Arch Environ Contam Toxicol (2014) 67:494–506
DOI 10.1007/s00244-014-0070-2

# Ecotoxicological Evaluation of Sewage Sludge Contaminated with Zinc Oxide Nanoparticles

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Received: 4 March 2014 / Accepted: 8 July 2014 / Published online: 4 September 2014


**Abstract** The objective of this work was to evaluate the ecotoxicological qualitative risk associated with the use of sewage sludge containing Zn oxide nanoparticles (ZnO-NPs) as soil amendment. A sludge-untreated soil and two sludge-treated soils were spiked with ZnO-NPs (0–1,000 mg/kg soil). Soil ecotoxicity was assessed with *Eisenia fetida* (acute and sublethal end points), and the unfilterable and filterable (0.02 μm) soil leachates were tested with a battery of biomarkers using *Chlorella vulgaris*, *Daphnia magna*, and the fish cell line RTG-2 (*Oncorhynchus mykiss*). The production of *E. fetida* cocoons in sludge-treated soils was lower than that in sludge-untreated soils. The highest effect in the algal growth inhibition test was detected in sludge-untreated soil, most likely caused by the loss of organic matter in these samples. The *D. magna* results were always negative. Toxic effects (lysosomal cell function and production of reactive oxygen species) in RTG-2 cells were only observed in sludge-treated soils. In general, the toxicity of ZnO-NPs in sludge-treated soils was similar to that of sludge-untreated soil, and the filterable leachate fraction [Zn salt ($Zn^{2+}$)] did not produce greater effects than the unfilterable fraction (ZnO-NPs). Thus, after the addition of ZnO-NP—enriched sewage sludge to agricultural soil, the risk of toxic effects for soil and aquatic organisms was shown to be low. These findings are important because repeated use of organic amendments such as sewage sludge may cause more and more increased concentrations of ZnO-NPs in soils over the long-term.

The substantial proliferation of nanotechnologies and the use of nanoparticles (NPs) in quantities that may lead to their relevant release into the environment raise environmental concerns about their adverse ecological effects (Gottschalk and Nowack 2011). Because of the present lack of experimental data, the disposal of nanomaterials is not regulated. To establish regulatory limits for NPs, additional research on biological responses to these substances is of special importance. The physical and chemical characterization of NPs in different media is important for toxicity studies. Sewage-treatment plants are considered to be intermediate stations that control the flow of NPs between anthropogenic and environmental compartments (Gottschalk et al. 2009). During wastewater-treatment processes, NPs may be integrated into the sewage sludge matrix and become concentrated over time (Benn and Westerhoff 2008). Little is known about the clearing efficiency of NPs in sewage-treatment plants. Studies have suggested that standard wastewater treatment is poorly suited to the capture of nanomaterials (Luo et al. 2014; Reijnders 2006). Therefore, the characterization of the environmental impact of NPs from environmental matrices should be investigated to predict their behaviour when they re-enter the environment. When sewage sludge materials re-enter the environment through application to agricultural land, contaminated sludge can have consequences not only

**Electronic supplementary material** The online version of this article (doi:10.1007/s00244-014-0070-2) contains supplementary material, which is available to authorized users.

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on terrestrial communities but also pose problems to aquatic species due to their leaching, erosion, and runoff process (Matejczyk et al. 2011). The ecotoxicological assessment of heterogeneous matrices, such as sewage sludge-treated soils, demands that tools cover the appropriate exposure pathways and that their is study of possible risks for a variety of organisms. From this perspective, the use of biological tests for the estimation of ecotoxicological effects of sewage sludges contaminated with NPs may provide a significant complement to analytical studies (Natal-da-luz et al. 2009).

Biological tests provide information not only from the presence of the contaminants, as well as the properties of sludge that may influence their toxicity, but also the toxicity resulting from potential interactions among the contaminants that appear in sludges (Domene et al. 2011). We choose for this study zinc oxide nanoparticles (ZnO-NPs) because ZnO nanomaterials are the most used in commercial production and represent the majority of the volume introduced to consumer products available on the market (Lovestam et al. 2010). To date, only Jośko and Oleszczuk (2013) have estimated the toxicity of sewage sludges polluted with ZnO-NPs involved in their application as fertilizers considering soil organisms (microorganisms and plants) and *Vibrio fischeri*.

An increasing number of studies have shown that ZnO-NPs may pose significant risks to soil and aquatic organisms (Cañas et al. 2011; Heggelund et al. 2014; Hooper et al. 2011; Ma et al. 2013; Zhu et al. 2009). Particle-induced generation of reactive oxygen species (ROS) and dissolution to ionic Zn represent the primary modes of action for ZnO-NP toxicity across all species tested (Ma et al. 2013).

The majority of test species employed in this research were chosen to model the receiving environment. The toxicity of the bioavailable fraction of the soil contaminants was assessed through mortality, body weight, reproduction, and changes in superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) enzyme activity assays with *Eisenia fetida.* The bioaccumulation of Zn by earthworms was also determined. To clarify the possible entrances of NPs into the aquatic environment, leachates were tested in vivo and in vitro for organisms from three different trophic levels. The tests included the inhibition of the growth of the alga *Chlorella vulgaris* (first producer) and the immobilisation of the cladoceran *Daphnia magna* (first consumer). Cell lysosomal (NRR assay) and membrane permeability [5-carboxyfluorescein diacetate (CFDA-AM) assay] functions, cell growth (Coomassie blue assay), reductase enzyme activity (Alamar blue assay) and production of ROS in cells (ROS assay) were studied in the cell line RTG-2, which is derived from the gonad of the second consumer rainbow trout (*Oncorhynchus mykiss*). In vitro systems have been used extensively for studying toxic mechanisms at the molecular and cellular levels with reproducible results (Castano et al. 2003). Similar approaches have been shown to be useful for the ecotoxicological evaluation of ZnO-NPs (Fernández et al. 2013).

**Table 1** Physical and chemical characteristics of reference soil and test sludges used in this study

| Parameter | Soil | SSA | SSB |
|---|---|---|---|
| pH | 7.8 | 7.8 | 7.0 |
| Sand (%) | 73.4 | ND | ND |
| Silt (%) | 18.8 | ND | ND |
| Clay (%) | 7.8 | ND | ND |
| EC (μS/cm) | 390 | 2,550 | 1,669 |
| Organic matter (%) | ND | 59.80 | 28.29 |
| Oxidizable organic C (%) | 1.9 | 31.21 | 20.32 |
| Total P (%) | ND | 7.34 | 1.02 |
| Total K (%) | ND | 0.66 | 1.13 |
| Total N (%) | ND | 3.61 | 3.09 |
| N-NH3 (mg/kg) | ND | 0.61 | 0.57 |
| Cd (mg/kg) | 0.2 | 3 | 5 |
| Cu (mg/kg) | 27 | 469 | 384 |
| Pb (mg/kg) | 31 | 82 | 108 |
| Zn (mg/kg) | 53 | 1288 | 868 |

*ND* not determined

The objective of this work was to evaluate the effects on soil and aquatic organisms associated with the disposal of sewage sludge amendments contaminated with ZnO-NPs to determine the potential qualitative ecotoxicological risk associated with the presence of this emerging contaminant in sewage sludges.

