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Effect of soil organic matter content and pH on the toxicity of ZnO nanoparticles to *Folsomia candida*

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ABSTRACT

Organic matter (OM) and pH may influence nanoparticle fate and effects in soil. This study investigated the influence of soil organic matter content and pH on the toxicity of ZnO-NP and $ZnCl_2$ to *Folsomia candida* in four natural soils, having between 2.37 and 14.7% OM and pH_{CaCl2} levels between 5.0 and 6.8. Porewater Zn concentrations were much lower in ZnO-NP than in $ZnCl_2$ spiked soils, resulting in higher Freundlich sorption constants for ZnO-NP. For $ZnCl_2$ the porewater Zn concentrations were significantly higher in less organic soils, while for ZnO-NP the highest soluble Zn level (23 mg Zn/l) was measured in the most organic soil, which had the lowest pH. Free Zn^{2+} ion concentrations were higher for $ZnCl_2$ than for ZnO-NP and were greatly dependent on pH (pH_{pw}) and dissolved organic carbon content of the pore water. The 28-d EC_{50} values for the effect of $ZnCl_2$ on the reproduction of *F. candida* increased with increasing OM content from 356 to 1592 mg Zn/kg d.w. For ZnO-NP no correlation between EC_{50} values and OM content was found and EC50 values ranged from 1695 in the most organic soil to 4446 mg Zn/kg d.w. in the higher pH soil. When based on porewater and free Zn^{2+} concentrations, EC_{50} values were higher for $ZnCl_2$ than for ZnO-NP, and consistently decreased with increasing pH_{pw}. This study shows that ZnO-NP toxicity is dependent on soil properties, but is mainly driven by soil pH.

Key words: zinc oxide nanoparticles; soil pH; organic matter content; *Folsomia candida*; bioavailability

1. Introduction

Nanotechnology is a fast growing technology of global economic importance, with special regard to the invention of new manufacturing methods and materials on the nanoscale (i.e. <100 nm) (Royal Society and Royal Academy of Engineering, 2004). The properties of engineered nanoparticles that make them useful in a wide range of industrial applications, however, have led to concerns regarding their potential impact on environmental health (Scown et al., 2010). Due to their small size and high reactivity engineered nanoparticles are an emerging class of contaminants with potential of damaging the environment (Manzo et al., 2013). Zinc oxide nanoparticles (ZnO-NP) have been used in a variety of products and applications such as semi-conductors, catalysts, and paints, and increasingly in consumer products such as sunscreens because of their strong ultraviolet absorption properties (Reed et al., 2012). Increased production and use of ZnO-NP suggest increased exposure for organisms living in the environment (Reed et al., 2012).

Release of ZnO-NP into the environment, such as through waste water treatment plant effluent, should increase environmental exposure, although this is difficult to quantify (Reed et al., 2012). Modelled data indicate that ZnO concentrations might be high enough to induce adverse effects on aquatic organisms (Gottschalk et al., 2009). Compared with other engineered nanoparticles, ZnO-NP has often been found to be among the most toxic ones (Adams et al., 2006; Aruoja et al., 2009). The general belief is that these particles dissolve relatively quickly and that the Zn^{2+} ion is the main contributor to ZnO-NP toxicity (Reed et al., 2012). Alternatively, however, the ZnO-NP may be toxic by themselves or toxicity may be due the combined effect of the mixture of ZnO-NP and released ions. Knowledge on the environmental fate and effects of ZnO-NP is growing, but mainly focussed on the aquatic environment. Soils are a sink for most environmental contaminants after sewage sludge applications and need to be studied as well (Tourinho et al., 2012).

Environmental conditions may act on ZnO-NP to change their size, shape and surface chemistry. Changing these basic characteristics may result in speciation products that are significantly different from the initial ZnO-NP. The interaction of nanoparticles with natural organic matter (NOM) is now receiving considerable interest, in order to better understand how these interactions might affect the stability, aggregation and dissolution in aquatic media (Quik et al., 2010; Scown et al., 2010). NOM originates from the breakdown of plant and animal tissue in the environment, and its main constituents are humic acids, fulvic acids, and a hydrophilic fraction (Quik et al., 2010). Generally NOM decreases nanoparticle aggregation

and increases the colloidal stability (Quik et al., 2010). Several metal oxide nanoparticles are stabilized in aqueous solutions by the adsorption of NOM, due to increased electrostatic repulsion (Domingos et al., 2009; Yang et al., 2009; Zhang et al., 2009). It has been demonstrated that NOM is able to coat nanoparticles (Lead and Wilkinson, 2006), resulting in a nanoscale coating of the particles (Lowry et al., 2012). The thickness of the coating increases with increasing humic acid concentration (Baalousha et al., 2008). Such a natural coating causes charge neutralization and colloidal stability, as the surface of metal-based nanoparticles is mostly positively charged at circum-neutral pH and humic acid molecules are partially deprotonated (Baalousha et al., 2008; Bian et al., 2011; Tourinho et al., 2012). This coating by humic acids could imply that the release of the toxic metal ions is diminished by blocking the nanoparticle surface, but so far this has not been studied in much detail.

Understanding the interactions of manufactured nanoparticles in soils is difficult, because complex interactions occur with the solid phase and the pore water. It is already known that a long time is needed to reach equilibrium for soil systems spiked with ZnO-NP (Scheckel et al., 2010; Waalewijn-Kool et al., 2013a). Complex interactions with the soil matrix could diminish the exposure of soil organisms to pristine ZnO-NP, but could lead to more complex exposures to coated NP, released Zn ions and mixtures of ZnO-NP and released ions at varying ratios (Tourinho et al., 2012). Unfortunately, appropriate characterization techniques for nanoparticles in soil do not exist and most techniques start with pre-treatment of the soil by preparing a suspension of soil extracts (Lead and Wilkinson, 2006). Similar to aquatic solutions, NOM is likely to affect the transformations and speciation of ZnO-NP in soils. It is hypothesized that with increasing OM content, ZnO-NP interacts stronger with OM by forming OM-ZnO clusters, resulting in a decreased bioavailability, dissolution and toxicity. Other soil properties, such as pH and cation exchange capacity (CEC) may also affect the bioavailability and toxicity of ZnO-NP. In a previous study, the effect of soil pH has been studied in amended field soils. Increased toxicity of ZnO-NP with decreasing pH was demonstrated for earthworms (Heggelund et al., 2013) and springtails (Waalewijn-Kool et al., 2013b), but pH effects were less consistent for the toxicity of ZnO-NP to isopods (Tourinho et al., 2013).

This study investigated how organic matter content, in addition to pH, influenced the bioavailability and toxicity of ZnO-NP and $ZnCl_2$ to the springtail *Folsomia candida*. Springtails are abundant in most natural soils and *F. candida* represents the collombolans in ecotoxicological tests (Fountain and Hopkin, 2005). Four natural soils were tested that provide a range of organic matter content (2.37 - 14.7%) and slightly different pH_{CaCl2} levels

(5.0 - 6.8). This study does not differentiate between types of NOM (quality), but just considers the total content of organic matter in soils. For both Zn forms, the 28-d EC_{50} values were expressed on the basis of total Zn, porewater Zn and free Zn^{2+} ion concentrations.

2. Material and methods

2.1. Soil properties

Four natural soils were collected from different countries within Europe: from Coimbra in Portugal, Speyer in Germany (Lufa standard soil 2.2, Sp 2121), De Kwakel in The Netherlands (grassland, soccer field) and from Rhydtalog in North-Wales, United Kingdom, hereafter called soils 1, 2, 3 and 4, respectively. These soils, which represent different organic matter contents and pH levels, were homogenized, sieved through a 5 mm mesh and air dried before spiking and toxicity testing.

