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Influence of soil pH on the toxicity of zinc oxide nanoparticles to the terrestrial isopod *Porcellionides pruinosus*

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Abstract

The effects of soil pH on the toxicity of ZnO nanoparticles (NPs) to the terrestrial isopod *Porcellionides pruinosus* were evaluated. Isopods were exposed to a natural soil amended with $CaCO_3$ to reach 3 different pH_{CaCl2} levels (4.5, 6.2, and 7.3) and to standard LUFA 2.2 soil (pH 5.5) spiked with ZnO NPs (30 nm), non-nano ZnO (200 nm), and ionic Zn as $ZnCl_2$. Toxicity was expressed based on total Zn concentration in soil, as well as total Zn and free Zn^{2+} ion concentrations in porewater. Compared with ZnO-spiked soils, the $ZnCl_2$-spiked soils had lower pH and higher porewater Ca^{2+} and Zn levels. Isopod survival did not differ between Zn forms and soils, but survival was higher for isopods exposed to ZnO NPs at pH 4.5. Median effect concentrations (EC50s) for biomass change showed similar trends for all Zn forms in all soils, with higher values at intermediate pH. Median lethal concentration (LC50) and EC50 values based on porewater Zn or free Zn ion concentrations were much lower for ZnO than for ionic zinc. Zn body concentrations increased in a dose-related manner, but no effect of soil pH was found. It is suggested not only that dissolved or free Zn in porewater contributed to uptake and toxicity, but also that oral uptake (i.e., ingestion of soil particles) could be an important additional route of exposure.

1. Introduction

Manufactured or engineered nanoparticles (NPs) have attracted industrial and scientific interest in the last decade because of their unique properties. Innovative products used in diverse fields have resulted in a substantial investment in the nanotechnology sector, which is estimated to be $1 trillion in 2015 [1]. Because of increasing annual production over the years, NPs have been regulated by the European Commission's regulation on the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) in Europe, under the same legislation as bulk compounds, even though nano and bulk materials have different properties [2].

Zinc oxide (ZnO) NPs are among the most produced NPs, with production volumes of more than 500 t/yr in 2010 [3]. The ZnO NPs are used mainly in cosmetics (as UV absorbants in sunscreens), paints, and coatings [3]. The use of NPs may result in emissions into the environment, with soil being an important sink [4].

Some attention has been paid to the behavior and effects of NPs in the environment [4]. The processes of dissolution and aggregation or agglomeration have been shown to be dependent on characteristics of both the exposure media and the NPs. The stability of ZnO NPs is affected by environmental conditions such as pH [5], organic matter content [6], and ionic strength [7]. In soils, pH is one of the most important factors to consider in toxicity tests, because it can change the NP surface charge and zeta potential [4]. As a consequence, the interactions between NPs and soil, as well as the interactions between particles, will change, influencing NP behavior, bioavailability, and toxicity.

The effect of soil pH on the bioavailability of ionic zinc to soil organisms has already been studied. Zinc toxicity to the potworm *Enchytraeus albidus* decreased with increasing soil pH [8]. For the springtail *Folsomia candida*, the median effect concentration (EC50) for effects on reproduction was lower in an acid soil than in a basic soil (pH_{KCl} of 3.4 and 6.0, respectively), and toxicity was mainly related to the water-extractable Zn fraction [9]. In another study [10], reproduction of *F. candida* decreased with decreasing pH (ranging from 4.5 to 6.0), but no clear relation between toxicity and soil pH was found. For the earthworm *Lumbricus rubellus*, soil pH did not affect zinc accumulation in the body; however, reproduction was affected by soil pH, being related to the soluble Zn fraction [11]. In this latter study, toxic responses could be predicted by free Zn^{2+} concentration and explained by the protective effect of H^+ ions (i.e., competition with Zn^{2+} ions) [11], which seems to agree with the biotic ligand model [12].

The bioavailability and toxicity of ZnO NPs has been evaluated for collembolans [13-15] and earthworms [16] by comparing the outcome with microsized ZnO, ionic Zn forms, or both. For terrestrial isopods, the toxicity of ZnO NPs has been assessed using contaminated food as the route of exposure [17]. Soil is also an important route of exposure to chemicals for isopods, and should be investigated for NPs [18-20]. Isopods can take up chemicals from the soil either by ingesting soil particles or by porewater inflow through the uropods. The influence of environmental conditions, such as pH, on the bioavailability of NPs in soils is an important issue and is far from being completely understood [4, 21].

The present study therefore aimed at evaluating the effects of soil pH on the toxicity of ZnO NPs to the terrestrial isopod *Porcellionides pruinosus*. For this purpose, a natural soil from Dorset (UK) was amended with $CaCO_3$ to reach 3 different pH levels. A standard soil (LUFA 2.2) was included for comparison. To better understand the contribution of particle size and ionic zinc to the toxicity of ZnO NPs in isopods, toxicity tests were also conducted with microsized ZnO and $ZnCl_2$.

2. Methodology

2.1. Soil treatment

Natural soil was collected at Wareham Forest (Dorset, UK) in May 2011. Soil was excavated from the 0-cm to 30-cm top soil layer. The soil originally had a pH_{CaCl2} of 3.0. After sieving (5-mm mesh) and air-drying, the soil was amended with calcium carbonate to adjust the pH_{CaCl2} to nominal values of 4.5 (soil 1), 5.9 (soil 2), and 7.3 (soil 3). Standard LUFA 2.2 soil (Sp 2121; LUFA-Speyer) was also used in the experiment. For details on pH adjustment, see Heggelund et al. [22]. The maximum water-holding capacity (WHC_{max}) of the Dorset soils was approximately 77%, and that of the LUFA 2.2 soil was 45%. Table 1 presents the soil properties and pH levels of the different test soils.

The soils were spiked with ZnO NPs (Nanosun ZnO P99/30; particle size 30 nm), non-nano ZnO (Microsun ZnO W45/30; 200 nm), and zinc chloride ($ZnCl_2$; Riedel-de Haën; purity 98%) at nominal concentrations of 250 mg Zn/kg dry soil, 500 mg Zn/kg dry soil, 1000 mg Zn/kg dry soil, 2000 mg Zn/kg dry soil, and 4000 mg Zn/kg dry soil. To spike the soils with ZnO, the dry

powders were added to 30 g of dry soil. After thorough mixing, the mixture was added to 270 g of dry soil. Milli-Q water (Millipore) was added to achieve a moisture content corresponding to 45% of the WHC_{max} of the soils. For $ZnCl_2$, 300 g of dry soil was mixed with a $ZnCl_2$ solution in Milli-Q water. If necessary, additional Milli-Q water was added to moisten the soil up to 45% of WHC_{max}. Nonspiked soils were moistened with Milli-Q water and tested as control soils. Soils were allowed to equilibrate for 1 wk before the toxicity tests began.

2.2. Toxicity test

Specimens of the isopod *Porcellionides pruinosus* were collected in Coimbra (Portugal) and kept under laboratory conditions for at least 1 mo before exposure. The animals were kept on a substrate of potting soil with alder (*Alnus glutinosa*) leaves provided ad libitum for food. Males and nongravid females (>12 mg) were exposed individually in plastic boxes containing 20 g of moist soil. Ten replicates were used for each treatment and control. Dry alder leaf disks (~10 mm diameter) were offered to the isopods as food ad libitum. The animals were kept at 20 °C ± 1 °C and a light:dark photoperiod of 16:8 h. Water loss was checked and adjusted after 7 d by weighing the test containers. After 14 d, survival and feeding activity were evaluated. The parameters used in this experiment were the consumption ratio and biomass change calculated as

$$Cr = (W_{li} - W_{lf}) / W_{isop}$$

$$B = (W_{isopf} - W_{isop}) / W_{isop} * 100$$

where *C*r is the consumption ratio (mg leaf/mg isopod), W_{li} is the initial leaf weight (mg dry wt), W_{lf} is the final leaf weight (mg dry wt), W_{isop} is the initial isopod weight (mg fresh wt), *B* is the biomass change (%), and W_{isopf} is the final isopod weight (mg fresh wt).

