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RESEARCH ARTICLE

Integrating ecotoxicity and chemical approaches to compare the effects of ZnO nanoparticles, ZnO bulk, and $ZnCl_2$ on plants and microorganisms in a natural soil

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Abstract This work compared the toxicity of ZnO nanoparticles (ZnO-NPs), ZnO bulk, and $ZnCl_2$ on microbial activity (C and N transformations and dehydrogenase and phosphatase activities) and their uptake and toxic effects (emergence, root elongation, and shoot growth) on three plant species namely wheat, radish, and vetch in a natural soil at 1000 mg Zn kg^{-1}. Additionally, plants were also tested at 250 mg Zn kg^{-1}. The effects of the chemical species on Zn extractability in soil were studied by performing single and sequential extractions. $ZnCl_2$-1000 presented the highest toxicity for both taxonomic groups. For microorganisms, ZnO-NPs demonstrated adverse effects on all measured parameters, except on N transformations. The effects of both ZnO forms were similar. For plants, ZnO-NPs affected the growth of more plant species than ZnO bulk, although the effects were small in all cases. Regarding accumulation, the total Zn amounts were higher in plants exposed to ZnO-NP than those exposed to ZnO bulk, except for vetch shoots. The soil sequential extraction revealed that the Zn concentration in the most labile forms (water soluble (WS) and exchangeable (EX)) was similar in soil treated with ZnO (NP and bulk) and lower than that of $ZnCl_2$-treated soil, indicating the higher availability of the ionic forms. The strong correlations obtained between WS-Zn fraction and the Zn concentrations in the roots, shoots, and the effects on shoot weight show the suitability of this soil extraction method for predicting bioavailable Zn soil for the three plant species when it was added as ZnO-NPs, ZnO bulk, or $ZnCl_2$. In this work, the hazard associated with the ZnO-NPs was similar to ZnO bulk in most cases.

Keywords Phytotoxicity · Soil microbial activity · ZnO nanoparticles · Zn extractions

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Introduction

The use of engineered nanoparticles (NPs) in consumer goods is rapidly increasing, and these NPs may be released into the environment during their life cycle. NPs may interact closely with environmental components. Hence, their fate, behavior, and the potential for harmful effects on ecosystems must be evaluated. Reviews on the toxicity of metal NPs to terrestrial plants have recently been published (Ma et al. 2013; Rico et al 2011). Because ZnO-NPs have widespread uses in cosmetics, pharmaceuticals, and photocatalyst pigments, they are found in sewage water and may be integrated into sewage sludge. Land applications of these residues may then be an important pathway for the introduction of ZnO-NPs into soils. Therefore, their ecotoxicological effects on crops are a significant concern (Rico et al. 2011; Fernandez et al. 2014).

Plants grow at the interface between different environmental compartments, e.g., soil/air and water/air, and are involved in the biogeochemical cycling of not only nutrients but also

pollutants in these habitats. As a result of their exposure to ZnO-NPs in contaminated soil and water, the potential of plants for the bioaccumulation and transference of ZnO-NPs through the food chain should be studied. Most nanotoxicological studies conducted on plants have used alternative exposure media in place of soil. These media include aqueous suspensions, nutrient solutions, and agar (Pokhrel and Dubey 2013; Lee et al. 2013; Lopez-Moreno et al. 2010). These studies allow for the investigation of the underlying mechanisms and the relevant physicochemical and biological parameters, but these assays do not mimic field conditions and, hence, may overestimate or underestimate toxicity. Additionally, some studies have examined the toxicity of ZnO-NPs to seed germination, plant uptake, and translocation in artificial or modified soils (Mukherjee et al. 2014; Zhao et al. 2013; Du et al. 2011). Studies of the phytotoxicity of ZnO-NPs under more relevant exposure conditions are scarce despite the importance of the medium and the environmental conditions on the availability and, hence, the toxicity and metal concentrations in crop plants (Peralta-Videa et al. 2014; Rico et al. 2011; Zhao et al. 2012a).

The microbiota plays an essential role in the cycling of elements and stabilization of soil structure. Several studies have reported adverse effects of ZnO-NPs on microbial diversity and community composition, but these studies did not examine the ecotoxicological relevance in terms of soil functions (Ge et al. 2011), although biochemical indices that assess transformations of C, N, and P are frequently used in the diagnosis of soil quality (Fraç and Jezierska-Tys 2011). Most of the processes occurring in soil are microbially mediated and are carried out by enzymes. The enzymes most frequently investigated in soils threatened with anthropogenic contamination are dehydrogenases (DH) and acid phosphatases (PH) because they respond the fastest to an increase in the content of metals in the environment (Bartkowiak and Lemanowicz 2014).

The toxicity of contaminants depends on their available fraction rather on their total soil concentration (Degryse et al. 2009). Currently, there is no universally accepted methodology to determine the bioavailability of metals and, thereby, predict their impacts on soil ecosystem. Single and sequential extraction schemes have been extensively applied with the aim of studying the environmental fate (leaching potential and bioavailability) of classic-sized metals in soils (Bacon and Davidson 2008; Hass and Fine 2010). Although it is unlikely that chemical extraction techniques can mimic the process of metal absorption or uptake in organisms, such techniques provide good estimates of the bioaccessibility of metals in the environment. Based on the experience and knowledge gained from studies of the bioavailability of trace metals in soils, the application of similar techniques to estimate bioavailability of metal oxide NPs holds promise (Coutris et al. 2012; Zhao et al. 2012b).

In this work, the phytotoxicity, the accumulation potential, and the translocation of ZnO-NPs, ZnO bulk, and $ZnCl_2$ spiked into an agricultural soil were studied on *Triticum aestivum*, *Raphanus sativus*, and *Vicia sativa.* Changes in the biological activity of the soil, such as alterations in respiration and nitrification functions and dehydrogenase and acid phosphatase activities, were also evaluated. Moreover, we determined Zn extractability in soil using single and sequential protocols to determine whether special characteristics of NPs could affect their mobility or availability and, consequently, their toxicity and accumulation in plants.

