content. Moreover, we assumed that the body earthworm concentrations measured in this study were bioavailable as a whole without taking into account the site or the route of toxic action. However, some investigators (Li et al. 2011; Hooper et al. 2011) found that the intracellular distribution of dissolved Zn and NP ZnO was different. This would partially explain the decreased effects found in the NP treatment despite the highest accumulation of this Zn species. However, further studies should be performed to clarify the results obtained.

The BCF_{worm} value was <1 with the exception of the control soil (Table 2), indicating that the chemicals were not bioconcentrated from the soil under the test conditions. The BCFworm value of salt and ZnO bulk were statistically similar ($p < 0.05$) but different from that for ZnO-NP, which presented the highest value within the contaminated samples. The BCFworm value found for NPs in this work (Table 3) was similar to the value found by Hu et al. (2010) of 0.26 (dw); they exposed *E. fetida* at the same ZnO-NP concentration of 1,000 mg kg^{-1} soil dw.

Conclusion

In a natural soil, ZnO-NPs had more effects on earthworm reproduction based on fertility than the classic bulk ZnO, although, $ZnCl_2$ salt was the most toxic compound. This observation is of concern because continuous exposure to ZnO-NPs would decrease the earthworm population over time. In addition, earthworms exposed to ZnO-NPs achieve the highest internal level of Zn, suggesting that vermivorous animals would have a greater exposure to this metal in NP-contaminated habitats compared with other chemical forms at the same Zn level. However, no risk of Zn biomagnification was found; all BCFworm values were <1. In terms of risk assessment, the data presented here are useful in suggesting that ZnO-NPs should be considered slightly differently from the larger-size ZnO molecules, and these results provide evidence for concern regarding earthworm protection. With respect to aquatic organisms, the results suggest a low risk as a result of the transfer of ZnO-NPs from contaminated soils to ground or surface waters. However, the $ZnCl_2$ extracts were toxic to Daphnia.

Sequential extraction showed that both ZnO-NPs and ZnO bulk had less availability in natural soils compared

with Zn salt. Moreover, NPs were the Zn species with the highest content in the nonreactive pool. These results were consistent with the highest toxicity shown by the salt, but they contrasted with Zn body concentrations.

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