## Materials and Methods

### Reference Soil, Sludges, and NPs

The soil (S) used in this study was collected from an untreated grassland area located 10 km northeast of Madrid at Global Positioning System coordinates N40°27′18″, WO3°44′55″. This soil is indicative of a typical agricultural soil with a well-known history: Pesticides and fertilisers have not been applied at least for the last 10 years. The soil sample was taken from within the top 10 cm of the soil layer and was air dried, sieved (2-mm mesh), homogenised, and stored at room temperature until use.

Two sewage sludge samples (namely, SSA and SSB) were supplied from two municipal wastewater-treatment plants located in the north of Spain. The sewage sludges were suitable for use as amendments to agricultural soils [Directive 86/278/EEC (limit values for heavy metals)]. The exposure containers were filled with 750 g of samples (soil samples or

sewage sludge-treated soil samples). Table 1 lists the physicochemical properties of both the soil and the amendments used in this study. Zn nanopowder (<100 nm) was obtained from Sigma-Aldrich (Germany). The primary particle size and shape analyses were performed by transmission electron microscopy (TEM). One drop of the aqueous ZnO-NP suspension (1 mg/mL ultra-pure water) placed on a carbon-coated copper (Cu) grid was air dried and observed with TEM (JEM-2100; JEOL, UK). For the hydrodynamic size measurements, the 1 mg/mL of ZnO-NP stock solution was dispersed with a homogeniser/disperser (T25 digital ULTRA-TURRAX; IKA-Werke, Germany) at 100 W and 40 kHz for 20 min to break up the agglomerates and to form a homogeneous suspension. The particle size (hydrodynamic diameter) distribution in the suspension was determined with a Nano-Zeta Sizer (Malvern Instrument; Madrid, Spain). Transmission electron micrographs showed that the size (mean ± SD) of the NPs was 58.40 ± 30.13 nm in diameter (see Supporting information). The NPs were often linked into aggregates, and isolated particles were rare. From dynamic light scattering (DLS), the average hydrodynamic diameter in aqueous solution (1 mg/mL) was 167 nm (98.3 % intensity). The zeta-potential was—13.7 mV. The differences between the TEM and DLS size analyses were likely caused by particle aggregation during the DLS analysis; this aggregation has been reported elsewhere for ZnO-NPs (Franklin et al. 2007), and is considered to be an inherent property of unmodified oxide NPs.

## Experimental Set-up and Zn Analysis

Two (SSA and SSB) ZnO-NPs-amended sludges (nominal range 2,500–20,000 mg ZnO-NPs/kg sludge) were prepared to have 125, 250, 500, and 1,000 mg ZnO-NPs/kg soil. It has been suggested that test organisms should be exposed to NPs in an environmentally relevant way (Crane et al. 2008). However, prediction of environmental concentrations of engineered NPs is still hindered by numerous problems, so we chose those high concentrations because if effects were observed at those concentrations, then we would perform in-depth studies using concentrations closer to (1) those reported in sludge-treated soils [2.98–23.1 μg/kg (Ma et al. 2013)] and (2) the predicted ZnO-NP concentrations in sludge [17–110 mg/kg (Sun et al. 2014)].

The dry amendments were thoroughly mixed with NPs as a powder of ZnO to obtain homogeneity, and dechlorinated water was added to the containers to make up 30–50 % of the water-holding capacity of sludges. Samples were left for 7 days at room temperature to allow them to equilibrate. The moisture of the samples was controlled by weight. The ZnO-NP—amended sludges were applied to soil at a 5 % w/w ratio. The toxicity experiment consisted of four ZnO-NP concentrations (125, 250, 500, and 1,000 mg/kg soil) spiked in a soil control (S) and in two different sludge amendments (SSA and SSB) with three replicates per group. Soil and sludge amendments without NPs were used as control samples.

The effect of each amendment on the contaminant extractability was evaluated with deionised water (pH 7) following the application of standard leaching method (DIN 38414-S4 1984). The pH and conductivity were also measured in these extracts. The unfiltered and filtered (Whatman alumina Anotop membrane, 0.02-μm pore size; Maidstone, Kent, UK) extracts containing ZnO-NPs and $Zn^{2+}$ ions, respectively, were tested for toxicity to aquatic organisms.

The measurement of the total Zn concentrations and zinc-extractable fractions for SSA and SSB sludge-amended soil were performed on sludge-amended soil and extract from all three replicates. To measure total Zn concentrations, soil, SSA, and SSB samples were digested in nitric acid (33 %), super-pure hydrofluoric acid (33 %), and double-deionised water (33 %) in a 1:1:1 (v:v:v) ratio in a microwave oven (MARS Multiwave; CEM Corp, Matthews, North Carolina, USA). This process required two steps at a maximum pressure of 170 psi. A certified reference soil provided by the Institute for Reference Materials and Measurements of the European Commission (ERM-CC141) was used to determine the quality of the results for the total Zn content. The Zn concentrations in the extracts were measured with flame or graphite furnace atomic absorption spectrometry (AAS) depending on the Zn concentration range (AAnalyst 700; Perkin-Elmer, Buckinghamshire, UK). Perkin-Elmer Pure standard checks were used for the quality-assurance system (certified by National Institute of Standards and Technology Standard Reference Materials). For each extraction, standard solutions of Zn were prepared in a background solution of the extracting agent (deionised water).

The *Eisenia* Zn concentration was measured (AAS) in a sample of nine earthworms (three earthworms from each replicate) after exposure. The earthworms were placed onto moist filter paper in Petri dishes for 24 h to void their gut contents. After being weighed, the samples were frozen at −20 °C for 24 h and were lyophilised (Cryodos; Telstar, Terrassa, Spain). The lyophilised earthworms were ground to a fine powder with an agate mortar and pestle and digested with nitric acid (33 %), super-pure hydrochloric acid (33 %), and double-deionised water (33 %) at 1:1:1 (v:v:v) ratio in a microwave oven (MARS Multiwave; CEM Corporation, NC, USA).