The objectives of the present study were to (i) compare the toxicity of ZnO-NPs to plants and microorganisms with the effects of bulk ZnO and the soluble salt $ZnCl_2$, which were used as reference compounds for size-dependent and solubility effects; (ii) assess the influence of the chemical form of Zn on its distribution in soil fractions; and (iii) relate the toxic effects to the internal concentrations of Zn in the plants.

Materials and methods

Chemicals

Uncoated ZnO nanopowder (advertised particle size <100 nm diameter) and the ZnO bulk form were purchased from Sigma-Aldrich (Germany), and the $ZnCl_2$ was from Panreac (Spain). The size and shape of the nanoparticles were determined with a transmission electron microscope (TEM) as described by Fernandez et al. (2013). A concentrated suspension of ZnO-NPs (1 mg mL^{-1}) was prepared in deionized water and dispersed with a homogenizer/disperser (T25 digital ULTRA-TURRAX, IKA®-Werke, Germany) at 100 W and 40 kHz for 20 min. A drop of this suspension was placed onto a carbon-coated copper grid, air-dried, and observed with a TEM (JEM-2100, JEOL).

The reagents NitriVer® 3 and NitriVer® 5 used for the determination of nitrites and nitrates in soil extracts were acquired from Hach (Spain).

Soil, plants, and microorganisms

Soil was collected from the top layer (0–20 cm depth) of a field located near Madrid (Spain), GPS coordinates N 40°27′ 18″, W 03°44′55″. The soil was air-dried and sieved (<2 mm mesh). The background concentration of zinc in soil was 52.6 mg kg^{-1}. The main physicochemical characteristics of this soil were as follows: clay, 7.8 %; silt, 18.8 %; sand, 73.4 %; pH, 7.48; and organic matter content (OM), 1.09± 0.07 %. OM, pH, and electrical conductivity (EC) were determined following the protocols of the Spanish Ministry of Agriculture (MAPA 1994). OM was determined by dichromate oxidation. pH and EC were measured in 1:2.5 and 1:5

soil/water (*w*/*v*) suspensions, respectively. Both the pH and EC tests were conducted at the beginning of the experiment and after 21 days.

Seeds of three plant species, *T. aestivum* (wheat), *R. sativus* (radish), *V. sativa* (vetch), were used. The seeds were obtained from the Spanish National Centre for Seeds and Vegetal Varieties (Madrid). The native microorganism populations in the soil were used to determine the functional and enzymatic activities.

Exposure treatments

Soil was contaminated with 1000 mg Zn kg^{-1} soil as ZnO-NPs, ZnO bulk, or $ZnCl_2$ to test for potential harmful effects on microorganisms and plants. Additionally, a fourth $ZnCl_2$ exposure treatment for plants at 250 mg Zn kg^{-1} soil ($ZnCl_2$-250) was prepared to determine if the solubility of ZnO-NPs could induce effects that were similar to a lower concentration of the salt after 21 days, assuming that the Zn^{2+} from the oxide would be responsible for toxicity. The Zn concentrations were always calculated on a dry soil (DW) basis. Untreated soil (natural Zn occurrence) was used as the control. The ZnO-NP and ZnO bulk powders were directly added to the soil and hand blended following previous studies (Hooper et al. 2011; Garcia-Gomez et al. 2014a). Moreover, Waalewijn-Kool et al. (2012) demonstrated that the distribution of ZnO-NPs in the soil was not influenced by the pathway of application (dry powder or suspension). Therefore, the powder application was selected because it was easier to make a homogenized mixing of the samples. The mixes were later sieved (2 mm) three times to ensure the homogenization of the samples. $ZnCl_2$ was added to soil as an aqueous solution to guarantee that Zn^{2+} was the chemical form added to the soil. The volume of water added was equivalent to that required to achieve 50 % of the water holding capacity (WHC) of the soil. Control and treatment samples were wet to 50 % WHC and stored in the dark at 20±2 °C for 24 h before filling pots or glass containers.

Performance of ecotoxicity test

Plant assays in pots

Five groups (control and four treatments) of plastic planting pots were prepared with three replicates per group. Each pot was filled with 1.80 kg of the control or treatment soil (DW), and then dechlorinated water was added to reach 80 % WHC. Twenty-one seeds of the three plant species (seven per species) were sown in sectors in each pot and incubated for 21 days in a climate-controlled room at 20±2 °C and 3000–3600 lux (16 h light). Moisture was maintained by weighing pots (twice a week) and adding water if needed.

The emergence of the seeds was registered at 7 and 14 days. After 21 days, the emerged plants were harvested, collecting the whole plant. The lengths of stems and roots and the fresh weights of the aerial parts were recorded. Plants were rinsed with tap water thoroughly (15 min) followed by deionized water (5 min). Subsequently, the roots were cut off and washed in deionized water in an ultrasound-assisted bath at 35 kHz for 15 min (Feng et al. 2005). Roots and shoots were dried at 60 °C for 24 h to a constant weight and separately stored in plastic bags until Zn content analysis.

Microorganism assays in glass containers

Four groups (control and three treatments) of glass containers with three replicates per group were used to assess the effects of Zn on microbial respiration rate and enzymatic activities, particularly DH and PH. Simultaneously, to determine the nitrification activity, a second set of identical containers that received a nitrogen source (500 mg Lucerne) was prepared. Every container was filled with 100 g (DW) of control or treatment soil. Containers were incubated for 28 days (20± 2 °C, dark, 40–60 % WHC). The moisture of the samples was controlled by weight. Microbial functions and enzymatic activities were evaluated at the beginning (24 h after the zinc addition to the soils) and end of the incubation. Duplicates were collected from each replicate for analysis.

Mineralization and nitrification effects were determined using standardized methods (OECD 2000a, b, 216, 217). DH and PH activities were measured according to Carbonell et al. (2000) and Freeman et al. (1995), respectively. Soil subsamples used for determining respiration rates were amended with 4 mg glucose/g soil (DW), and the carbon dioxide released was measured using a BacTrac 4300 SY-Lab (Microbiological Analysers). Effects on nitrifying bacteria were determined by measuring nitrate concentration in soils. Nitrate was extracted from the soil samples by shaking with a 0.1 M potassium chloride solution and then reduced to nitrite, which was determined by a colorimetric method using the NitriVer® 3 and NitriVer® 5 reagents. The nitrification activity was obtained as the difference between the nitrate content at day 28 and at day 1. The DH and PH activities were analyzed photometrically by the absorbance of 1,3,5-triphenylformazan at 490 nm and the fluorescence intensity of 4-methylumbelliferone at the excitation and emission wavelengths of 360 and 465 nm, respectively. These measurements were collected using a microplate spectrofluorometer GENios (Tecan, Switzerland).

