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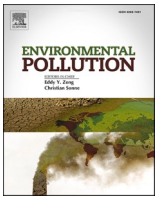

Soil properties influence the toxicity and availability of Zn from ZnO nanoparticles to earthworms☆

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ABSTRACT

To develop models that support site-specific risk assessment for nanoparticles (NPs), a better understanding of how NP transformation processes, bioavailability and toxicity are influenced by soil properties is needed. In this study, the influence of differing soil properties on the bioavailability and toxicity of zinc oxide (ZnO) NPs and ionic Zn to the earthworm *Eisenia fetida* was investigated. Earthworms were exposed to ZnO_NPs and ionic Zn, between 100 and 4400 mg Zn/kg, in four different natural soils (organic matter content: 1.8–16.7%, soil pH: 5.4–8.3, representing sandy loam to calcareous soils). Survival and reproduction were assessed after 28 and 56 days, respectively. Zn concentrations in soil pore waters were measured while labile concentrations of Zn were measured using an in-situ dynamic speciation technique (diffusive gradient in thin films, DGT). Earthworm Zn tissue concentrations were also measured. Soil properties influenced earthworm reproduction between soil controls, with highest reproductive output in soils with pH values of 6–7. Toxicity was also influenced by soil properties, with EC_{50}s based on total Zn in soil ranging from 694 to >2200 mg Zn/kg for ZnO_NP and 277–734 mg Zn/kg for ionic Zn. Soil pore water and DGT measurements showed good agreement in the relative amount of Zn extracted across the four soils. Earthworms exposed to ZnO_NPs survived higher Zn concentrations in the soils and had higher tissue concentrations compared with ionic Zn exposures, particularly in the high organic content calcareous soil. These higher tissue concentrations in ZnO_NP exposed earthworm could have consequences for the persistence and trophic mobility of Zn in terrestrial systems and need to be further investigated to elucidate if there any longer-term risks associated with sustained input of ZnO_NP to soil.

1. Introduction

Zinc oxide nanoparticles (ZnO_NPs) can reach soil through direct addition (e.g. application of Zn incorporated into fertilisers) (Milani et al., 2015; Rodrigues et al., 2017; Sun et al., 2020) or as trace constituents of industrial or domestic waste materials and sewage sludge applied as a soil conditioner (Gottschalk, Sonderer et al., 2009, Ma et al., 2014). Thus, soil can act as a sink for these released NPs. Soils vary in a number of key properties such as pH, organic matter content (OM), cation exchange capacity (CEC), clay content, and manganese and iron oxide levels, all of which have been shown to affect chemical bioavailability and toxicity (Lock and Janssen, 2003; Smolders et al., 2004; Smolders et al., 2009; Qiu and Smolders, 2017). For a number of metals (e.g. Zn, Cu, Ni, Cd), understanding of soil property effects on toxicity has been used to derive models for effect prediction that integrate bioavailability concepts (e.g. terrestrial biotic ligand model, t-BLM, tissue residue approach, TRA) (Lock et al., 2006; Thakali et al., 2006a; Thakali et al., 2006b; Lock et al., 2007; Meador et al., 2011; Lofts et al., 2013), including for formal risk assessment (Sauvé et al., 2000; Smolders et al., 2009). The influence of soil properties on NPs bioavailability and toxicity has been less well studied, and the appropriateness of the models developed for trace and other metals to be applied to NPs has not yet been verified.

On entering soils, metal-based NPs may undergo transformation

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reactions including solid-phase homo- and hetero-agglomeration and dissolution to release ions (Spurgeon et al., 2020; Svendsen et al., 2020). Bioavailability–based models can describe the effects of released ions. However, the extent to which processes governed by the specific properties of a given soil affect NP form, bioavailability and toxicity for soil organisms needs to be fully elucidated. There is evidence that NP toxicity and bioaccumulation are driven by particle dissolution (Heggelund et al., 2014; Talaber et al., 2020; Song et al., 2022). This assertion is based on relating observed toxicity to measured soil pore water metal concentrations, assuming that exposure from soil pore water is the dominant uptake route for both NPs and released metal ions (Kool et al., 2011; Heggelund et al., 2014; Diez-Ortiz et al., 2015a). If the NP toxicity is driven by the release of metal ions, then existing bioavailability–based models could be adapted to model effects of NPs, given information on dissolution rates (Khan et al., 2015; Qiu and Smolders, 2017). However, when investigating Ag NP availability in soils this assumption has been questioned, where pore water concentrations do not reflect uptake patterns (Baccaro et al., 2021). Other studies have pointed instead towards the importance of intestinal uptake of both ionic Ag and Ag NPs in the earthworm, *Lumbricus rubellus* (Diez-Ortiz et al., 2015b; Makama et al., 2016). Indeed the use of biotic ligand models (BLM) and tissue residue approaches (TRA) has been confounded by this ingestion of NPs, which complicates the relationship between tissue concentrations and acute toxicity (Khan et al., 2015).

To date only a limited number of studies have examined the relationship between soil properties and NP toxicity and bioavailability with a view towards developing a mechanistic understanding of NP toxicity. Soil pH has been shown to be a key variable in determining ZnO_NP toxicity to earthworms, springtails and soil bacterial communities in soils (Waalewijn-Kool et al., 2013b; Heggelund et al., 2014; Read et al., 2016; García-Gómez et al., 2018; García-Gómez et al., 2020). Two studies have used diverse natural soils, rather than artificial amendment of a single soil property (Waalewijn-Kool et al., 2014; Romero-Freire et al., 2017). These two studies, however, reached different conclusions, one showing pH and dissolved organic carbon (DOC) in the pore water have greater influence on ZnO_NP behaviour and toxicity while the other did not find clear relationships between toxicity and soil properties. This highlights the need for further systematic studies to address the uncertainties about how varying soil properties drive of bioavailability and toxicity for different NPs, and whether the observed trends are similar between metallic NPs and their ionic counterparts.

To address this topic, this study investigates the bioavailability and toxicity of ZnO_NPs to the earthworm, *Eisenia fetida*, in four natural soils with differing major properties (e.g. pH, %OM). Earthworms are key species in soil ecosystems and are continually exposed to the soil solid phase by ingestion (oral) and the pore-water phase of the soils through both ingestion and dermally via their skin. Hence, these taxa are an ideal group with which to investigate the effects of soil properties on metal NPs and their resulting bioavailability and toxicity in different soil types. The effects of ZnO_NP and ionic Zn on earthworm survival and reproduction were assessed in four soils and Zn tissue concentrations measured to evaluate the bioavailability of Zn forms in the soils. To track exposure form in the test medium soil pore water Zn concentrations were measured via two extraction methods: 1) centrifugation and filtration and 2) diffusive gradients in thin-films (DGT). DGT is an established, *in situ* method for measuring labile concentrations of metals and metalloids in terrestrial and aquatic systems (Zhang and Davison, 2015). Here we assess its suitability for measuring ZnO_NPs and released Zn ions (Pouran et al., 2014; Pouran et al., 2021). Collectively, the quantification of total Zn soil concentrations, pore water concentrations and dissolution will help elucidate the effect of soil properties on relative Zn availability across soils and Zn forms. Finally, we can use these measurements, together with the toxicity measurements, to compare how well the modelled relationship between soil properties, pore water metal concentrations and toxicity for ionic Zn can predict these relationships for ZnO_NP (Lofts et al., 2004).