## Toxicity Testing

### *Eisenia fetida*

Three replicate test containers were used for each ZnO-NP concentration and the control to test for *E. fetida* toxicity, Zn concentration in the earthworms, total Zn concentration in the soil, and extractable Zn fractions. Each replicate was

performed in an individual glass container containing 750 g of moist soil, which is equivalent to 80 % of the water-holding capacity. Ten adult *E. fetida* (total wet weight range 300–500 mg/10 earthworms with an analogous mean for all exposure replicates) were added to each replicate. The test containers were incubated at 20 °C under constant illumination during 28 days. Once per week, all of the test containers were opened for aeration, weighed to correct for water loss, and had the addition of 2 g of rolled oats.

The tests with the earthworms followed Organisation for Economic Co-operation and Development (OECD) guideline 222 [2004 (reproduction ratio)] and OECD guideline 207 [1984 (survival ratio)]. *Eisenia* CAT, SOD, and GST enzymatic activities were measured for a sample of three earthworms (three replicates in each treatment) after exposure. The earthworms were homogenised in sodium (Na)–potassium (K) buffer [phosphate-buffered saline (pH 7.2)] in a ¼ w/v ratio for 1 min with a Polytron tissue processor (CH-6010; Switzerland). After centrifugation of the homogenate, the supernatants were collected and were stored at −80 °C until analysis. CAT activity was determined according to Claiborne (1985). SOD activity was assayed with water-soluble tetrazolium salt (Peskin and Winterbourn 2000) from a commercial kit (Sigma-Aldrich, Germany). GST activity measurement was performed according to Habig et al. (1974) with 1-chloro-2,4-dinitro-benzene. The total protein content was determined by Bradford's method (Bradford 1976) using albumin as a standard.

### *Chlorella vulgaris*

The algae growth-inhibition test was modified for 96-well microtitre plates (Ramos et al. 1996) and was used in this study. *C. vulgaris* was exposed to 10 and 25 % dilutions of each replicate extract in reconstituted water with hardness of 250 mg $CaCO_3$/L and pH 7.9 ± 0.3 [International Organization for Standardization 6341 1996], and six replicate wells were used per concentration. After 72 h, the in vivo fluorescence of algae was recorded with a microplate spectrofluorometer GENios (Tecan, Switzerland).

### *Daphnia magna*

An acute 48-h immobilisation bioassay with *D. magna* (21–25 days) from the same generation and with similar sizes was performed under static nonrenewal conditions (T = 25 °C ± 1 °C under a 16:8-h light-to-dark photocycle) according to the methodology described in the ISO 6341:1996 guideline (1996) with some modifications. Ten *D. magna* were placed in a 25-mL volume extract (three replicates for each treatment at each concentration) in duplicate. Control medium (solution without ZnO-NP sludge-amended soil) was performed in duplicate. The numbers of viable and dead individuals were noted each day, and mortality was calculated at the end of the exposure.

### *RTG-2 Cell Line (Oncorhynchus mykiss)*

Cells were seeded in 96-well microplates at a density of $2.5 \times 10^4$ cells/mL. At least three independent experiments were performed, and three replicate wells were employed per concentration per plate in each experiment. After 24 h of cell attachment, the cells were treated with a 75 % dilution of each sample for 24 h. Negative (EMEM medium) and positive (10 % DMSO or SIN-1) controls were run in parallel. Five end points were used to measure the effects of the extracts obtained from soils spiked with ZnO-NPs. The NRRA and CBA assays were performed consecutively on the same plate. The tests were performed according to Vevers and Jha (2008). The metabolic activity was monitored with AB (Invitrogen, USA), and the membrane integrity was evaluated with CFDA-AM (Invitrogen, USA) according to the method of Schirmer et al. (1997). Generation of ROS was measured by the intracellular oxidation of 2′,7′-dichlorodihydrofluorescein diacetate through the method by Wang and Joseph (1999). The results were recorded as relative absorbance or fluorescent units with a microplate spectrofluorometer GENios (Tecan, Switzerland).

### Data Analyses

The bioaccumulation factor (BAF) of the earthworms was calculated from the Zn concentration in the body related to the dried weight divided by the total Zn in the soil. The toxicity effects were calculated from the percentages of effect (inhibition or induction) at the given concentration values. The percentages of inhibition were determined relative to the control samples without ZnO-NPs. In the acute earthworm test and the *Daphnia* test, the percentage of mortality or immobilisation, respectively, was determined relative to the number of test organisms (10 organisms/exposure container). The toxicity responses obtained in the soil and aquatic tests were compared with those in the control by one-way analysis of variance (ANOVA). Two-way ANOVAs were performed with ZnO-NP concentrations and experimental groups as fixed factors. All data passed the Fisher's least significant difference test (LSD, $p < 0.05$) for normality. All calculations were performed with the Statgraphics software package (StatPoint Technologies, USA).

## Results and Discussion

### Chemical Properties of Soil and Leachates

The major chemical parameters of the sludges used to prepare the nano-ZnO mixtures are listed in Table 1. SSA

**Table 2** pH, EC, and Zn concentration of soil samples

| Samples | Parameter | Days | Mean ± SD (variation coefficient) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Application rate (mg ZnO-NPs/kg soil) | | | | |
| | | | Control | 125 | 250 | 500 | 1,000 |
| Soil | PH | 0 | 7.94 | 7.98 | 7.99 | 8.06 | 8.19 |
| | | 35 | 7.90 ± 0.03 (0.0005) | 7.79 ± 0.02 (0.0024) | 7.64. ± 0.05 (0.0061) | 7.85 ± 0.02 (0.0030) | 7.94 ± 0.02 (0.0021) |
| | EC (μS/cm) | 0 | 428 | 524 | 459 | 471 | 551 |
| | | 35 | 440 ± 105 (0.2395) | 482 ± 23 (0.0477) | 523 ± 63 (0.1213) | 508 ± 24 (0.0484) | 601 ± 24 (0.0409) |
| | Zn (mg/kg) | 0 | 52.60 ± 0.44 (0.0084) | 163.04 ± 11.55 (0.0708) | 266.43 ± 60.34 (0.2265) | 509.59 ± 18.22(0.0358) | 1,012.96 ± 98.56 (0.0973) |
| SSA | PH | 0 | 7.55 | 7.56 | 7.88 | 7.97 | 8.17 |
| | | 35 | 6.98 ± 0.03 (0.0044)[a] | 6.76 ± 0.02 (0.0026)[b] | 6.65 ± 0.03 (0.0052)[c] | 6.54 ± 0.01 (0.0020)[d] | 6.41 ± 0.01 (0.0015)[e] |
| | EC (μS/cm) | 0 | 882 | 806 | 757 | 867 | 849 |
| | | 35 | 1,136 ± 60 (0.0528) | 1,087 ± 16 (0.0151) | 1,131 ± 27 (0.0236) | 1,125 ± 7 (0.0060) | 1,085 ± 14 (0.0127) |
| | Zn (mg/kg) | 0 | 83.88 ± 7.08 (0.0844) | 244.13 ± 7.25 (0.0297) | 342.17 ± 31.97 (0.0934) | 609.79 ± 57.68 (0.0947) | 1,071.62 ± 70.97 (0.0662) |
| SSB | PH | 0 | 7.29 | 7.24 | 7.27 | 7.32 | 7.63 |
| | | 35 | 6.98 ± 0.03 (0.0043)[a] | 6.91 ± 0.02 (0.0024)[b] | 6.69 ± 0.02 (0.0028)[c] | 6.61 ± 0.01 (0.0014)[d] | 6.57 ± 0.02 (0.0020)[e] |
| | EC (μS/cm) | 0 | 1,055 | 1,042 | 1,033 | 1,049 | 1,037 |
| | | 35 | 1,132 ± 43 (0.0386) | 1,134 ± 26 (0.0024) | 1,117 ± 40 (0.0361) | 1,176 ± 36 (0.0310) | 1,141 ± 6 (0.0056) |
| | Zn (mg/kg) | 0 | 82.62 ± 13.57 (0.1642) | 190.98 ± 5.64 (0.0295) | 313.43 ± 45.86 (0.1463) | 520.40 ± 28.28 (0.0543) | 1,018.88 ± 15.16 (0.0149) |