Characterization of Zn in soil and plant material

The chemical analyses for Zn were performed on all replicates of the control and treated soils at the beginning and immediately after the toxicity assays and in the plant material after

exposure. The acid digestions of solid samples were conducted following one of the following methods.

The soil was digested in Teflon bombs in a microwave oven (CEM Corporation, model-Mars, Matthews, NC, USA) equipped with a rotating tray using an acid mixture of HNO_3/HF/double deionized water (1:1:1). This involved a two-step process at 1200 W (heating ramp up to 170 psi in 20 min, followed by a 100-min plateau at 170 psi pressure). A certified reference soil provided by the Institute for Reference Materials and Measurements of the European Commission (ERM-CC141) was used to verify the quality of the results for the total Zn content. In the case of plant material, the dry roots and aerial portions were digested separately. The digestion of plant tissues was conducted following the above-described process but with an acid mixture of HNO_3/HCl/double deionized water (1:1:1) and a shorter plateau step of 20 min. The soil and plant extracts were filtered (no. 42 filter paper, Whatman).

Zinc distribution in the different soil fractions from the microorganisms assay was determined by the sequential extraction procedure (SEP) proposed by Pietrzak and McPhail (2004). The metal pools related to biological effects (reactive fractions) were sequentially determined in three extraction steps: F1, with double-deionized water for 2 h (WS, water soluble); F2, with 1 M $MgCl_2$ for 2 h (EX, exchangeable) (using a soil/extractant ratio of 1:10 for both F1 and F2); F3, with 1 % NaCaHEDTA in 1 M NH_4AcO for 2 h (SORB, sorbed) (soil/extractant ratio of 1:20). Zinc was extracted from soil in each step using a 50-mL tube centrifuge with the corresponding extracting solutions. The samples were then agitated at room temperature (24±2 °C) in a rotative shaker. It was important to avoid delays between adding the extracting solution and beginning of shaking. After each successive extraction, the supernatant was obtained by centrifugation (4500 rpm, 15 min), decantation, filtering through 0.45 μm cellulose acetate paper, and acidification with HNO_3.

To assess the soil Zn available for plants, a slightly modified version of the one-step rhizosphere-based extraction method (the soil/extractant ratio was reduced to 1:4 to increase analytical precision) was used: 5.00 g of soil in 20 mL of a 0.01 M mixture of low-molecular-weight-organic acids (LMWOAs, combined organic acid solution of acetic, lactic, citric, malic, and formic acids in a molar ratio of 4:2:1:1:1, respectively) (Feng et al. 2005). A one-step extraction was conducted with the aim of evaluating the potential of Zn losses by leaching from soils contaminated with ZnO-NPs, ZnO bulk, and dissolved Zn^{2+}. The easily leachable Zn fraction in the soil was extracted using $BaCl_2$ as the extractant (3.00 g of soil in 30 mL of 0.01 M $BaCl_2$) (Schultz et al. 2004), which is a modified version of Normal ISO 11260 (1994). Both single extracts were filtered through 0.45 μm cellulose acetate paper and acidified with HNO_3.

All soil samples (three for control and nine for Zn treatments) were extracted and analyzed in triplicate using each of the procedures. Zn concentrations in the extracts were determined using flame/graphite furnace atomic absorption spectrometry depending on the Zn concentration range (Perkin-Elmer, AAnalyst 700). "Perkin-Elmer Pure" standard checks were used for the Quality Assurance System (certified by NIST-SRM). External standards including blanks were prepared in the corresponding extracting solutions. Ulterior studies to determine the nature of Zn (Zn ion, ZnO, or ZnO NPs) in each extract were not performed.

Data analysis

The data were analyzed statistically using the STAT GRAPHICS software (Version 5.0). Statistically significant differences between individual means for chemical and toxicological data were identified by one-way analysis of variance (ANOVA) with Fisher's least significant difference procedure (LSD, $p<0.05$).

Total Zn uptake was calculated as the product of dry matter production and the total Zn concentration in roots or shoots. The transference factor (Tf) was defined as the ratio of the Zn concentration in shoots to that in roots. The bioconcentration factor (BCF) was calculated as the ratio of the Zn concentration measured in roots or shoots to that of the Zn in soil. Zinc concentrations are reported on a DW basis.

Results and discussion

ZnO nanoparticles and physicochemical soil properties

The TEM image revealed that the ZnO-NPs were mainly elongated in shape. The mean size and SD calculated by observing 200 ZnO-NPs in random view fields was 58.40±30.13 nm. The particle size distribution showed that 75 % of the particles (by number) had diameters from 20 to 80 nm.

The pH and EC values of the control and spiked natural soils are included in Table 1. Initially, the pH of soils treated with ZnO-NPs and ZnO bulk increased relative to the control; however, in agreement with other authors, these differences decreased with time in the presence of plants (Zhao et al. 2013; Heggelund et al. 2014). These decreases could be associated with the slight increase of Zn^{2+} in the solution (it is a strong Lewis acid) released from the ZnO (Zhao et al. 2013; Dimkpa et al. 2013). However, the pH of soils spiked with $ZnCl_2$ (at both concentrations) initially decreased, likely because the excess of Zn ions caused a release of protons from sorption sites in the soil (Kool et al. 2011), and continued decreasing with time. Statistically significant differences ($p<0.05$) were found among the pH values of the soils treated with the different Zn forms at 1000 mg Zn kg^{-1} after 21 days. Regarding EC values, after the addition of both ZnO oxides, the EC values were lower than that of the control, probably as

Table 1 Total Zn content, pH, and electrical conductivity (EC) of the soils used for the plant assays at the initiation and end of the exposure period. Values are the mean±SD (n=3). The soil/water (w/v) ratio used for the measurements is included in parentheses