2. Materials and methods

2.1. Soils

Four natural soils with a range of different pH levels (pH 5.4–8.3) and organic matter contents (1.8–16.7%) were used. Three soils were collected from an agricultural (Woburn), pasture (North Wales) and calcareous grassland (Chiltern) sites in the UK and the fourth was a commercially-sourced agricultural soil (Lufa 2.2) from Germany (LUFA Speyer, Germany) widely used for toxicity testing. As both pH and organic matter content were viewed as possible key variables affecting bioavailability, the values for these two parameters are indicated for clarity throughout the text hereafter for each soil as ^{pH}Soil name$_{Organic\ matter\%}$ (e.g. as $^{6.9}$Woburn$_{1.8\%}$) (Table S1). After field collection, all soils were homogenized, air-dried and sieved through a 2 mm mesh. An amount of 550 g dry weight (d.w.) of soil, held in a 183 × 120 × 70 mm polypropylene container, was used for each test replicate for all treatment levels. Soils had only relatively small differences in bulk density; hence total soil volume was similar between the soils.

2.2. Experimental animals

Eisenia fetida obtained originally from a commercial source (Blades Biological, Kent, UK) were maintained in a culture soil constituting 33% loamy soil, 33% peat and 33% composted bark on a volume basis at 20 ± 1 °C in a 12:12 h light:dark cycle. Earthworm cultures were fed fresh horse manure free from contamination or veterinary medication. All earthworms used were reared under these conditions for eight weeks to ensure that they were fully-clitellated adults and of a suitable size (300–600 mg) for testing (OECD, 2004).

2.3. Nanomaterials and metal salt

The ZnO_NP selected for this experiment was NanoSun ZnO P99/30 obtained from Microniser Pty Ltd (Dandenong, Australia). The particle has a stated average particle size of 30 nm. The NanoSun P99/30 ZnO has no coatings or surface modifications and was supplied as a dry white powder of particles of near spherical shape. The material is in commercial use in products such as sunscreens and has also been proposed for use as a soil fertilizer for zinc deficient agricultural soils (Milani et al., 2015). The ZnO_NPs used in these sets of experiments were the same as those used by Heggelund et al., 2014) and were the same batch (Heggelund et al., 2014). To confirm that the ZnO_NP batch had not changed during storage, samples were analysed using TEM to confirm that primary particle size and aggregation state (Fig. S1). Zinc nitrate ($Zn(NO_3)_2$) (Sigma Aldrich, Poole, UK) was used as the zinc metal ion (ionic Zn) treatment.

2.4. Experimental design and dosing

The toxicity test procedure followed the OECD guideline 222 for assessing effects on earthworm reproductive output. Exposure concentrations used for both Zn forms were 0, 100, 225, 500, 1100, 2200, mg Zn kg^{-1} (d.w. soil), with the exception of $^{5.9}$Lufa 2.2$_{4.8\%}$ exposed to ZnO_NP which had a concentration range 0, 225, 500, 1100, 2200, 4400 mg Zn kg^{-1} (d.w. soil). Three replicate containers were set up for each treatment concentration. Since the ionic and NP exposure in each soil were always run concurrently, effect could be benchmarked against a universal control treatment; six replicates of soil without Zn amendment.

ZnO_NPs were dosed to the test soils as dry powders to avoid NP transformations occurring during suspension in a stock solution, so that only processes in the soil were included. (Waalewijn-Kool, Diez Ortiz et al., 2012). To ensure a homogenous distribution of NPs throughout the soil, initially the amount of NP powder required was added to 50 g of test soil and thoroughly mixed. This spiked soil aliquot was then added

to the remaining test soil and further mixed. In order to dose the ionic Zn, stock solutions of $Zn(NO_3)_2$ were added to each soil to give the required soil concentrations. For all replicates, MilliQ water was added to the soil to raise the moisture content to 50% of the water holding capacity (WHC). Soils were then left for one week to allow interactions with the soil solid phase and pore water components before the test organisms were added, in accordance with OECD 222 guideline for metal salts (Heggelund et al., 2014; Diez-Ortiz et al., 2015a).

2.5. Toxicity test procedure

The toxicity test procedure followed OECD guideline and (Diez-Ortiz et al., 2015a). In brief, ten adult earthworms (average weight = 0.451 ± 0.064 g, n = 145) were added to each replicate. Ten earthworms per replicate were rinsed, blotted dry and weighed as a batch before being placed onto the soil surface. As food, 10 g dry weight of horse manure wetted to 80% WHC was added to the soil surface. All containers were then placed in a controlled temperature (CT) room (20 ± 1 °C in a 12:12 h light:dark cycle) for a total of 56 days. Over the duration of the test, soils were checked for moisture loss every two weeks and additional water added as needed to maintain a consistent soil moisture level over the exposure. After 14 days and 28 days, the containers were sorted and the numbers of earthworms alive in each counted and all earthworms weighed as a batch (see SI). At the end of the 28 days, three earthworms from each replicate were allow them to purge their gut contents for 24 h to allow at least three full gut transit periods, based on available information of gut transit times for *Eisenia andrei,* a closely related species to *Eisenia fetida* (Cosín et al., 2002; Fleuren et al., 2003), while limiting potential losses due to the fast elimination Zn by *Eisenia fetida* (Spurgeon and Hopkin, 1999). After washing, these purged earthworms were frozen at −20 °C for tissue metal analysis.

Following adult removal, all test soils were returned to the constant temperature (CT) facility for a further 28 days to allow any laid cocoons to hatch. On removal, soil samples were collected for measuring total metal in the soils, as well as for soil pore water extraction and DGT. To count juvenile numbers, the containers were placed in a water bath at 60 °C for 15 min to force individuals to the soil surface (OECD, 2004). Based on the number collected, reproduction could then be expressed as a juvenile production rate (juveniles/earthworm/week).

2.6. Soil pore water extraction and DGT deployment

Pore water samples were collected from all soils at the end of the 56-day exposure period. A 25 g (d.w. equivalent) aliquot of the sampled soil was initially saturated to 100% WHC. After overnight equilibration, a pore water sample was extracted from this soil by a centrifugation filtration at 4000*g* for 1.5 h (J2-HC, Beckman Coulter, California, USA) following a published method (Diez-Ortiz et al., 2015a) but without Cu-soaking of the filter unit. Once extracted, the three replicate samples per treatment were pooled for analysis to give a single pore water total Zn measurement for each treatment. To separate the particulate fraction from the soluble metal in the pore water, a sub-sample of extracted pore water was placed in a 10 kDa ultra-filtration device (Amicon Ultra-15 Filters, Millipore, Ireland) and centrifuged for 1.5 h at 4000 g. The ultra-filtrate (UF) was collected to measure soluble Zn concentrations in the pore water.

