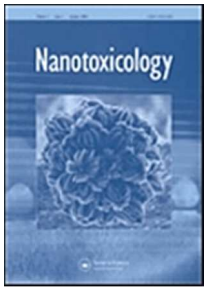

Soil pH effects on the comparative toxicity of dissolved zinc, non-nano and nano ZnO to the earthworm Eisenia fetida

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Soil pH effects on the comparative toxicity of dissolved zinc, non-nano and nano ZnO to the earthworm *Eisenia fetida*

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Abstract

To determine how soil properties influence nanoparticle (NP) fate, bioavailability and toxicity, this study compared the toxicity of nano zinc oxide (ZnO NPs), non-nano ZnO and ionic $ZnCl_2$ to the earthworm *Eisenia fetida* in a natural soil at three pH levels. NP characterisation indicated that reaction with the soil media greatly control ZnO properties. Three main conclusions were drawn. Firstly that Zn toxicity, especially for reproduction, was influenced by pH for all Zn forms. This can be linked to the influence of pH on Zn dissolution. Secondly that ZnO fate, toxicity and bioaccumulation were similar (including relationships with pH) for both ZnO forms, indicating the absence of NP specific effects. Finally earthworm Zn concentrations were higher in worms exposed to ZnO compared to $ZnCl_2$, despite the greater toxicity of the ionic form. This observation suggests the importance of considering the relationship between uptake and toxicity in nanotoxicology studies.

Keywords

Zinc oxide, nanoparticle, nanotoxicology, soil pH, earthworms

Introduction

The field of nanotechnology has received increasing attention in the last decade resulting in a range of new innovations. This development is driven by the potential for industries to improve goods and processes by exploiting the unique properties of materials when fabricated at the nanoscale (Christian, 2009). The potential of engineered NPs is in great part due to their increased surface area and, thereby, their high surface energy and reactivity. These properties can set them apart from their bulk (i.e non nanoscale) counterparts.The fact that NPs can be produced in a highly specific manner to fit a certain purpose is very attractive to industry and underpins their applications and growth potential (Christian, 2009). In 2004 the estimated production volume of NPs was only 2000 tons per year. This production is, however, predicted to increase to more than 50,000 tons per year by 2020 (PROSPECT, 2009).

As a direct consequence of their current commercial aplications, e.g. in cosmetics, antimicrobial applications and industrial processing, manufactured NPs are increasingly being released into waste water systems. In many countries (including the UK), sewage sludge is routinely spread to agricultural land as fertilizer. This application creates a direct exposure route for NPs to terrestrial ecosystems (Benn & Westerhoff, 2008; Johnson et al., 2011). Very few analytical techniques for measuring NPs in natural systems are available. This results in a lack of data on their occurrence in the environment (Nowack & Buchelli, 2007). However, modelling studies have suggested that concentrations of NPs reaching soil could result in concentrations ranging from the low nanogram (fullerenes) to approximately 100 µg/kg (nano TiO_2) concentrations depending on the assumptions made concerning market development and post use release fate (see Gottschalk et al. 2009, Johnson et al. 2011).

Once in the environment the exposure to, and toxicological effects of, NPs are intimately associated with the processes that decide their environmental fate and behaviour. It is widely acknowledged that the composition, size, shape, and surface energy of NPs are key properties that determine the surface and speciation chemistry, stability and aggregation behaviour. Under certain conditions some core materials may react resulting in the transformation of the surface layer (e.g. sulpherisation of silver under reducing conditions (Choi et al., 2009) or alternatively surface entropy may favour the dissolution of the particle leading to the production of free metal ions (Puzyn et al., 2011). For particles that remain intact, it is known from classic Derjaguin-Landau and Verwey-Overbeek (DLVO) theory that the stability of a colloidal dispersion is determined by the combined forces of the attractive Van der Waals forces and the repulsive energy of the surrounding electric double layer (Shaw, 1970). Additionally non-DLVO forces such as steric stabilization are known to affect dispersion stability (Baalousha et al., 2009). These properties themselves are affected by abiotic factors in the environment such as pH and the presence of organic matter, which can change electrostatic conditions in the media (e.g. ionic strength or pH) or exert steric stabilization influencing fate and behaviour of NPs and as a result organism exposure (Christian, 2009; Bian et al., 2011; Baalousha et al., 2008; Handy et al., 2008).

Among the current range of NPs in production, ZnO NPs are already among the most widely used in cosmetics products and in some countries (not currently the EU) in sunscreens. ZnO NPs have been found to be some of the most harmful in aquatic exposures (Kahru & Dubourguier, 2010) and have also been found to be toxic to soil living organisms (Kool et al. 2011, Dimkpa et al. 2011). Studies to date on the toxic effects of NPs towards earthworms have generally found that NPs are less toxic than equivalent ionic exposure (Unrine et al., 2010a; Coleman et al., 2010; Hooper et al., 2011). However, to date these studies have largely been conducted in standardised test media.

From studies conducted with trace metals, it is widely acknowledged that soil properties, such as soil pH, can modify the bioavailability and toxicity of metals in soils systems. Soil pH is the most

important soil property affecting Zn partitioning between the solid phase and the pore water. For NPs, the situation may be even more complex because not only will soil properties affect the speciation and availability of desorbed ions, but soil solution properties such as pH, ionic strength and concentrations of humic substances will affect NP behaviour in a manner dependent on the intrinsic properties of the particle. For example, humic substances have been found to coat NP surfaces exerting stabilizing and destabilizing effects depending on the thickness and nature of the coating and media pH (Baalousha et al., 2008; Bian et al., 2011). Similarly pH has also been found to influences NP properties. Thus, decreasing pH has been found in water suspensions with ZnO nanoparticles (Dimkpa et al. 2011). To date, however, the relevance of such process to soil have yet to be investigated, identifying this topic as relevant to understanding the long-term behaviour of NP in the terrestrial compartment.

To understand how media properties influence Zn and ZnO behaviour, bioavailability and toxicity, comparative effects of nano, non-nano, and dissolved zinc need to be investigated in detail. Currently there is an absence of studies performed in natural soil and at different pH levels. To address this knowledge gap, this study investigates how soil pH affect the fate, behaviour and toxicity of three forms of zinc: 1) ionic zinc dosed as the chloride salt, 2) a 30 nm ZnO NP and 3) a larger non-nanoscale ZnO material. The following hypotheses are addressed within the study: First that Zn ions taken up from soil solution by the worms are primarily responsible for observed toxicity independent of exposure form . If this is the case, it can be expected that the toxicity of the ionic Zn and nano and non-nano ZnO will be greater at lower pH since Zn sorption to the soil and ZnO solubility will be negatively correlated with pH. It would also be expected that toxicity will be independent of Zn-form and pH when expressed according to dissolved Zn ion pore water concentrations. Further that ZnO will be more toxic than non nano scale ZnO when expressed on a Zn mass basis, since NPs have a larger surface area for ion desorbtion and greater surface reactivity than the bulk Zn. Finally that Zn accumulation into earthworm tissues is related to the soluble metal

concentration and if compared on that basis will be independent of Zn form. Furthermore, in worms with similar total Zn burden, similar toxic effects would be anticipated.

Materials and methods

Soil

The test soil was collected from an open heathland site in Wareham forrest (Ordnance Survey Grid Reference: SU108058, Dorset , United Kingdom). The vegetation on site was dominated by heather (*Erica sp.*) with small trees. This soil was chosen for sampling because of its acidity (around pH 4) which made it ideal for constituting a low pH treatment, while higher pH treatments could be obtained by soil ammendment to raise pH. During sampling, large roots were removed, and soil was collected from 0-30 cm depth. The soil was homogenised, sieved through a 5 mm mesh and air dried (initial moisture content was approximately 14% w/w).

From samples of the sieved dried material, texture (hydrometer method), pH (determined in water and 0.01M $CaCl_2$), conductivity, total carbon and nitrogen (Dry combustion and Kjeldahl method respectively), cation exchange capacity (Kjeldahl method), along with oxalate and citrate-bicarbonate-dithionite (CBD) extractable iron and aluminium was determined according to standard operating procedures set out in Møberg et al. (1994) and Borggaard et al. (2003). A summary of the soil properties can be found in Table S1 . Soil water holding capacity (WHC) was determined using a volumetric standard method (Rothamsted Research, 2011).

Soil pH adjustment

The exposures were conducted at a range of pH values encountered in natural soil solutions as selected based on the result of a UK national soil survey (Emmett et al., 2010). The sieved homogenised soil was divided into three batches of approximately 50 kg dry weight. To adjust the

pH of the soil to three levels 0.2%, 0.45%, and 1% w/w calcium carbonate ($CaCO_3$, Sigma Aldrich) was added to the soil (Table S2). These pH amendments gave values of 4.8, 5.9, and 7.2 (range $\pm$ 0.3 pH units for 3 replicate samples in all cases) as measured in a 1:3 slurry of test soil made in distilled water. These are hereafter refered to as the low, medium and high pH soils, respectively. For each replicate, 450 g dry weight of the respective pH soil was added to the test container (1 lt glass Kilner jars).

Chemicals

The ZnO based nanomaterial selected for this experiment was NanoSun zinc oxide P99/30 obtained from Microniser Pth Ltd (Dandenong, Australia) with a nominal average particle size of 30nm. The NanoSun P99/30 ZnO has no coatings or surface modifications and is a white odourless dry powder of particles with a close to spherical shape. The stated/nominal water solubility is 0.0016 g/l at 20ºC, the melting point 1975°C and the purity 99.5%. In addition to the NanoSun ZnO NP, two further Zn forms were studied. These were dissolved Zn^{2+} from $ZnCl_2$ (BHDChemicals, Poole, UK) and a non-nano scale ZnO material also obtained from Microniser Pth Ltd. This non-nano scale ZnO powder had an average nominal primary particle size of 200 nm, but was otherwise identical to the 30 nm material.