The organic matter content was determined as loss on ignition at 500 °C in an ashing oven. Since previous studies showed little change (no more than 0.2 units) of soil pH during 28-day toxicity tests on ZnO-NP with *F. candida*, the pH_{CaCl2} of the soils was measured in the middle of the toxicity test, after two weeks exposure, in two replicate samples of each treatment. Soils were shaken with 0.01 M $CaCl_2$ solution (1:5) for 2 hours at 200 rpm. After settlement of the particles, the pH of the soil solution was recorded using a Consort P907 meter. The Water Holding Capacity (WHC) was determined following ISO (1999). The cation exchange capacity (CEC) was determined by the Silver Thiourea Method (Dohrmann, 2006). Approx. 2 g dry soil was shaken with 25 mL 0.01 M silver thiourea complex cation (AgTU) solution for 3 hours at 200 rpm to achieve a complete exchange of all cations. Four blanks without soil were included. Ag was measured in the supernatant solution by flame Atomic Absorption Spectrometry (AAS) (Perkin Elmer AAnalyst 100). The decrease in Ag concentration is a measure for the CEC of the soil.

2.2. Test compounds

ZnO nanoparticle powders (Nanosun Zinc Oxide P99/30) with a reported diameter size of 30 nm were tested. Transmission Electron Micrographs and Particle Size Distribution of the ZnO nanoparticles are shown by Waalewijn-Kool et al. (2012). Primary particle size of the nanoparticle powder was in agreement with the size reported by the manufacturer. The

effect of dissolved Zn was investigated by running tests with the soluble salt $ZnCl_2$ (Merck, zinc chloride pure).

Seven concentrations of ZnO-NP (nominal range 100 – 6400 mg Zn/kg d.w.) and five concentrations of $ZnCl_2$ (nominal 100-1600 mg Zn/kg d.w.) were tested. Test concentrations were based on toxicity data found in earlier studies (Kool et al., 2011). ZnO-NP powder was mixed in with 200 g dry soil to reach nominal test concentrations. After mixing, water was added to reach 50% of the WHC. $ZnCl_2$ was added to the soil using solutions in Milli-Q water, and soils were thoroughly mixed. The amount of water added with the $ZnCl_2$ was sufficient to reach a moisture content equivalent with 50% of the WHC. Controls received water only. All spiked soils were equilibrated for five days before starting the toxicity tests.

2.3. Soil and porewater analysis

Two samples per test concentration (± 100 mg dried soil) were taken from the spiked soils and digested in a mixture of Milli-Q water, concentrated HCl and concentrated HNO_3 (1:1:4 by vol.) using an oven (CEM MDS 81-D). After digestion for 7 hours at 140 °C, solutions were analysed for total zinc concentrations by flame AAS (Perkin Elmer AAnalyst 100). Certified reference material (ISE sample 989 of River Clay from Wageningen, The Netherlands) was used to ensure the accuracy of the analytical procedure. Detection limit of the analysis was 0.003 mg Zn/L.

Pore water was collected at the beginning of each toxicity test by centrifugation. For that purpose, 30 g soil portions were sampled, saturated with Milli-Q water and incubated for another three days equilibration. Then the soils were centrifuged for 45 min. (Centrifuge Falcon 6/300 series, CFC Free) with a relative force of 2000 g over two round filters (S&S 597 Ø 47 mm, pore size 11 µm) and a 0.45 µm membrane filter (S&S Ø 47 mm), placed inside the tubes (method cf. Hobbelen et al., 2004). Approximately 7 mL pore water per sample was collected for Zn analysis by flame AAS. Since we expected the grassland soil to be limed, we also measured Ca concentrations in the porewater samples from the controls of soil 3 by flame AAS. For the sake of comparison, Ca concentration was also measured in the porewater of the control Lufa 2.2 soil (soil 2), which we always use as our reference soil. The dissolved organic carbon (DOC) concentration in the pore water was measured by high temperature oxidation at 850°C - 900°C and detection of the formed CO_2 (liquiTOC). The pH of the pore water (pH_{pw}) was measured using a Consort P907 meter.

The Zn^{2+} ion concentrations in the pore water from the four soils spiked with ZnO-NP and $ZnCl_2$ were calculated with the speciation model WHAM7 using H^+, Ca, Zn and DOC concentrations (mg/L) of the pore water and soil pH.

2.4. Toxicity tests

The springtail *F. candida* (Berlin strain; VU University Amsterdam) was cultured in pots with a base of moist plaster of Paris mixed with charcoal at 20 ± 1 °C at a light/dark regime of 12/12 h. The experiments were initiated with juveniles of the same age (10 - 12 days) that were obtained by synchronising the egg laying of the culture animals, fed with dried baker's yeast (Dr. Oetker).

Each soil was tested in a separate toxicity test, including the two Zn forms, control soil without added Zn and Lufa 2.2 soil as a control for springtail performance. The ISO guideline 11267 for testing for chemical effects on the reproduction of springtails was followed (ISO, 1999). Tests were conducted in 100 mL glass jars containing 30 g moist soil with five replicates for each treatment. At the start of the test, ten synchronised animals were transferred into each test jar. The jars were filled randomly and before introduction the animals were checked under the microscope for a healthy appearance. The animals were fed a few grains of dried baker's yeast (Dr. Oetker). The jars were incubated in a climate room at 20 ± 1 °C at a light/dark regime of 12/12 h. Once a week, the moisture content of test soils was checked by weighing the jars, and moisture was replenished with Milli-Q water when necessary. The jars were also aerated by this procedure.

After four weeks, the jars were sacrificed for determination of springtail survival and reproduction. Each jar was emptied into a 200 mL beaker glass and 100 mL tap water was added. The mixture was stirred carefully to let all the animals float to the surface. The number of surviving adults and juveniles produced were counted manually after taking a picture of the water surface using a digital camera (Olympus, C-5060).

2.5. Data analysis

Using the soil and porewater concentrations, sorption of zinc to the test soil was described by a Freundlich isotherm:

$$C_s = k_f * C_w^{\,n}$$

where,

C_s = concentration in the soil (mg Zn/kg d.w.)

k_f = Freundlich sorption constant ($(L/kg)^{1/n}$)

C_w = concentration in the pore water (mg Zn/L) and

n = shape parameter of the Freundlich isotherm

EC_{50} values for the effect on reproduction were estimated applying the logistic model of Haanstra et al. (1985) and were determined based on total Zn concentrations in soil and dissolved Zn and free Zn^{2+} concentrations in the pore water. A generalized likelihood ratio test (Sokal and Rohlf, 1995) was applied to compare EC_{50} values based on total Zn concentration obtained for both Zn forms and for each soil. Calculations were performed in SPSS Statistics 20.

3. Results

3.1. Soil properties

Table 1 summarizes the properties of the four test soils used in this study. The organic matter content measured in the different soils increased from 2.37% in soil 1 up to 14.7% in soil 4. The DOC concentrations in the pore water increased with increasing OM content from approx. 64 mg/L in soils 1 and 2 up to 3605 mg/L in soil 4. Soils 1 and 2 had nearly the same pH_{CaCl2} (around 5.7 - 5.8), while the pH_{CaCl2} of soil 3 was slightly higher (6.8) and soil 4 was more acidic (pH_{CaCl2} 5.0). This was probably due to liming of soil 3 in the field, because more than a 3-fold higher Ca level was measured in the pore water of this soil (221 mg/L) compared to Lufa 2.2 (64.3 mg/L). Clay contents ranged between 4.2 and 13% and was highest in the most organic soil 4. CEC increased with increasing OM content for soils 1, 2 and 3, but was lower in the more acidic soil 4 (11.8 mval/100g) compared to soil 3 (20.0 mval/100g).