2.7. DGT deployment

A diffuse gradients in thin films (DGT) device is comprised of two key components; a diffusive hydrogel layer and a binding layer, which are protected by an external filter membrane and placed in a piston type of plastic housing (Zhang and Davison, 1995). The standard DGT devices were modified to add a dialysis membrane layer with a known molecular weight cut-off in front of the diffusive layer, acting in a similar way to the ultra-filtration devices used for the pore waters (nano-DGT). This study used Chelex® as the binding resin/layer and were equipped with 1 kDa dialysis membranes (thickness ≈0.05 mm) (Spectrum Biotech). These nano-DGT membranes were deployed along with standard DGT devices to determine both the particulate (DGT-labile) and dissolved Zn (nano-DGT) concentrations in the soil samples.

At the 56-day time point, 50 g soil from each replicate was collected before juvenile counting and stored at 4 °C in the dark until they were prepared for DGT deployment. To prepare the soil for DGT deployment, three replicate soils per treatment were wet to 90% WHC. Following 24 h equilibration, the DGT devices (with and without dialysis membranes) were deployed for about 18 h at 21 °C. At the end of the deployment, each DGT device was collected, and its binding layer was retrieved. The DGT concentration was calculated according to the methods that have been extensively described in other publications and can be found in the SI (Pouran et al., 2014; Davidson, 2016, Pouran et al., 2021). In these calculations, the nano-DGT devices with dialysis membrane provided the concentration of ionic Zn in the soil samples, and the standard DGT devices determined the total concentrations of Zn, including both NP and ionic forms.

2.8. Soil, soil pore water, DGT and earthworm chemical (including metal) analysis

Soil pore water pH was measured by Sartorius Professional Meter PP-25 (Sartorius AG, Goettingen, Germany; combination pH probe, filled with 3 M KCl). The total Zn concentrations in the soils and the earthworm tissue concentrations were digested and analysed as described in Lahive et al., 2017. The extracted pore water and ultra-filtered pore water were analysed for Zn using inductively coupled plasma mass spectrometry (ICP-MS) (Lahive et al., 2017). After DGT deployments, the binding layers were retrieved using acid-cleaned tweezers and immersed in 1 mL of 1.0 M ultrapure nitric acid for elution. After 24 h, eluted samples were diluted at least 10 times using MilliQ prior to Zn being measured using inductively coupled plasma mass spectrometry (ICPMS, Thermo X7 series). The mass of Zn in the binding layer, M, as well as time-averaged concentration, C_{DGT}, were obtained using available equations for DGT technique (Pouran et al., 2014). In these calculations the combined thickness of the filter membrane and the diffusive layer, the diffusion coefficient of the analyte, the duration of deployment and the area of the sampling window of the DGT device are considered (see SI).

2.9. Data analysis

Data for survival and reproduction were first checked for normal variance structure using the Anderson-Darling test. Concentration-specific effects on the proportion of surviving individuals and reproduction (as juvenile production rates) for each of the Zn forms in different soils were analysed using analysis of variance (ANOVA). Where significant differences were found, the Tukey test was used to identify significant differences between treatments (Minitab 16). If data were found to be non-normal, such as tissue concentrations, tests were performed on log-transformed data. To assess the influence of different factors (nominal concentration, Zn form and soil type) on earthworm reproductive output a generalised linear model was performed, including interactions between the factors (Minitab 16).

To estimate response parameters, data for survival and reproduction was used for least square fitting of a three-parameter log-logistic model (Equation (1)) to obtain estimate LC_{50} and/or EC_{50} values with standard errors in SigmaPlot 13.0.

$$y = y(max)/(1 + (c / EC(50))exp(b)) \quad (1)$$

where *ymax* is the upper asymptote, *c* is concentration in soil/pore water/earthworm, EC_{50} is the concentration resulting in a 50% effect on the measured endpoint and *b* the slope parameter. For the analysis of

survival data, a binominal distribution of data within each treatment was assumed, while for reproduction, a normal distribution was assumed. All concentration-response relationships were fitted using total soil Zn concentration, soil pore water or labile Zn concentration and earthworm tissue concentrations.

The conceptual model of ionic zinc toxicity in soils of Lofts and co–workers was extended to consider patterns of toxicity for ZnO_NPs (Lofts et al., 2004). To investigate the relationship between effect concentrations for ZnO_NP and ionic Zn exposures across soil types, a simple function expressing the log EC_{50} (μg/g) for ionic Zn exposure to the soil porewater pH and %OM content was fitted:

$$log\ EC50\ (\mu g\ /\ g) = a + b \bullet log(\%OM) + c \bullet pH_{pw} \quad (2)$$

and applied it to 12 literature EC_{50} values from Spurgeon and Hopkin (1996) and Lock and Janssen (2001) (see SI) (R Core Team, 2018).

3. Results

3.1. Earthworm survival and reproduction

Earthworm survival was greater than 90% in all soil control treatments, and more than 30 juveniles produced in each control replicate, meeting the validation criteria for both test endpoints (OECD, 2004). Control juvenile production was significantly influenced by the soil type ($F = 34.5$, $P < 0.05$). The two sandy loam soils $^{5.9}$Lufa $2.2_{4.2\%}$ and $^{6.9}$Woburn$_{1.8\%}$ showed the highest control production rates, while reproduction rates in the highest %OM soil $^{5.4}$North Wales$_{16.7\%}$ and calcareous soil $^{8.3}$Chiltern$_{14.7\%}$ were lowest (Fig. S2). To account for the influence of soil type on control reproduction, observed effects were expressed relative to the reproduction rate for the control treatments of the relevant soil.

The ZnO NPs showed lower toxicity relative to ionic Zn in all the soils, based on total soil concentrations (Table 1). For ionic Zn, LC_{50} values could be calculated in all soils except $^{8.3}$Chiltern$_{14.7\%}$ (Tables S6 and S7) and reproduction was significantly reduced by ionic Zn in all four soils (ANOVA: Zn: $^{8.3}$Chiltern$_{14.7\%}$ $F = 38.6$, $P < 0.05$; $^{5.9}$Lufa $2.2_{4.2\%}$ $F = 13.7$, $P < 0.05$; $^{6.9}$Woburn$_{1.8\%}$ $F = 4.58$, $P < 0.05$; $^{5.4}$North Wales$_{16.7\%}$ $F = 42.9$, $P < 0.05$ (Fig. S3). For the ZnO_NPs, no significant effects on survival were found in any soil up to the maximum concentrations tested. Earthworm reproduction was significantly reduced by ZnO_NP exposure in all soils, except $^{8.3}$Chiltern$_{14.7\%}$ (ANOVA: $P < 0.05$ all soils for Zn except for ZnO_NP in $^{8.3}$Chiltern$_{14.7\%}$: $F = 1.36$, $P = 0.293$) (Fig. S3). For both Zn forms, lowest toxicity was observed in the $^{8.3}$Chiltern$_{14.7\%}$ soil.