We verified the primary particle size reported by the manufacturer by TEM (JEOL 2010 analytical TEM operating at 200kV). Results of particle size determination with different methods are collected in table S3. The hydrodynamic diameter of the particles in the stock suspension and the zeta potential were determined by Dynamic Light Scattering (DLS) and Laser Doppler Electrophoresis (LDE) respectively using a Malvern Zetasizer Nano ZS. Density measurements were carried out with a helium pycnometer (Micromeritics AccuPyc, model 1340). Specific surface area (SSA) of powders was measured by Brunauer-Emmett-Teller (BET) method (Micromeritics AccuPyc, model Gemini 2360). Having the specific surface area, and assuming that all particles were spherical and identical, the average diameter of the particles was calculated. X-ray diffraction (XRD) with a Philips X'pert Pro

Diffractometer (PANalytical) was performed to confirm the NPs' crystallographic phase as ZnO. The solid powders were placed in sample holders at room temperature and analyzed with Cu Kα radiation at 2Theta angles from 10 to 100 with 0,03º step. Particle size was determined by Scherrer equation. Stock solutions of all materials were prepared in equal Zn concentrations (nominal concentration 7.5 mg Zn/ml), taking into account the purity of the materials and the composition of the material.

Dosing procedure

Particles were dosed into the soils as a dispersion in a soil suspension made from each of the three pH soils to produce the required test concentrations in the test soil. This method is based on the dosing technique described by van der Ploeh et al. (2011) and adapted by Kool et al. (2011). The method was selected because the particles are introduced into the soil in a way that mimics their state in soil pore water. The soil suspensions were prepared by mixing air dried $CaCO_3$ ammended Dorset soils with MilliQ water in a 1:2.5 soil:water ratio (w/v). The slurries were then shaken at 200 rpm at room temperature for 1 hour and then filtered through Whatmann No. 1 paper. The different Zn forms were then dispersed in the soil suspension to produce a highly concentrated stock dispersion/solution. The dispersions of nano ZnO and non-nano ZnO in the soil solution were then prepared for soil dosing following an established protocol (PROSPECT, 2010). Zn spiked suspensions were sonicated for 30 seconds in a sonication bath and then continously stirred before application to soils. Aliquots of unspiked and spiked soil suspensions were taken for immediate characterization and measurement of pH, zeta potential and hydrodynamic radius. After dosing the soil, pure MilliQ water was added to all containers to reach appropriate soil moisture content (45% of maximum water holding capacity, WHC) in the test soils; his being within the recommended range of 40-60% WHC recommended by the OECD (2004) test guideline. The soils were then thoroughly mixed to ensure a homogeneous distribution of zinc through the soil. The freshly spiked soils were

equilibrated for seven days before organism exposure. The control containers for all soil pH levels received MilliQ water only.

Experimental animals and toxicity test procedure

Eisenia fetida were initially obtained from Blade's Biological (Kent, UK) and reared in a sheltered outdoor culture consisting of a mix of clean horse manure and composted bark. Immediately prior to the test, adult worms with a well developed clitellum were collected from the cultures and acclimatised to the test temperature (20±1°C) for 24 hours in a loamy soil with manure provided as food. The average weight of individual worms used was 0.36 ± 0.03g (SD, *n* = 2100). The study was conducted using a procedure based on the OECD guideline 222 (OECD, 2004). The study comprised nine parallel similarly designed toxicity tests conducted with ZnO NPs, non nano scale ZnO and $ZnCl_2$, each with a seperate test series for each material performed at each of the low, medium and high soil pH levels. The tests series with zinc chloride and nanoparticulate ZnO each had 6 exposure levels of 238, 381, 610, 976, 1520, and 2500 mg Zn/kg dw soil. For logistic reasons, the non-nano ZnO test was conducted using a reduced number of exposure levels of 381, 976 and 2500 mg Zn/kg dw soil. All treatments (i.e. each material at each tested concentration and soil pH) had four replicates. The overall controls for the experiment comprised ten separate replicates per soil pH level. This gave a total of 210 containers in the full experiment.

For practical reasons the experiment was started over a period of ten days with all jars fully randomised. This meant that a maximum of 21 jars were handled per day at any stage. During the exposure, each replicate was fed 5 g dry weight of horse manure in two batches, with 2.5 g fed at the start and after two weeks of the experiment. The ratio of water and dry manure was 4:1 by weight. The food was placed in a small hole in the middle of the test soil as described by Van Gestel et al. (1989).

For each replicate, 10 worms were collected from the preincubated culture. The worms were washed, blotted dry and weighed as a batch of ten before being put onto the surface of the test soil. The worms were incubated for 28 days at 20 ± 1°C under constant light. Weekly the test containers were opened to aerate the test soils and add de-ionized water to correct for moisture loss. After four weeks the soils were hand sorted and the surviving worms collected and weighed as a batch. The worms were left over night to egest their gut contents on moist filterpaper according to Arnold & Hodson (2007) after which they were frozen at -18°C. Due to batch variation within the worm cultures, some mortality was observed within some test containers at the end of the four week period. This mortality was not treatment (dose and pH) related and occurred only in replicates prepared in days 4-8. Therefore, to avoid inclusion of data from worms that were not in adequate condition during the experiment, it was decided only to exclude results from affected containers to provide data for two replicates per treatment in all cases. For selected replicates, earthworm reproduction, expressed as number of cocoons/worm/week, was recorded by wet sieving the soil and retrieving earthworm cocoons. Retrieved cocoons were then inclubated in a 50 g sample of the corresponding contaminated soil and maintained at 20 ± 1°C for four weeks, whereafter the total number juveniles alive in the soil were forced to the surface using a 60°C water bath and counted to allow juvenile production to be assessed as the product of cocoon production and hatching traits.

NP characterisation in soil suspensions

Particle size distribution and zeta potential of unspiked (control) and nano ZnO spiked soil suspensions were assessed using dynamic light scattering (DLS) (Zetasizer, Malvern Insruments Ltd, Malvern, UK). Samples of the nano ZnO dosed suspension were also prepared for transmission electron microscopy analysis by drying 1 drop on a TEM grid for 1 hour followed by examination on a JEOL 2010 analytical TEM equipped with Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS).

Extraction of pore water from spiked soil

A separate soil sample of 200 g dry weight of soil of each pH was spiked with same quanities of the three Zn forms to give concentrations equivalent to the test treatments for all ZnO treatments and for three of the six $ZnCl_2$ treatments (381, 987, and 2500mg/kg). This single soil per treatment was used to enable the extraction of soil pore water to allow an an assessment of the influence of pH and Zn concentartion and form on Zn bioavailability at the beginning (day 0) of the experiment without impact on the replicate number of the main exposure. After an initial aging period following the same procedure as for the test soils, the soils were saturated with Milli Q water and left for 10 days to equilibrate. Subsequently the soil pore water was extracted by centrifugation (Beckman coulter Allegra^{TM} 25R Centrifuge) at 2,482 g for two hours, and the supernatant centrifuged at 18,330 g for a further hour to minimise the number of remaining soil particles. The total organic carbon content of the soil pore water was measured by DC-190 TOC-analyzer (Rosemount Analytical, Solon, USA) calibrated with a 1000 ppm sucrose solution in Milli Q water. Soil pore water was analysed for pH (measured in control soils and soils spiked at 235 and 2500 mg/kg nano ZnO), zeta potential and size distribution (Zetasizer, Malvern Insruments Ltd, UK). For Zn concentrations analysis, the centrifuged soil pore water extracts were acidified by addition of 1µl/ml 1M nitric acid and zinc concentration in the pore water analysed by Atomic Absorption Spectroscopy (AAS) (Perkin Elmer 1100B). After 56 days this procedure was repeated (excluding pH measuring and particle characterization), this time using a pooled sample comprising an equal quantity of soil taken from the test containers, to allow an assessment of the trends of pH and Zn treatment and form on water extractable zinc concentration and organic carbon content at the point at which juvenile production was assessed.

Metal analysis in soils, pore waters and earthworm tissues

Approximately 100 mg oven-dried soil samples from all considered experimental replicates in the test with ZnO materials and for three of the six $ZnCl_2$ treatments (381, 987, and 2500mg/kg) were used for analysis of total Zn concentrations. These samples were digested in 2 ml of a 4:1 mixture of nitric acid (65% p.a., Riedel-de-Haen, Seelze, Germany) and hydrochloric acid (37% p.a., Baker, Grainger, USA) in tightly closed Teflon® bombs and oven heated at 140 °C for 7 hours. These digests were diluted with 8 ml of DI water and total Zn measured by AAS (Perkin Elmer 1100B). A certified reference soil (International Soil-Analytical Exchange, WEPAL River clay, ISE sample 989, containing a certified concentration of 1060 mg Zn/kg dry weight) was included for quality control purposes. The averages of measured Zn concentrations were within 99% of the certified reference value. No zinc was detected in the blank samples.

Tissue zinc concentrations were measured in three of the surviving earthworms for each replicate. For this analysis, worms depurated as described previously were lyophilisated and then digested using the same acid mixture and Teflon® bombs as for soil samples. Following digestion, samples were diluted with 6 ml of distilled water to a total volume of 8 ml, to keep the analysed concentrations within calibration range and analysed for Zn concentration by AAS. Certified reference material (NRC Canada, DOLT-4, fish liver) was included in the analysis. The mean recovery of Zn was 100.1 ± 0.36% from DOLT 4.

Data handling

Metal data

Pore water Zn concentrations in the $ZnCl_2$ treated soil for the measured treatments were used to estimate the remaining concentration for the unmeasured test concentrations using the Freundlich isotherm (Equation 1).

$$C_s = K_f \cdot C_{pw}^{\ n} \qquad (1)$$

Where C_s is the zinc concentration in the soil (mg Zn/kg); K_f is the Freundlich sorption constant (L/kg); C_{pw} is the zinc concentration in the pore water (mg Zn/L), and n is the shape parameter of the Freundlich isotherm.

Toxicity data

Concentration specific effects on the proportion of survivors, weight change as a percentage of initial weight and reproduction as cocoon production and juvenile production rates were analysed for each of the nine separate treatment series (i.e. three Zn types at three pH levels) were analysed using analysis of variance (ANOVA). Where significant differences were found the Tukey test was used to identify the pattern of significant differences between treatments (SPSS version 17). To estimate response parameters, data for survival and reproduction (cocoon and juvenile production rate) was used to fit a three parameter log logistic model (Equation 2) to obtain estimate LC_{50} and/or EC_{50} value.

$$y = \frac{y_{max}}{1 + exp\{b \cdot (log(x) - log(e))\}} \quad (2)$$

Where y_{max} is the upper asymptote, e is the concentration resulting in a 50% effect on the measured endpoint (EC_{50}) and b the slope parameter. For the analysis of survival data, a binominal distribution of data within each treatment is assumed, while for weight change and reproduction, a normal distributions is assumed. Model fits to derive parameters with associated standard errors were completed using the drc package in R version 2.13.1 (R-project, 2011). All concentration response relationships were fitted using either measured total zinc in soil or pore water zinc concentrations.