3.2. Total Zn analysis and soil pH

Total Zn analyses of the four test soils showed recoveries of Zn ranging from 66.9 to 127% (Supplementary Data, Tables S1-S4). Zinc concentrations in the soil were corrected for

the zinc measured in the controls, which may explain the variability in the Zn recovery. Total Zn concentrations in the untreated soil were 75.6, 17.1, 39.4 and 71.5 mg Zn/kg d.w. for soils 1, 2, 3 and 4, respectively. As the measured zinc concentrations in the reference material were within 6% of the certified concentrations, the measured Zn concentrations were considered reliable. All calculations used measured Zn concentrations.

Soil pH_{CaCl2} and porewater pH_{pw} increased with increasing ZnO-NP concentrations, and decreased with increasing concentrations of $ZnCl_2$ (Supplementary Data, Tables S5-S8).

3.3. Porewater Zn concentration and sorption

The Zn concentrations in the pore water of the four test soils are presented in Table S7 for ZnO-NP and in Table S8 for $ZnCl_2$. In soils 2, 3 and 4 the Zn concentrations in the pore water increased with increasing ZnO-NP concentrations, up to a maximum of 2.83, 3.52 and 23.8 mg Zn/L, respectively. So, the highest porewater concentration was measured in soil 4 having the highest organic matter content and the lowest pH_{CaCl2}. For soil 1, the maximum porewater concentration of 3.09 mg Zn/L was measured at an intermediate nominal concentration of 800 mg Zn/kg d.w. The porewater Zn concentrations for $ZnCl_2$ were much higher than for ZnO-NP spiked soils. For the $ZnCl_2$-spiked soils 1, 2, 3 and 4 the porewater Zn levels increased with increasing soil concentrations up to 1021, 720, 10.4 and 63.7 mg Zn/L, respectively. The highest porewater concentrations were found for the soils 1 and 2 with the lowest OM content, while for the more organic soils 3 and 4 higher Zn porewater concentration was found in the more acidic soil 4.

The porewater Zn levels were translated into Freundlich sorption constants for ZnO-NP and $ZnCl_2$, indicating zinc availability in the four soils (Table 2). The k_f value for $ZnCl_2$ increased with OM content except for soil 4, and with CEC for all soils. The highest k_f value of 458 $(L/kg)^{1/n}$ (n = 0.572) was calculated for soil 3 having the highest pH. For ZnO-NP, k_f values did not correlate with OM content. The highest k_f value of 1146 $(L/kg)^{1/n}$ (n = 1.08) was calculated for soil 3, while the lowest k_f value of 97.1 $(L/kg)^{1/n}$ (n = 1.45) was estimated for the most organic soil (soil 4). No k_f value could be derived for ZnO-NP sorption in soil 1. For the other soils, k_f constants increased with increasing soil pH, suggesting higher availability in the more acidic soil 4. In all soils spiked with ZnO-NP the shape parameter n of the Freundlich isotherm was higher than one, suggesting saturation of the pore water or Zn precipitation. For all soils spiked with $ZnCl_2$ the shape parameter n was below one, suggesting saturation of the solid phase at high porewater concentrations.

3.4. *Free Zn^{2+} ion concentrations*

The free Zn^{2+} ion concentrations in the pore water calculated using WHAM7 are presented in the Supplementary Data Table S9 for ZnO-NP and in Table S10 for $ZnCl_2$. The free Zn^{2+} ion concentrations were much higher for $ZnCl_2$ than for ZnO-NP spiked soils. For $ZnCl_2$, the free Zn^{2+} ion concentrations increased with increasing total Zn concentration up to 5501, 4189, 15.0 and 176 µM for soils 1, 2, 3 and 4, respectively. The free Zn^{2+} ion concentrations for $ZnCl_2$ were lowest in soil 3, having the highest pH_{pw}. In the other soils , having lower pH_{pw} (4.7-5.0 at 1600 mg Zn/kg dry soil; Table S8), free Zn^{2+} ion concentrations decreased with increasing soil OM content. For ZnO-NP, the free Zn^{2+} ion concentrations increased with increasing soil concentration for soil 3 with a maximum of 2.70 µM. In the other three soils, the free Zn^{2+} ion concentrations showed a peak at intermediate soil concentrations of 11.2, 6.18 and 1.50 µM in soils 1, 2 and 4, respectively. For higher soil ZnO-NP concentrations the free Zn^{2+} ion concentrations showed a more scattered pattern which did not always coincide with changes in pH_{pw}.

3.5. *Toxicity*

Control performance of the collembolans was little affected in the most organic soil with the lowest pH_{CaCl2}. Control survival after 28 days exposure in control soils 1, 2, 3 and 4 was 86, 96, 90 and 68%, respectively. The average number of juveniles in the controls was 263, 475, 514 and 114 for soils 1, 2, 3 and 4, respectively, with coefficients of variance of 68, 60, 6.4 and 26%, respectively.

No effect on springtail survival was found in the four test soils spiked with ZnO-NP up to 6400 mg Zn/kg d.w. or with $ZnCl_2$ up to 1600 mg Zn/kg d.w. Reproduction was reduced in a dose-dependent manner for the two Zn forms in all four soils (See Supplementary Data Figures S1 and S2 for all dose-response curves). Table 3 shows the EC_{50} values for the effect of ZnO-NP and $ZnCl_2$ on the reproduction of *F. candida* after 28 days exposure to the four soils. The EC_{50} values are presented as total Zn concentrations (mg Zn/kg d.w.), as porewater Zn concentrations (mg Zn/L) and as free Zn^{2+} ion concentrations (also in mg Zn/L in order to compare them with the EC_{50} values based on porewater Zn concentration).

The EC_{50} values for $ZnCl_2$ increased with increasing OM content from 356 mg Zn/kg d.w. in soil 1 up to 1592 mg Zn/kg d.w. in soil 4. Linear regression of the relation between

these EC_{50} values and soil OM content resulted in an R^2 of 0.967 (Figure 1). According to a generalized likelihood-ratio test, the EC_{50} for soil 3 was significantly higher than the ones for soils 1 and 2, ($\chi^2_{(1)}$ = 24.6 (soil 1 vs 3) and 21.7 (soil 2 vs 3), $p < 0.05$). No significant relation of EC_{50}s with soil OM contents was seen for ZnO-NP. The estimated EC_{50} values ranged between 1695 (soil 4) and 4446 (soil 3) mg Zn/kg d.w. The highest toxicity was observed in the most organic soil, but its EC_{50} value (1695 mg Zn/kg d.w.) was not significantly lower than the ones for the other three soils with lower OM content. The lowest toxicity was observed in soil 3 with the highest pH_{CaCl2}, but also this EC_{50} was not significantly higher than the ones for the other three soils with lower pH levels. Regression of EC_{50} values with soil pH resulted in an R^2 of 0.787, showing a good correlation between toxicity and pH for ZnO-NP. For $ZnCl_2$ the relation between EC_{50} values and soil pH based on total Zn concentrations was poor. The EC_{50} values for $ZnCl_2$ were significantly lower than for ZnO-NP in soils 1 and 2, according to a generalized likelihood-ratio test ($\chi^2_{(1)}$ = 18.9 (soil 1) and 21.2 (soil 2), $p < 0.05$). In soil 4 the EC_{50} values for ZnO-NP and $ZnCl_2$ were almost similar, namely 1695 and 1592 mg Zn/kg d.w., respectively.