Treatment	[Zn] (mg kg^{-1} DW)			pH (1:2.5)		EC (1:5) (μS cm^{-1})	
	Nominal	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
Control	–	52.6±1.5	56.4±2.0 a	7.48±0.03	7.33±0.02 a	291±6.0	315±4.5 c
ZnO-NP	1000	977.3±2.9	986±29.3 c	8.10±0.01	7.65±0.03 b	207±5.2	263±20.2 b
ZnO bulk	1000	964.4±19.0	978.2±10.5 c	8.29±0.02	7.83±0.02 c	202±7.9	229±14.8 a
$ZnCl_2$	1000	955.6±17.7	976.6±10.3 c	6.48±0.02	6.32±0.01 d	946±17	1037±32.5 d
$ZnCl_2$	250	268.5±13.1	277.6±8.5 b	7.24±0.02	6.78±0.02	497±9.1	561±10.0

Different letters indicate significant differences within columns (LSD, $p<0.05$)

result of ZnO molecules combining with cations or anions from soil components (Table 1). In contrast, the EC strongly increased with the addition of $ZnCl_2$ because of the incorporation of the dissolved salt. After 21 days, the EC values in the oxide treatments, especially in the case of ZnO-NPs, had significantly increased as a result of their solubilization in the medium but remained lower than the control. In the $ZnCl_2$ treatments, the final EC values continued to increase during the exposure period.

Toxic effects on plants

The nominal test concentration of 1000 mg Zn kg^{-1} was selected because it is high enough to provoke undesirable effects on plants (Rico et al. 2011; Zhao et al. 2013) and it is well above the solubility of the ZnO (both NPs and bulk). Hence, the proportion of the ions is low compared to the non-dissociated molecule.

In Fig. 1, the toxic effects of Zn treatments on wheat, radish, and vetch, expressed as the percentage of inhibition relative to control, at the given concentration are included. Neither ZnO-NPs nor ZnO bulk had a significant effect on the emergence of any of the three plant species. Only $ZnCl_2$ at the highest dose ($ZnCl_2$-1000) inhibited seed germination in vetch probably due to a higher permeability of the seed coat of vetch by Zn ion. This result agrees with the findings of other authors who have reported no effect of exposure to ZnO-NP suspensions up to 2000 mg L^{-1} on this parameter for six different crops, even under hydroponic conditions (Lin and Xing 2007; Lopez-Moreno et al. 2010).

The lengths of roots demonstrated a low sensitivity as only the plants exposed to $ZnCl_2$-1000 were affected, with a 37 to 47 % reduction compared to the control depending on the

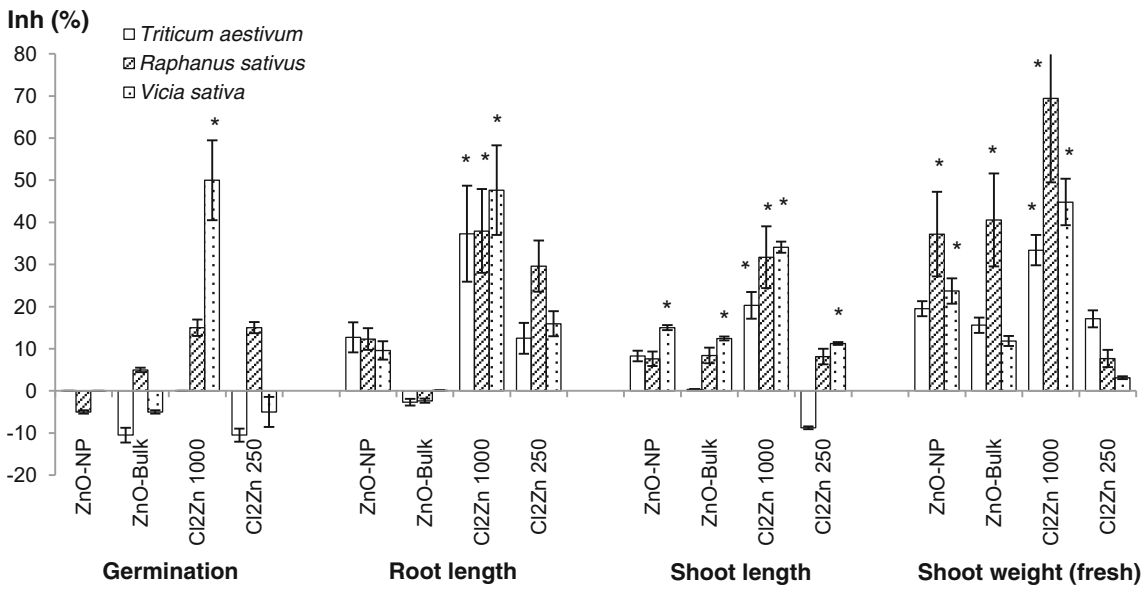

Fig. 1 Phytotoxic effects on plants grown in soil contaminated with ZnO-NPs, ZnO bulk, and $ZnCl_2$ at 1000 mg Zn kg^{-1} soil and $ZnCl_2$ at 250 mg Zn kg^{-1} soil after 21 days of exposure. Values are expressed as the percentage of inhibition relative to the control. *Asterisk*, significantly different from controls (LSD test, $p<0.05$). *Error bars* represent±SD (n=3)

species (Fig. 1). Previous studies were limited to wheat (Dimkpa et al. 2013) and radish (Lin and Xing 2007), and exposure conditions differed from those in this work. Both of these studies reported phytotoxicity towards root elongation, the first by ZnO-NPs and by bulk ZnO (in a lesser degree) on wheat at 500 mg Zn kg^{-1} sand and the second for radish at 20 mg ZnO-NPs L^{-1} aqueous suspension. The lack of sensitivity in this work could be associated to the use of a natural soil that affects the mobility and, hence, toxicity of metals in soil (Thomson and Frederick 2002). These results highlight the importance of the growing media on plant toxicity and the need to conduct more realistic assays employing a natural environment such as soil.

Regarding effects on growth, the shoot weight was more sensitive than the shoot length. Thus, considering shoot length, wheat and radish were resistant to the treatments, except for $ZnCl_2$-1000. Only vetch had shoot lengths that were significantly shorter than the control, with the effects ranging between 13 and 34 % for all three Zn forms. The results from the ZnO-NPs, ZnO bulk, and $ZnCl_2$-250 treatments were statistically similar.