Bioaccumulation data

Worm tissue Zn concentrations were analyzed using one-way ANOVA followed by Tukey test. Bioaccumulation factors (BAFs) for Zn were calculated by comparing measured tissue concentrations with measured total soil Zn concentrations in the soil. Since BAFs for metals such as Zn can tend to

decrease at higher exposure levels especially when toxicity occurs (McGeer et al., 2003) BAF (and BCF) values were only calculated for spiked treatments resulting in effect concentrations below EC_{50} for reproduction. Controls were also omitted, since bioavailability in the control soil was likely to be lower, and so not fully comparable, with the spiked soils (Hobbelen et al., 2006). Bioconcentration factors (BCFs) were calculated by comparing measured tissue concentrations with pore water Zn concentrations measured in the soil pore water.

Results

Material Characterization

TEM analysis indicated that the particles were spherical and relatively monodisperse in the case of NanoSunP99/30. The non nano scale material P99/200 contained a higher proportion of faceted rod-shaped material . Characteristic images of the particles in distilled water and dosing solution are presented in the Supporting Information (Figure S1). The average primary particle diameters of the ZnO material batches as measured by TEM were 29.8nm ± 9.4 (mean ± standard deviation) for NanoSun P99/30 ZnO and 300nm ± 164 (length) and 188 nm ± 102 (width) for non nano scale ZnO (Waalewijn-Kool et al. 2012). Crystallographic phase purity, crystallite size from XRD, grain size and specific surface area from BET measurements and Zeta potential in DI water, and density measurements are presented in Table S3.

Soil properties

The unadjusted Dorset soil was of sandy texture with a mean composition of 51.5% coarse sand, 40.2% fine sand, 4.7% silt and 3.5% clay (Table S1). The soil had a low organic carbon content of 4% and a total nitrogen content of 0.13%. The pH of the unadjusted soil was 4.2 when measured in H_2O and 3.1 in 0.01M $CaCl_2$. Soil conductivity was 422 µS and the maximum WHC was determined as 766 ml/kg dry weight.

Characterization in soil suspensions for dosing

The pH of the unspiked and nano ZnO dosed soil suspension used for the dosing of the NPs are shown in Table 1. Measurements of the soil suspensions indicated a rise of approximately one pH unit after dosing.

The zeta potential of unspiked soil suspensions is determined by the presence of natural colloids. Measurements indicated values between -10 and -20mV (Table 1). These values indicate an potentially unstable dispersion of natural colloids in the suspension as the measured values did not differ greatly from a neutral charge (Baalousha et al., 2009). Only slight pH effects were observed, with the zeta potential being closest to the point of zero charge at higher pH. Comparing spiked and unspiked soil suspensions, a positive shift in zeta potential towards the point of zero charge in the presence of NPs was observed at all pH levels. This indicates the likelihood of an unstable dispersion, with the particles likely to become agglomerated and settle out of suspension. Hence dosing suspensions were continuously stirred prior to spiking.

Average hydrodynamic sizes in the unspiked soil suspensions was over 1000 nm, reflecting the presence of natural colloids. When ZnO NPs were added to the soil suspension for spiking, average particle size was reduced to 600-700 nm. This analysis suggests the presence of an increased presence of particles and agglomerates smaller than the average hydrodynamic size of the natural colloids in solutions to which the ZnO NPs have been added. Size distribution by intensity in spiked samples showed a high degree of polydispersity in all the samples. Thus, DLS analysis indicated that the spiking solutions are dominated (on a mass basis) by the presence of larger structures within these high concentration dispersions. These may represent at least partly the presence of homo- and/or hetero agglomerates of ZnO NPs with clays and humic substances. However, size distribution also confirmed the presence of NPs around or below 100nm. With regards to effects of soil pH on measured hydrodynamic radius in the soil suspensions, no significant effects were observed despite

a slight tendency towards an increase in the number of smaller particles at higher pHs both in spiked and unspiked suspensions.

Particle imaging by TEM confirmed the DLS measurements showing ZnO NPs present primarily as hetero- and homo-agglomerates associated with organic material and NPs in the stock soil suspensions (Figure S3). Even though many particles were present as large agglomerates, a number of loosely associated small agglomerate and single particles were also present in the suspensions. TEM visual inspection provided no clear evidence of a trend for an effect of pH on the range of agglomerate sizes observed.

Total soil Zn and soil pore water Zn and organic carbon concentrations

Recoveries of total Zn from the spiked soils ranged between 64 and 108% (with an average recovery of 86 % for all Zn forms). This confirms that actual concentrations were in close agreement with nominal values. Hereafter, within this article concentratration levels will be reffered to by their nominal concentration, although effect concentrations are in all cases calculated based on measured Zn concentrations.

The highest Zn pore water concentrations were measured in the $ZnCl_2$ treated soils (Figure 1). These were in the range of 20 to 50-fold higher than the pore water concentrations of ZnO-spiked soils at the same nominated concentration. For all Zn forms, measured concentrations in the pore water increased with increasing nominal concentrations, ranging between 0.4 and 14.8 mg/l for nano ZnO, between 0.7 and 14.4 mg/l for non nano ZnO and between 3.2 and 809 mg/l for $ZnCl_2$ (Figure 1). Higher Zn pore water concentrations were found in the low pH soil for all Zn forms. After 56 days, zinc concentrations in the soil pore water were between 55-89% higher than initial measured values in the nano and non nano ZnO dosed soils especially at the high concentrations. This suggests a release of soluble Zn from the ZnO materials over the exposure period. The extracted soil pore water

collected at Day 0 and Day 56 was analysed for total organic carbon content. At the later time point, pore water carbon concentrations were consistently lower than at the start of the experiment for samples taken from all treatment and pH levels (Figure S3). This may reflect a reduction in organic carbon solubility associated with factor such as the lower pH resulting as Zn^{2+} ions are liberated from dissolving ZnO particles.

Characterization of NP fate in soil pore water

To investigate the fate of the NPs in the soil and in particular on the supply of zinc ions to the labile pool and soil solution, soil pore water extracted from the NP spiked soils at a low (235 mg Zn/kg) and a high (2500 mg Zn/kg) Zn concentration were analysed using DLS (Table 2). A pH analysis of the soil pore water indicated that NP addition increased the pH in the low and medium pH treatments, but caused a small decrease at high pH. These changes may be related to NP surface reactions consuming or liberating protons from the soil solution. Measurements at different pH values indicated that in soil solution there was a predominant negative NP surface charge which was largely independent of pH. Size measurements indicated the presence of small ZnO agglomerates and/or a better dispersion of colloids in the soil solutions as compared to the highly concentrated spiking solutions.

Toxicity to *E. fetida*

Survival and weight change

Survival of *Eisenia fetida* was reduced in soils spiked with the highest concentrations of $ZnCl_2$ at all pH levels. In contrast, effects on survival were less evident when worms were exposed to either nano or non-nano scale ZnO under any of the three soil pH regimes (Figure S4). For the ionic Zn exposures, a concentration response relationship was clearly evident at each soil pH and LC_{50} values of 718, 590 and 1983 mg Zn/kg were estimated for $ZnCl_2$ at the low, medium and high pH soils

respectively (see Table 3). Non-nano ZnO had an effect on earthworm survival in the low and medium soil pHs. This effect could be described by a logistic model for the medium pH soil, although survival was not reduced below 50%. At low pH a lower survival was found at 910 mg/kg, however, this was not dose related. For earthworms exposed to ZnO NPs survival was only reduced compared to control in the high pH soil at 610 mg/kg. This effect was not dose dependant since higher survival at higher ZnO NP concentrations was found. Weight change data generally confirmed the trends seen within the survival data. Thus, a dose related effects on weight loss was only observed in the $ZnCl_2$ treated soils, but not in the ZnO spiked soils. Consequently since comparison between Zn forms is not feasible, these data were not dealt with any further.

Reproduction

Cocoon production was reduced in *Eisenia fetida* in a dose dependent manner by all three zinc forms and in each case for all of the soil pHs tested (Figure 2). This shows that reproduction is a more sensitive endpoint than survival for earthworm exposed to ZnO, since effects on survival were not seen for these materials. Juvenile production data confirmed trends seen for cocoon production, hence these responses can be seen as effectively equivalent suggesting no Zn specific effects on cocoon viability.

Based on the cocoon production data (n.b. calculations using juvenile data would produce similar results), EC_{50} values for all Zn forms and soil pH values could be calculated from both measured total soil and soil pore water Zn concentrations (Table 4). When calculated using total soil Zn, a clear difference between estimated EC_{50} values for the ionic and the two particulate ZnO forms was evident. Ionic metal was approximately 2.5 fold more toxic than the particulate material. The comparison between the nano and non-nano ZnO indicated that these two forms gave comparable toxicity values. This suggests there is no strong particle size specific effect on ZnO toxicity within this test system. The influence of pH on toxicity was broadly consistent across all three Zn forms. The

EC_{50} value based on total Zn concentrations in the high pH soil being higher than those in the medium and low pH soil. The trend was particularly evident in the ionic and ZnO NP tests (Table 4).

When cocoon production effect concentrations were calculated based on soil pore water Zn concentrations, EC_{50} values showed different trends than values estimated from total soil Zn levels. Thus, pore water EC_{50} values were up to three-fold lower for both ZnO compounds compared than values in the $ZnCl_2$ test (Table 4). Again the toxicity of both ZnO forms are broadly comparable providing no indication of size specific toxicity. A clear effect of pH on Zn toxicity for all three Zn forms was also observed for the pore water Zn calculated values. The EC_{50} values found in the low pH soils generally being higher than those in the medium and high pH soils; the only exception being nano ZnO in the high pH soil (Table 4).

Zinc bioaccumulation and bioconcentration

Figure 3 displays the average internal Zn concentrations measured in *Eisenia fetida* after the 28 day exposure. Internal Zn concentrations measured in the control earthworms ranged between 100-200 µg/g dry weight and were on average 132±13, 123±2 and 124±0.4 µg/g dry weight for the low, medium and high pH soil respectively. This is in good agreement with previous values (Lock & Janssen, 2001). Tissue Zn concentrations were significantly dependent on Zn form (ANOVA, $p<0.05$). Post-hoc analysis indicated that earthworms exposed to nano and non-nano ZnO had significantly higher tissue Zn concentrations than the $ZnCl_2$ exposed worms (Tukey, $p<0.05$). Indeed the highest average internal concentrations were observed for worms exposed to non nano ZnO in the low pH soil spiked at 2500 mg/kg d.w.