Based on porewater Zn concentrations, the EC_{50} values for ZnO-NP ranged between 2.60 and 7.01 mg Zn/L and increased with increasing DOC concentrations of the pore water (R^2 = 0.991). The highest EC_{50} value (7.01 mg Zn/L) was estimated for soil 4, but this was not significantly higher than the EC_{50} of 2.60 and 3.03 mg Zn/L estimated for soils 2 and 3, respectively. For all soils, higher EC_{50} values based on porewater concentrations were estimated for $ZnCl_2$ than for ZnO-NP. The lowest EC_{50} of 10.2 mg Zn/L for $ZnCl_2$ was estimated for soil 3, higher EC_{50} values for the other three soils ranged from 33.8 to 55.8 mg Zn/L. The EC_{50} for $ZnCl_2$ related poorly with DOC concentrations of the pore water, but clearly decreased with increasing pH_{pw} (R^2 = 0.846). Taken all EC_{50}s based on porewater concentrations together for both Zn forms, toxicity of dissolved Zn consistently increased with increasing pH_{pw} (Figure 1).

For both Zn forms, the toxicity was higher on the basis of free Zn^{2+} concentrations than on the basis of porewater concentrations, as shown by the lower EC_{50} values in Table 3. For $ZnCl_2$ the EC_{50} based on free Zn^{2+} concentrations was 0.969 mg Zn/L for soil 3 and ranged from 10.3 to 27.0 mg Zn/L for the other three soils. For ZnO-NP, the estimated EC_{50} values based on free Zn^{2+} concentrations were 0.368 and 0.147 mg Zn/L for soils 2 and 3, respectively. It was not possible to estimate EC_{50} values on the basis of free Zn^{2+} concentrations for soils 1 and 4, due to the peak in free Zn^{2+} concentrations at intermediate

soil concentrations making that reduction in reproduction was not consistently related with free Zn^{2+} concentration.

4. Discussion

In light of nanoparticle risk assessment, soil properties need to be taken into account to predict the bioavailability and toxicity of ZnO-NP. The most important soil properties determining equilibrium partitioning of metals in soils are the adsorption phases (clay, OM and hydroxides), the amount of available sorption sites (CEC) and pH (Janssen et al., 1997). The aim of this study was to investigate the effect of soil OM content and pH on ZnO-NP dissolution and toxicity to *F. candida* in four natural soils, ranging in OM content from 2.37 to 14.7% and in pH_{CaCl2} from 5.04 to 6.78. The EC_{50} values for $ZnCl_2$ based on total soil concentrations increased with increasing OM content, but contrary to our hypothesis no correlation between toxicity and OM content was found for ZnO-NP. Soil pH had a major influence on the EC_{50} values estimated on the basis of porewater Zn concentrations.

For $ZnCl_2$, the porewater Zn concentrations were much higher in less organic soils (containing approx. 2.37-3.09% OM) than in soils containing approx. 10.6 to 14.7% OM. This is explained by the amount of binding sites available to bind zinc, because it is known that humic and fulvic acids form chelates with Zn ions (Alloway, 1990). The porewater Zn levels in the more organic soils also showed that the CEC influenced the soluble Zn concentrations. The lowest porewater Zn concentrations were measured in the soil with the highest CEC. For both zinc forms, the lowest dissolved Zn concentrations and the highest sorption constants were estimated for the Dutch grassland soil with the highest pH. This can be explained by the absence of competition with H^+ ions for Zn binding at the negatively charged phases in the soil when pH levels are increasing (Alloway, 1990).

The solubility of ZnO-NP, measured as dissolved Zn centrifuged over a 0.45 µm filter, was much lower than for the soluble metal salt $ZnCl_2$ in all four soils. For ZnO-NP, the highest soluble Zn levels (max. 23.8 mg Zn/L) were measured in the most organic soil. It cannot be excluded that the dissolution of ZnO-NP in this soil was stimulated by the lower soil pH. The larger amount of water needed to reach 50%WHC of this soil (320, 255, and 115 mL water more than in soils 1, 2 and 3, respectively) may have triggered a greater dissolution of ZnO-NP. Nevertheless, this is the first study that shows that ZnO-NP dissolution results in different available Zn concentrations in soils with different soil properties. The aggregation and dissolution of ZnO-NP in different soils have not been explored much. Aquatic studies

393 demonstrated that OM can have a "masking" effect, either by direct coating of the
394 nanoparticle surface or by minimizing dissolution (Lowry et al., 2012). Once coated and
395 aggregated in either the solid phase or in the pore water, it can be more difficult for the ZnO-
396 NP to release Zn into the solution. In soils, the equilibrium processes of metals between pore
397 water and solid phases are rather complex (Tipping et al., 2003). Although increasing
398 solution pH will decrease metal solubility, it may increase dissolved metal concentrations
399 because of the formation of metal complexes with DOC in the porewater. DOC may coat
400 reactive adsorption sites, inactivating them and therefore indirectly inhibit metal adsorption.
401 The shape parameter *n* of the Freundlich isotherm was higher than one for ZnO-NP spiked
402 soils, supporting this idea of saturation of the pore water with Zn complexes. In our soils the
403 OM could have enhanced the release of Zn from the ZnO-NP, for example by assisting in
404 breaking up large aggregates (de-agglomeration). Several aquatic studies with ZnO-NP and
405 humic acids or clay minerals support this hypothesis (Bian et al., 2011; Li et al., 2011a; Miao
406 et al., 2010; Scheckel et al., 2010).
407 Our experimental data showed that both soil pH and DOC concentrations influenced
408 ZnO-NP dissolution. The measured porewater Zn concentrations reflected to some extent the
409 effect of pH on ZnO-NP solubility. The pH levels in all soils increased with increasing total
410 Zn concentration, confirming that spiking ZnO into the soils did cause an increased soil pH.
411 It should be noted that, to enable the creation of full dose-response curves and compare
412 toxicity among different soils, we had to test rather high ZnO-NP concentration, by far
413 exceeding levels expected to occur in the environment (Gottschalk et al., 2009). Porewater Zn
414 concentrations generally increased with increasing total Zn concentration in the soils, but
415 peaked at intermediate soil concentrations for soil 1. The pH where the net surface charge of
416 ZnO-NP is zero and highest stability of the particles is expected (point of zero charge, pzc),
417 ranges between 7.5 and 9.8 in water (Keller et al., 2010; Kosmulski, 2004) and above 8 in
418 soil (Collins et al., 2012). The pH_{CaCl2} values of our test soils were below this pzc, suggesting
419 low stability of the particles. The pH values of the pore water of soils 1, 2, and 3, however,
420 were closer to this pzc (in between 7 and 8). In theory, an increase in stability could lead to
421 an increase in Zn release from the nanoparticles due to a larger surface area of the individual
422 particles compared to aggregated nanoparticles. The peak in porewater and free Zn^{2+}
423 concentrations for soils 1, 2 and 4 suggested a reduction of Zn release above a pH_{pw} of 7.
424 Such a peak has been observed before in Lufa 2.2 soil for ZnO-NP as well as for non-nano
425 ZnO in aged soils (Waalewijn-Kool et al., 2013a). The present study showed that also in
426 freshly spiked soil ZnO-NP dissolution may be reduced at high exposure concentrations. This

phenomenon was however, restricted to soil 1, which seemed strange as in soils 2 and 3 pH_{pw} reached higher levels. When plotting porewater Zn levels, measured at the same total Zn concentrations (Table S7) against pH_{pw} in the four soils, a linear decrease with increasing pH_{pw} was seen for all ZnO-NP treatment levels. This seems to confirm that the Zn release from ZnO-NP decreased with increasing pH_{pw}. At all exposure levels, Zn concentrations in the pore water however, also linearly increased with increasing DOC content (Table 1). This shows that organic matter content also played a role in the dissolution of ZnO-NP, probably by binding (part of) the released Zn, and making that not all Zn in the pore water was freely available. The latter was accounted for when calculating free Zn ion activities.