The shoot weights of the three species were reduced when they were grown in $ZnCl_2$-1000. The effects of ZnO-NPs and ZnO bulk depended on the plant species. The weights of radish shoots in both soil oxide treatments were highly affected (38–40 % inhibition), and the vetch weight was sensitive to ZnO-NPs but not to the bulk partner (Fig. 1). By contrast, wheat shoot weight was not affected by ZnO-NPs in accordance with the result from Du et al. (2011). None of the three plant species were affected by $ZnCl_2$-250. These results seem indicate that the amounts of Zn^{2+} from ZnO-NPs or Zn bulk were higher than the corresponding amount from the salt at 250 mg kg^{-1} or that the toxicity of the oxides was not only associated to the Zn^{2+} released from ZnO-NPs.

In the plant assay, the shoot weight was the only parameter that differed significantly between NPs and bulk for vetch and between ZnO-NPs and $ZnCl_2$-250 for radish and vetch ($p<0.05$). In both cases, ZnO-NPs were of higher concern than the other forms.

Effects on soil microbial activity

The cultures of microbial populations were tested independently from those of plants to avoid the influence of enzyme exudates from the roots on the microbial activity measurements. Although soil enzymes are primarily derived from microorganisms, they can also originate from plants and animals (Frederick et al. 2014).

In Fig. 2, the results obtained for the inhibitory effects are presented as the percentage relative to the respective control. The organic carbon mineralization in all soil treatments at both test times was reduced compared to the respective controls, although the differences were less than 30 %. The maximum effect was observed for $ZnCl_2$, and there was no difference between ZnO-NPs and Zn bulk. Similarly, N transformation processes were only affected by $ZnCl_2$, which inhibited N turnover by more than 30 % compared with the control. Both DH and PH (to a lesser extent) activities were significantly affected by the Zn treatments (Fig. 2). DH activity was reduced immediately following the addition of any Zn chemical to the soil, and this reduction increased with the incubation period. Among treatments, there were not significant differences in DH activity between microorganisms exposed to ZnO-NPs and those exposed to both ZnO bulk or $ZnCl_2$ after 28 days; however, the responses of the later (ZnO bulk and $ZnCl_2$) were statistically different from each other ($p<0.01$). The PH activity at day 1 was not affected by any ZnO form (NP or bulk), but it was reduced by the Zn ion. After 28 days, all treatments inhibited PH activities by a similar amount. The amount of soil N available to plants mainly depends on N mineralization-immobilization turnover, which is conducted by "keystone species" that do not appear to be affected by the ZnO forms (NPs or bulk), whereas the mineralization of organic matter is carried out by a large number of

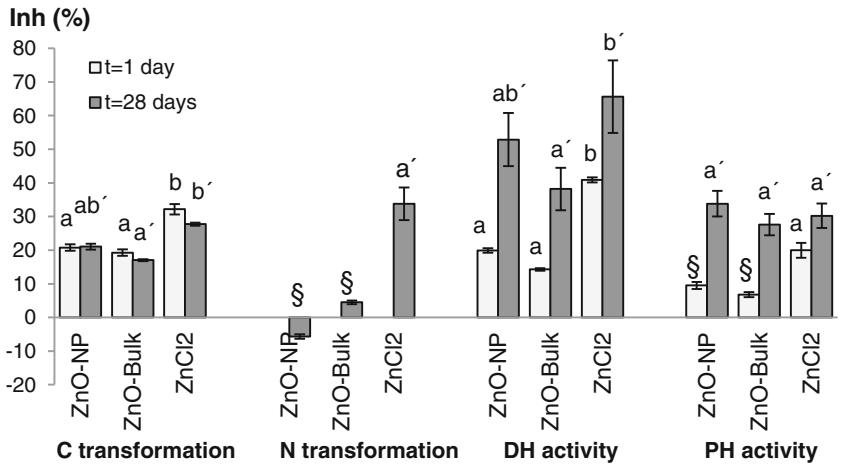

Fig. 2 Effects of Zn on soil microorganisms exposed to ZnO-NPs, ZnO bulk, and $ZnCl_2$ at 1000 mg Zn kg^{-1} soil. Values are expressed as the percentage of inhibition relative to the control. *Section sign*, no significantly different from controls. *Different letters* indicate significant differences between treatments for each period (LSD test, $p<0.01$). *Error bars* represent± SD ($n=3$)

microorganisms. The lower C transformation rates and DH and PH activities after 28 days of incubation could be ascribed to the suppression of a sensitive soil biota component in the Zn-treated samples. Previously, Collins et al. (2012) found that ZnO-NPs caused a reduction in microbial diversity. Moreover, the results from this study have confirmed ecological consequences of ZnO-NPs in terms of soil functions, except for N transformations. In the Collins et al. (2012) study, the authors placed the blame on the Zn^{2+} from ZnO-NPs and suggested a rapid dissolution/transformation of the ZnO-NPs over a 30-day period. This would explain the similarities of ZnO-NPs with $ZnCl_2$ after 28 days in this work. Based on this result, the release of Zn^{2+} from bulk ZnO would be slower.

Zinc concentrations in soil and distribution in soil fractions

The initial determinations of Zn in the treated soils ranged from 97 to 107 % of nominal values. This confirmed the correct spiking of soil samples. A good distribution of Zn within the soil was obtained based on the low variation among replicates (less than 8 %) (Table 1).