In all treatments except the low pH ionic Zn test, a significant effects of exposure concentration on internal Zn concentration was found (ANOVA , $p<0.05$). Post-hoc testing indicated a number of exposure concentrations were significantly different from controls predominantly at the higher test

concentrations (see Figure 3). Based on the total zinc concentrations measured in the soil or pore water and the internal zinc concentrations, bioaccumulation factors using total Zn and bioconcentration factors using pore water Zn were calculated (Table 5). BAFs estimated for control worms were on average 33.2 ± 7.05, 28.8 ± 0.87 and 24.1 ± 1.09 for the low, medium and high pH soil, respectively. All BAFs for spiked soils (estimated as explained in material and methods) were below one, suggesting that as an essential metal, Zn can be excluded or regulated by earthworms. Analysis of the calculated bioaccumulation factors indicated a significant effect of pH, with BAFs significantly lower in the high pH soil when compared to the low and medium pH treatements (one way ANOVA, $p<0.05$). No significant effect of Zn type on BAF was, however, evident. BCFs were also found to be significantly dependent on soil pH (one way ANOVA, $p<0.05$). Highest BCFs were found at the high soil pH level. Differences in BCFs for the different Zn compounds were aparent across the experiment with the BCF being lower for $ZnCl_2$ than the ZnO forms which in turn were comparable. Within each soil pH, this trend was significant only in the low pH soil. This suggests that Zn uptake is not solely related to soluble Zn concentration in pore water, since if this was the case, an effect of Zn form would not be expected in any soil.

Discussion

This study is, to our knowledge, the first assessment of comparative toxic effects of dissolved Zn^{2+} ($ZnCl_2$), NP ZnO and nonnano ZnO to earthworms in a natural soil and also one of the few studies to explicitly incorporate the influence of soil pH. The toxicity tests were combined with particle characterization techniques and supporting soil and tissue metal analysis to comprehensively assess the exposure scenario, fate, and availability of the ionic Zn and ZnO form in the soil at three different soil pHs tested.

Characterizarion of NPs in soil suspension and pore water

NPs were characterized in the spiked soil suspensions used for dosing to assess the exposure scenario and determine the effect of the different pHs. In the unspiked solution, soil pH affected the natural colloidal particles that were present within the extracted soil suspensions. Average size changed from 2000 mm to 1500mm and zeta potential from -16 to -12 between the pH 5.1 and 8 soils. In the ZnO spiked solutions zeta potential was near neutral regardless of pH (Table 1). At the observed pH range (Table 1), ZnO would be expected to have neutral ranging to negative zeta potential (Geert Conelis, personal communication) The absence of pH specifiic effects on zeta potential did not correlate with expections derived from DLVO theory (Shaw, 1970). Natural organic matter tends to coat the surface of environmental and manufactured NPs (Baalousha et al., 2009; Fabrega et al., 2009). When particles are coated with organic matter, charge neutralization can occur (Ghosh et al. 2009). Indeed Bian et al. (2011) tested the role of humic acids on the aggregation of ZnO NPs and reported that lower humic acid concentrations (1.7 mg/L) resulted in a higher sedimentation rate compared to suspensions without humic acid and attributed this effect to charge neutralization by adsorption of humic acids. Since the present study were conducted across a similar pH range (6.2 – 8, as compared to the circumneutral pH used from Bian et al. (2011)), such charge neutralisation can be expected to have occurred in the present study given that natural organic matter is present in the soil suspension. Indeed TEM images confirm that the ZnO NPs are associated with organic matter in the soil suspension (Figure S2), as also reported previously (Kool et al., 2011).

To provide exposure characterisation and assess the fate of the NPs in the soil after spiking, particle characterization was performed in pore water extracted from the NP spiked soils (Table 2). Measurement of particle size distributions and zeta potentials (of natural NPs and possibly ZnO NPs) were in agreement and accorded to DLVO theory and a dependence on pH. Further dissolved zinc in the soil pore water can affect the surface charge of natural colloids by forming organo-metallic complexes (Tipping et al., 2003). This is a further considerations that might account for the changes

in zeta potential and particles sizes observed in the the extracted pore water. The DLS measurements made, at exposure start indicated small ZnO agglomerates, however it could not be confirmed to what extent well disperse ZnO NPs were present in the extracted pore water. The total amount of zinc measured, demonstrated that the dosing did provide good uniform pH and Zn form dependent gradients of Zn in the pore water.

Fate and availability of zinc

To assess zinc and ZnO fate and availabillity under the three pH regimes, total zinc and dissolved carbon was measured in soil pore water extracts. Pore water carbon concentrations were found to decrease over the 56 day course of the experiment (Figure S3). Kalbitz et al. (2000) reported a complex relationship between DOC and soil pH, however, it was concluded that generally DOC solubility is reduced at lower pHs. Analysis of the soil pH in the present study indicated a general drop in soil DOC over the 56 days exposure, across both soil pH and Zn dose. For example, at the top concentrations (2500 mg/kg), pH was reduced by between 0.5 and 1.2 pH units, to 5.4 and 5.9, in the medium and high pH soils respectively (related possibly to $CaCO_3$ speciation and/or earthworm activity). This general pH shift could explain the decreasing concentration of total carbon observed in the extracted soil pore water between the start of the exposure and day 56.

The amount of total zinc in pore water extracted from the soil, was negatively correlated to soil pH, with the low pH soils (pH 4.5) having the highest pore water Zn concentrations. Although the trends seen here were based on analysis of a pooled soil samples for each treatment, similar trend have been observed in relation to the effects of pH on ZnO toxicity for springtails (P. Kools. et al submited) supporting the validity of our analysis. Further, a similar correlation was also found by Tipping et al. (2003) who found higher soil solution concentrations in lower pH field soils when compared to more neutral systems providing support for our observation. For ZnO, a higher Zn concentration in solution can generally be expected below pH 6 compared to pH 6-9 as a result of both increased ZnO

dissolution and pH influences on Zn solubility (Yamabi & Imai, 2002). At low pH, soluble ionic forms will include Zn^{2+} and $Zn(OH)^{+}$. However, between pH 6-9, it is expected that solid $Zn(OH)_2$ will precipitate from solution (Yamabi & Imai, 2002). This speciation behaviour can explain the trends observed in this study where higher soluble Zn concentrations were found particularly in the low pH soil. Bian et al. (2011) observed the lowest dissolution of ZnO NPs within the pH 6-9 range. A finding similar to that observed in the present study for which lowest pore water concentrations were observed in the 7.2 pH soil (Fig. 1).

In this study there was no obvious difference, in terms of dissolution and resulting soluble Zn concentrations between the ZnO NPs and non-nano scale ZnO. The same trend was found by Milani et al. (2010). Over the exposure time, zinc concentrations in the extracted pore water from the nano and non nano ZnO treated soils decreased slightly over time in both the low and high pH soils, but conversely increased at medium pH. Reductions in soluble Zn at low pH can be attributed to the well known processes of aging which in turn act to increase the non-labile fraction. The increased solubility in the medium pH soil may be related to the decrease in the pH from 5.9 to 5.4 of this soil. This pH shift is unfavourable for the production of $Zn(OH)_2$ precipitate forms, with possible consequences for soluble Zn concentrations.

Toxicity to *E. fetida*

Survival and reproduction of the control worms among included replicates generally meets the validity criteria of the OECD standard test protocol (OECD, 2004). The survival in controls overall was 98%, reflecting the loss of only two worms in a single low pH control replicate. The average number of cocoons in the controls for the low, medium, and high pHs were 28.7±3.5, 37.3±6.7 and 36±7.5 cocoons/container, respectively. These values are close to or exceed the validity criteria of ≥30 juveniles per control as defined by the standard test guidelines (OECD, 2004). The cocoon production rates are also in agreement with results from other studies using the same earthworm

species (e.g. Spurgeon et al., 2000; Spurgeon & Hopkins, 1996a). Across the soils, cocoon production was lowest in the low pH soil. This suggests that pH itself acts as a mild stressor in the test. This effect was anticipated since the lowest pH used (4.8) is close to the limit of the normal biological pH range for *E. fetida* (Edwards & Bohlen, 1996). The presence of a direct pH effect on reproduction is also consistent with results of previous studies of pH effects on earthworm reproduction and Zn toxicity (e.g. Spurgeon & Hopkins, 1996a).

As anticipated, $ZnCl_2$ had a clear negative impact on the survival and reproduction of *E. fetida* exposed to concentrations comparable to those known to be toxic for earthworms (e.g. Spurgeon and Hopkin, 1996a; Spurgeon et al., 1994; van Gestel et al., 1993). For example, Spurgeon et al. (1994) found an LC_{50} value of 1010 mg/kg for 14 days zinc exposure of *E. fetida*. While in the present study, LC_{50} values for $ZnCl_2$ based on total soil Zn increased from 700 to 2000 mg Zn/kg with increasing pH (Table 3). For both ZnO form, a lower toxicity was found when compared to ionic Zn with LC_{50} values being above approximately 2000 mg/kg for all soil pHs. These results indicate a lower lethality from NP and non nano ZnO compared to $ZnCl_2$ when evaluated based on total Zn concentrations. The same trend has also been found in other studies on metal based NPs (Hooper et al., 2011; Unrine et al., 2010a).

Reproduction rate was the most sensitive of the life-cycle endpoints studied, being significantly affected by all Zn forms in all soils tested (Table 4). Based on total soil Zn concentrations, highest toxicity was observed in the $ZnCl_2$ treatment at low pH (EC_{50}: 420 mg Zn/kg). This compares compared to a Zn EC_{50} value of 919 mg /kg for ZnO NPs and 985 mg/kg for non-nano ZnO in the same soil. As for effects on survival, the lower reproductive toxicity of NP and non-nano scale materials when compared to the ionic form is in agreement with previous results (Hooper et al., 2011; Unrine et al., 2010b). For example, Hooper et al. (2011) found that *Eisenia veneta*

reproduction after 21 days exposure to 750 mg/kg of ZnO NPs (<-100nm) was reduced by 50%, while the matching the total Zn concentration of $ZnCl_2$ completely inhibited cocoon production.