For ZnO-NP no significant effect (increase or decrease) of soil OM content on the toxicity to springtail reproduction was detected, while the EC_{50} values for $ZnCl_2$ increased with increasing OM content. The EC_{50} for ZnO-NP in Lufa 2.2 soil was 3493 mg Zn/kg d.w. which was comparable to the EC_{50} of 3159 mg Zn/kg d.w. estimated in previous experiment with this type of ZnO-NP (Waalewijn-Kool et al., 2012). The higher pH in the Dutch grassland soil most probably resulted in the lower toxicity observed for ZnO-NP and $ZnCl_2$ based on total Zn concentrations. Zinc toxicity has been shown to be related to OM and clay content in freshly contaminated OECD artificial soil with 10% OM (Smit and Van Gestel, 1998). The effect of OM on ZnO-NP toxicity is still poorly understood, with many studies producing contradictory results. Li et al. (2011b) found a reduced toxicity of ZnO-NP to bacteria with increasing DOC concentrations in aquatic media, while Blinova et al. (2010) reported that DOC could not decrease the acute toxicity of ZnO-NP to crustaceans in natural water. Li et al. (2011a) exposed *Eisenia fetida* for 96 hours to soil extracts and found that addition of humic acids enhanced ZnO-NP dissolution, but reduced toxicity.

Released Zn may form steady organic complexes, which is likely to occur when more DOC is present in the soil pore water (Fang et al., 2009). From our study, it remained unclear if the effect of DOC concentrations on the EC_{50} values based on porewater concentrations (for both Zn forms) was significant. A protective effect of DOC in the pore water may have occurred in our most organic soils, as could be seen from the increase of porewater Zn concentrations with increasing DOC level in the case of ZnO-NP. But toxicity seemed most affected by pH, as EC_{50}s expressed on the basis of dissolved Zn concentrations in the pore water consistently decreased with increasing pH (see Figure 1). This suggests that at the lower pH_{pw} levels, which especially occurred in the $ZnCl_2$-spiked soils, H^+ ions may have competed with dissolved Zn species resulting in lower toxicity. According to the theory of the terrestrial Biotic Ligand Model the reduction of competition with H^+ ions may lead to

higher toxicity data (Thakali et al., 2006). This effect was also observed for the EC_{50} values based on free Zn^{2+} ion concentrations, in particular for $ZnCl_2$, which suggests that the ionic Zn was a good predictor for toxicity.

In conclusion, the toxicity of $ZnCl_2$ was related to soil OM content but this was not the case for ZnO-NP, suggesting that ZnO-NP have to dissolve to make Zn bioavailable. Soil pH and DOC play an important role in the dissolution of ZnO-NP and its release into the pore water. Further research is necessary on interactions between ZnO-NP and soil components such as OM, affecting ZnO-NP toxicity, but it cannot be seen separately from the role of other soil properties, such as pH.

Supplementary data

Supplementary data include dose-response curves for ZnO-NP (Figure S1) and $ZnCl_2$ (Figure S2) in the four test soils, measured Zn concentrations and pH values in the four test soils (Table S1-S6) and in pore water (Tables S7 and S8) and free Zn^{2+} ion concentrations in the pore water calculated with WHAM7 (Tables S9 and S10).

Acknowledgment

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Figures:

Figure. 1. Estimated EC_{50} values for the effect of Zn on the reproduction of *Folsomia candida* based on total Zn concentrations (mg Zn/kg d.w.) and porewater Zn concentrations (mg Zn/l) in relation to pH_{CaCl2}, pH_{pw}, soil organic matter content and DOC concentrations in the pore water (see Table S-5 to S-8 for measured pH values and Table 1 for soil properties). Data used are from 28-d toxicity tests in the four test soils freshly spiked ZnO-NP and $ZnCl_2$ (see Table 3).

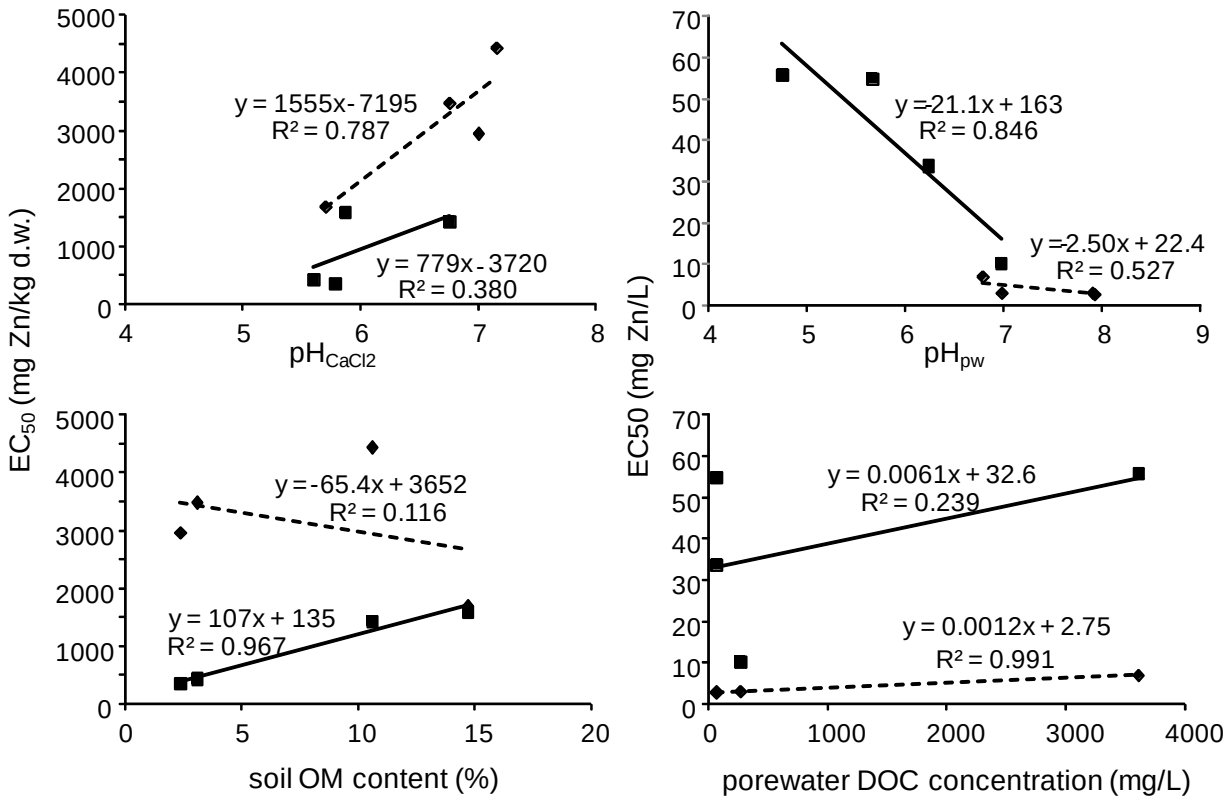

Fig. 1. Estimated EC_{50} values for the effect of Zn on the reproduction of *Folsomia candida* based on total Zn concentrations (mg Zn/kg d.w.) and porewater Zn concentrations (mg Zn/l) in relation to pH_{CaCl2}, pH_{pw}, soil organic matter content and DOC concentrations in the pore water (see Table S-5 to S-8 for measured pH values and Table 1 for soil properties). Data used are from 28-d toxicity tests in the four test soils freshly spiked ZnO-NP and $ZnCl_2$ (see Table 3).