The amounts of extractable Zn from treated and untreated soil were measured immediately after both the plant harvest (21 days) and the soil microbial activity measurements (28 days) (Table 2). In all cases, multifactor variance analyses revealed differences among treatments ($p<0.0001$); the extractable Zn concentration in soil depended on the chemical extraction procedure and the source of Zn pollution. In this work, a SEP was applied to the soils from the microorganism incubation, following an established procedure. The first two fractions extracted (WS and EX) are the most accessible to living organisms and, according to Coutris et al. (2012), can be referred to as the potentially bioaccessible fraction (the directly available pool). The EDTA-extractable fraction (SORB) represents sorbed metal reacting with binding sites located on surfaces of clay, amorphous metal oxides, and soil organic matter. Only the fractions involved in displacement processes were obtained because the difference between the total and these pools (WS plus EX and SORB) is considered to be non-reactive or inert (Römkens et al 2009). In the control soil, only 8.5 % of total Zn was potentially bioaccessible (WS+EX), while more than 70 % was found in a non-reactive pool. In contrast, for ZnO-NPs, ZnO bulk, and $ZnCl_2$, much of the applied Zn existed in a bioavailable form: 43.6, 41.8, and 56.4 %, respectively. The maximum WS-Zn concentration corresponded to the $ZnCl_2$-spiked soil treatment that differed significantly from the ZnO-NPs and ZnO bulk ($p<0.0001$), while the WS-Zn from both the ZnO bulk and ZnO-NPs did not differ significantly. Considering the EX-Zn, the $ZnCl_2$ salt again represented the highest portion of the total (51.9 %), and the values for the bulk material and NPs were similar. Overall, the sizes of the WS and EX fractions suggest that the amount of Zn from ZnO-NPs that is bioavailable to microorganisms should be similar to that of ZnO bulk and that both are less bioavailable than the corresponding $ZnCl_2$ treatment.

In the plant assay, the amounts of Zn extracted by the LMWOAs reagent (which assesses potential Zn availability to plants) were considerably higher than those obtained for the

Table 2 Total and Zn-extractable concentrations measured in soil after the application of different Zn treatments at the end of the corresponding assay. All values are mean±SD ($n=3$)

Treatment	Zn (mg kg^{-1} soil DW)							
	Plant assay $t=21$ days				Microorganisms assay $t=28$ days			
	Total Zn	Single extractions			Total Zn	Sequential extraction		
		WS[a]	Potentially[b] available	Easily[c] leachable		WS[a]	EX[d]	SORB[e]
Control	52.6±2.6 a	0.95±0.01 a	2.19±0.27 a	0.86±0.12 a	52.6±2.6 a	0.75±0.11 a	3.74±0.48 a	9.77±1.1 a
ZnO-NP	986±29.4 c	8.63±1.7 c	176.87±14.6 c	13.50±1.1 c	969.4±28.1 b	7.23±0.39 b	397.4±14.4 b	385.9±39.1 c
ZnO bulk	978±10.5 c	9.71±1.0 c	192.37±19.4 c	13.15±0.45 c	985.3±14.4 b	8.11±0.74 b	386.0±21.8 b	450.2±43.8 c
$ZnCl_2$-1000	976±10.3 c	14.00±0.03 d	192.44±6.3 c	55.73±2.8 d	998.1±69.5 b	19.34±3.0 c	518.5±9.9 c	302.9±13.1 b
$ZnCl_2$-250	277.6±8.5 b	2.66±0.17 b	26.39±1.8 b	5.47±1.7 b	No assayed	No assayed	No assayed	No assayed

Different letters indicate significant differences between treatments and the control for the same extractant (LSD, $p<0.0001$)

[a] Zn-extractable with deionized water

[b] Zn-extractable with a mixture of low-molecular-weight-organic acids

[c] Zn-extractable with $BaCl_2$

[d] Zn-extractable with $MgCl_2$

[e] Zn-extractable with NH_4Ac/NaCaHEDTA

WS fraction. Moreover, the LMWOAs-extractable Zn exhibited a different behavior from the WS-extractable Zn for the various treatments applied (Table 2). The amount of Zn extracted by the LMWOAs reagent was as follows: $ZnCl_2$-1000~ZnO bulk~ZnO-NPs>$ZnCl_2$-250>control. Therefore, according to this soil test, the quantities of potentially available Zn to plants were similar when Zn was applied to soil at 1000 mg kg^{-1} as ZnO-NPs, ZnO bulk, or $ZnCl_2$. However, the Zn extracted in the WS fraction from the $ZnCl_2$-1000 treatment produced the highest amounts of Zn, and both Zn oxides had similarly lower values. Although the order for Zn extracted with the $BaCl_2$ reagent (easily leachable Zn) followed the same order as WS-Zn, the treatment with the highest dose of $ZnCl_2$ produced a rather high amount of easily leachable Zn (more than four times higher) and, therefore, a higher risk for potential Zn loss by leaching from contaminated soils with this salt compared with the two types of oxides, which again exhibited a very similar behavior.

Zn in plants

The total Zn concentrations in shoots and roots (DW material basis) are presented in Fig. 3. The Zn contents in roots were higher than those in shoots for all Zn treatments and the control, except for radish. Taken as a whole, regardless of the plant species, the total Zn in roots and shoots followed the order $ZnCl_2$-1000>ZnO-NPs>ZnO bulk>$ZnCl_2$-250>control. Considering species, the potential of plants for Zn accumulation in roots (wheat>vetch>radish) and in shoots (radish>wheat>vetch) followed the same order in all treatments irrespective of the chemical form of Zn applied to the soil. Thus, all soils showed an analogous profile of Zn accumulation for the three plant species, indicating that the incorporation of Zn into plants occurred in a similar manner with a similar regulation mechanism regardless of the chemical form.

In roots, total Zn levels (2100–3400 mg kg^{-1} root) from ZnO-NPs soil were appreciably higher than those from ZnO bulk (1700–2500 mg kg^{-1} root) and more similar to the Zn values for the $ZnCl_2$-1000 treatment (3000–3700 mg kg^{-1} root) than to those from the $ZnCl_2$-250 treatment (750–1600 mg kg^{-1} root) (Fig. 3). Similarly, applications of ZnO-NPs to this soil always resulted in greater total Zn uptake by roots than ZnO bulk additions.