Across all Zn forms, observed toxic effects were found to be influenced by soil pH. When effect concentrations were derived based on total soil Zn levels, the tendency was for higher toxicity (i.e. lower effect concentrations) at lower pH. This pH effect can be explained by the higher Zn availabillity resulting from the increased solubility of the Zn at low pH values. When effect concentrations were calculated based on pore water Zn concentrations, this relationship with pH was reversed. Thus, the lowest pore water based effect concentrations, indicative of higher toxicity were observed in the high pH for ionic Zn and the non-nano scale material and at the medium pH for ZnO NPs. According to the biotic ligand model (Paquin et al., 2002; Santore et al., 2002), low pH is characterised by a high concentration of H^+ ions and a resulting high competition between Zn^{2+} and other cations, most notably H^+, in soil solution for binding to key organisms receptors. That the relationship between effect concentrations derived from soluble Zn levels and soil pH follows similar trends, suggests ionic Zn governs a large part of the toxicity caused, and confirms the importance of considering existing paradigms of metal toxicity with NP effect assessment (van Gestel et al. 2010).

While in some respects toxicity followed ion based paradigms as above, then comparing EC_{50}s calculated based on pore water Zn concentration between Zn forms indicated that both NP and non-nano ZnO generally produced greater effect at lower soluble concentration than for ionic Zn (Figure 3). These results suggest a complex relationship between toxicity and soluble Zn concentration for particulate forms of zinc, that cannot be related to Zn ion derived toxicity only. It has been suggested that metal based NPs, such as ZnO NPs, can cause a high localised exposure of toxic dissolved species, since the concentration of ions close to the particle surface will be greater than in the bulk solution (Apte et al. 2009). Such localised NP specific effects could explain the high toxicity for the ZnO forms. A further mechanism of NP toxicity that can result in nano specific toxic effects can occur via the formation of reactive oxygen species (ROS). Dimkpa et al. (2011) observed that association of

Cu and ZnO NPs with soil bacterial cells, led to an accumulation of ROS on the inside of the cell and resulting dysfunction. Since the ZnO, either as well dispersed material or as agglomerates, can be expected in the soil solution and so come into contact with body surfaces, this mechanism can potentially explain the higher ZnO toxicity based on zinc pore water concentrations. The endocytosis of these surface associated particles can also lead to uptake with the further potential for greater toxicity resulting from local hotspots (Apte et al., 2009).

Zinc bioaccumulation and bioconcentration

Bioaccumulation factors (BAFs) for the three Zn forms were all in the range reported for zinc by Suthar & Singh (2009) for *E. fetida.* All BAFs were below one indicating that biomagnification of Zn does not occur under the conditions tested. This is in good agreement with the fact that zinc is an essential metal and is highly regulated by earthworms (Spurgeon and Hopkin, 1996b, Lock & Janssen, 2001; Hobbelen et al., 2006). For example, Lock & Janssen (2001) found that internal Zn concentrations were frequently regulated to a level between 100-200 mg/kg by earthworms. An observation that corresponds well with the findings of the current study at low soil Zn levels. Indeed for $ZnCl_2$ only in some of the higest Zn concentrations did internal zinc concentrations differ significantly from the control. While for both forms of ZnO a more general trend of significant increases in internal Zn concentrations were observed at lower concentrations (Figure 4).

Comparisons between the BAFs (calculated relative to total soil Zn) and BCF (calculated by relation to pore water soluble Zn) for the different Zn forms indicated higher values in worms exposed to ZnO (as either NP or non-nano form) than for worms exposed to $ZnCl_2$ when exposed to similar total Zn concentrations (Fig. 4). Hooper et al. (2011) also noted a similar trend for increased internal concentration, but lower toxicity in *Eisenia veneta* exposed to a ZnO NP. Such increase accumulation in ZnO exposed earthworms may be related to the presence of internalised particles (Unrine et al., 2010b; Hooper et al., 2011). For example, crystaline ZnO was detected in *E. veneta* tissue after an

aqueous ZnO NP exposure (Hooper et al., 2011). Taken together, these data suggest that the fundamental paradigm of a link between body burden and toxic effect that is inherent in models such as the critical body residue hypothesis (which assume a direct relationship between internal concentration and effect) could be more complex for ZnO NPs than is the case for ionic metal or indeed other chemicals. However, the fact that the same trend for increased internal concentration was seen for both ZnO forms suggests that this increased assimilation is not a nano size specifc effect, but more likely related to Zn ion availability, delivery and resupply in the pore water phase.

Conclusion

The comparative toxic effects of dissolved Zn ($ZnCl_2$), nano ZnO and non nano scale ZnO to the earthworm *Eisenia fetida* has been thoroughly investigated in natural soil at three different soil pHs. Particle characterization in spiked soil suspension and extracted pore water indicated changes in zeta potential, size distributions and soluble Zn concentrations that reflect the interactions of the different Zn form with soil constituents under the range of tested soil pH conditions. Based on the characterisation and toxicity data obtained it was possible to test the three hypotheses set out previously: First that Zn ions taken up from soil solution by the worms are primarily responsible for observed toxicity independent of exposure form. This hypothesis was partly validated, since greatest toxicity was found at low pH where particle disolution can be expected. However, interestingly calculation of effect concentration based on pore water Zn concentrations indicated lower values in the ZnO test. This suggest the presence of a particle specific toxicity in tests conducted with these materials. Our second hypothesis was that ZnO would be more toxic than non-nano ZnO when expressed on a total Zn mass basis, due to factors such as increased dissolution and greater surface reactivity. In this case we were not able to find evidence supporting this ascertion. Thus for the ZnO materials considered (30 nm vs 200 nm) similar solubility relationships with pH, toxicity and bioaccumulation patterns were found. Finally we assessed how accumulation into body tissue were

related to the soluble concentration and toxicity. Contrary to our expectation body burdens were higher in ZnO exposed worm than worms exposed to $ZnCl_2$, even though toxicity was greatest for the ionic form. These results suggest the importance of carefully considering the relationship between uptake and toxicity in future nanotoxicology studies.

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Tables

Table 1. Characterization data for the dosing soil suspensions. The pHss refers to the pH measured in the soil suspension before and after spiking with 30nM ZnONPs. The size measurement is the z-average (nm) ± SE (n=3) of hydrodynamic size of particles present in the media and ζ- potential is the average (mV) ± SE (n=3) zeta potential of the NPs.

Table 2. Pore water characterization data from the dynamic light scattering analysis. At the start of exposure, pore water samples were extracted from: unspiked control soils, soils spiked with low (235 mg Zn/kg), and high concentration (2500 mg Zn/kg) concentrations of 30nmZnONPs. The pH_{pw} refers to pH measured in the pore water extract. The size measurement is the z-average of hydrodynamic size (nm) ± SE (n=3) of particles present in the media and ζ- potential (mV) ± SE (n=3) is the zeta potential of these particles.

Table 3. LC50 values (+/- SE) for the effect of $ZnCl_2$, ZnO NPs and non-nano ZnO on the survival of the earthworm *Eisenia fetida* after 28 days exposure in a natural sandy soils at a low, medium and high pH. The (-) indicates that data did not allow calculating standard error.

Table 4. EC50 values (+/- SE) for the effects of $ZnCl_2$, ZnO NPs, and non-nano scale ZnO on the cocoon production of the earthworm *Eisenia fetida* after 28 days exposure in a natural sandy soils at a low, medium and high pH.

Table 5. Calculated bioaccumulation factors (BAF) +/- SD calculated as earthworm tissue Zn concentrations / total soil Zn concentration and bioconcentration factors (BCF) +/- SD calculated as earthworm tissue Zn concentrations / soil pore water Zn concentration. Values are based on BAFs and BCFs derived for all treatment resulting in effects below EC50 for reproduction with control

treatments excluded as difference in speciation can be expected in these unspiked soils compared to the spiked treatment.

Figures

Figure 1. The measured total zinc concentrations in a single sample of soil pore water collected for each concentrations of A) $ZnCl_2$, B) ZnO NPs and C) non-nano scale ZnO for thelow pH soil (◆), medium pH soil(■), and high pH soil (△), including separate analysis for samples at the beginning of the toxicity test (t=0, solid line) and at experiment completion (t=56, dashed line).

Figure 2. The cocoon production rate of *Eisenia fetida* in low pH soil (◆), medium pH soil (■), and high pH soil (△) after 28 days exposure to A-B) $ZnCl_2$, C-D) ZnO NPs and E-F) non-nano scale ZnO. The left hand colum of graphs (A, C, E) plots cocoon production rate in relation to total nominal zinc concentration in the soil, and the right hand column of graphs (graphs B, D, F) plots rate as a function of pore water zinc concentration. Solid, dashed and punctured lines show fit obtained with a logistic model for the low, medium and high pH soils, respectively.

Figure 3. Internal Zn concentration (µg Zn/g d.w.) in the earthworm *Eisenia fetida* exposed in low pH (◆), medium pH (■), and high pH (△) soil to $ZnCl_2$ (A), ZnO nanoparticles (B) and non-nano ZnO (C) after 28 days of exposure. Bars represented standard errors. Letters indicate significant difference from the respective control soil (Tukey, $p < 0.05$) for (a) low, (b) medium and (c) high pH soil. Note variation for y-axis scale.

Supplementary Material

Table S1. Summary of the soil properties of the unchanged Dorset soil used for all toxicity tests prior to pH amendment. Dorset soil texture, pH (in water and 0.01M $CaCl_2$), conductivity, total carbon (% Total C) and nitrogen (% Total N), cation exchange capacity (CEC), percent base saturation (% BS), major cations concentrations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) along with oxalate (% Fe_{ox}, % Al_{ox}) and citrate-bicarbonate-dithionite (% Fe_{CBD} and % Al_{CBD}) extractable iron and aluminium are shown.

Table S2. Levels of calcium carbonate addition and final pH in Dorset sandy soil with original properties shown in Table S1 as used for the low, medium and high pH soils.

Table S3. Characterisation results for Nanosun P99/30 and non nano scale ZnO P99/200 with XRD, BET and density measurements

Figure S1. NanoSun P99/30 ZnO (A) and non nano scale ZnO (B) observed in a transmission electron microscope.

Figure S2. TEM images of the three ZnO nanoparticle stock soil suspensions. A) low pH, B) medium pH, C) high pH.