623 **Table 1.** Properties of Coimbra (soil 1), Lufa 2.2 (soil 2), Dutch grassland (soil 3) and North
624 Wales (soil 4) test soils used to determine the influence of soil properties on the toxicity of
625 ZnO nanoparticles to *Folsomia candida*. Reported OM, DOC and CEC values are the mean
626 of two measurements.
627

Soil	OM (%)	DOC (mg/L)	pH_{CaCl2}	% clay	WHC (g/100g)	CEC (mval/100g)
1	2.37	64.9	5.9	4.2	32	5.17
2	3.09	64.1	5.7	7.2	45	6.34
3	10.6	265	6.8	5.1	73	20.0
4	14.7	3605	5.0	13	96	11.8

628 OM = organic matter; WHC = water holding capacity; CEC = cation exchange capacity;
629 DOC= dissolved organic carbon, measured in the pore water;
630

631 **Table 2.** Freundlich sorption constants k_f ($(L/kg)^{1/n}$) and shape parameters *n* for Zn
632 partitioning in four soils spiked with ZnO-NP and $ZnCl_2$. All values are given with
633 corresponding SE. See Table 1 for soil properties.
634

Soil	ZnO-NP		$ZnCl_2$	
	k_f	*n*	k_f	*n*
1	-	-	37.1 ± 1.24	0.535 ± 0.048
2	329 ± 1.35	2.10 ± 0.397	83.2 ± 1.10	0.424 ± 0.023
3	1146 ± 1.09	1.08 ± 0.016	458 ± 1.13	0.572 ± 0.072
4	97.1 ± 1.14	1.45 ± 0.073	146 ± 1.47	0.608 ± 0.161

635 - k_f and *n* values could not be estimated, because the porewater concentrations did not
636 increase linearly with soil concentrations
637

638 **Table 3.** EC_{50} values (with 95% confidence intervals) for the effect on reproduction of
639 *Folsomia candida* after 28-d exposure to ZnO-NP and $ZnCl_2$ in the four soils. See Table1 for
640 soil properties. EC_{50} values are presented as total Zn concentrations in mg Zn/kg d.w. (left) as
641 porewater Zn concentrations in mg Zn/L (middle) and as free Zn^{2+} ion concentrations in mg
642 Zn/L (right)
643

	Total soil (mg Zn/kg d.w.)		Pore water (mg Zn/L)		Free Zn (mg Zn/L)	
	ZnO-NP	$ZnCl_2$	ZnO-NP	$ZnCl_2$	ZnO-NP	$ZnCl_2$
Soil 1	2962[1a]	356[1b]	> 3.09*	54.9	> 0.728*	27.0
(2.37 % OM)	(1389-4534)	(24-5378)	(-)	(-)	(-)	(-)
Soil 2	3493[1a]	439[1b]	2.60	33.8	0.368	14.8
(3.09% OM)	(358-6628)	(316-561)	(2.37-2.84)	(-)	(0.259-0.476)	(-)
Soil 3	4446[1]	1433[2]	3.03	10.2	0.147	0.969
(10.6% OM)	(2830-6061)	(-)	(2.53-3.53)	(-)	(0.118-0.176)	(-)
Soil 4	1695[1a]	1592[12a]	7.01	55.8	> 0.093*	10.3
(14.7% OM)	(784-2605)	(-)	(5.22-8.81)	(-)	(-)	(-)

644 [1,2] indicate significant differences between EC_{50} values of ZnO-NP and $ZnCl_2$ for the different soils
645 according to a generalized likelihood-ratio test ($\chi^2_{(1)} > 3.84$; $p < 0.05$)
646 [a,b] indicate significant differences between EC_{50} values based on total Zn concentration for the
647 different Zn forms according to a generalized likelihood-ratio test ($\chi^2_{(1)} > 3.84$; $p < 0.05$)
648 - Data did not allow calculating reliable 95% confidence intervals
649 * EC_{50} is above highest porewater Zn/free Zn^{2+} ion concentration measured/calculated
650

Supplementary Data

Effect of soil organic matter content and pH on the toxicity of ZnO nanoparticles to *Folsomia candida*

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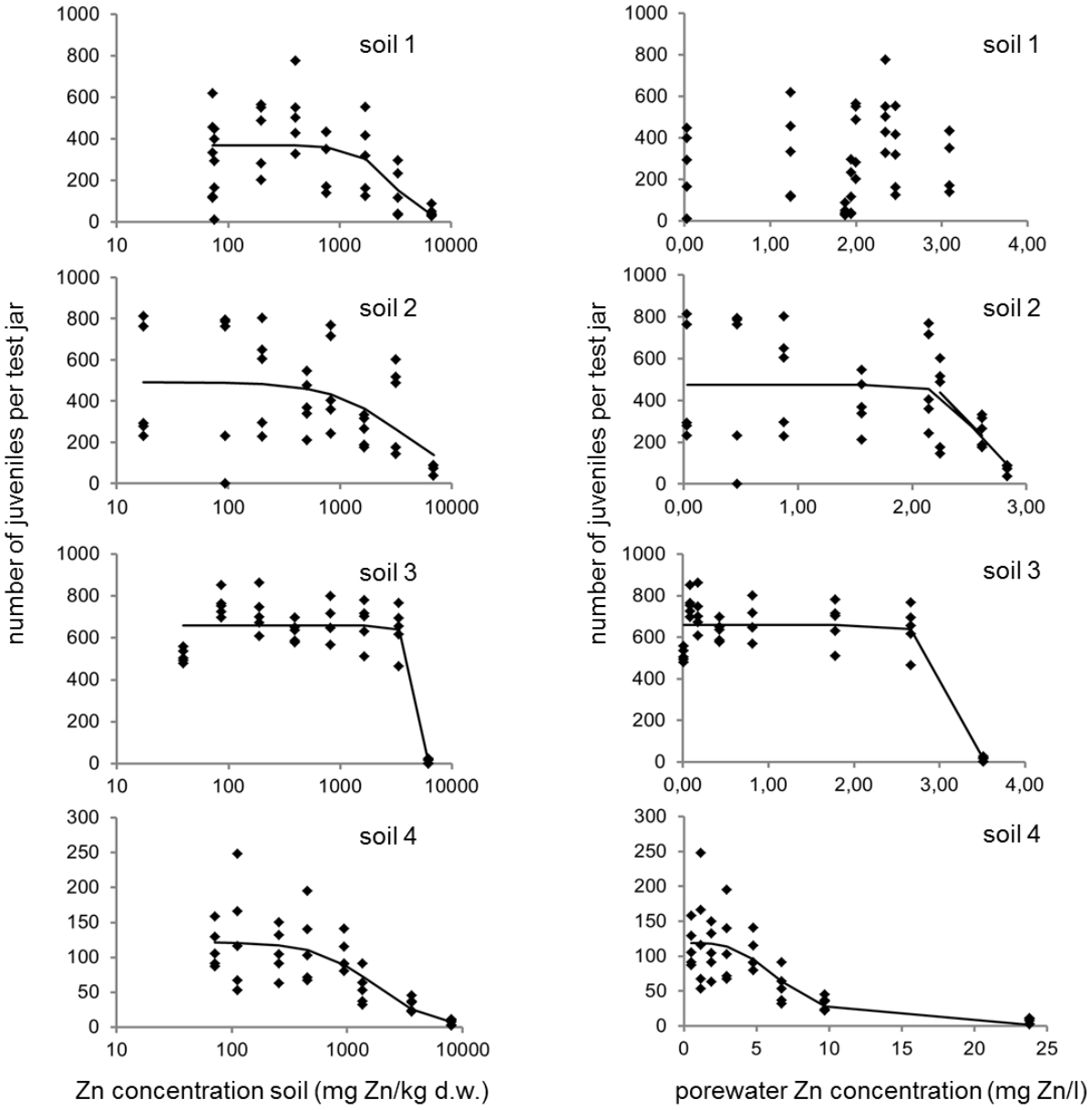