Total Zn concentration in the soil was found to be the primary factor influencing the observed effects on reproduction (GLM, $P < 0.01$) but there was an interaction between soil concentration and Zn form ($P < 0.001$), meaning Zn form was also driving the observed toxicity. Soil type alone was not a factor driving reproductive effects ($P > 0.05$), but soil type also interacted with Zn form and concentration to drive toxicity, mainly associated with the lower toxicity observed in the calcareous $^{8.3}$Chiltern$_{14.7\%}$ soil ($P < 0.05$).

3.2. Zinc concentrations in soil and soil pore waters (centrifugation extraction and DGT measurements)

Average recovery of Zn from the spiked soils was 96 ± 6.6% of the nominal concentrations for ZnO_NP and ionic Zn, validating the dosing. The total concentration of Zn in the pore waters and the total DGT-labile concentrations increased with increasing Zn concentration, for both Zn forms (Tables S1 and S2). For both pore water and standard DGT measurements, concentrations of Zn were lower in soils spiked with ZnO NP compared to ionic Zn. The relationship between the pore water zinc concentration and the standard DGT-labile concentration is consistent

Table 1
56-day reproduction EC_{50} values calculated for earthworms exposed in four different soils to ZnO_NP or ionic Zn. EC_{50} values were based on the total Zn soil concentration, Zn tissue concentration of the earthworms and the pore water concentrations.

	$^{5.4}$North Wales$_{16.7\%}$	$^{5.9}$Lufa $2.2_{4.2\%}$	$^{6.9}$Woburn$_{1.8\%}$	$^{8.3}$Chiltern$_{14.7\%}$
ZnO NP				
Total soil concentration (mg/kg)	842 ± 103	694 ± 136	751 ± 128	> 2200
Total pore water concentration (mg/l)	0.98 ± 0.08	1.78 ± 0.15	0.39 ± 0.01	n.d.
UF pore water concentration (mg/l)	1.06 ± 0.07	1.88 ± 0.06	0.34 ± 0.02	n.d.
Total DGT (mg/l)	8.34 ± 0.95	18.9 ± 2.87	1.88 ± 0.23	n.d.
UF DGT (mg/l)	7.03 ± 0.99	11.9 ± 2.09	1.10 ± 0.13	n.d.
DGT – NP (mg/l)	2.15 ± 1.74	11.9 ± 1.81	0.70 ± 0.17	n.d.
Tissue concentration (μg/g)	200-222	160 ± 13.6	210 ± 16.3	> 316
Ionic Zn				
Total soil concentration (mg/kg)	345 ± 36.2	374 ± 72.2	277 ± 31.4	734 ± 267
Total pore water concentration (mg/l)	5.06 ± 0.15	26.3 ± 2.66	5.37 ± 2.32	0.46 ± 0.45
UF pore water concentration (mg/l)	4.65 ± 0.197	27.9 ± 2.21	5.64 ± 2.51	1.07 (n.d.)
Total DGT (mg/l)	5.5 ± 0.786	18.8 ± 3.76	1.74 ± 0.68	0.83 ± 0.49
UF DGT (mg/l)	4.46 ± 0.59	5.1 ± 3.12	1.5 ± 0.61	0.48 ± 0.45
Tissue concentration (μg/g)	> 190	151 ± 3.6	159 ± 3.9	147 ± 5.4

across all the soils for both ionic Zn and ZnO_NP additions, with no indication of an effect of soil type or chemistry. However, the relationship between pore water and DGT-labile concentrations differs depending on the form of the added zinc (Fig. 1). In the case of the ionic Zn exposures, there is good agreement between porewater and DGT-labile zinc up to a porewater concentration of ~50 mg Zn/L, above which the DGT labile concentration becomes smaller than the porewater concentration. At the highest porewater concentration found (2320 mg Zn/L in $^{6.9}$Woburn$_{1.8\%}$ at an addition of 2200 mg/kg), the corresponding DGT-labile concentration is over an order of magnitude lower (73.6 mg Zn/L). In contrast, DGT-labile concentrations in the nanoparticle-spiked soils largely exceed those in the porewater, although the range of concentrations is smaller (up to 10 mg Zn/L, versus up to > 1000 mg Zn/L in the ionic Zn-spiked soils).

Of the four soils, the calcareous $^{8.3}$Chiltern$_{14.7\%}$ soil had the lowest Zn concentrations in pore water. The two sandy-loam soils with the lowest % OM, $^{6.9}$Woburn$_{1.8\%}$ and $^{5.9}$Lufa 2.2$_{4.2\%}$, had the highest pore water concentrations when spiked with ionic Zn. $^{5.9}$Lufa 2.2$_{4.2\%}$ had the highest pore water concentrations when spiked with ZnO NP of all the soils, followed by the soil with the lowest pH, $^{5.4}$North Wales$_{16.7\%}$. For soils spiked with ionic Zn, the pore water extracted by standard DGT had similar or lower Zn concentrations compared to the centrifugation extraction method, as expected (Fig. 1). Conversely, the standard DGT extracted more Zn from the soils in the ZnO NP spiked soils compared to the pore water extraction for all four soils, although to a lesser extent in the $^{8.3}$Chiltern$_{14.7\%}$ soil (Fig. 1).

The Zn concentrations in the soil pore waters (total) and the concentrations in ultra-filtered (UF) samples were similar (Table S3). However, although the concentrations in the DGT extracts were higher, there was no significant differences between the standard DGT extract and the Zn concentration in the nano-DGT extract, indicating both methods were extracting Zn in a similar form, mainly dissolved Zn (Table S4, Fig. 2).

EC_{50} values for the effects of both Zn forms on reproduction were calculated based on the exposure metrics of total pore water concentrations and total DGT-labile concentrations, in both cases with and without ultrafiltration (Table 1, Figs. S4 and S5). For both Zn forms and both measurement approaches (with/without UF), the highest reproduction EC_{50}s were calculated for $^{5.9}$Lufa 2.2$_{4.2\%}$ soil. For ionic Zn, the EC_{50} values determined from pore water or DGT measurements were comparable in the soils with higher OM content ($^{8.3}$Chiltern$_{14.7\%}$ and $^{5.4}$North Wales$_{16.7\%}$). In soils where the %OM content was low ($^{6.9}$Woburn$_{1.8\%}$ and $^{5.9}$Lufa 2.2$_{4.2\%}$), the EC_{50} value calculated based on pore water was higher than that calculated based on the DGT measurements, reflecting the higher concentrations measured in the pore water compared to the DGT for these soils. For the ZnO_NP spiked soils, the standard and nano-DGT EC_{50}s also reflected the higher concentration extracted via DGT, with the EC_{50} values exceeding those for the pore water by 5–10 fold.