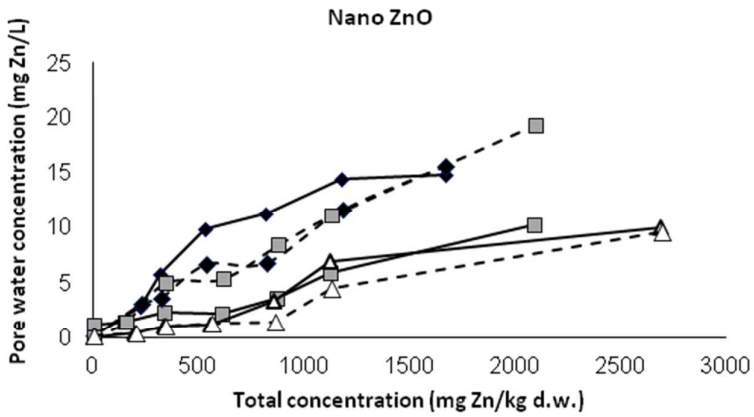
Figure S3. The measured total organic carbon concentration in the soil pore water of the low pH soil (◆), medium pH soil (■), and high pH soil (△) spiked with ZnO nanoparticles as a function of Zn pore water concentration (mg Zn/L). The solid lines reflect time zero and punctured lines reflect time 56 days.

Figure S4. The survival of *Eisenia fetida* in low pH soil (◆), medium pH soil (■), and high pH soil (△) after four weeks exposure to A-B) $ZnCl_2$, C-D) ZnO nanoparticles and E-F) ZnO. The left hand column

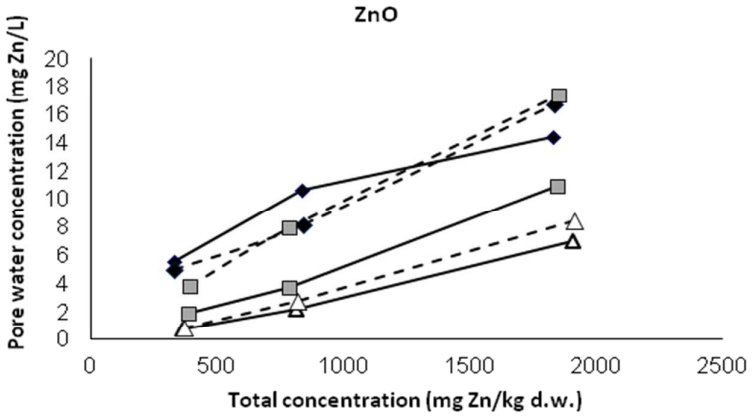
(graphs A, C, E) express the survival as a function total nominal zinc concentration in the soil, and the right hand column survival (graphs B, D, F) as a function of zinc concentration in the pore water. Small letters (a) indicate significant difference between control and treatments (Tukey, $p < 0.05$). Solid, dashed and punctured lines show fit obtained with a logistic model for the low, medium and high pH soils, respectively.

Figure 1.

A)



B)



C)

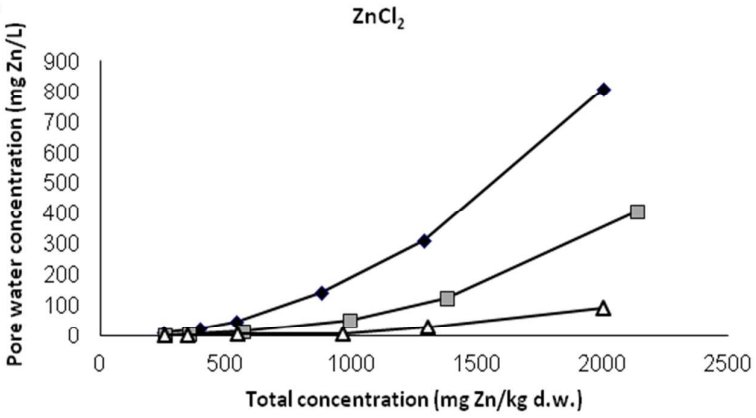


Figure 2.

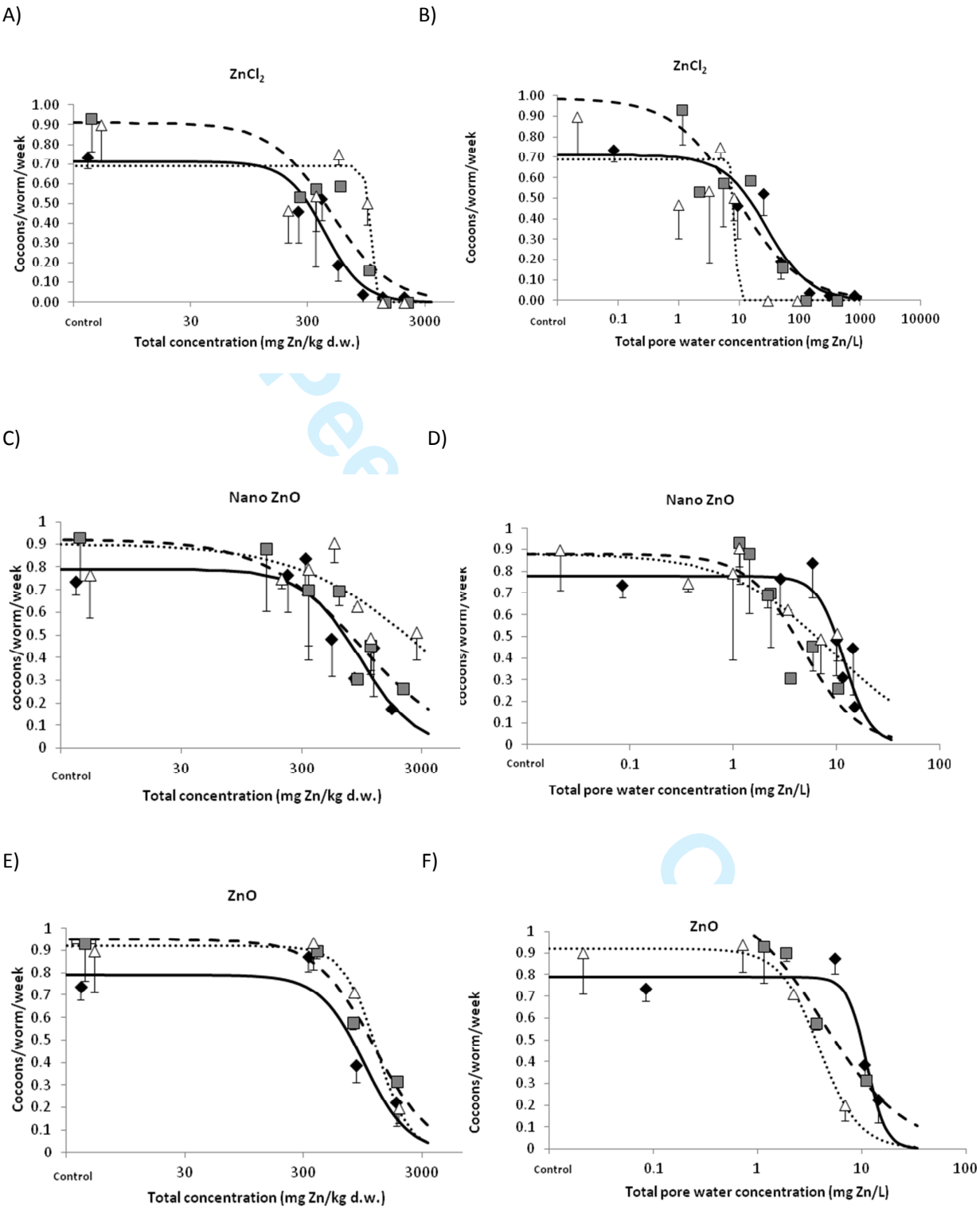
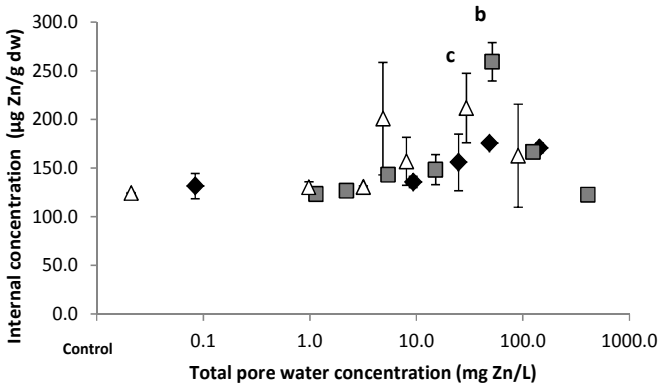
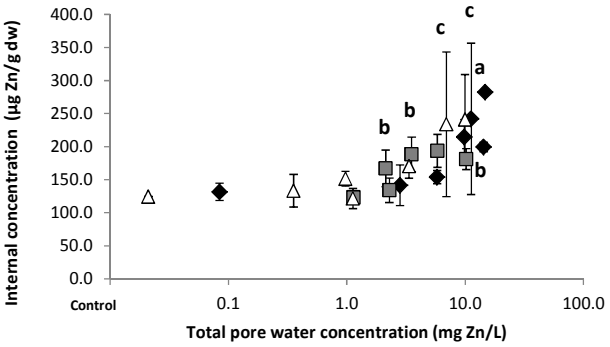

A)
B)
ZnCl2
Cocoons/worm/week
Control
30
300
3000
Total concentration (mg Zn/kg d.w.)
0.1
1
10
100
1000
10000
Total pore water concentration (mg Zn/L)
C)
D)
Nano ZnO
cocoons/worm/week
E)
F)
ZnO

Figure 3.

A. $ZnCl_2$



B. Nano ZnO



C. ZnO

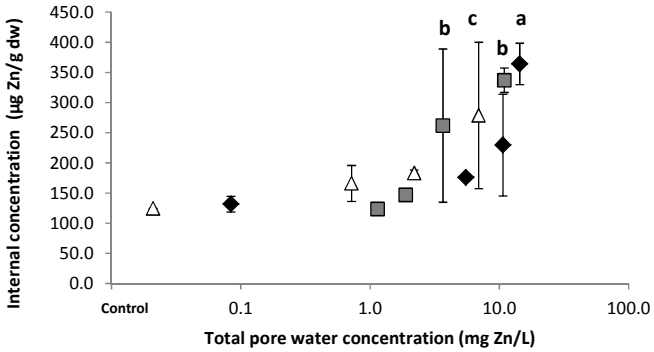


Table 1. Characterization data for the dosing soil suspensions. The pH_{ss} refers to the pH measured in the soil suspension before and after spiking with 30nM ZnONPs. The size measurement is the z-average (nm) ± SE (n=3) of hydrodynamic size of particles present in the media and ζ- potential is the average (mV) ± SE (n=3) zeta potential of the NPs.