2.3. Chemical analysis

Soil pH was measured in 0.01 M $CaCl_2$ extracts at the beginning of the test, in accordance with International Organization for Standardization guideline 10390 [23]. For determining total Zn concentrations in soil, dry soil samples were digested for 7 h in a mixture of ultrapure water, concentrated HCl (J.T. Baker, purity 37%), and HNO_3 (J.T. Baker, purity 70%; 1:1:4, v/v) at 140 °C in an oven (CEM MDS 81-D). After digestion, the samples were analyzed for zinc by flame atomic absorption spectrometry (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. Measured zinc concentrations in the reference material were within 10% of the certified concentrations.

Porewater was collected by saturating 50 g of soil with ultrapure water for 1 wk. Samples were centrifuged at a relative centrifugal force (RCF) of 2862 *g* for 90 min (Eppendorf 5810R centrifuge). The supernatant was collected and filtered using a cellulose nitrate filter (Whatman, 0.45-µm pore size). Total zinc concentration in porewater was analyzed by flame AAS (Perkin-Elmer AAnalyst 100), after dilution with distilled water. Calcium concentrations in the porewater samples were determined after dilution with 1% $La(NO_3)_3$ in 0.1 nHNO_3 and analyzed by flame AAS (Perkin-Elmer AAnalyst 100).

After 14 d of exposure, total zinc body content in the surviving isopods was analyzed in triplicate for each exposure concentration. After freeze-drying, isopods were individually weighed and digested with a mixture of concentrated HNO_3:$HClO_4$ (7:1, v/v; J.T. Baker, ultrapure). The samples were evaporated to dryness and the residues were taken up in 300 µl 0.1 M HNO_3. Zinc content was determined by graphite furnace AAS (Perkin-Elmer 5100 PC).

2.4. Statistical analysis

Zinc concentrations causing 50% mortality (median lethal concentration [LC50]) of *P. pruinosus* were calculated by probit analysis. Consumption ratio (log-transformed) was analyzed by one-way analysis of variance (ANOVA), followed by a Dunnett's test. Data homoscedasticity and normality were tested by Levene's test and the Kolmogorov–Smirnov test, respectively. For biomass change, EC50s were estimated by applying a four-parameter logistic model:

$$Y = Ymin+(Ymax-Ymin)/(1+(X/EC50)^{-b}$$

where Y_{min} is the minimum biomass gain (%); Y_{max} is the maximum biomass gain (%); X is the Zn concentration in soil (mg Zn/kg) or porewater (mg Zn/L), or the free Zn^{2+} ion concentration (µM) in porewater; and b is the slope parameter. The free Zn ion concentrations were estimated from total Zn concentrations in porewater using the speciation model WHAM7.
Slopes of the probit regression and EC50 values were compared between the different soils by a generalized likelihood ratio test. Zinc body content in the isopods was analyzed by a two-way ANOVA, using zinc concentration in soil and soil pH as the independent variables. When necessary, data were log-transformed to reach homoscedasticity and normality. Statistical analyses were performed with SPSS software (Ver 20).

3. Results

3.1. Soil characteristics

Soil pH changed in the presence of zinc in all soils, mainly in a dose-related manner. A great difference was found between soils spiked with ZnO particles (30 nm and 200 nm ZnO) and $ZnCl_2$ (Figure 1). For ZnO particles, the pH increased up to 2 units with increasing Zn concentration in soils 1 and 2 and in LUFA soil, whereas a slight dose-related decrease of up to 0.3 pH units at the highest test concentration was found in soil 3. For $ZnCl_2$, pH decreased in all soils in a dose-related fashion up to 0.9 units at the highest Zn concentration. Porewater pH levels showed the same trends as soil pH (Supplemental Data, Table S1).

Total measured Zn concentrations in the soil ranged between 68% and 130% of the nominal ones (Supplemental Data, Table S1). All effect concentrations reported are based on measured concentrations.

Calcium concentrations in porewater also varied between the different Zn forms and concentrations (Figure 1). For 30 nm and 200 nm ZnO, calcium levels remained approximately constant in soils 1 and 2, ranging between 11.7 mg Ca/L and 22.1 mg Ca/L. In soil 3, calcium levels slightly decreased with increasing ZnO concentration in soil, whereas in LUFA 2.2 soil, they slightly increased. For $ZnCl_2$, calcium levels increased with increasing Zn concentration and were between 30 times and 50 times higher than in Dorset soils and 10-fold higher than in LUFA soil spiked with ZnO.

Zinc concentrations in porewater were similar in soils spiked with nano- and microsized ZnO particles, showing a slight increase with increasing Zn concentration (Supplemental Data, Table S1). The $ZnCl_2$-spiked soils showed strong dose-related increases in Zn levels in porewater, reaching concentrations approximately 100-fold higher than in ZnO-spiked soils.

3.2. Toxicity to Porcellionides pruinosus

3.2.1. *Mortality*

Control survival of isopods was 80%, 100%, 90%, and 100% for soils 1, 2, and 3 and LUFA 2.2 soil, respectively. The LC50 values could be calculated for all 3 Zn forms in the different soils, except for 30 nm ZnO in soil 1, where only 30% mortality occurred at the highest concentration. The LC50 values for the effects of 30 nm ZnO on survival of the isopods ranged from 1757 mg Zn/kg dry soil to >3369 mg Zn/kg dry soil in the different soils (Table 2). The LC50 values ranged from 2169 mg Zn/kg dry soil to 2894 mg Zn/kg dry soil and from 1792 mg Zn/kg dry soil to 3732 mg Zn/kg dry soil for 200 nm ZnO and $ZnCl_2$, respectively (Table 2). The LC50 values decreased with increasing soil pH for ZnO NPs (Table 2). No significant difference in slopes of the probit regressions were found between soils for 30 nm ZnO ($X^2_{(2)}$= 5.56, $p > 0.05$), 200 nm ZnO ($X^2_{(3)}$=0.86, $p > 0.05$), and $ZnCl_2$ ($X^2_{(3)}$=7.68, $p > 0.05$).

The LC50 values were also calculated based on Zn concentration (mg/L) and free Zn2+ ion concentration in porewater (µM) for CaCO3-amended Dorset soils (Supplemental Data, Tables S2 and S3). Values found for ZnO were found to be much lower than for ZnCl2, ranging from 1 µM to 32 µM and 2000 µM to 24 000 µM for ZnO and ZnCl2, respectively.

Dead animals were excluded from further analysis of sublethal responses and Zn body content.