3.3. Relationship between effect concentration and soil type

The fit of the expression in Eq (2), applying it to the literature EC_{50} values for ionic Zn, obtained values of 1.26, 0.48 and 0.15 were obtained for *a*, *b* and *c* respectively and were all significant ($P < 0.05$, R v4.1.3) (R Core Team, 2018). There was a good prediction of the EC_{50} values for the ionic Zn exposures in this study and previous work in our laboratory (Heggelund et al., 2014) (Fig. 3A, closed circles), with a root mean squared difference in log EC_{50} of 0.014 compared to 0.026 for the fitted data (Fig. 3A, open circles) and a mean bias (observed - calculated) of 0.019. We also used the regression to predict log EC_{50} in exposures to ZnO_NPs using the data in this study and those of Heggelund et al., 2014. The EC_{50}s for ZnO_NPs were consistently underestimated, with a mean bias of 0.36 (Fig. 3B), which is consistent. However, a significant correlation between the observed and predicted values was found ($r = 0.85$, $P < 0.05$).

3.4. Zn tissue concentrations

Earthworms exposed to ionic Zn did not significantly differ in their tissue concentrations across all the exposure concentrations (ANOVA: $F = 1.81$, $P = 0.172$) (Fig. 4). The highest body concentrations found were 251 ± 32.1 μg Zn/g for earthworms exposed to ionic at 2200 mg Zn/kg in the calcareous $^{8.3}$Chiltern$_{14.7\%}$ soil (Fig. 4). In contrast, earthworms exposed to ZnO NPs in three of the soils, $^{5.9}$Lufa 2.2$_{4.2\%}$, $^{6.9}$Woburn$_{1.8\%}$ and $^{8.3}$Chiltern$_{14.7\%}$, all showed significant increases in body concentration with increasing soil concentration. Only earthworms in the $^{5.4}$North Wales$_{16.7\%}$ soil did not have increased Zn body concentrations

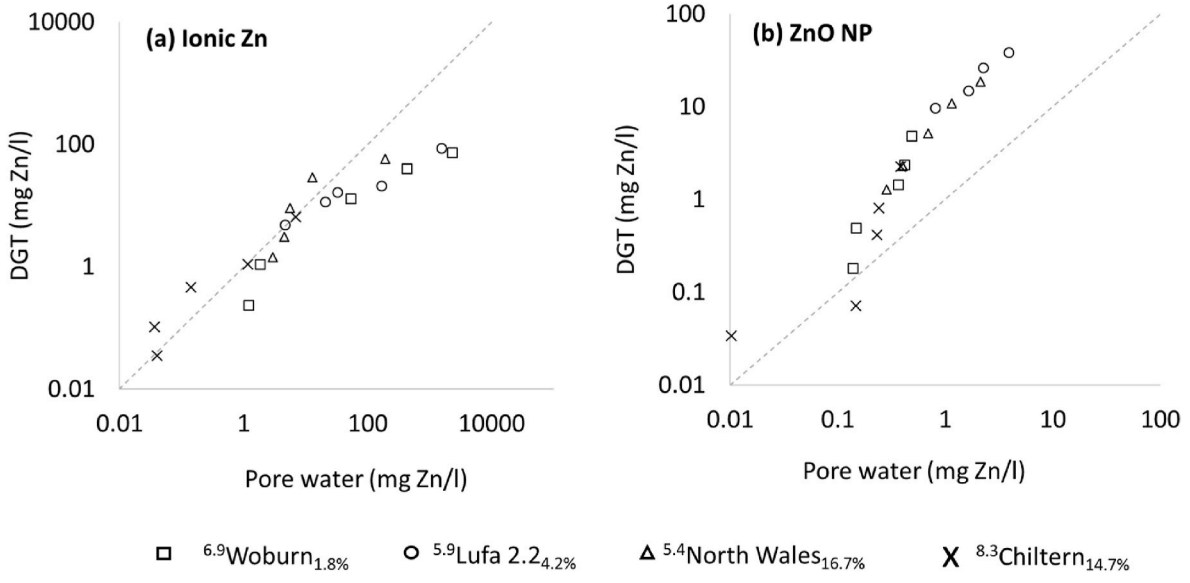

Fig. 1. The total labile Zn concentrations measured by standard DGT and the total Zn measured in the soil pore water in the four soils spiked with (a) ionic Zn and (b) ZnO_NP at the end of the 56 day exposure. The DGT measurements are the average of three replicates ± standard deviations; the pore water extract is one measurement from a pooled sample of three replicates; the broken grey line is a one-to-one line (if the ratio between DGT and pore water measurements was equal to 1); data points show the average measurement from DGT (n = 3) or soil pore water (n = 1) for each soil and Zn treatment.

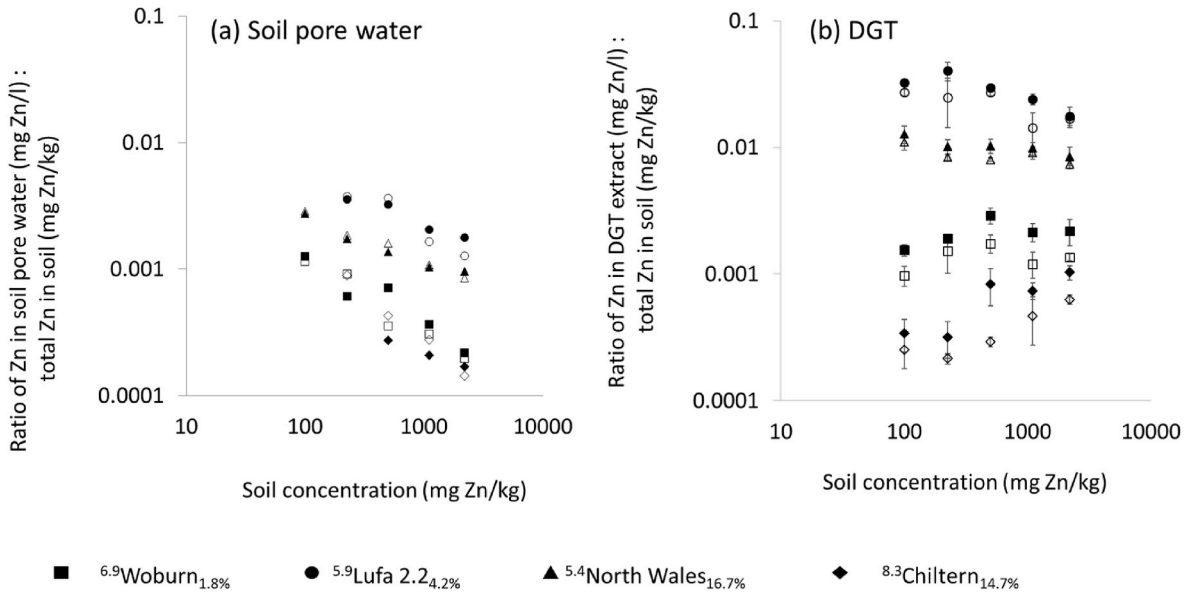

Fig. 2. The proportion of total zinc in the soil that was measured in (a) the pore water or (b) using the DGT (total Zn = solid symbols and ultra-filtered Zn = open symbols) for the four different soils spiked with ZnO_NPs. The DGT measurements are the average of three replicates ± standard deviations: pore water extract measure for one pooled sample from three replicates.