	Unspiked soil suspensions			Spiked soil suspensions		
Soil	pH_{ss}	Size	ζ- potential	pH_{ss}	Size	ζ- potential
Low pH	5.1	2228 ± 172	-16.3 ± 0.5	6.2	720 ± 1036	0.7 ± 0.05
Medium pH	6.4	1107 ± 90	-16 ± 0.9	7.5	614 ± 192	0.8 ± 0.5
High pH	7	1476 ± 133	-12.4 ± 0.6	8	653 ± 142	1.3 ± 0.2

Table 2. Pore water characterization data from the dynamic light scattering analysis. At the start of exposure, pore water samples were extracted from: unspiked control soils, soils spiked with low (235 mg Zn/kg), and high concentration (2500 mg Zn/kg) concentrations of 30nmZnONPs. The pH_{pw} refers to pH measured in the pore water extract. The size measurement is the z-average of hydrodynamic size (nm) ± SE (n=3) of particles present in the media and ζ- potential (mV) ± SE (n=3) is the zeta potential of these particles.

	Pore water (control soils)			Pore water - low Zn concentration			Pore water - high Zn concentration		
Soil	pH_{pw}	Size	ζ- potential	pH_{pw}	Size	ζ- potential	pH_{pw}	Size	ζ- potential
Low pH	5.2	199 ± 3	-20.3 ± 0.5	5.3	237 ± 4	-17.6 ± 1.2	6.3	202 ± 4	-9.9 ± 1.7
Medium pH	6.4*	402 ± 5	-17.2 ± 1.7	7.2	285 ± 49	-14 ± 0.7	7.5	119 ± 6	-14.1 ± 2.4
High pH	8.2	604 ± 67	-11.4 ± 0.7	8.1	429 ± 15	-14.2 ± 1.8	7.7	247 ± 28	-12.1 ± 0.7

*Due to a very small volume of available sample a pH measurement could not be obtained. This value refers to the pH in the unspiked soil suspension made from the same soil which is assumed to be similar.

Table 3. LC_{50} values (+/- SE) for the effect of $ZnCl_2$, ZnO NPs and non-nano ZnO on the survival of the earthworm *Eisenia fetida* after 28 days exposure in a natural sandy soils at a low, medium and high pH. The (-) indicates that data did not allow calculating standard error.

	pH	$ZnCl_2$	Nano ZnO	ZnO
LC_{50} based on Zn in soil (mg Zn/kg)	Low	718 ± 73.6	>> 1669 (-)	>> 1832 (-)
	Medium	589.5 ± 72.7	>> 2094 (-)	4147 ± 2751
	High	1983 ± 285.8	>> 2689 (-)	>> 1908 (-)
LC_{50} based on Zn in pore water (mg Zn/L)	Low	89.4 ± 16.4	>> 14.8 (-)	>> 14.38 (-)
	Medium	20.1 ± 4.8	>> 10.2 (-)	13.8 ± 5.5
	High	76.9 ± 24.2	>> 9.98 (-)	>> 6.93 (-)

Table 4. EC_{50} values (+/- SE) for the effects of $ZnCl_2$, ZnO NPs, and non-nano scale ZnO on the cocoon production of the earthworm *Eisenia fetida* after 28 days exposure in a natural sandy soils at a low, medium and high pH.

	pH	$ZnCl_2$	Nano ZnO	ZnO
EC_{50} based on Zn in soil (mg Zn/kg)	Low	420.4 ± 97	918.6 ± 222	985.4 ± 194.7
	Medium	472.2 ± 110	901 ± 243.2	1171.5 ± 225.3
	High	1002 ± 308	2874 ± 1799	1241 ± 188
EC_{50} based on Zn in pore water (mg Zn/L)	Low	28.5 ± 14.6	11.8 ± 1.4	11.1 ± 0.9
	Medium	9.3 ± 16	4.7 ± 1	4.2 ± 2.8
	High	8.5 ± 4.2	11.4 ± 6	3.9 ± 0.7

Table 5. Calculated bioaccumulation factors (BAF) +/- SD calculated as earthworm tissue Zn concentrations / total soil Zn concentration and bioconcentration factors (BCF) +/- SD calculated as earthworm tissue Zn concentrations / soil pore water Zn concentration. Values are based on BAFs and BCFs derived for all treatment resulting in effects below EC_{50} for reproduction with control treatments excluded as difference in speciation can be expected in these unspiked soils compared to the spiked treatment.

	BAF			BCF		
Soil	$ZnCl_2$	Nano ZnO	ZnO	$ZnCl_2$	Nano ZnO	ZnO
Low pH	0.47 ± 0.12	0.46 ± 0.16	0.41 ± 0.19	10.4 ± 5	29.9 ± 14	26.7 ± 11
Medium pH	0.39 ± 0.11	0.48 ± 0.27	0.36 ± 0.13	31.2 ± 21	77.7 ± 22	74.7 ± 27
High pH	0.34 ± 0.14	0.33 ± 0.2	0.3 ± 0.16	56.8 ± 49	134.4 ± 127	139.9 ± 90

Supplementary Material

Table S1. Summary of the soil properties of the unchanged Dorset soil used for all toxicity tests prior to pH amendment. Dorset soil texture, pH (in water and 0.01M $CaCl_2$), conductivity, total carbon (% Total C) and nitrogen (% Total N), cation exchange capacity (CEC), percent base saturation (% BS), major cations concentrations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) along with oxalate (% Fe_{ox}, % Al_{ox}) and citrate-bicarbonate-dithionite (% Fe_{CBD} and % Al_{CBD}) extractable iron and aluminium are shown.

	% Coarse sand	% Fine sand	% Clay	% Silt	% Total C	% Total N
Texture, total carbon and nitrogen	51.5	40.2	3.5	4.7	4	0.1
	pH_{H2O}	pH_{CaCl2}	Conductivity (µS)			
pH and conductivity	4.2	3.1	422			
	Ca	Mg	K	Na	CEC	% BS
Exchangeable cations (cmol(+)/kg)	1.4	0.6	0.1	0.1	5.4	41
	% Fe_{ox}	% Al_{ox}	% Fe_{CBD}	% Al_{CBD}		
Extractable Fe and Al	0.04	0.03	0.11	0.03		

Table S2. Levels of calcium carbonate addition and final pH in Dorset sandy soil with original properties shown in Table S1 as used for the low, medium and high pH soils.

Soil	$CaCO_3$ (w/w %)	pH[a]
Low pH	0.2	4.5
Medium pH	0.45	5.9
High pH	1	7.2

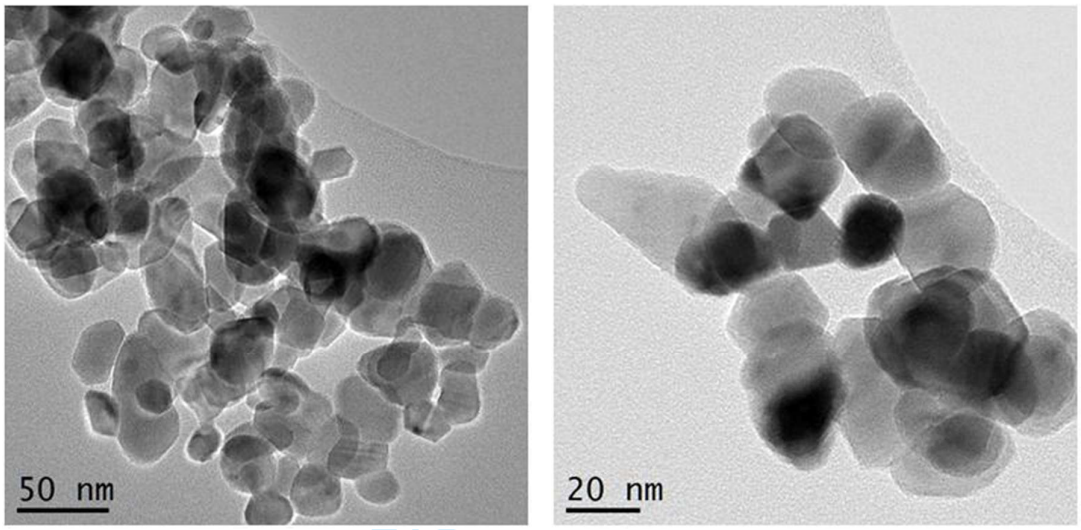
[a] pH measured in 0.01M $CaCl_2$, n=4

Table S3. Characterisation results for Nanosun P99/30 and non nano scale ZnO P99/200 with XRD, BET and density measurements

Manufacturer name	NanoSun P99/30 ZnO	Non nano scale ZnO
XRD	No alienphases	No alien phases
Average crystallite size from Scherrer's formula (XRD):	a = 25 nm c = 27 nm	a = 72 nm c = 77 nm
Grain size from SSA BET:	29 nm	296 nm
Specific Surface Area (BET):	38 m^2/g	3,59 m^2/g
Density:	5,52 g/cm^3	5,67 g/cm^3
Zeta potential in DI water	20.7 ±2.0	18.75±1.3

Figure S1.

A)



B)

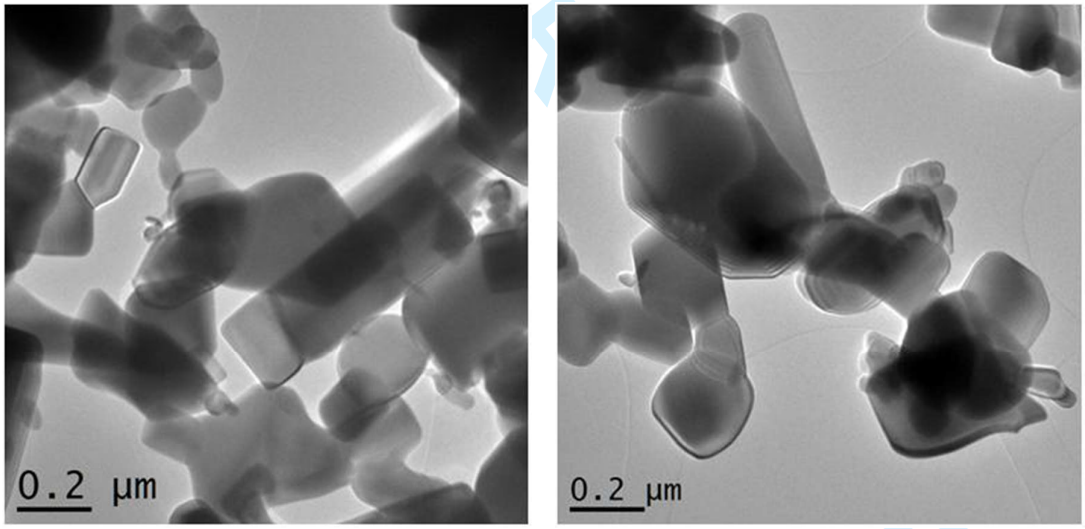


Figure S2.