[a–e] Statistically significant differences for application rate in one-way ANOVA ($p < 0.05$)

had an organic matter content of 59.80 %, whereas SSB contained 28.29 %. Organic matter content of the sludges plays an important role in the bioavailability and toxicity of ZnO-NPs (Lee et al. 2011; Yang et al. 2009). The concentration of macronutrients differed between the two sludges and was most pronounced in the phosphorous concentrations, which were 7.34 and 1.02 % in SSA and SSB, respectively. In SSA, the concentration of K was <1 %. Jośko and Oleszczuk (2013) and Lv et al. (2012) showed that the interaction between ZnO-NPs and the other compounds of sewage sludge (phosphorus, magnesium, potassium) may result in a decrease of their toxicity.

The data for the pH and electrical conductivity (EC) of the soils (S, SSA, and SSB samples) at the beginning and at the end of the study and the Zn content are listed in Table 2. The pH values decreased significantly ($p < 0.001$) in the sludge-treated soils spiked with ZnO-NPs at the end of the study. The addition of sewage sludge to soil slightly decreased the pH values, whereas the EC value on sewage-amended soils was 1.5–2 and 1.8–2.5 times for SSA and SSB, respectively, compared with S. After 35 days of exposure, the pH values had decreased in amended soils compared with unamended control soil, and the EC values had slightly increased. Soil pH is the most essential factor managing Zn accessibility, which decreases with the increment of pH (Li and Shuman 1996).

The leachate characteristics (pH and electric conductivity) and Zn content are listed in Table 3. The pH values decreased significantly ($p < 0.001$) in the leachates from sludge-treated soils spiked with ZnO-NPs. The EC values of the leachates did not show significant differences between the control and the samples with ZnO-NPs. In this study, sewage sludge applied to agricultural soil (SSA and SSB samples) produced a decrease of Zn content in leachate compared with unamended soil (S samples) (Table 3). This agrees with Lee et al. (2009) where the application of soil amendments decreased the amount of soluble and extractable heavy metals in the soil.

One restriction in assessing the toxic impact of NPs is the interaction of the NPs with the media in which they are dissolved or distributed. However, Jassby et al. (2012) concluded that aggregation exhibits only minor changes,

**Table 3** pH, EC, and Zn concentration of leachates

| Samples | | Mean ± SD (variation coefficient) | | | | |
|---|---|---|---|---|---|---|
| | Parameter | Control | Application rate (mg ZnO-NPs/kg soil | | | |
| | | | 125 | 250 | 500 | 1,000 |
| Soil | pH | 7.66 ± 0.12 (0.0157) | 7.30 ± 0.07 (0.0099) | 7.97 ± 0.19 (0.0233) | 7.85 ± 0.18 (0.0233) | 7.92 ± 0.07 (0.0091) |
| | EC (uS/$^{c}$m) | 277 ± 4 (0.0127) | 254 ± 8 (0.0298) | 301 ± 6 (0.0183) | 268 ± 4 (0.0149) | 296 ± 18 (0.0614) |
| SSA | Zn (mg/L) | 0.06 ± 0.00 (0) | 0.22 ± 0.04 (0.1644) | 0.40 ± 0.05 (0.1223) | 0.90 ± 0.04 (0.0461) | 2.54 ± 0.30 (0.1731) |
| | pH | 7.59 ± 0.22 (0.0289)$^{a}$ | 7.42 ± 0.19 (0.0262)$^{a}$ | 7.09 ± 0.01 (0.0016)$^{b}$ | 6.99 ± 0.04 (0.0062)$^{b}$ | 6.87 ± 0.04 (0.0051)$^{b}$ |
| | EC (uS/$^{c}$m) | 626 ± 37 (0.0598) | 682 ± 12 (0.0177) | 659 ± 3 (0.0031) | 642 ± 49 (0.0764) | 643 ± 21 (0.0323) |
| | Zn (mg/L) | 0.06 ± 0.01 (0.4385) | 0.16 ± 0.02 (0.1368) | 0.34 ± 0.02 (0.0618) | 0.73 ± 0,03 (0.0481) | 1.76 ± 0.03 (0.0164) |
| SSB | pH | 7.34 ± 0.15 (0.0204)$^{a}$ | 7.14 ± 0.03 (0.0045)$^{b}$ | 7.12 ± 0.02 (0.0032)$^{b}$ | 7.03 ± 0,02 (0.0033)$^{bc}$ | 6.95 ± 0.03 (0.0046)$^{c}$ |
| | EC (uS/$^{c}$m) | 657 ± 20 (0.0309) | 738 ± 16 (0.0216) | 745 ± 14 (0.0194) | 748 ± 7 (0.0094) | 714 ± 15 (0.0207) |
| | Zn (mg/L) | 0.03 ± 0.00 (0) | 0.12 ± 0.00 (0) | 0.23 ± 0.02 (0.0918) | 0.56 ± 0.03 (0.0542) | 1.55 ± 0.06 (0.0366) |

$^{a-c}$ Statistically significant differences for application rate in one-way ANOVA ($p < 0.05$)

regardless of the aggregation rate, because the packing of primary particles within an aggregate is not affected by further aggregation. Chen et al. (2011) showed that the aggregation of NPs significantly decreases their toxic effect. The aggregation behaviour of ZnO-NPs in this study was not monitored. Overall, the study was performed under laboratory experimental exposure conditions using an holistic approach (Hubal 2009). In the environment, naturally occurring and anthropogenic surface modifiers exist, which facilitate the formation of stable NP suspensions through a combination of steric and electrostatic effects (Hyung et al. 2007).