3.2.2. Feeding inhibition

Feeding activity measured as the consumption ratio of control animals differed significantly between soils (ANOVA, $p < 0.05$). In soil 3, the consumption ratio was significantly higher than in all other soils, whereas the consumption ratio

in soil 1 was significantly higher than in LUFA 2.2 soil (ANOVA, $p < 0.05$). The consumption ratio did not change in the isopods exposed to 30 nm and 200 nm ZnO in soils 1 and 2 and LUFA 2.2 soil (ANOVA, $p > 0.05$). However, the consumption ratio decreased significantly in soil 3 at 2000 mg Zn/kg soil and 1000 mg Zn/kg soil for 30 nm and 200 nm ZnO, respectively (Dunnett's test, $p < 0.05$; Figure 2). Due to high mortality at these concentrations, the sample sizes were 3 and 1 at 2000 mg Zn/kg soil and 4000 mg Zn/kg soil, respectively, for 30 nm ZnO. Sample sizes were 8, 2, and 2 at 1000 mg Zn/kg soil, 2000 mg Zn/kg soil, and 4000 mg Zn/kg soil, respectively, for 200 nm ZnO. For $ZnCl_2$, the consumption ratio decreased in a dose-related manner in all tested soils at concentrations ≥ 500 mg Zn/kg soil (Dunnett's test, $p < 0.05$).

3.2.3. Biomass change

Biomass change of the isopods, calculated as the difference between final and initial fresh weights, did not differ between control soils (ANOVA, $p > 0.05$). The EC50s ranged from 713 mg Zn/kg soil to 1479 mg Zn/kg soil for 30 nm ZnO, from 119 mg Zn/kg soil to 1951 mg Zn/kg soil for 200 nm ZnO, and from 331 mg Zn/kg soil to 1478 mg Zn/kg soil for $ZnCl_2$ (Table 2). The corresponding logistic dose–response relationships based on total Zn concentrations can be found in Supplemental Data, Figure S1. The 3 Zn forms showed similar trends in EC50 values with soil pH. Soil 2 showed the highest EC50 values for all Zn forms; however, significant differences between EC50s were found only for 200 nm ZnO ($X^2_{(3)} = 69.82$, $p < 0.001$) and $ZnCl_2$ ($X^2_{(3)} = 23.10$, $p < 0.001$).

The EC50 values for effects on biomass change based on total porewater Zn concentrations ranged from 4.21 mg Zn/L to 9.06 mg Zn/L and from 2.28 mg Zn/L to 3.23 mg Zn/L for 30 nm and 200 nm ZnO, respectively. For $ZnCl_2$, EC50 values ranged from 35.9 mg Zn/L to 250 mg Zn/L (Supplemental Data, Table S2). Biomass change in isopods exposed to ZnO particles was not dose-related to free Zn^{2+} ion concentration in the porewater of soils 1 and 2, therefore making it impossible to obtain EC50s based on Zn^{2+} concentration. In soil 3, EC50 values were 0.59 µM and 0.42 µM for 30 nm and 200 nm ZnO, respectively. For $ZnCl_2$, EC50 values were 449 µM, 3000 µM, and 37.8 µM in soils 1, 2, and 3, respectively (Supplemental Data, Table S3). Logistic dose–response relationships based on total soil Zn concentrations, and on total Zn and free Zn^{2+} concentrations in the porewater can be found in Supplemental Data, Figure S2.

3.3. Zinc body content

Zinc body content in the isopods showed a dose-related increase in all soils and for all 3 Zn forms tested (Figure 3). Zn body concentrations of isopods exposed to 30 nm ZnO were affected by zinc concentration in soil (ANOVA, $p < 0.01$), but not by soil pH (ANOVA,$p > 0.05$) or the interaction between soil concentration and pH (ANOVA, $p > 0.05$). Similarly, for 200 nm ZnO, a significant effect of soil concentration on Zn body content of the isopods was observed (ANOVA, $p < 0.01$) with no significant effect of soil pH or their interaction (ANOVA, $p > 0.05$). For $ZnCl_2$, zinc body content significantly increased with soil concentration (ANOVA, $p < 0.01$) and soil pH (ANOVA, $p < 0.05$); however, the interaction was not significant (ANOVA, $p > 0.05$). In $ZnCl_2$-exposed isopods, zinc body content differed between soil 1 and LUFA soil (Tukey test, $p < 0.05$).

4. Discussion

4.1. Soil characteristics

Soils spiked with 30-nm ZnO NPs and 200 nm ZnO showed similar characteristics in terms of soil pH and Ca^{2+} levels in the porewater. Also, zinc concentrations in the porewater were similar for both ZnO forms. The solubility of ZnO NPs has been shown to be very similar in comparison with 200 nm ZnO in LUFA 2.2 soil [13]. A different result was found for $ZnCl_2$-spiked soils (i.e., much higher Zn concentrations in the porewater were measured), as expected for a soluble metal salt. A decrease in Zn porewater concentrations with increasing soil pH was observed, whereas the Ca^{2+} concentrations in the porewater increased with increasing pH. The latter may be because $CaCO_3$ was used to adjust soil pH. The addition of Zn^{2+} cations in the case of $ZnCl_2$ led to competition with protons and Ca^{2+} bound to the negatively charged soil particles, resulting in a decrease in soil pH and an increase in porewater Ca concentrations [24]. Zinc solubility is affected by soil pH, which is well described by the competitive adsorption model [25]. In line with this, the competition between Zn^{2+} and Ca^{2+} ions in our tests resulted in an increase in Ca concentrations in solution (Figure 1), and the presence of protons and cations also affected zinc partitioning in soils.

The addition of NPs resulted in either a decrease or an increase in soil pH, depending on the nature of the soil. This finding is probably related to the buffer capacity of the soil as well as the nature of the particles. Zinc oxide seems to increase rather than decrease soil pH, which may be related to its chemistry. The NP surface charge changes depending on the pH of the surrounding medium. The particles can reach the point of zero charge (pH_{pzc}), in which the positive and negative charges of the NPs are equally balanced [26]. The dissolution reaction of Zn^{2+} ions from the NPs will consume protons and increase soil pH. However, most ZnO particles will not dissolve, and other reactions will take place on particle surfaces, more specifically on ZnOH groups that can undergo 2 reactions, depending on (porewater) pH. Below the pH_{pzc}, the NP surface will adsorb protons, giving rise to a net positive surface charge and increasing pH. Above pH_{pzc}, a second reaction dominates in which the surface will release protons, giving rise to a net negative surface charge and acidifying the soil.
This explains why pH increased in acid or neutral soil (pH 4.5–6.2) and slightly decreased at more basic soil (pH 7.3). In practice, however, the explanation is more complex than that, because the change in pH brought about by adding the oxide will itself modify the oxide surface charge. So the pH at which the effect of the oxide switches from increasing the soil pH to decreasing it is not necessarily exactly the same as the pH of the point of zero charge.

A similar pH increase after ZnO addition was also seen in LUFA 2.2 soil by Waalewijn-Kool et al. [27], but they observed a decrease later on when equilibrating the soils for up to 1 yr. The reason for such a pH decrease remains unclear, but it might simply be the result of soil microbial activity.
Zinc oxide NPs were found to have a pH_{pzc} above 7.5 in water [28, 29] and above 8 in soil [30]. At the highest pH_{CaCl2} of 7.3 in soil 3 (i.e., closer to pH_{pzc}), the attraction between NPs was increased, and consequently a greater diameter size would be expected. However, Heggelund et al. [22] performed transmission electron microscopy analysis on $CaCO_3$-amended Dorset soils spiked with 30 nm ZnO and did not find an effect of soil pH on NP aggregate size. Dynamic light scattering analysis showed that the zeta potential was close to neutral for all soils, which was caused by the binding of organic matter to the NP surface, neutralizing the charge [22].