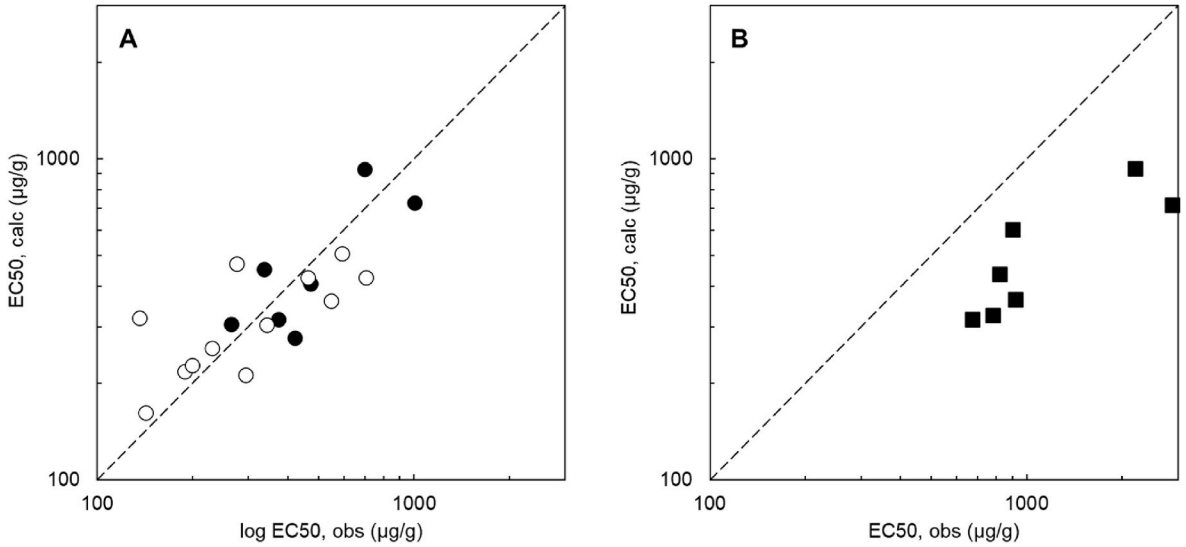

Fig. 3. Fitting and prediction of EC_{50} values for ZnO_NP and ionic Zn effects on 56-day E. fetida reproduction rates. Panel A: observed literature EC_{50}s for ionic exposure plotted against a fit to the expression $logEC_{50}$ (µg/g) = a+b•log (%OM)+c•pH_pw ⟧ (open circles) with a = 1.26, b = 0.48 and c = 0.15 and the observed EC_{50}s for ionic exposure in this study plotted against predictions (closed circles); panel B: observed EC_{50}s for ZnO_NP exposure plotted against predicted EC_{50}s using the regression for ionic Zn exposure. The dashed line is the 1:1 line.

when exposed to ZnO NPs (Fig. 4). The highest body concentrations were reported for the earthworms exposed to Zn NPs in $^{6.9}Woburn_{1.8\%}$ soil (421 ± 81 µg Zn/g) followed by $^{5.9}Lufa\ 2.2_{4.2\%}$, (417 ± 145 µg Zn/g), $^{8.3}Chiltern_{14.7\%}$ (302 ± 22 µg Zn/g) and $^{5.4}North\ Wales_{16.7\%}$ (205 ± 65 µg Zn/g). The calculated EC_{50}s for earthworm reproduction based on tissue concentrations were between 147 and 190 µg Zn/g for ionic Zn and 160 and 360 µg Zn/g for ZnO NP (Table 1, Fig. S6).

4. Discussion

Environmental factors (e.g. pH, carbon content, clay content), physiochemical properties (i.e. the chemical form) and biological components (e.g. route of exposure, toxicokinetic and toxicodynamic pathways) all need to be brought together in order to holistically describe bioavailability and to use this information to attribute cause to effects (Baker et al., 2003). The chemical forms of the metal in the soil, and how this affects bioavailability, will be an important determinant of how the total concentration links to observed effects (Lock and Janssen, 2001; Meyer, 2002; Smolders et al., 2009; Qiu and Smolders, 2017). In this current study, earthworms were exposed in four natural soils representing pH and %OM content ranges covering almost all cases of agricultural soils in a temperature country such as the UK, except those for lowland peat systems (Emmett et al., 2010). Earthworm behaviour and reproduction are already known to be affected by soil properties (Hund-Rinke and Wiechering, 2001; Römbke et al., 2005; van Gestel, Borgman et al., 2011; Romero-Freire et al., 2017) and earthworms typically thrive in mostly neutral soil conditions (~ pH 7). This is consistent with the higher reproduction rates found for earthworms

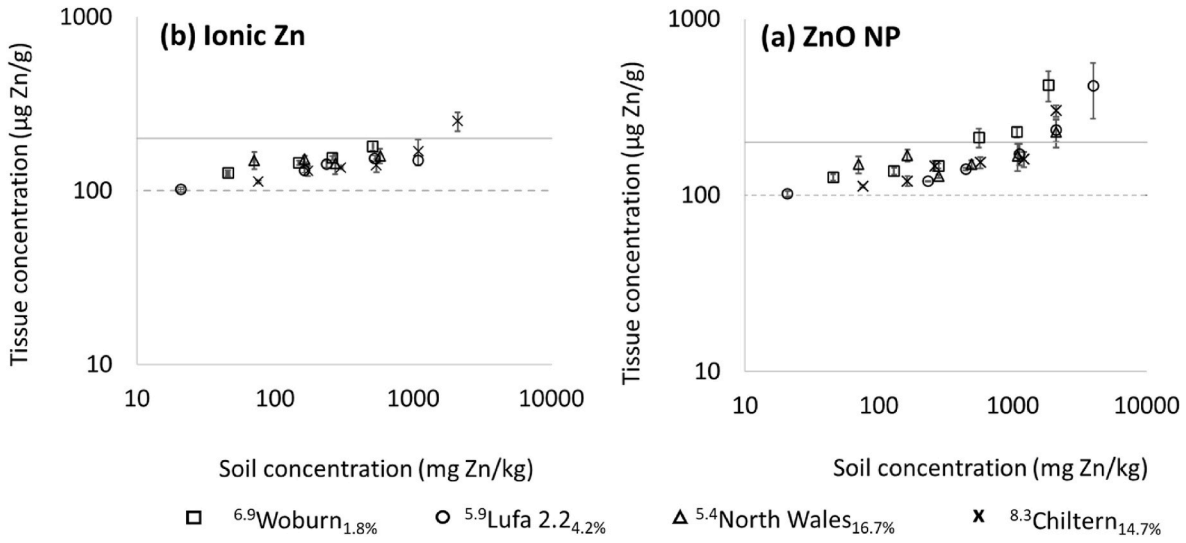

Fig. 4. Tissue Zn concentrations for earthworms exposed in four different soils spiked with ionic Zn and ZnO_NP for 28 days; grey lines indicate the usual lower (broken) and upper (solid) limits for Zn regulation (100–200 µg Zn/g) by earthworms (Lock and Janssen, 2001).

exposed in the $^{6.9}$Woburn$_{1.8\%}$ in this study. In contrast, low pH and OM content have been reported to result in lower earthworm reproduction (van Gestel et al., 2011; Heggelund et al., 2014). Here earthworms in the soils with the highest ($^{8.3}$Chiltern$_{14.7\%}$) and lowest ($^{5.4}$North Wales$_{16.7\%}$) pH values, but a relatively high OM content, showed lower control reproduction rates than did the intermediate pH soils. The lower rate was particularly evident in the highest pH soil ($^{8.3}$Chiltern$_{14.7\%}$) (0.4 ± 0.1 juveniles/earthworm/week compared with 0.75 juveniles/earthworm/week according to the OECD guideline criteria). This indicates that *E. fetida* has an optimal pH range, outside of which rates of reproduction are more limited; this is most evident in high pH soil.