675

676

677 **Fig. S1.** Effect of ZnO-NP on the reproduction (number of juveniles) of *Folsomia candida*
678 after 28 days exposure in four different test soils. See Table 1 for soil properties. Measured
679 concentrations of zinc in the soil (left) and the pore water (right) are provided on the x-axis.
680 Dots represent the results of different replicates. Lines show the fit obtained with a logistic
681 model.

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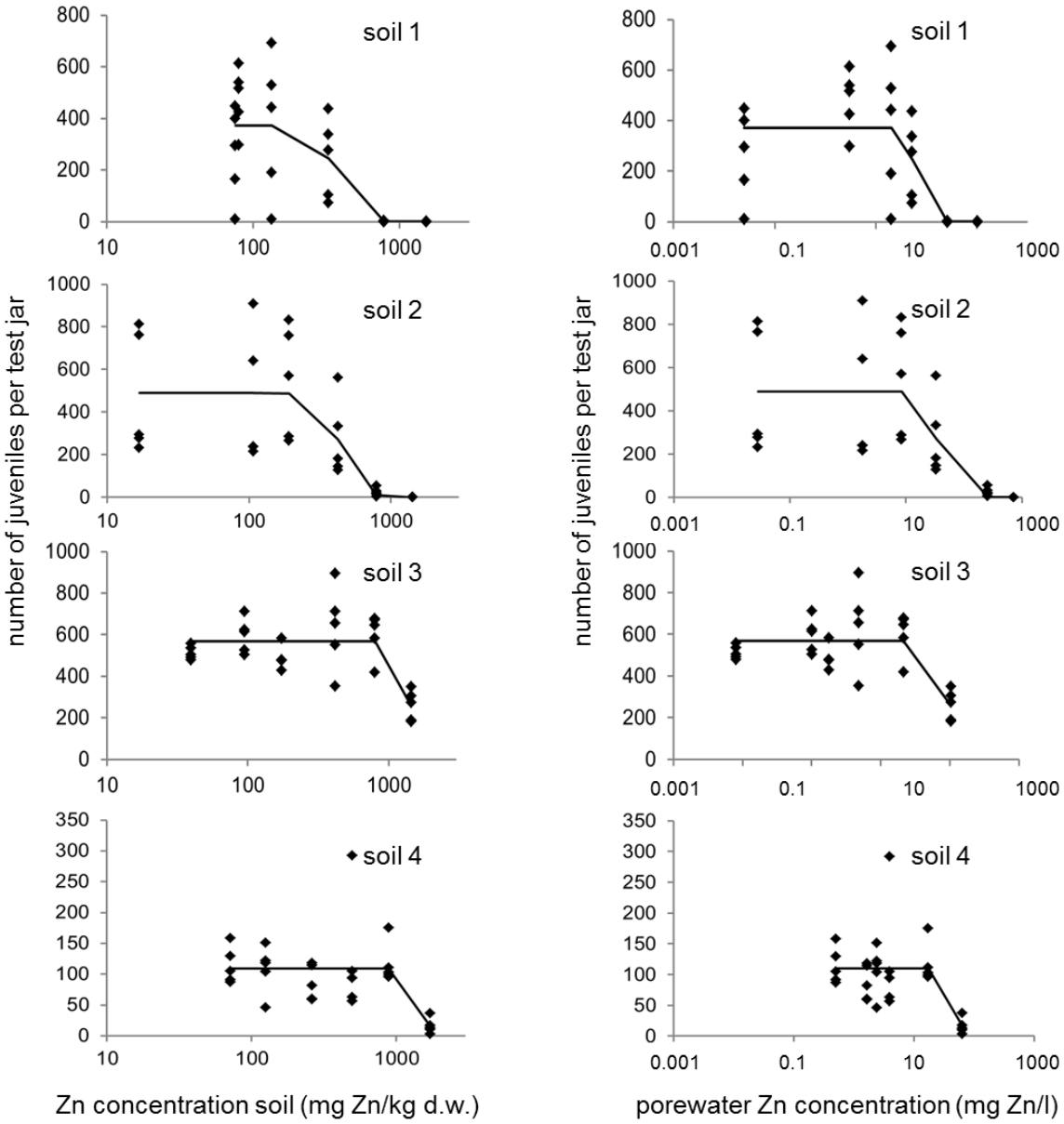

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684

685 **Fig. S2.** Effect of $ZnCl_2$ on the reproduction (number of juveniles) of *Folsomia candida* after
686 28 days exposure in four different test soils. See Table 1 for soil properties. Measured
687 concentrations of zinc in the soil (left) and the pore water (right) are provided on the x-axis.
688 Dots represent the results of different replicates. Lines show the fit obtained with a logistic
689 model.

690

691 **Table S1.** Average zinc concentrations (n=2) measured in soil 1 (Coimbra soil) spiked with
692 30 nm ZnO and $ZnCl_2$. Zinc concentrations in the soil are corrected for the zinc levels
693 measured in the controls. The amount of added Zn recovered (%) is presented in brackets.

Nominal conc. (mg Zn/kg)	30 nm ZnO	$ZnCl_2$
Control		75.6
100	72.7 (72.7)	79.9 (79.9)
200	199 (99.3)	134 (66.9)
400	405 (101)	328 (82.0)
800	766 (95.8)	785 (98.1)
1600	1721 (108)	1523 (95.2)
3200	3361 (105)	-
6400	6722 (105)	-

694 - test concentrations not included in this study

695

696

697 **Table S2.** Average zinc concentrations (n=2) measured in soil 2 (Lufa 2.2) spiked with 30
698 nm ZnO and $ZnCl_2$. Zinc concentrations in the soil are corrected for the zinc levels measured
699 in the controls. The amount of added Zn recovered (%) is presented in brackets.

Nominal conc. (mg Zn/kg)	30 nm ZnO	$ZnCl_2$
Control		17.1
100	93.6 (93.6)	107 (107)
200	202 (101)	192 (95.8)
400	507 (127)	424 (106)
800	830 (104)	788 (98.5)
1600	1661 (104)	1420 (88.8)
3200	3164 (98.9)	-
6400	6925 (108)	-

700 - test concentrations not included in this study

701

Table S3. Average zinc concentrations (n=2) measured in soil 3 (Dutch grassland) spiked with 30 nm ZnO and $ZnCl_2$. Zinc concentrations in the soil are corrected for the zinc levels measured in the controls. The amount of added Zn recovered (%) is presented in brackets.