4.2. Toxicity to Porcellionides pruinosus

In the present study, LC50 values for total Zn concentration in soil were comparable for all 3 Zn forms, with the exception of soil 1, where ZnO NPs were less toxic. Zinc oxide NPs (>100 nm) and $ZnCl_2$ had no significant effect on survival of the isopod *Porcellio scaber* exposed for 28 d via contaminated food in concentrations up to 5000 mg/kg [17]. For the earthworm *Eisenia fetida*, $ZnCl_2$ had a greater effect on survival compared with 30 nm and 200 nm ZnO [22]. For the springtail *F. candida*, 30 nm and 200 nm ZnO had no effect on survival after 28 d of exposure to up to 6400 mg Zn/kg in LUFA 2.2 soil [13]. For $ZnCl_2$, the same authors [13] found an LC50 value of 1000 mg Zn/kg. It therefore seems that isopods responded differently to ZnO and $ZnCl_2$ than other soil invertebrates, with less difference in sensitivity to the different Zn forms. This also means that for isopods, unlike other soil invertebrates, particles are not less toxic than ionic Zn. This could also mean that particulate Zn contributes more to the toxicity of ZnO NPs or non-nano ZnO than the free Zn ions. Whether this is due to a fast dissolution of particulate Zn in the isopod's intestinal tract leading to an increased exposure to free Zn ions or a direct effect of the particles cannot be concluded from these data.

Zinc exposure induced a decrease in isopod biomass independent of the Zn form present. Effects of $ZnCl_2$ on growth (mg/wk) of the isopod *P. scaber* exposed via contaminated food have been reported in the literature. The EC50 value found by van Straalen et al. [31] of around 30 µmol/g (corresponding to 1980 mg Zn/kg dry food) was closely related to the EC50 found by Donker et al. [32] of approximately 33 µmol/g (corresponding to 2230 mg Zn/kg dry food). These values are higher than the values found in the present study for effects on biomass change, suggesting that soil exposure is more effective at reducing isopod growth compared with food exposure. However, it is in fact hard to compare both routes of exposure.

In the present study, EC50 values reached the highest values at an intermediate pH of approximately 6.0. The lowest EC50 value was found in soil 1 (pH_{CaCl2} 4.5). Even at the lowest concentration (i.e., 250 mg Zn/kg), the isopods showed a drastic decrease in biomass when exposed to soil 1 for all Zn forms, which could be due to a physiological response of the animals to the low soil pH. Litter acidification has been shown to decrease microbial density on leaf material [33], whereas optimal microbial colonization was found at pH 5.0 (see references in [33]). Moreover, growth of the isopod *P. scaber* was influenced by leaf litter–colonizing microbiota when the organisms were fed on alder leaf [33]. Although we have no data on microbial

communities in the tested soils, the trend observed seemed to be more related to a physiological effect on the biomass. It was observed for isopods that the preference for soil pH was species-specific ($n = 5$) and that the preference ranged from pH 5 to pH 7 [34]. At present, no data on pH preference of *P. pruinosus* is available under laboratory conditions, making it hard to draw firm conclusions as to why ZnO and $ZnCl_2$ toxicity was lowest at the intermediate soil pH. Moreover, potential confounding factors may influence the results found. As indicated above, when soil pH is increased by addition of $CaCO_3$, the soil solution will contain less H^+, but at the same time, higher Ca^{2+} levels and increased Zn solubility. So, the effects of pH may be confounded by changes in the ionic strength in soil solution due to pH adjustment.

The competition of Zn with Ca may have resulted in lower toxicity for $ZnCl_2$ than for ZnO based on porewater concentrations. Calcium plays an important role in terrestrial crustaceans, especially in the formation of the exoskeleton [35]. Such organisms can absorb calcium either from food or from the cuticle itself (i.e., exuviae) [35]. During the premolting period, the calcium from the old cuticle is transported and stored as $CaCO_3$ deposits, until reuse to form a new cuticle [36]. However, in most soils, calcium is generally available in sufficient levels for isopods [37]. In terms of toxicity, Ca^{2+} may provide a protective effect by competing with Zn^{2+} in soil solution, decreasing metal toxicity, according to the biotic ligand model [11]. In accordance with the biotic ligand model, the free concentration of metal ions and other cations in soil solution, and their competition to bind to the receptors on the organisms, will be the factors driving toxicity [12, 38, 39]. The biotic ligand model considers the free metal ion to be the main metal form, being available for uptake and causing effects, with other cations reducing toxicity by competing for the same uptake sites. When the activities of different cations in the soil porewater are known, the biotic ligand model can help to explain the toxicity and uptake of metals. The model therefore can be applied to environmental risk assessment studies, enabling comparison of soils with different characteristics. However, one should keep in mind that not all organisms are equally exposed to soil solution and that other routes of exposure might also be important. When the 2 main routes of chemicals to soil invertebrates (oral and dermal) are considered, dermal uptake is less important for organisms with exoskeletons compared with oral uptake via the porewater [40]. Exchange of ions with the surrounding medium occurs through the uropods, located on the ventral abdomen. Uptake of ionic zinc ($^{65}ZnCl_2$) in *P.*

scaber was shown to be similar in food (gut route) and soil exposures (gut and pleopod routes), indicating a low contribution of the pleopods as an uptake route [41]. For oral uptake, it is hard to distinguish the contributions of porewater and soil particles. Isopods mainly obtain water needs from the diet, but they can also absorb water from humid surfaces [42]. From our data, it seems that porewater concentrations do affect toxicity, at least when the EC50 values are considered, but that other routes of exposure cannot be excluded.

For the collembolan *F. candida*, which is mainly exposed through the soil porewater [9], toxicity decreased with increasing soil pH for all Zn forms (30 nm and 200 nm ZnO, and $ZnCl_2$), and the EC50 for effects on reproduction was significant lower for $ZnCl_2$ than for ZnO particles[43]. However, when EC50 values were based on Zn in porewater or free Zn^{2+} ion concentrations, the same authors found that the EC50 for $ZnCl_2$ was higher than that for ZnO particles. The results were attributed to the protective effect of calcium, competing with Zn^{2+} ions and reducing toxicity (once calcium levels in porewater were much higher for $ZnCl_2$-spiked soils), combined with the decrease in pH values of $ZnCl_2$-spiked soils, which resulted in competition between Zn^{2+} and H^+ ions [43].

Differences between LC50 or EC50 based on Zn concentration in soil and porewater were also found for survival and reproduction of *E. fetida* [22]. In general, LC50 and EC50 values based on total concentration in soil were higher for ZnO particles than for $ZnCl_2$; however, when such values were based on porewater concentrations, ZnO particles showed lower results than $ZnCl_2$. The results provided by Waalewijn-Kool et al. [43] and Heggelund et al. [22] are in accordance with the present results. It is possible that such differences in EC50s between total soil concentrations and porewater concentrations might suggest a particle effect [22, 43], but these differences could also be explained by a protective effect of calcium in $ZnCl_2$ exposures following the principles of the biotic ligand model [43]. The EC50 values for both ZnO NPs and ionic Zn found for the isopods in the present study were much lower than the values found by Waalewijn-Kool et al. [43] for the collembolans, possibly due to the dual potential uptake routes by soil ingestion and porewater.

The effect of pH on the bioavailability of metals is less pronounced for organisms with complex uptake routes (e.g., ingestion of soil particles) compared with soil solution exposure [38]. Vijver et al. [41] showed that the main route of exposure to ionic zinc (as $ZnCl_2$) for the isopod *P. scaber* was

the ingestion of contaminated soil particles (i.e., oral). In the case of NPs, an even more complex scenario could be expected, as not only dissolution into ions but also aggregation or agglomeration of NPs will occur.