To determine whether it is possible to establish a relationship between the accumulation of Zn in plants and the observed effects, both parameters were compared. There was no clear correlation between total Zn concentrations in roots and effects on root length for all species. Significant differences in Zn-root concentrations among treatments (Fig. 3) did not lead to significant root length reductions (Fig. 1). Although the Zn concentrations of roots of plants exposed to ZnO-NP were similar or only slight lower than those for $ZnCl_2$-1000, the effects on root length were observed only in plants exposed to the ion at the highest concentration. This could be explained having in mind that the total Zn concentration measured in the roots consisted of both internal Zn and Zn adsorbed to the root surface. The ultrasonic washing of the roots minimized but did not eliminate the Zn adsorbed (Zhou et al. 2011) and hence the Zn root concentration could overestimate the Zn uptake depending on the Zn compound. Although NPs adsorbed to plant roots can also exert physical or chemical toxicity on the plants (Capaldi Arruda et al. 2015), the differences observed between ZnONPs and $ZnCl_2$ could

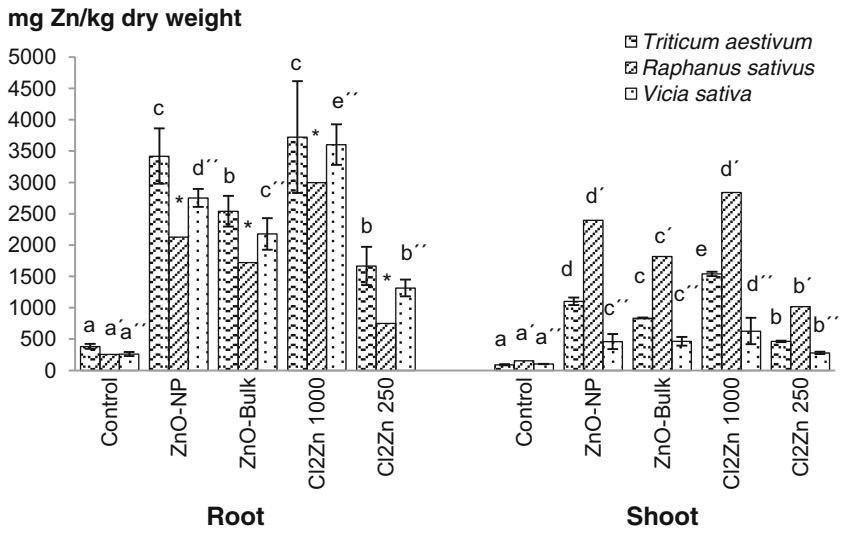

Fig. 3 Zn concentration in shoots and roots of plants grown in soil contaminated with ZnO-NPs, ZnO bulk, and $ZnCl_2$ at 1000 mg Zn kg^{-1} soil and $ZnCl_2$ at 250 mg Zn kg^{-1} soil after 21-day exposure. *Different letters* indicate significant differences among Zn treatments for each plant species (LSD test, $p<0.0001$). *Error bars* represent±SD ($n=3$). *Asterisk*, SD values could not be calculated in radish roots because the root material from a single replicate was not substantial enough for Zn determination; hence, the root replicates were combined for Zn measurements

be explained by differences in the ratio between absorbed and adsorbed Zn in roots exposed to these Zn molecules.

Regarding Zn concentrations in shoots, radish had the highest values of the three species regardless of the chemical form (Fig. 3), which is consistent with the maximum effect on shoot weight observed in this plant. However, in vetch, the effects on weight were higher than in wheat, a result that is opposite to the Zn shoot concentrations in the two plants.

Simple correlation analyses were performed to determine the relationships between the plant parameters in roots and shoots (DW, total Zn, and Zn uptake) and extractable soil Zn contents (WS- and LMWOAs-). For all plant species, root DW did not correlate with any of the extractable soil Zn concentrations. In contrast, in all cases, significant and negative correlations were found between plant shoot DW and WS-extractable Zn (r ranged between −0.904 for vetch and −0.967 for radish, $p<0.036$–0.007) and between wheat shoot DW and LMWOAs-extractable Zn ($r=-0.905$, $p<0.035$). In relation to total Zn uptake by shoots, a positive relationship between total Zn uptake by shoots and both WS- ($r=0.947$, $p<0.015$) and LMWOAs-extractable Zn ($r=0.904$, $p<0.036$) was only found for wheat. There were no correlations between total Zn in roots and any of the extractable soil Zn contents for any case. In contrast, for all plant species, the Zn concentrations in roots and shoots were positively correlated with the WS-Zn (r ranged between 0.919 for Zn in wheat roots and 0.978 for Zn in vetch shoots, $p<0.028$–0.004) and more weakly correlated with LMWOAs-extractable Zn (r ranged between 0.883 for Zn in wheat shoots and 0.924 for Zn in vetch shoots, $p<0.047$–0.025).

The few (and poor) correlations obtained in this soil between LMWOAs-extractable Zn and plant parameters were mainly because, according to this test, similar quantities of potentially available Zn to plants should occur when Zn was applied to soil at 1000 mg kg^{-1} as ZnO-NPs, ZnO bulk, or $ZnCl_2$ (Table 2). The lack of consistency with the results indicates the unsuitability of this extraction method for predicting the Zn availability to plants in this soil when it was contaminated with ZnO-NPs and the other Zn compounds. In contrast, it was possible to predict the Zn concentration in plants with a significant degree of accuracy by determining the WS-Zn in the soil. Considering the much higher Zn concentration extracted with the LMWOAs reagent compared with deionized water, it could be possible that the LMWOAs reagent extracts larger amounts of the metal than the plants are able to accumulate, thus overestimating phytoavailability and, therefore, assessing long-term availability rather than immediate availability. In contrast, deionized water may only extract amounts of the metal that would tend to represent the short-term available pool.

The Tf of wheat and vetch were lower than 1 regardless of the Zn treatment or control. However, radish had a Tf>1, with the exception of the $ZnCl_2$-1000 treatment (Fig. 4). Vetch was the plant with the lowest capacity to translocate Zn from roots to stem, with Tf values in treatments lower than the corresponding control. By contrast, radish was able to translocate Zn from roots to aerial parts more efficiently than the other species. Radish roots were likely permeable to not only Zn ions but also to other Zn compounds with higher capacities to migrate into the aerial portion of the plant.

The BCFs of Zn in the shoots and roots of the tested species are included in Fig. 5. In general, the BCF values were the highest in control soils, and $ZnCl_2$-250 had a higher value compared to $ZnCl_2$-1000. This result can be explained because the transference rate of metals from soils to plants decreases with an increase in soil concentration. Comparing plants, the BCF values were the highest in the roots and shoots of wheat and radish, respectively. In the roots, all of the BCF values were much greater than 1, demonstrating that the three plant species are able to bioconcentrate Zn in their roots.