## Toxicity Testing

### *Eisenia fetida*

Earthworm Zn concentrations were greater at greater Zn-exposure levels (Fig. 1). The measured Zn in the *Eisenia* samples was significantly greater at 125–1,000 mg/kg in soil not treated with sludge and at 500 and 1,000 mg/kg in the sludge-treated soils. However, it is possible this effect is to some extent related to the Zn concentration in the gut content. Significant differences ($p < 0.001$) were observed between the treatments at the tested ZnO-NP concentrations of 250, 500, and 1,000 mg/kg. In studies with a number of invertebrate species, high excretion rates were found for Zn, and these results indicated that this metal is detoxified primarily by excretion (Spurgeon and Hopkin 1999). However, after 28 days of exposure to soil spiked with ZnO-NPs, the loss of Zn content reported in other invertebrate species was not found in *E. fetida*. The ability of earthworms to remove excess Zn is most likely dependent on the nature of the metal. Thus, the physiological pathways to control Zn could be a less competent carrier system in the control of ZnO-NPs. The Zn content found in *E. fetida* tissues exposed to sludge-treated soils was lower than those from sludge-untreated soil, and this result could be attributed to the binding capacity of the dissolved or particulate organic matter to NPs. Metals are strongly bound to the compost matrix and organic matter, and this strong binding limits the solubility and potential bioavailability of metals in sludge-treated soils (Ghosh et al. 2008). In our study, the BAF for the earthworms ranged between 0.15 and 0.64 if the control soils without ZnO-NPs are omitted.

No significant differences were observed in the survival and growth rates of the adult earthworms between treatments. After 28 days of exposure, all adult worms survived except four individuals from the soil treated with sludge A at 1,000 mg/kg. We observed no statistically significant effects on the body mass of the earthworms for any of the treatments. Worms slightly lost weight in the control soil

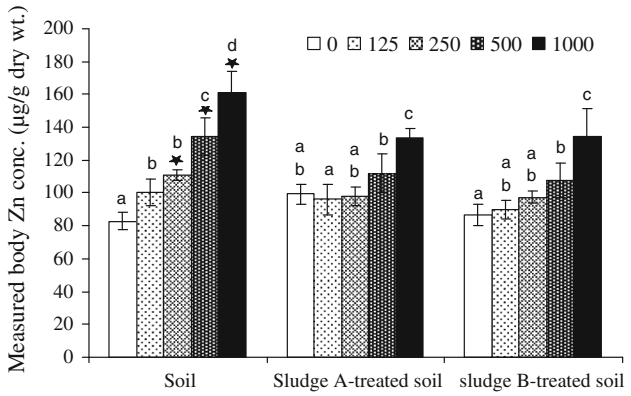


**Fig. 1** Zn concentrations for *E. fetida* after 28 days of exposure to ZnO-NPs dosed in soil at the nominal concentrations shown (0–1,000 mg/K soil) (*error bars* = ±SE, $n = 3$). *Significantly different from sludge-amended soil (SSA and SSB) (LSD test, $p < 0.05$). Within each treatment group, means sharing the same letter are not significantly different (LSD test, $p < 0.05$)

and slightly gained weight in the sludge-treated soils (S = 0.48 mg, SS = 0.53 mg, and SSB = 0.63 mg at the tested ZnO-NP concentration of 250 mg/kg), and these results could indicate that some aspect of the structure or behaviour of the ZnO-NPs may attenuate their toxicity even as the concentration increases in the organism. However, these result may also come from the use of soil amendments that can change the tendency of ZnO-NPs to aggregation (Hotze et al. 2010), thus decreasing the bioavailable fraction and modified the toxicity (Hotze et al. 2010; Phenrat et al. 2009). A qualitative analysis of the earthworm tissue identified localisation of Zn and Cu within the worm tissues and confirmed that some of the accumulated metals remained as particles and did not dissolve in the worm tissues to release metal ions (Hooper et al. 2011; Unrine et al. 2010). The toxicity that was found for ZnO-NPs in earthworms was similar to the results of studies performed with Cu-based NPs (Unrine et al. 2010).

Earthworms exposed to sludge-treated soils spiked with ZnO-NP concentrations of 500 and 1,000 mg/kg produced significantly fewer cocoons ($p < 0.001$) than those exposed to the soil not amended with sludge (Fig. 2). The effects on the suppression of cocoon production, hatchability, and juvenile survival were assessed from the total production of juveniles after 56 days of exposure (Fig. 2). Differences in both end points, *i.e.*, number of cocoons and juvenile worm production, were statistically significant ($p = 0.014$ and $p = 0.032$, respectively) at the highest ZnO-NPs concentration (1,000 mg/kg) in soil (S treatment). Studies in soil have already described decreased worm reproduction at increased ZnO-NP concentrations (Cañas et al. 2011; Hooper et al. 2011). Heggelund et al. (2014) described Zn toxicity on reproduction and found a stronger influence from soil pH than for all Zn forms (nano, bulk, or ions) in natural soils, and these results indicated the absence of nano-specific effects. In this study, the soil pH values (S treatment) were analogous for all of the nano-ZnO concentrations. The cocoon production of the worms and the production of juveniles in the SSA treatment was lower than those in the other treatments (S and SSB) even for the control concentration without ZnO-NPs. Multivalent cations and natural organic matter (NOM) are present in soil porewater, and dissolved organic carbon concentrations are usually approximately 10 mg/L (Tao and Lin 2000). Soluble Zn present in the soil porewater was not measured in this study because the soils were used to collect the cocoons. The high affinity of NOM for metal ions affects the stability of the ZnO-NPs in the porewater of the soils. High levels of multivalent cations could lead to the aggregation NPs and decreased risk for intracellular uptake. Santore et al. (2002) found decreased bioavailability and toxicity of Zn when calcium (Ca), Mg, and sodium were present in high concentrations; they ascribed this decrease to the competition with $Zn^{2+}$ in binding to the receptor. Ideally, particle dissolution and aggregation over time within the soil matrix would have been characterised. This characterisation in solid-phase media remains an outstanding demand for routine use in environmental nanotoxicology (Lead 2009).

Metabolic GST and oxidative SOD and CAT stress enzymes were negative in earthworms after 28 days. After a relatively long exposure period (28 days), earthworms exposed to soil treated with ZnO-NP sludge could be acclimatised to the disturbed environment through active metabolism of the xenobiotic and control of the oxidative stress. Other investigators have already described that the Zn concentration in earthworm tissue is controlled through physiological regulation; thus, body load is the result of a balance between uptake and excretory kinetics (Olchawa et al. 2006).

