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Influence of soil pore water properties on the fate and toxicity of silver nanoparticles to *C. elegans*

Running title: Fate and toxicity of Ag nanoparticles in soil pore waters

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ABSTRACT

Engineered nanoparticles entering the environment are subject to various transformations that in turn influence how particles are presented to, and taken up by organisms. To understand the effect of soil properties on the toxicity of nanosilver to *C. elegans* toxicity assays were performed in pore water extracts from natural soils with varying organic matter content and pH using 3-8 nm un-functionalised (Ag 3-8Unf), 52 nm PVP-coated silver nanoparticles (Ag 52PVP) and $AgNO_3$ as ionic silver. Effects on nanoparticle agglomeration and stability were investigated using UV-vis spectroscopy and asymmetric flow field-flow fractionation (AF4). Ag^+ showed greater overall toxicity than nanosilver with little difference between the nanoparticle types. Increasing soil organic matter content significantly decreased the toxicity of Ag 3-8Unf while it increased that of $AgNO_3$. The toxicity of all silver treatments significantly decreased with increasing pore water pH. Dissolution of both nanoparticles in the pore water extracts was too low to have contributed to their observed toxic effects. UV-vis spectroscopy revealed low levels of agglomeration/aggregation independent of soil properties for Ag 3-8Unf, while higher organic matter as well as low pH appeared to stabilise Ag 52PVP. Overall both soil organic matter content and pH affected nanoparticle fate as well as toxicity to *C. elegans*, however, there appears to be no clear connection between the measured particle characteristics and their effect.

Keywords: Nanotoxicology, Silver, Bioavailability, Dissolved organic matter, pH

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1. INTRODUCTION

Engineered nanoparticle (ENP) emissions to the environment occur through direct input (e.g. as biocide, pesticides, fertilizers and remediation agents), accidentally from spills and unintentionally through e.g. soil amendment with waste. Once in the soil environment, the potential for exposure of a range of soil dwelling taxa exists. A number of studies with soil species have indicated the potential toxicity of ENP, ranging from effects on survival, to more subtle changes in traits such as behaviour and gene expression [1-3].

Soils can be heterogeneous for a range of key properties including organic matter (OM) content, pH, elemental concentrations and mineralogy. Variations in such properties have been widely shown to influence nanomaterial toxicity. For example, a comparison of exposure of the earthworm *Eisenia fetida* to Ag ENP in an artificial soil and sandy loam found greater accumulation and avoidance behaviour in the sandy loam: the soil with the lower pH and organic matter content [4, 5]. This finding was consistent with observation of pH dependent effects of ZnO-ENPs on the earthworm *E. fetida* (highest toxicity at lower pHs) [6] and soil microbial communities where greatest changes in the community composition were observed at lower pHs [7].

The effects of soil properties on ENP toxicity can, in addition to their direct impact on organisms, result from their influence on nanomaterial fate and consequently bioavailability and exposure. These soil property effects can take a number of forms. For example, the attachment of dissolve organic matter (DOM) to ENPs can both stabilise or destabilise ENPs depending on the pristine surface charge, the type of DOM and presence of divalent cations [8]. Negatively charged DOM makes the ENP surface potential more negative, thus decreasing agglomeration of already negatively charged ENPs [9]. Adsorption of humic acid has also been found to disagglomerate small Ag ENP agglomerates [10]. When homo- and

heteroagglomerates/aggregates occur ENP mobility tends to decrease, yet the attachment of DOM itself can lead to increased transportation through electrosteric stabilisation [8].

In soils agglomeration/aggregation can also be pH-dependent; it increases when soil pH approaches the point of zero charge (PZC), while stable suspensions are usually found for zeta potentials >30 mV [2, 11]. Following this principle, the absence of pH effects on ENP size has in one study been linked to no or only small shifts in electrophoretic mobility of the tested citrate stabilised Ag ENPs [10]. Further, where particle dispersion is based on steric stabilisation, e.g. through a PVP coating, surface charge effects have also been shown to play no role in the aggregation of Ag ENPs [12]. The effects of the different soil properties are often confounded e.g. DOM can affect PZC, which can in turn affect agglomeration/aggregation state, thus establishing causation can be difficult. Additionally, the chemical nature of ENPs can change after entering into the environment. The most studied process is their dissolution, which can be influenced by pH, OM coating, deposition, agglomeration/aggregation and mineral transformation e.g. Ag^0 to Ag_2S [2, 13, 14].