Nominal conc. (mg Zn/kg)	30 nm ZnO	$ZnCl_2$
Control	39.4	
100	86.0 (86.0)	94.7 (94.7)
200	189 (94.5)	173 (86.7)
400	392 (98.1)	415 (104)
800	820 (102)	799 (99.9)
1600	1644 (103)	1441 (90.1)
3200	3356 (105)	-
6400	6174 (96.5)	-

- test concentrations not included in this study

Table S4. Average zinc concentrations (n=2) measured in soil 4 (North Wales soil) spiked with 30 nm ZnO and $ZnCl_2$. Zinc concentrations in the soil are corrected for the zinc levels measured in the controls. The amount of added Zn recovered (%) is presented in brackets.

Nominal conc. (mg Zn/kg)	30 nm ZnO	$ZnCl_2$
Control	71.5	
100	113 (113)	125 (125)
200	257 (128)	261 (130)
400	455 (114)	497 (124)
800	948 (118)	884 (111)
1600	1365 (85.3)	1705 (107)
3200	3612 (113)	-
6400	7997 (125)	-

- test concentrations not included in this study

Table S5. pH_{cacl2} of the four test soils spiked with ZnO-NP. Soil pH was measured approx. three weeks after spiking the soils, which is halfway the toxicity test with *Folsomia candida*

Nominal conc. (mg Zn/kg)	Coimbra (soil 1)	Lufa 2.2 (soil 2)	Dutch grassland (soil 3)	North Wales (soil 4)
Control	5.9	5.7	6.8	5.0
100	6.0	5.7	6.8	5.0
200	6.1	5.8	6.8	5.1
400	6.3	6.0	6.9	5.1
800	6.4	6.2	6.9	5.2
1600	6.7	6.5	7.0	5.7
3200	7.0	6.7	7.1	6.1
6400	7.2	7.3	7.3	6.6

Table S6. pH_{cacl2} of the four test soils spiked with $ZnCl_2$. Soil pH was measured approx. three weeks after spiking the soils, which is halfway the toxicity test with *Folsomia candida*

Nominal conc. (mg Zn/kg)	Coimbra (soil 1)	Lufa 2.2 (soil 2)	Dutch grassland (soil 3)	North Wales (soil 4)
Control	5.9	5.7	6.8	5.0
100	6.0	5.8	6.9	5.0
200	5.8	5.7	6.9	5.0
400	5.8	5.6	6.8	5.0
800	5.7	5.5	6.8	4.9
1600	5.6	5.3	6.7	4.9

Table S7. Zinc concentrations measured in the pore water (mg Zn/L) of the four test soils spiked with ZnO-NP. The pH of the pore water is also reported (pH_{pw}). Pore water was collected three days after saturation of the soils with Milli-Q water and approx. two weeks after spiking the soils

Nominal conc. (mg Zn/kg)	Coimbra (soil 1)		Lufa 2.2 (soil 2)		Dutch grassland (soil 3)		North Wales (soil 4)	
	mg Zn/L	pH_{pw}	mg Zn/L	pH_{pw}	mg Zn/L	pH_{pw}	mg Zn/L	pH_{pw}
Control	0.025	6.3	0.028	7.1	0.008	7.5	0.501	5.9
100	1.24	6.2	0.466	6.9	0.082	7.6	1.17	5.9
200	2.00	7.0	0.875	6.9	0.178	7.7	1.90	6.0
400	2.35	6.9	1.56	7.0	0.426	7.6	2.96	6.0
800	3.09	7.0	2.14	7.2	0.811	7.6	4.76	6.2
1600	2.46	7.6	2.61	7.4	1.78	7.6	6.74	6.7
3200	1.94	7.7	2.24	7.9	2.66	7.8	9.70	7.3
6400	1.88	7.7	2.83	7.9	3.52	7.9	23.8	7.0

Table S8. Zinc concentrations measured in the pore water (mg Zn/L) of the four test soils spiked with $ZnCl_2$. The pH of the pore water is also reported (pH_{pw}). Pore water was collected three days after saturation of the soils with Milli-Q water and approx. two weeks after spiking the soils

Nominal conc. (mg Zn/kg)	Coimbra (soil 1)		Lufa 2.2 (soil 2)		Dutch grassland (soil 3)		North Wales (soil 4)	
	mg Zn/L	pH_{pw}	mg Zn/L	pH_{pw}	mg Zn/L	pH_{pw}	mg Zn/L	pH_{pw}
Control	0.025	6.3	0.028	7.1	0.008	7.5	0.501	5.9
100	3.08	6.2	1.81	6.8	0.101	7.6	2.40	5.2
200	20.4	6.0	8.40	6.5	0.179	7.5	1.63	5.5
400	52.6	5.7	33.3	6.2	0.477	7.5	3.88	5.3
800	260	5.4	259	5.7	2.14	7.2	17.2	5.0
1600	1021	4.8	720	5.0	10.4	7.0	63.7	4.7

Table S9. Predicted free Zn^{2+} concentrations in the pore water (µM) of the four test soils spiked with ZnO-NP using WHAM7. Calculations were based on total Zn concentrations (mg Zn/kg d.w.), pH_{CaCl2}, porewater Zn concentrations (mg Zn/L), pH_{pw}, cation exchange capacity (mval/100g), organic matter content (%), water holding capacity (mL/100g), dissolved organic carbon (mg/L), colloidal fulvic acid (mg/L) and calcium concentrations in the pore water (mg/L). Percentage free Zn^{2+} concentrations of the dissolved Zn fraction measured in the pore water are presented in between brackets.

Nominal conc. (mg Zn/kg)	Coimbra (soil 1)	Lufa 2.2 (soil 2)	Dutch grassland (soil 3)	North Wales (soil 4)
Control	0.076 (19.8)	0.032 (7.48)	0.003 (2.69)	0.047 (0.610)
100	5.76 (30.3)	0.822 (11.5)	0.032 (2.54)	0.108 (0.595)
200	6.37 (20.7)	1.78 (13.2)	0.068 (2.48)	0.133 (0.454)
400	8.12 (22.5)	3.58 (15.0)	0.210 (3.21)	0.150 (0.329)
800	11.2 (23.6)	4.96 (15.0)	0.453 (3.63)	0.120 (0.163)
1600	6.58 (17.4)	6.18 (15.4)	1.21 (4.39)	0.016 (0.016)
3200	4.53 (15.1)	3.84 (11.1)	1.93 (4.72)	0.0001 (0.0001)
6400	4.38 (15.2)	5.48 (12.6)	2.70 (4.99)	0.018 (0.005)

Table S10. Predicted free Zn^{2+} concentrations in the pore water (µM) of the four test soils spiked with $ZnCl_2$ using WHAM7. Calculations were based on total Zn concentrations (mg Zn/kg d.w.), pH_{CaCl2}, porewater Zn concentrations (mg Zn/L), pH_{pw}, cation exchange capacity (mval/100g), organic matter content (%), water holding capacity (mL/100g), dissolved organic carbon (mg/L), colloidal fulvic acid (mg/L), chloride and calcium concentrations in the pore water (mg/L).

Nominal conc. (mg Zn/kg)	Coimbra (soil 1)	Lufa 2.2 (soil 2)	Dutch grassland (soil 3)	North Wales (soil 4)
Control	0.076 (19.8)	0.032 (7.48)	0.003 (2.69)	0.047 (0.610)
100	15.2 (32.2)	4.70 (16.9)	0.039 (2.52)	1.51 (4.10)
200	143 (45.5)	41.4 (32.0)	0.076 (2.77)	0.547 (2.18)
400	383 (47.3)	224 (43.7)	0.229 (3.12)	2.06 (3.46)
800	1735 (43.3)	1822 (45.6)	1.63 (4.96)	25.0 (9.45)
1600	5501 (35.0)	4189 (37.8)	15.0 (9.43)	176 (18.0)