All of these results suggest a complicated relation between total body concentration and toxic effect for earthworms exposed to sludge-treated soils spiked with ZnO-NPs. To obtain a better understanding of the behaviour of NPs as a function of sludge properties, further studies with multiple amendments are needed.

### *Chlorella vulgaris*

As primary producers, green algae are a key indicator of the health of aquatic environments. Leachates of sludge-untreated and sludge-treated soils showed different results for algal growth with unfilterable (ZnO-NPs) and filterable ($Zn^{2+}$ ions) fractions (Fig. 3). Overall, algal viability decreased as the Zn concentration of leachates increased (Table 3). Unfiltered leachates from the S and SSA

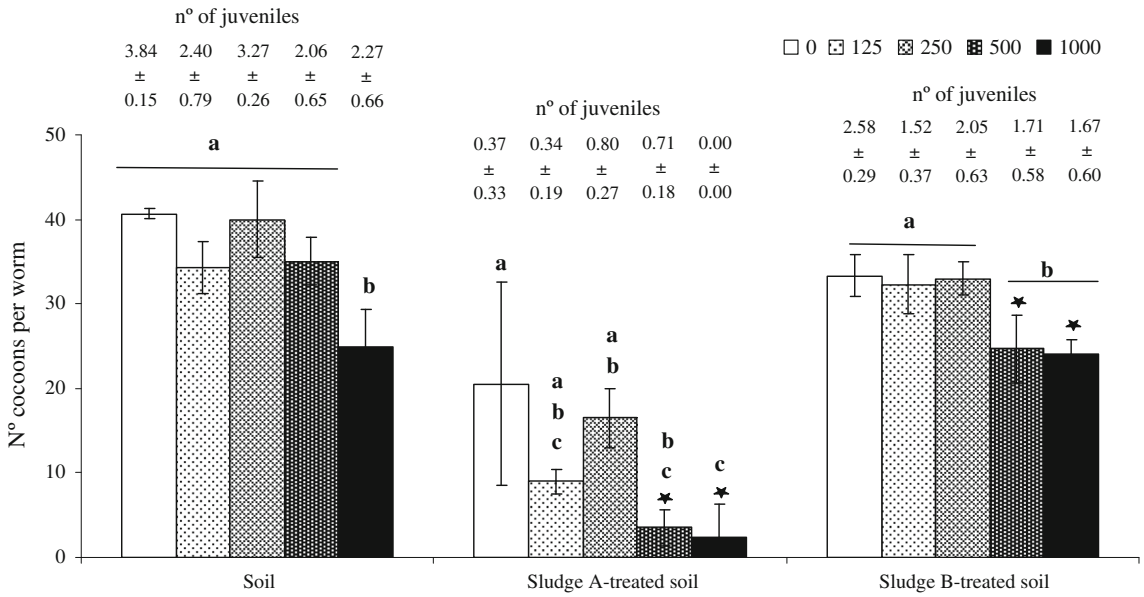


**Fig. 2** Population development of the earthworm *E. fetida* exposed for 28 days (cocoons) and 56 days (juveniles) to the different concentrations (0–1,000 mg/K soil) of ZnO-NP sludge-untreated and -treated soils (*error bars* = ±SE, $n = 3$). *Significantly different from sludge-untreated soil (LSD test, $p < 0.05$). Within each treatment group, means sharing the same letter are not significantly different (LSD test, $p < 0.05$)

treatments inhibited algal growth by 39.96 % (2.54 mg Zn/L) ($p < 0.001$) and 13.35 % (1.76 mg Zn/L) ($p = 0.04$), respectively, whereas the leachate from the SSB treatment showed maximum growth promotion by 15.56 % (0.23 mg Zn/L) on day 4.

The filtered leachates from the SSB treatment inhibited algal growth by 18.53 % (1.55 mg Zn/L) ($p < 0.05$), but no reproducible concentration–response relationships could be established for the SSA treatment, which showed growth promotion by 22.03 % (0.34 mg Zn/L) or for the S treatment in which the values, 95.81 % (0.40 mg Zn/L) to 101.55 % (0.90 mg Zn/L), were similar to those of the control (0.06 mg Zn/L) (Fig. 3; Table 3). The damage to *C. vulgaris* on exposure to ZnO-NPs has been attributed to the composition of the algal walls, which consist of cellulose, polysaccharides, and glycoproteins and could provide many binding sites for the ZnO-NPs through nonspecific interactions (Chen et al. 2012).

Significant differences were observed between treatments: The ZnO-NPs were toxic to *C. vulgaris* in unamended soil compared with amended soils ($p < 0.001$). The different toxicological responses to ZnO-NPs in sludge-untreated and -treated soils may be interpreted by the different characteristics of the soils, which could influence algal growth and the quality and behaviour of the NPs. The influences of the sludge chemical characteristics deserve more specific investigation.

The 72-h values for the $Zn^{2+}$ ions between treatments were not significantly different. These values could be attributed to the negatively charged algal cells, which self-aggregated in response to ZnO-NPs to decrease their exposed surface area (Chen et al. 2012). This decreased algal surface area most likely minimises uptake of the released $Zn^{2+}$ ions from the ZnO-NPs and thereby limits algal cell damage. Other investigators have suggested that dissolved $Zn^{2+}$ ions from ZnO-NPs were not the dominant mechanism for algal growth inhibition (Ji et al. 2011). Accordingly, factors other than $Zn^{2+}$ ions must be responsible for the algal growth inhibition.

Only a few studies on algae are available in the literature. Algal growth-inhibition data obtained by other investigators showed EC50 values for *Pseudokirchneriella subcapitata* to be approximately 0.068 mg/L (Aruoja et al. 2009; Franklin et al. 2007), which is much lower than our results. The highest Zn concentration detected in the leachate in this study was 2.54 mg/L (Table 3) and corresponded to the soil spiked with 1,000 mg/kg of ZnO-NPs. At this concentration, algal growth inhibition was 39.96 %. The decrease in effects could be related to the pH values. A 10 % volume of leachate added to the algal growth medium modified the pH. The aqueous solubility of ZnO is extremely pH-dependent and extends from several thousand mg/L at pH 6 to approximately 1 mg/L at pH 8 (Franklin et al. 2007). The pH variation could interfere