4.3. Zinc body content

Zinc body content was found to increase in a dose-dependent way in isopods exposed to 30 nm ZnO, 200 nm ZnO, and $ZnCl_2$ in all tested soils. The increase in internal concentration indicates that the animals are storing zinc. The hepatopancreas of isopods is composed of small (S) and big (B) cells, whose functions are absorption and absorption/secretion, respectively [44]. While B cells secrete granules containing metals into the hepatopancreas tubules to be excreted through the feces, S cells will accumulate the metals [45]. It has been shown that zinc will form granules in both B and S cells of *P. scaber* [44]. However, the capacity to excrete metals is species-dependent[46].

Effects of pH on Zn body content were not observed when the data were analyzed by ANOVA. The only significant difference for $ZnCl_2$ was found to be between soil 1 (pH_{CaCl2} 4.5) and LUFa soil (pH_{CaCl2} 5.5), indicating that soil properties other than soil pH may also be responsible for the difference. Similarly, soil pH did not affect ionic Zn accumulation in the earthworm *L. rubellus* [11]. For *E. fetida*, however, the bioaccumulation factor was lower at a higher pH of 7.3 (e.g., soil 3) for all Zn forms (30 nm and 200 nm ZnO and ionic zinc) than at a lower pH [22]. The absence of a clear pH effect might suggest that soil ingestion is an important route of exposure in isopods.

Reductions in growth or reproduction occur when energy must be diverted to detoxification processes [45]. Effects of zinc on isopod growth are rather dependent on the fluxes of zinc between pools, which are divided into an active pool and a storage pool [31]. Metabolic processes in the active pool transfer zinc from the active to the storage pool, up to a limit at which storage is no longer possible and free zinc ions can cause damage to the animal, resulting in growth reduction [31]. Thus, no relation could be found between growth of isopods and zinc content in the hepatopancreas, once growth reduction was dependent on the fluxes of zinc between the pools rather than total Zn body content [31]. Mortality, however, could be related to zinc concentration in the hepatopancreas [31].

Our results are in agreement with these findings. No difference in mortality and zinc body content between soils was found for any zinc form. Growth,

however, was affected by soil pH, and could not be related to zinc body content. Zinc body content did not differ between different soil pH levels, which may indicate that the isopods were able to store similar Zn quantities, but yet the effects (EC50s) occurred at different soil concentrations. Zinc body content therefore could not predict sublethal toxicity, being in agreement with previous studies on the growth rate of *P. scaber* exposed to $ZnCl_2$-contaminated food [31, 32]. In the earthworm *Eisenia veneta*, $ZnCl_2$ was found to be more toxic than ZnO NPs (e.g., reproduction and immune activity); however, Zn body content was comparable between the 2 Zn forms [16]. Similarly, greater Zn uptake was found with ZnO (30 nm and 200 nm) compared with ionic Zn in *E. fetida*; however, ionic Zn was more toxic[18]. The relation between sublethal effects and Zn uptake is more complex for ZnO NPs than for ionic zinc [16, 22].

The critical body concentration was found to be 25 g Zn/kg in the hepatopancreas of *P. scaber* before causing death by poisoning [47]. Van Straalen et al. [31] transformed these data and found an equivalent critical total body concentration of 1660 mg Zn/kg dry weight. This critical value is comparable to the maximum Zn levels found in *P. pruinosus* in the present study (Figure 3).

Although zinc body content was found to be slightly higher in animals exposed to $ZnCl_2$, the levels were quite comparable to those of ZnO particles. Likewise, *P. scaber* feeding on ZnO NPs and $ZnCl_2$-contaminated food showed no differences in zinc body content, which was dose-dependent [17]. Pipan-Tkalec et al. [17] concluded that the isopods accumulated zinc in the same manner for both zinc forms [17]. In the case of soil exposure, even though the distributions of NPs and ionic zinc are completely different in the soil matrix, Zn accumulation in the isopods was similar independent of the Zn form (ZnO and $ZnCl_2$).

The present study showed that soil pH affected the toxicity of ZnO NPs, non-nano ZnO, and $ZnCl_2$ to the isopod *P. pruinosus* in a similar way, with the lowest toxicity generally found at intermediate soil pH. Uptake of Zn did not seem to be affected by soil pH. There was little difference in Zn toxicity and Zn uptake between the different Zn forms, suggesting either a role of particulate ZnO in toxicity or a different contribution of routes of exposure, dependent on the Zn form. It seems that oral ingestion may contribute more to

uptake and effects of particulate ZnO, whereas the toxicity of ionic Zn will also be influenced by properties of the porewater.

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Tables list

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Figure 1- Soil pH_{CaCl2} and calcium (Ca^{2+}) levels (mg/L) in pore water of soils spiked with 30 nm ZnO, 200 nm ZnO and $ZnCl_2$. See Table 1 for soil properties.

Figure 2 – Food consumption (expressed as consumption ratio) of the isopod *Porcellionides pruinosus* exposed to different concentrations of 30 nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ in four different soils after two weeks (see Table 1 for soil properties). * represents significant differences with Dunnett's test ($p<0.05$).

Figure 3 – Zinc body concentrations (µg Zn/g dry body weight) of the isopod *Porcellionides pruinosus* as a function of total Zn soil concentrations after 2 weeks exposure to 30 nm ZnO, 200 nm ZnO and $ZnCl_2$ in four different soils (See Table 1 for soil properties). Each data point is the mean of three replicate samples. (•) soil 1, (❑) soil 2, (Δ) soil 3, and (X) Lufa 2.2 soil.

Table 1: Properties of Dorset soil with different amounts of calcium carbonate (w/w%) added and standard Lufa 2.2 soil[a].

Soil sample	$CaCO_3$ (w/w%)	Nominal pH_{CaCl2}	Measured pH_{CaCl2}	Organic matter (%)	CEC ($cmol_c$/kg soil)
Soil 1	0.20	4.5	4.5	7.39 ± 0.00	8.19 ± 0.74
Soil 2	0.45	5.9	6.2	7.63 ± 0.14	9.09 ± 0.05
Soil 3	1.00	7.2	7.3	7.65 ± 0.27	10.8 ± 0.73
Lufa 2.2 soil	-	-	5.1	4.35 ± 0.09	8.24 ± 0.34

a Data refer to nominal and actual measured pH values, organic matter content, and cation exchange capacity (CEC) (mean ± standard deviation; n = 2) before soils were spiked.

Table 2: Median lethal and effective concentration (LC50 and EC50, respectively; mg Zn/kg dry soil) values for the effects of 30 nm ZnO nanoparticles, 200 nm ZnO, and ZnCl2 on the survival and biomass change of *Porcellionides pruinosus* in four different soils[a]

Soil	LC50			EC50		
sample[b]	30 nm ZnO	200 nm ZnO	$ZnCl_2$	30 nm ZnO	200 nm ZnO	$ZnCl_2$
Soil 1	>3,369	2,277	2,352 [c]	713 A	119 [c] A	312 A
		(1,505-4,334)		(127-1,300)		(97-528)
Soil 2	2,586 [c]	2,551	3,732	1,479 A	1,951 [c] B	1,400 B
		(2,017-3,491)	(3,013-6,751)	(913-2,046)		(886-1,913)
Soil 3	1,757	2,169	1,792 [c]	904 A	974 [c] C	783 [c] C
	(1,339-2,351)	(1,628-2,899)		(533-1,274)		
Lufa 2.2 soil	3,361	2,894 [c]	2,292	788 A	1,405 [c] B,C	687 A,B,C
	(2,593-4,839)		(1,698-3,229)	(117-1,458)	(670-2,141)	(332-1,042)

a All values are based on measured Zn concentrations; 95% confidence intervals are given in parentheses. Letters (A, B, C) indicate significant differences between EC50 values for the different soils as determined by a generalized likelihood ratio test.
b See Table 1 for soil properties.
c Not possible to calculate reliable 95% confidence intervals.