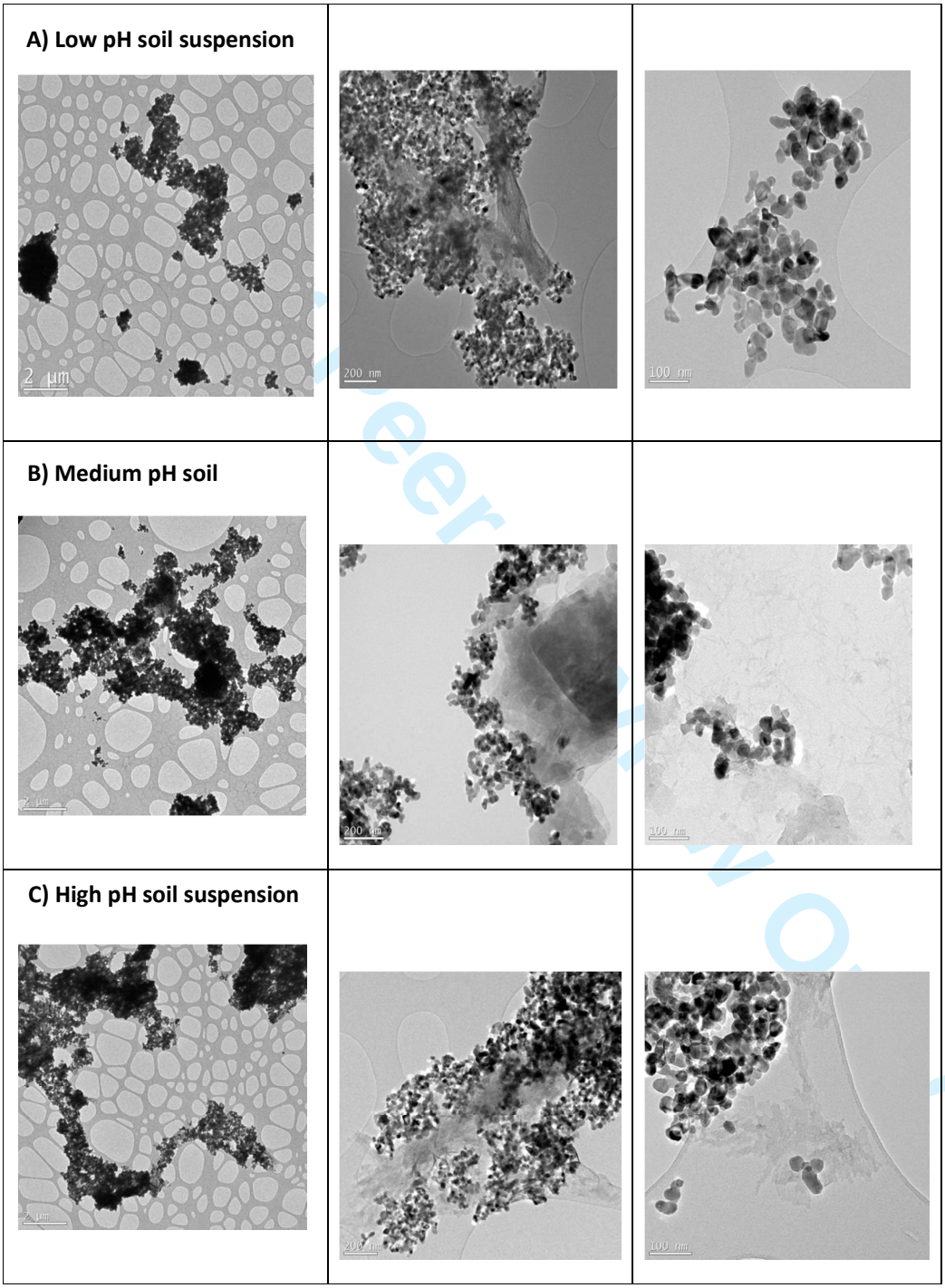

A) Low pH soil suspension
2 µm
200 nm
100 nm
B) Medium pH soil
2 µm
200 nm
100 nm
C) High pH soil suspension
2 µm
200 nm
100 nm

Figure S3.

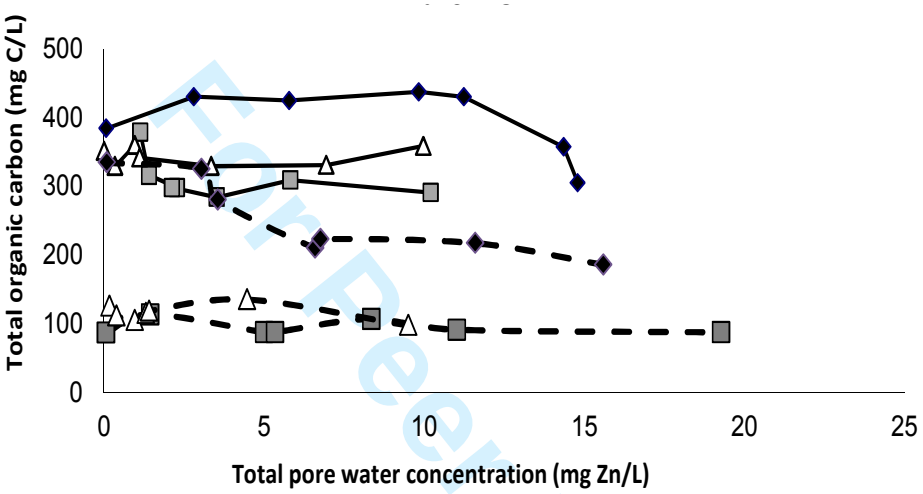
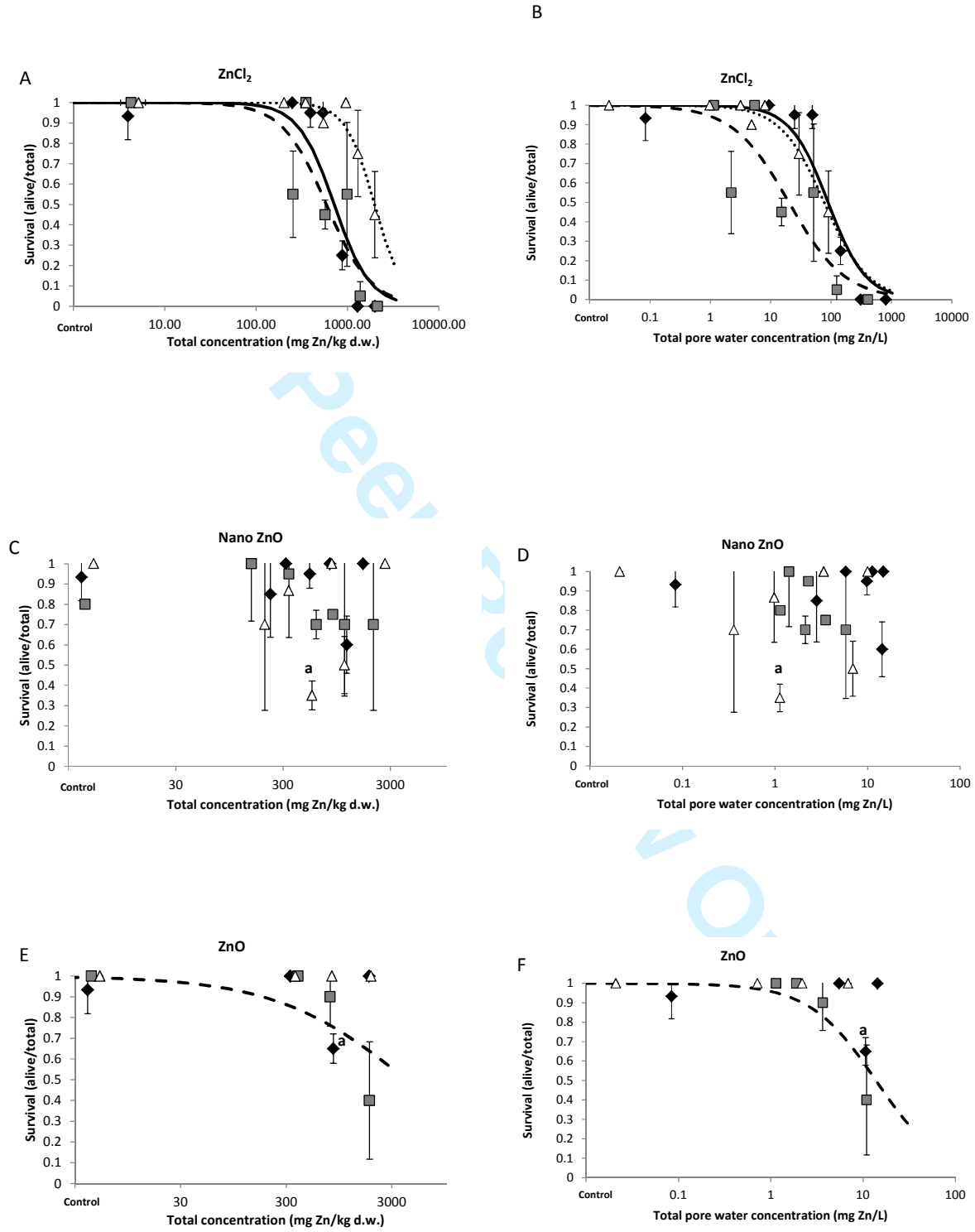


Figure S4.


A
ZnCl2
Survival (alive/total)
Control
10.00
100.00
1000.00
10000.00
Total concentration (mg Zn/kg d.w.)
B
ZnCl2
Survival (alive/total)
Control
0.1
1
10
100
1000
10000
Total pore water concentration (mg Zn/L)
C
Nano ZnO
Survival (alive/total)
a
Control
30
300
3000
Total concentration (mg Zn/kg d.w.)
D
Nano ZnO
Survival (alive/total)
a
Control
0.1
1
10
100
Total pore water concentration (mg Zn/L)
E
ZnO
Survival (alive/total)
a
Control
30
300
3000
Total concentration (mg Zn/kg d.w.)
F
ZnO
Survival (alive/total)
a
Control
0.1
1
10
100
Total pore water concentration (mg Zn/L)