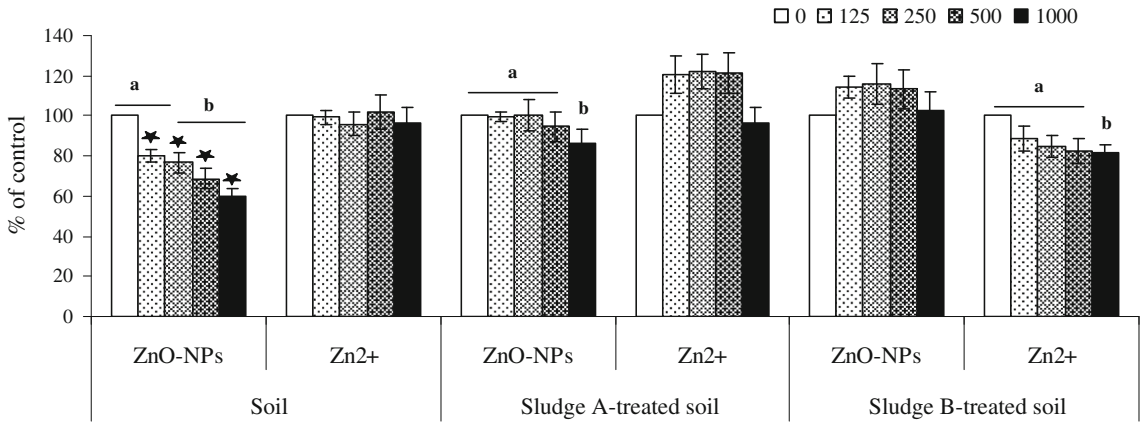


**Fig. 3** Growth rate of *C. vulgaris* exposed to 10 % dilution of unfilterable (ZnO-NPs) and filterable ($Zn^{2+}$) leachates of sludge-untreated and -treated soils spiked with ZnO-NPs (*error bars* = ±SE, $n = 3$). *Significantly different from sludge-untreated soil (LSD test, $p < 0.05$). Within each treatment group, means sharing the same letter are not significantly different (LSD test, $p < 0.05$)

with dissolution by generating kinetic obstruction to the diffusion process (Borm et al. 2006).

### *Daphnia magna*

*Daphnia* species, primary consumers, are incorporated into a number of guidelines and international standards for acute tests, so they are an evident first option for aquatic test organisms when conducting toxicological tests. Related to body size, *D. magna* (primary consumer) can filter proportionately large volumes of water and have been established to feed on algal cells, larger bacteria, and other inorganic and organic particles in the size range of 0.4–40 μm (Gophen and Geller 1984). Cladocerans have been considered a high-sensitivity model organism to predict the toxicity of pollutants including the metal Zn (Evens et al. 2011).

Acute toxicity studies in *D. magna* have reported EC50 ZnO-NP values ranging from 0.5 to 18 mg/L (Lopes et al. 2014; Poynton et al. 2011; Zhu et al. 2009), and EC50 for $Zn^{2+}$ values between 0.1 and 14 mg/L (Lopes et al. 2014; Poynton et al. 2011). In this study, exposure to leachates at maximum concentrations [100 % of solvent; Zn content between 0.03 and 2.54 mg/L (Table 3)] caused no effects on *Daphnia* mobility, and no concentration–response relationships could be shown. To our knowledge, *Daphnia* toxicity was never tested with leachates obtained from sludge-treated soils spiked with NPs. In daphnids, Ca mitigation of acute metal toxicity has been shown for Cu (Schamphelaere and Janssen 2002), Ni (Kozlova 2009), and Zn (Clifford and McGeer 2009). Mitigation of Zn toxicity could reflect increased competitive interactions between $Zn^{2+}$ and $Ca^{2+}$ and/or $Mg^{2+}$ induced by concentrations of these elements in leachates.

Most studies have been performed in sample water systems that do not reflect the complexity of environmental samples. The use of drastic mixing methods and toxic solvents in the preparation of NP suspensions leads to model dispersion levels that are probably not realistic in natural waters. A *Ceriodaphnia dubia* acute toxicity study, using selected nanomaterials (i.e., fullerenes, nano-Cu, and nanosilver) suspended directly in natural river waters through simple and gentle mechanical mixing methods produced results suggesting that nanomaterials could be considerably less toxic if released directly under natural conditions and could produce results that are far from what might occur under laboratory conditions (Gao et al. 2009). This result is in agreement with our data, which suggested that the complexity of leachates, in which ZnO-NPs co-occur with electrolytes, could result in less toxicity. Our results are of great importance in the perspective of understanding the changes and biological effects of ZnO-NPs in natural processes.

### *RTG-2 Cell Line (Oncorhynchus mykiss)*

In vitro assays are a common first step in assessing the toxicity of environmental pollutants through biochemical biomarkers that show the initiation of specific biotransformation systems or the beginning of cellular alterations (Viarengo et al. 2007).

Leachates of sludge-untreated and sludge-treated soils showed different results for the NRRA with unfilterable (ZnO-NPs) and filterable ($Zn^{2+}$ ions) fractions (Fig. 4a). There was a relationship between ZnO-NP and $Zn^{2+}$ concentrations and the decrease in cell viability (NRRA) in RTG-2 cell exposed to leachates of sludge-treated soils. Unfiltered leachates of the SSA and SSB treatments decrease in NRRA dye by 15.68 and 22.23 % ($p = 0.041$),