Both Zn form and soil properties were found to interact to play a role in governing the bioavailability and toxicity of Zn to earthworms, with ZnO NP exposure resulting in lower toxicity compared to ionic Zn, and overall toxicity decreasing with increasing soil pH. The lower toxicity of ZnO NP compared with ionic Zn is in agreement with observations for other organisms including isopods (Tourinho et al., 2013), collembolans (Waalewijn-Kool et al., 2013b; Waalewijn-Kool et al., 2014) and other earthworm species (Romero-Freire et al., 2017) and the overall trend of relatively low toxicity of metal-containing nanoforms compared with equivalent ionic forms (Notter et al., 2014). This lower toxicity has largely been attributed to lower pore water metal concentrations found in soils spiked with ZnO_NP compared to those with ionic Zn. This is associated with the incomplete dissolution of the NP and the component remaining in the particulate form assumed to be less readily available to the exposed species than the dissociated ions. This suggests that Zn availability in the pore water may underlie the observed toxicity. Hence, a range of chemical measurements were subsequently undertaken in this study to link these observations with the Zn chemistry within the different soils.

Soil pH was altered by the addition of ZnO NP and ionic Zn, but in divergent ways. The contrasting effect of Zn form on soil pH following spiking results from the different chemical influences of the ionic and nanoparticulate Zn. Addition of ionic Zn acidifies the soil via the Lewis acid action of the Zn salt. Addition of Zn in oxide form has a more complex and mixed set of effects. Firstly, the dissolution of ZnO to form ionic Zn is a proton consuming process and will tend to cause pH to rise overall, although this may be counteracted to a degree by hydrolysis of released Zn^{2+}:

$$ZnO + 2H^+ \rightarrow Zn^{2+} + H_2O \rightarrow ZnOH^+ + H^+ \quad (3)$$

Secondly, the amphoteric nature of the reactive –OH sites on the surface of the oxide will tend to shift the pH of the soil towards the point of zero change of the oxide ($pH_{PZC} \sim 6.9$; Pouran et al., 2021). It is not possible to distinguish the relative importance of these phenomena. The observed trend of lower ionic Zn toxicity with increasing pH, when expressed as a total soil concentration, is in agreement with previously reported results, emphasising the role pH has in governing Zn toxicity to earthworms (Spurgeon and Hopkin, 1996; Lock and Janssen, 2001, García-Gómez et al., 2020). However, it is established that total zinc concentration alone does not predict toxicity and bioaccumulation to soil organisms (Elhaj Baddar et al., 2019). Lofts and co–workers interpreted the effect of soil chemistry on cationic metal toxicity by using the free metal ion concentration in the soil pore water as the most appropriate indicator of toxicity, combined with an effect of pH representing the protective effect of pore water cations such as H^+ and Ca^{2+} against direct uptake of the metal (in the case of earthworms, via the dermis) (). The toxicity of Zn along a gradient of soil pH is controlled by two opposing factors, both of which decrease as the pH increases: the proportion of Zn present as the free ion and the cation protective effect. If these two factors were to completely cancel each other, no overall effect of pH on toxicity would be observed. In the case of Zn, the effects do not cancel, resulting in a general trend towards higher toxicity (lower effect concentrations) in soil of relatively low pH. Soil properties that influence the extent of Zn complexation in the soil – for example the %OM – will also influence toxicity.

The conceptual model of Lofts and co–workers was extended from ionic Zn to consider patterns of toxicity for ZnO_NPs. We hypothesised two possible pathways by which ZnO_NPs may exert toxicity: firstly, by dissolution and release of Zn into the ionic pool, and secondly following direct uptake of the NPs into tissues. It is well known that ZnO NPs are generally soluble in aqueous solutions (Miao et al., 2010; Lopes et al., 2014). Indeed, in this study, the Zn concentrations in the soil pore waters (total) and the concentrations in ultra-filtered (UF) samples were similar for ZnO NP spiked soils, indicating Zn in the pore water was mainly in the dissolved form. In soils, long-term incubations have demonstrated gradual, concentration–dependent dissolution rates (Waalewijn-Kool, Diez Ortiz et al., 2013a). Dissolution of ZnO_NPs has also been shown to decrease with increasing soil pH (Waalewijn-Kool, Diez Ortiz et al., 2013a, Heggelund et al., 2014, Pouran et al., 2021). If the observed toxicity of ZnO-NPs is due solely to dissolution and toxicity driven by the ionic form, then unless dissolution is rapid relative to the exposure duration, the toxicity endpoint (e.g. EC_{50}) should be higher than under a comparable exposure to ionic Zn in the same soil. When investigating the relationship between effect concentrations across soil

types by fitting Eq (2) the positive values of *b* and *c* indicate decreasing toxic effect (higher EC_{50}) at higher %OM and pH respectively, in accordance with expectations. There was a good prediction of the EC_{50} values for the ionic Zn exposures in this study and previous work in our laboratory (Heggelund et al., 2014) but EC_{50}s for ZnO_NPs were consistently underestimated which is also consistent. However, the significant correlation between the observed and predicted values suggests that differences in soil pH and %OM may play a role in influencing the toxicity of Zn applied in NP form to *E. fetida.* This observation is aligned with the hypothesis that toxicity is controlled by dissolution to the ionic form, but it does not preclude the possibility of other mechanisms of toxicity involving direct effects of the zinc in nanoparticulate form.

The DGT-labile concentrations depend on the soil solution concentration and resupply from labile pools in the solid phase (Zhang et al., 2001). Ordinarily the pore water concentrations are determined from the same samples as for the DGT and are found to have higher concentrations in comparison to DGT measured concentrations (Zhang et al., 1998). Although not determined in the same sample in this study, the consistency of the relationships between porewater and DGT-labile zinc concentrations across all four soils for ionic Zn suggests that there are no issues of Zn concentration depletion in the porewater adjacent to the DGT device due to uptake by the DGT, particularly for lower labile concentrations. This is not surprising in soils spiked with ionic Zn where there will be a large pool of adsorbed Zn present to replenish the loss from pore water caused by DGT uptake and the supply of Zn from solid phase to pore water is fully sustained due to the very weak binding of Zn ions on the soil particles (Zhang et al., 2006). The decline in DGT-labile concentration relative to the porewater concentration, at higher porewater concentrations in the ionic Zn-spiked soils, may be due to saturation of the DGT device at ~50 mg Zn/l.