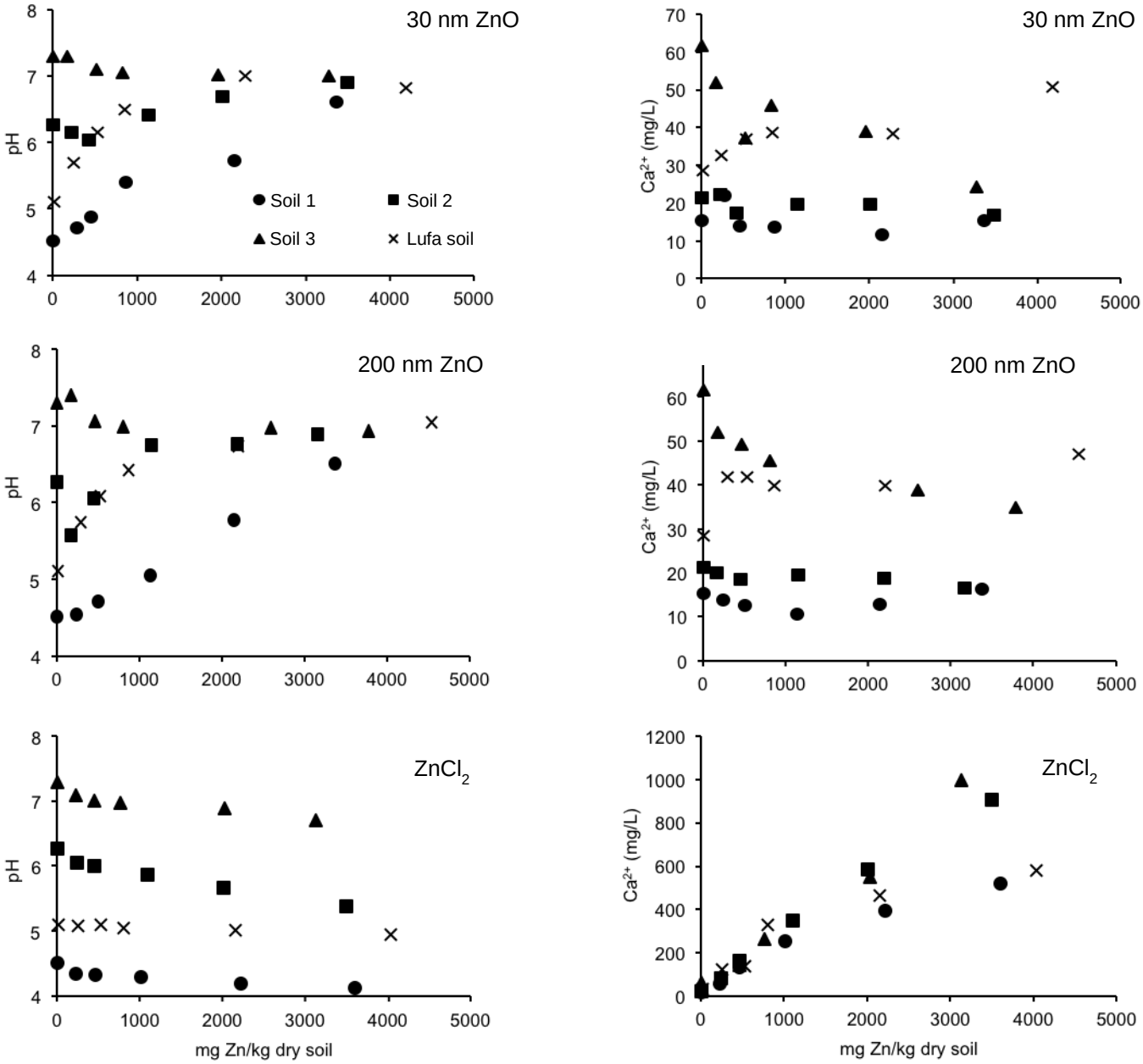

Figure 1- Soil pH_{CaCl2} and calcium (Ca^{2+}) levels (mg/L) in pore water of soils spiked with 30 nm ZnO, 200 nm ZnO and $ZnCl_2$. See Table 1 for soil properties.

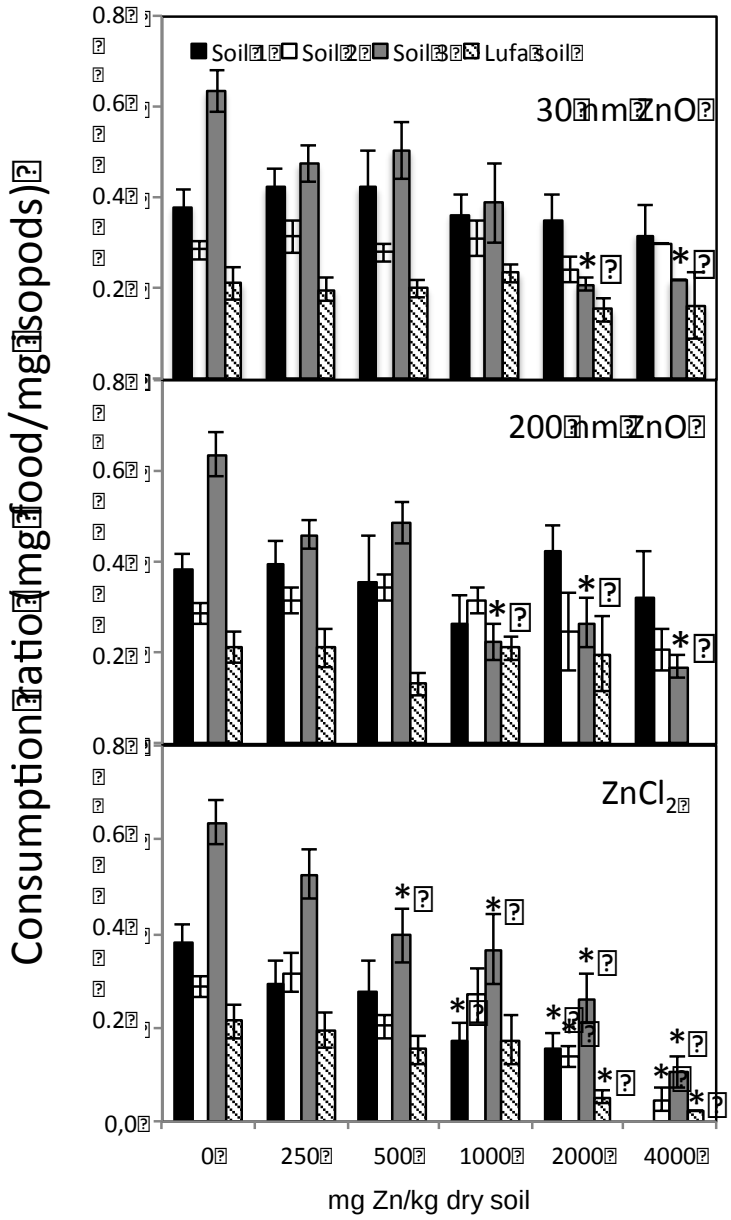

Figure 2 – Food consumption (expressed as consumption ratio in mg food/mg isopod) of the isopod *Porcellionides pruinosus* exposed to different concentrations of 30-nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ in 4 different soils after 2 wk (control soil [CT]; Soil 1, pH 4.5; Soil 2, pH 6.2; Soil 3, pH 7.3; LUFA 2.2 soil, pH 5.1). *Represents significant differences by Dunnett's test ($p < 0.05$).