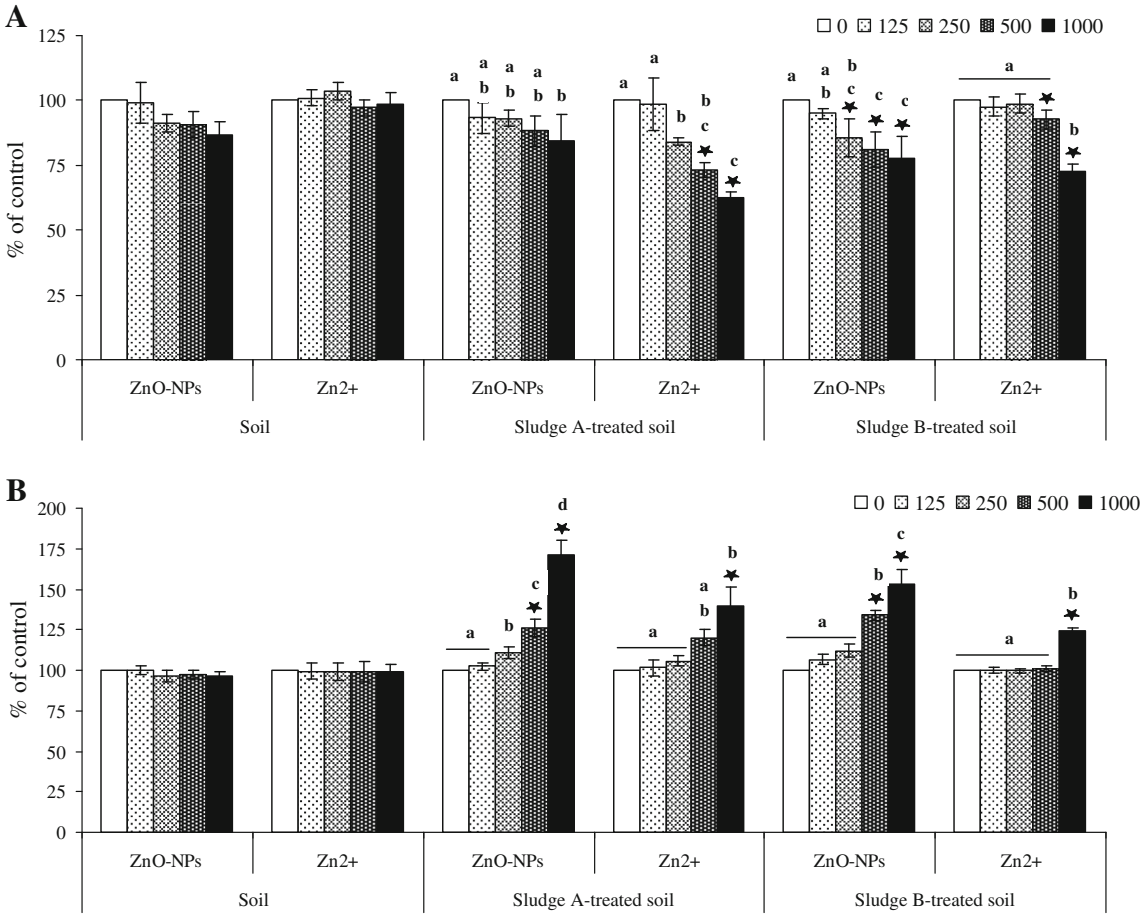


**Fig. 4** Dosage-dependent cytotoxicity to RTG-2 cells exposed to unfilterable (ZnO-NPs) and filterable ($Zn^{2+}$) leachates of untreated and sludge-treated soils spiked with nano-ZnO. After 24 h of cell exposure, **a** cell viability (NRR assay) and **b** ROS production (ROS assay) were measured (*error bars* = ±SE, $n = 3$). *Significantly different from sludge-untreated soil (LSD test, $p < 0.05$). Within each treatment group, means sharing the same letter are not significantly different (LSD test, $p < 0.05$)

whereas the filtered leachates showed cytotoxicity by 37.4 % ($p < 0.001$) and 27.29 % ($p = 0.005$), respectively, on exposure to 1,000 mg/kg of ZnO-NPs. Significant differences were observed between treatments for the 250–1,000 mg/kg unfiltered leachate concentrations (SSB treatment) ($p = 0.040$) and for the 500 to 1,000 mg/kg of filtered leachate concentrations (SSA and SSB treatments) ($p < 0.001$). The in vivo or in vitro bibliographic data were found for the NRRA in any of the aquatic species, but the in vitro alteration of ß-galactosidase enzyme activity, another test of lysosomal function, has already been described with different fish cell lines (Fernández et al. 2013).

The effect of ZnO-NPs on different aspects of cellular activity—such as the amount of protein present in the cells (CB assay), basic cellular metabolism (AB assay), and cell membrane integrity (CFDA-AM assay)—were negative for all of the unfilterable and filterable leachates of sludge-untreated and sludge-treated soils (data not shown). This could be because the highest Zn concentration content in the leachates was 2.54 mg/L [1,000 mg/kg ZnO-NPs spiked soil (Table 3)] lower than the cytotoxic ZnO-NP concentrations described for other investigators after 24 h in the RTG-2 cells exposures [6.25 and 3.12 µg/mL (AB and CFDA-AM assays, respectively)] (Fernández et al. 2013).

The intracellular ROS levels were significantly greater after 24 h of exposure to the unfiltered and filtered leachates of the sludge-amended soils (Fig. 4b). After exposure to 1,000 mg/kg, intracellular ROS levels increased for SSA and SSB treatments to 71.3 % (ZnO-NPs; $p < 0.001$), 39.21 % ($Zn^{2+}$; $p = 0.047$), 52.77 % (ZnO-NPs; $p < 0.001$), and 23.94 % ($Zn^{2+}$; $p = 0.001$), respectively. Significant differences were observed between treatments for the 500–1,000 mg/kg unfiltered leachate concentrations (SSA and SSB treatments; $p < 0.001$) and for the 1,000 mg/kg filtered leachate concentration (SSA and SSB treatments; $p < 0.001$). Fernández et al. (2013) described

an increase in % ROS generation for ZnO-NPs to a concentration of 3.12 μg/mL after 24 h of treatment for RTG-2 cells. The Zn content of the sludge-untreated and -treated soils (Table 3) was always <3.12 μg/mL. However, ROS increased in unfiltered and filtered samples in SSA and SSB treatments. This could be an additive and/or synergistic effect between other contaminants present in the sludges and the ZnO-NPs. Potential synergistic effect of the $Fe_2O_3$ and $TiO_2$ NPs with pro-oxidative compound has already been described (Aranda et al. 2013; Zheng et al. 2012). In addition, the 24-h ZnO-NP exposure values were greater than the $Zn^{2+}$ ion exposure values. It has been reported that ZnO-NPs are embedded in leachate solids matrices (Reinhart et al. 2012). In our study, filtration of the leachates was performed to examine the extent of the effects of $Zn^{2+}$ ions relative to ZnO-NPs. Our negative results, as well as the low Zn concentration in the leachates, could suggest that only a small proportion of the ZnO-NPs passed to the leachates; thus, $Zn^{2+}$ concentration in the leachates was low. The dissolved $Zn^{2+}$ ions could be easily chelated by cell proteins, the function of which includes binding of $Zn^{2+}$ (Maret 2012).

## Conclusion

One of the restrictions in assessing the toxic impact of NPs is their interaction with the media in which they are dispersed or dissolved. This work gives an overview in the interaction of ZnO-NP—enriched sewage sludge with natural soil and water components. After the addition of sewage sludge enriched with high concentrations of ZnO-NPs to agricultural soils, the risk of qualitative toxic effects for soil and aquatic organisms was shown to be low. These findings are important with respect to the consideration of the environmental risk of routine additions of sewage sludge amendments to soil.

**Acknowledgments** We thank Ana Obrador and José Manuel Alvarez (Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica, Madrid, Spain) for the metal analysis. We thank Francisco J. García (Instalación Científico Tecnológica Singular, Universidad Complutense, Madrid, Spain) for the TEM analysis. We thank Daniel Alonso and Carmen del Rio for technical assistance. This work was supported by Spanish Project No. RTA2010-00018-00-00.

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