For the ZnO_NP spiked soils, the DGT-labile concentrations measured exceeded those for the pore water concentrations for all except the lowest spiked concentrations in the $^{8.3}Chiltern_{14.7\%}$ soil (Fig. 2). This suggests an additional source of DGT-labile Zn in these soils which causes the DGT-labile concentration to exceed the porewater concentration. A similar relationship between porewater and DGT-labile Zn is seen for ultrafiltered porewater and nano DGT-labile concentrations in the ZnO-spiked soils (data not shown). This shows that the observed relationship is due to uptake of ionic Zn, not ZnO, by the DGT devices, supported by the similarity between the total and ultrafiltered zinc concentrations in the soil pore waters and those in DGT-labile concentrations obtained using standard and nano DGT devices. ZnO_NPs spiked into the soils freshly will undergo processes such as dissolution and attachment over time. As such, they may be releasing ionic Zn over a period longer than that for which the DGT was deployed (18 h). We hypothesise that local depletion of the pore water in ionic Zn in the vicinity of the DGT device induces dissolution from soil-attached ZnO into the pore water. This continued ion release means there will be no depletion of Zn from the soil close to the DGT device surface due to this re-supply. This phenomenon deserves more detailed research as it may suggest enhanced exposure of organisms to Zn from ZnO compared to that predicted by pore water concentrations.

If soluble Zn (i.e. ultra-filtered (UF) Zn) in the ZnO_NP-spiked soils was the main driver of the observed toxicity, it could be expected that EC_{50}s based on UF pore water or nano-DGT concentrations would be comparable across the different soils. However, there was 2 to 5-fold difference between these EC_{50}s calculated for the soils for ZnO_NPs. Comparable differences between the soils were found when UF-EC_{50}s were based on the nano-DGT measurements (Table 1). Converse to the earlier hypothesis that toxicity is controlled by dissolution to the ionic form, this finding would indicate that soluble Zn in the pore water does not alone explain all the observed toxicity in the exposure. As previously asserted, this could be because the NPs themselves are exerting an effect on the earthworms. Indeed, the EC_{50} values estimated based on DGT-NP concentrations (standard DGT minus nano-DGT measurements) for the soils with the lowest OM content were similar to those based on nano-DGT, which would suggest each Zn form could be contributing to toxicity (Table 1). Earthworms are exposed to soils both dermally and through their diet, and so are exposed to metals both in the soil pore water and associated with or bound to the soil solid phase (Vijver et al., 2003; Diez-Ortiz et al., 2015b). There has been speculation as to whether once ingested, further dissolution of NPs could occur in tissues, resulting in differential toxicity (Tsyusko et al., 2012). Because of this combined exposure, we have to conclude that the soluble Zn measured in the pore water does not explain the relative toxicity observed in the different soils.

Bioavailability can be further understood by measuring the concentration of metal accumulated in the biological tissue of interest (Meyer, 2002). Zn as an essential metal playing an important role for many biological functions such as in the cell cycle and in enzymes co-factors (Vallee and Falchuk, 1993). Zn is highly regulated in earthworms and the tissue concentrations measured (151 ± 35 μg Zn/g) here are in line with previously observed physiological limits for earthworms (~100–200 μg Zn/g) (van Gestel et al., 1993; Lock and Janssen, 2001). The only anomaly was earthworms exposed to ionic at 2200 mg Zn/kg in the $^{8.3}Chiltern_{14.7\%}$ soil with tissue concentrations, 251 ± 32.1 μg Zn/g (Fig. 3). Typically, the availability of Zn is lower in calcareous soils, supported in this study by the lower pore water measurements in the $^{8.3}Chiltern\ soil_{14.7\%}$ (Heggelund et al., 2014; Romero-Freire et al., 2017; García-Gómez et al., 2020) but it could be that the higher concentrations could also be explained by some soil residues in the gut if depuration was incomplete. It could also be attributed to the relatively high %OM in the soil, which could also have confounded the trend associated with high pH soils.

The higher tissue concentrations in earthworms for the ZnO_NP treatments compared with the ionic Zn treatments are in line previous findings (Heggelund et al., 2014; Romero-Freire et al., 2017). This consistent with findings of increased tissue concentrations, alongside lower toxicity of ZnO_NP compared to ionic Zn. As toxicity is expected to be related to metals that are biologically active, rather than total tissue burden, it can be proposed that the uptake of Zn from ZnO NP must be different, or must act by a different mechanism, compared with ionic Zn. However, when investigated further using global gene expression profiling of the earthworms exposed to $^{5.9}Lufa\ 2.2_{4.2\%}$ soil from this same study, a significant overlap in the pathway terms affected by exposure to the two Zn forms was revealed (Novo et al., 2020). This overlap suggests that the toxic effects of ZnO_NPs occur through the same mechanisms as for ionic Zn, most likely following ion release from the NPs through dissolution. Whether this occurs externally in the pore water or internally following uptake of ZnO_NPs, or both, has been difficult to unravel and warrants further investigation. Nevertheless, it is important to note that higher tissue concentrations do potentially have implications for the trophic transfer of Zn following ZnO_NP exposure which may need to be considered.

5. Conclusions

- Soil properties influenced earthworm reproductive traits, particularly for the calcareous $^{8.3}Chiltern_{14.7\%}$ soil, where juvenile numbers in the control were lower compared with other soils.
- Ionic Zn was consistently more toxic than ZnO_NP in all four soils.
- Current risk assessment needs to consider ZnO_NPs, but overall hazard assessment based on ionic Zn can already be protective for ZnO_NP in the environment.
- There are competing interactions which influence earthworm reproduction when exposed to different Zn forms and there was no one soil pool measurement alone which could explain the differences in toxicity among soil types or initial metal forms.
- This study highlights the higher accumulation of Zn in earthworms exposed to ZnO_NPs compared to corresponding ionic exposures. This could have consequences for the persistence and trophic

mobility of Zn in terrestrial systems and the mechanisms which result in this observation need further investigation.

Credit author statement

Elma Lahive: Conceptualisation, Methodology, Data curation, Visualization, Original draft, Marianne Matzke: Investigation, Methodology, Writing - Review & Editing, Claus Svendsen: Conceptualisation, Investigation, Methodology, Writing - Review & Editing. David J Spurgeon: Conceptualisation, Investigation, Writing - Review & Editing, Hamid Pouran: Writing - Investigation, Methodology, Data curation Review & Editing, Hao Zhang: Conceptualisation, Writing - Review & Editing, Alan Lawlor: Investigation, Methodology, Data curation, M. Gloria Pereira: Methodology, Data curation, Writing - Review & Editing, Stephen Lofts: Conceptualisation, Methodology, Data curation, Visualization, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120907.

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