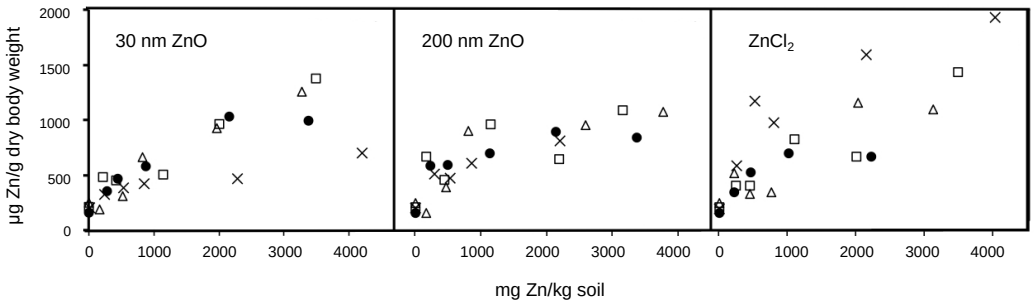

Figure 3 – Zinc body concentrations (mg Zn/kg dry body wt) of the isopod*Porcellionides pruinosus* as a function of total Zn soil concentrations after 2-wk exposure to 30 nm ZnO, 200 nm ZnO, and $ZnCl_2$ in 4 different soils (see Table 1 for soil properties). Each data point is the mean of 3 replicate samples. (•) Soil 1, pH 4.5; (□) Soil 2, pH 6.2; (Δ) Soil 3, pH 7.3; and (X) LUFA 2.2 soil, pH 5.1.

Supporting information

Influence of soil pH on the toxicity of zinc oxide nanoparticles to the terrestrial isopod *Porcellionides pruinosus*

Paula S. Tourinho, Cornelis A.M. van Gestel, Stephen Lofts, Amadeu M.V.M. Soares, and Susana Loureiro

Table S1: Measured total soil and pore water Zn concentrations in four different soils spiked with ZnO NPs, non-nano ZnO and $ZnCl_2$ for determining the toxicity to *Porcellionides pruinosus*. Also included is pH of the pore water.

Table S2: LC50 and EC50 based on zinc concentration in porewater (mg Zn/L) for the effects of 30 nm ZnO NPs, 200 nm ZnO and $ZnCl_2$ on the survival and biomass change in *Porcellionides pruinosus* after 14 days exposure in four different soils. Differences between soils and Zn forms could not be estimated, as confidence intervals could not be calculated. See Table 1 for soil properties.

Table S3: LC50 and EC50 based on free zinc ion concentration in pore water (uM) for the effects of 30 nm ZnO NPs, 200 nm ZnO and $ZnCl_2$ on the survival and biomass change in *Porcellionides pruinosus* after 14 days exposure in three different soils. Differences between soils and Zn forms could not be estimated, as confidence intervals could not be calculated. See Table 1 for soil properties.

Table S1: Measured total soil and pore water Zn concentrations in four different soils spiked with ZnO NPs (30 nm), non-nano ZnO (200 nm) and $ZnCl_2$ for determining the toxicity to *Porcellionides pruinosus*. Also included is pH of the pore water.

	Nominal concentration (mg Zn/kg)	Measured concentration (mg Zn/kg)				Porewater concentration (mg Zn/L)				Porewater pH			
		Soil 1	Soil 2	Soil 3	Lufa 2.2	Soil 1	Soil 2	Soil 3	Lufa 2.2	Soil 1	Soil 2	Soil 3	Lufa 2.2
	Control	4.2	4.0	4.4	11.8	1.93	0.10	0.57	3.68	5.09	6.69	7.03	6.22
30 nm ZnO	250	281	219	169	243	9.29	5.11	0.37	8.61	4.94	6.65	6.95	6.39
	500	451	425	518	532	7.01	2.62	1.23	6.35	5.55	6.59	7.25	6.48
	1000	871	1,142	828	848	9.11	4.39	2.96	7.37	5.80	6.89	7.14	7.35
	2000	2,161	2,011	1,961	2,285	13.3	6.00	6.47	7.37	6.51	7.26	7.25	7.70
	4000	3,369	3,492	3,279	4,196	18.1	12.1	13.7	8.21	7.14	7.35	7.43	6.5
200 nm ZnO	250	239	168	173	293	2.85	1.39	0.37	4.64	5.38	6.15	6.73	6.61
	500	503	453	470	531	5.82	2.41	1.16	5.99	5.42	6.51	7.07	6.74
	1000	1,138	1,153	805	866	9.23	3.36	2.9	6.78	5.79	6.95	7.10	7.41
	2000	2,142	2,193	2,595	2,201	13.5	7.75	7.10	6.75	6.55	6.97	7.26	7.74
	4000	3,369	3,160	3,780	4,542	21.6	12.1	17.8	8.28	6.94	7.19	7.35	7.73
$ZnCl_2$	250	228	244	221	256	9.72	1.66	0.74	25.6	4.47	6.07	7.1	5.5
	500	467	457	457	524	254	8.98	1.59	105.5	4.34	6.04	6.94	5.26
	1000	1,016	1,099	768	805	178	54.6	8.88	320	4.12	5.56	6.77	5.1
	2000	2,221	2,011	2,031	2,154	803	300	70.8	1,147	3.81	5.20	6.79	4.85
	4000	3,601	3,499	3,136	4,034	2,890	1,660	370	-	3.64	4.91	6.51	4.60

Table S2: LC50 and EC50 based on zinc concentration in porewater (mg Zn/L) for the effects of 30 nm ZnO NPs, 200 nm ZnO and $ZnCl_2$ on the survival and biomass change in *Porcellionides pruinosus* after 14 days exposure in four different soils. Differences between soils and Zn forms could not be estimated, as confidence intervals could not be calculated. See Table 1 for soil properties.

	LC50			EC50		
	30 nm ZnO	200 nm ZnO	$ZnCl_2$	30 nm ZnO	200 nm ZnO	$ZnCl_2$
Soil 1	-	15.3	1,194	9.06	2.28	250
Soil 2	9.96	9.46	1,829	5.91	2.84	204
Soil 3	6.56	8.72	169	4.21	3.23	35.9
Lufa soil	9.27	7.53	2,022	-	-	99.9

Table S3: LC50 and EC50 based on free zinc ion concentration in pore water (uM) for the effects of 30 nm ZnO NPs, 200 nm ZnO and $ZnCl_2$ on the survival and biomass change in *Porcellionides pruinosus* after 14 days exposure in three different soils. Differences between soils and Zn forms could not be estimated, as confidence intervals could not be calculated. See Table 1 for soil properties.