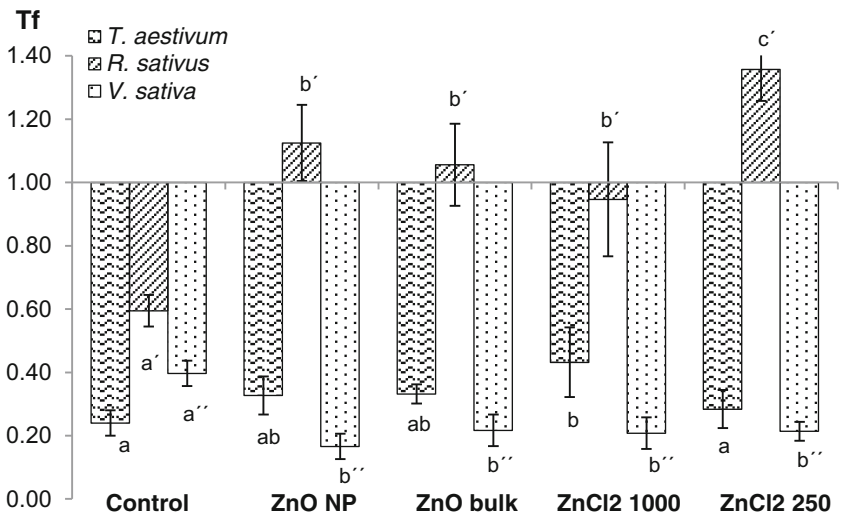

Fig. 4 Translocation factors (Tf) of Zn in the three plant species grown in soil spiked with ZnO NPs, ZnO bulk, and $ZnCl_2$ at 1000 mg Zn kg^{-1} soil and $ZnCl_2$ at 250 mg Zn kg^{-1} soil after 21-day exposure. *Different letters* indicate significant differences among Zn treatments for each plant species (LSD test, $p<0.0001$). *Error bars* represent ±SD ($n=3$)

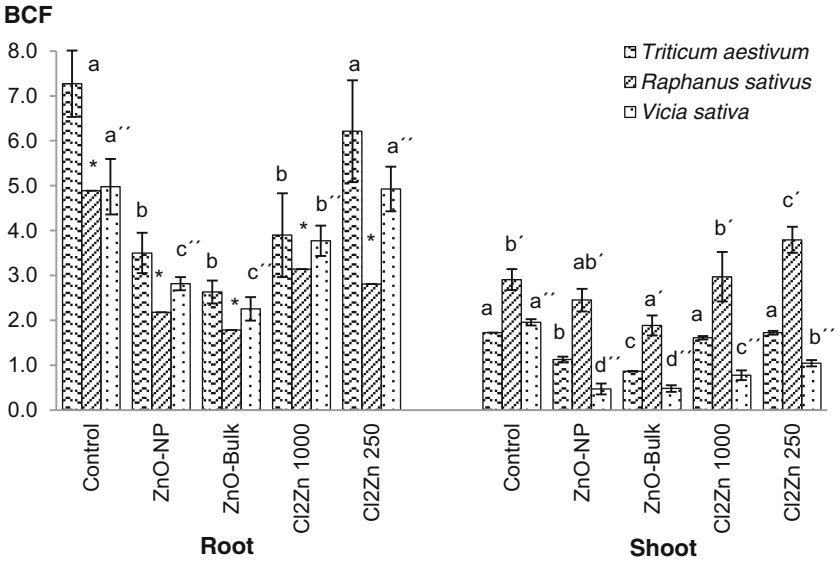

Fig. 5 Bioconcentration factors (BCF) measured in plants grown in soil spiked with ZnO-NPs, ZnO bulk, and $ZnCl_2$ at 1000 mg Zn kg^{-1} soil and $ZnCl_2$ at 250 mg Zn kg^{-1} soil after 21-day exposure. *Different letters* indicate significant differences among treatments for each plant species (LSD test, $p<0.0001$). *Error bars* represent±SD ($n=3$). SD values could not be calculated for Zn in radish roots because the root material from the single replicate was not substantial enough for Zn determination; hence, the root replicates were combined for Zn measurements

However, in the shoots, differences occurred among plant species. Thus, vetch presented the lowest shoot BCF values (<1), except in the control, in which the shoot BCF reached a value of 2. In contrast, radish had the highest shoot BCF values (>2) of any case, likely because of the high capacity of this plant species to incorporate Zn from soils into shoots, as observed in a previous study (Garcia-Gomez et al. 2014b).

Comparing the chemical forms at 1000 mg Zn kg^{-1}, the root BCFs did not differ among plants and treatments, except for vetch grown in $ZnCl_2$-1000. Shoots grown in the $ZnCl_2$ treatment had the highest BCF values. The values for the ZnO-NPs and bulk partner were only different in the case of wheat shoots exhibiting a higher potential for Zn accumulation from ZnO-NPs than Zn bulk.

Conclusions

ZnO-NPs can affect the growth of plants depending on plant species as well as soil functionality because impacts on C mineralization and DH and PH activities were detected. In most cases, the ZnO-NPs showed effects lower than $ZnCl_2$ and statistically similar to ZnO bulk with the exception of vetch shoot weight. In this case, ZnO-NPs exhibited higher toxicity. In this work, the hazard associated with the bulk partner could be assimilated to the risk linked to the presence of ZnO-NPs in the environment. The particle size does not seem to affect the distribution of Zn in soil; both oxides presented similar concentrations in the most accessible fractions. In contrast, differences were observed in the accumulation potential between the two oxides, although this difference was not associated with the difference in toxicity. This finding is of utmost importance because the accumulation of Zn or metals in edible portions of plants is a significant concern. The results of this study indicate that the WS fraction is a better predictor of Zn concentration in plants tissues than the LMWOAs fraction, and the LMWOAs reagent extracted larger amounts of Zn than the plants were able to accumulate.

The low toxicity observed on root lengths compared with data from published studies performed in other artificial matrices showed that the use of a natural soil could mitigate the toxicity of the NPs. This reinforces the importance of using natural soil as the exposure medium to increase the reliability of results. However, these findings should be confirmed with additional studies examining various natural soils because soil characteristics may influence the behavior and, hence, the toxicity of chemicals.

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