	LC50			EC50		
	30 nm ZnO	200 nm ZnO	$ZnCl_2$	30 nm ZnO	200 nm ZnO	$ZnCl_2$
Soil 1	-	6.00	15,000	-	-	449
Soil 2	32.0	4.00	24,000	-	-	3,000
Soil 3	1.00	1.00	2,000	0.59	0.41	37.8

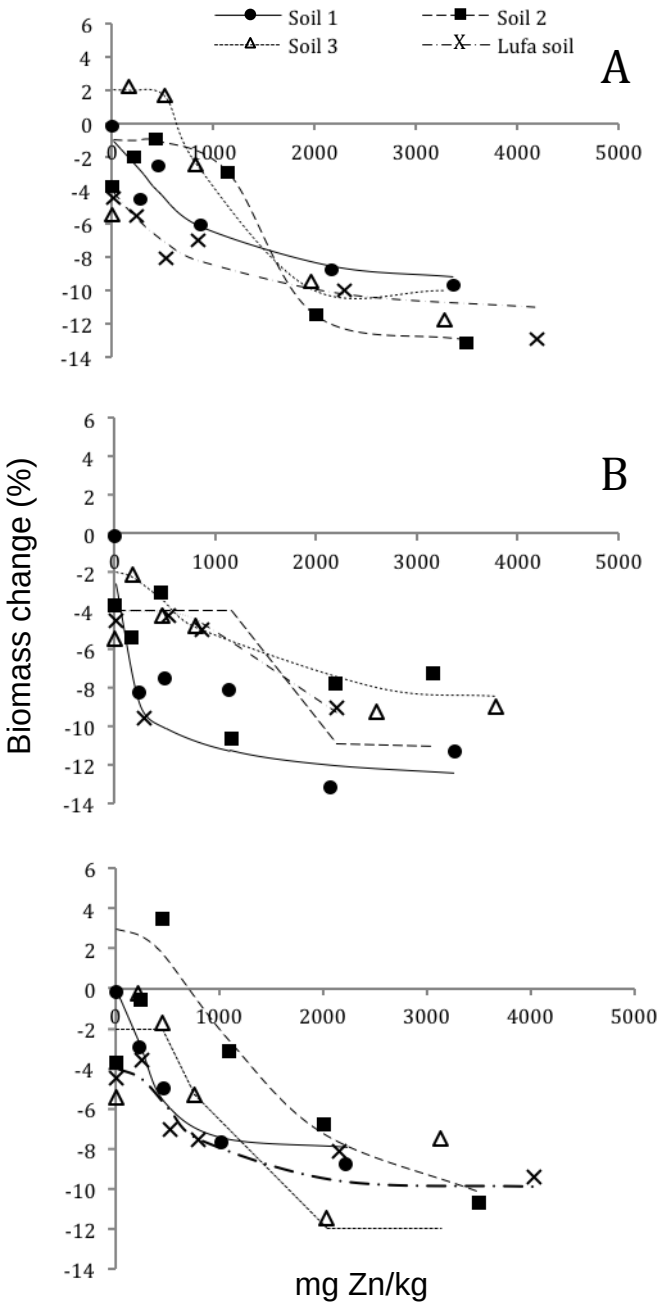

Figure S1 - Effects of 30 nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ on biomass change of the isopod *Porcellionides pruinosus* after 14 days exposure in four different soils (see Table 1). Lines represent the fit obtained with a logistic model for the different soils.

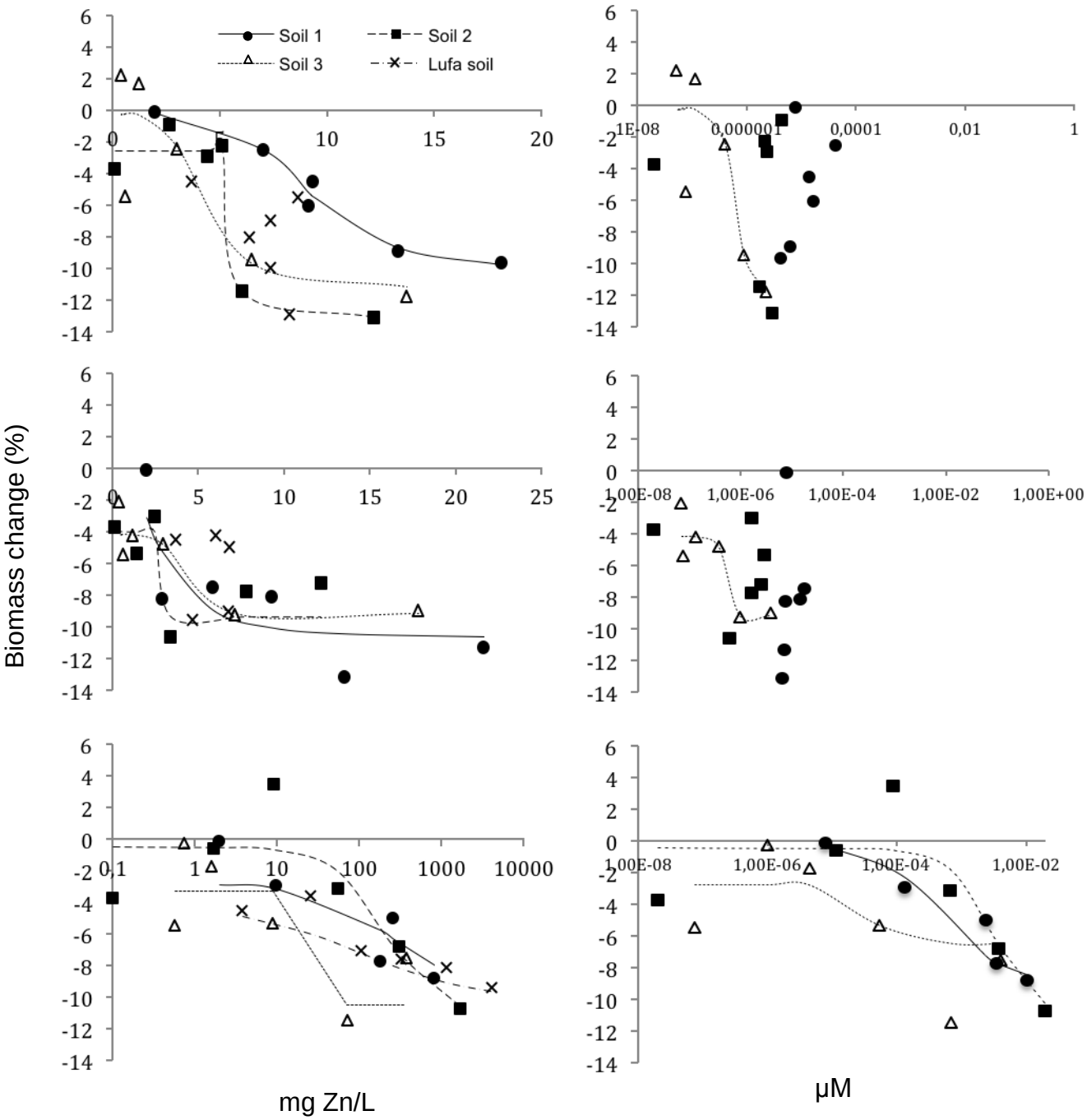

Figure S2 - Effects of 30 nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ on biomass change of the isopod *Porcellionides pruinosus* after 14 days exposure in four different soils (see Table 1). Biomass change is related to Zn concentrations in porewater (left) and free zinc ion concentrations calculated by WHAM7 (right) (plot in log scale for $ZnCl_2$) Lines represent the fit obtained with a logistic model for the different soils.