Understanding the processes involved in the environmental fate of ENPs is essential when interpreting the results of toxicity tests since dissolution and agglomeration/ aggregation can change ENP uptake and toxicity [15]. In this study the effect of soil properties on the fate and toxicity of two types of Ag ENPs and ionic silver to *C. elegans* was assessed in pore waters extracted from soils with variant properties. Two of the chosen soils differed in their OM content and another soil was adjusted to 3 different pH values (pH 4.8, 6.1, 7.2). This allowed for a thorough investigation of the effect of soil properties on both the fate of Ag ENPs in a relevant soil compartment in accordance with best practice for bioavailability assessment [16].

2. MATERIALS AND METHODS

2.1 Particles

The Ag ENPs tested were a commercial 3 - 8 nm un-functionalised ENP (Ag 3-8Unf), that appears as individual particles and in clusters of 50-100 nm, and a 52 nm polyvinylpyrrolidone coated ENP (Ag 52PVP). These materials were taken from the same batches used by Tourinho et al. 2015 [17], Waalewijn-Kool et al. 2014 [18] and Starnes et al. 2015 [19] with initial material characterisation details contained therein.

2.2 Soils

Two soil parameters were selected to establish their effects of Ag ENP fate and toxicity: soil OM content and pore water pH. A low ionic strength artificial test medium (Simulated Soil Pore Water – SSPW, modified without fulvic acid addition) that has previously been used for nanotoxicity studies with *C. elegans* was also tested as a reference [20]. To assess the effects of OM content, two natural soils varying in this parameter were selected for testing: "lowOM": LUFA 2.2, a well characterised standard soil (LUFA Speyer, Germany) which has an OM content of 4.2% and "highOM": a pasture soil collected in North Wales, UK (OS Grid Reference SJ224564), with 16.7% OM, previously used in the development of a BLM for terrestrial species [21]. For the experiments investigating pH effects of ENP behaviour and toxicity, a soil collected from an open acidic heath land in Wareham forest, Dorset, UK (OS Grid Reference SU108058) was used and its pH adjusted with $CaCO_3$ (2, 4 and 8 g/kg) to give soils with the same properties that varied in pore water pH over three values: 4.76 ± 0.02, 6.05 ± 0.02 and 7.24 ± 0.04, following the approach of Heggelund et al. 2014 [6]. The measured levels were in good agreement with the target values and the published soil bulk pHs reported previously for the same method [6, 22]. All soils were homogenized, 2 mm sieved and air dried prior to use. The properties of the three soils are summarised in Table 1.

2.3 Pore water extractions

Toxicity tests were conducted in pore waters extracted from the field soil described above. The extracts were obtained by wetting the soils with Milli-Q water to 50% of their respective water holding capacity (WHC) for 7 days to allow for pH equilibration and incubated before wetting to the full 100% of their WHC for an additional 24 h. After incubation, the wetted soils were centrifuged (Beckman Coulter, Avanti J-E, rotor JS.5.3) for 90 min at 4000 g in 50 mL centrifuge tubes. The pore water supernatant was collected, and exposed to UV radiation (254nm, 10000 µJ/cm^2, 60 min). Extracted pore waters were treated this way since initial tests with non-UV radiated pore waters revealed decreased nematode survival and reproduction. Despite incomplete sterilisation this UV treatment was sufficient to ensure normal levels of reproduction. ENPs and $AgNO_3$ were subsequently added to the pore waters and toxicity tests and exposure characterisation performed. Comparisons were drawn between lowOM and highOM for OM effects and between the three pH adjusted field soil pore waters for the influence of pH.

2.4 Nematode toxicity assays

Experiments were carried out using *C. elegans* wild type strain N2 obtained through the *C. elegans* Genetics Center (CGC), University of Minnesota, USA. Cultures were maintained on nematode growth medium plates seeded with *Escherichia coli* strain OP50 at 20°C in the dark [23]. Cultures were age synchronised twice weekly by transferring gravid adults onto fresh plates letting them lay eggs for 2 hours and subsequently removing the adults.

The effect of the varied soil properties on the toxicity of the selected Ag 3-8Unf and Ag 52PVP for *C. elegans* was investigated in a 72 h adult reproductive toxicity test conducted in the pore water extracts. In the bioassay 72 h old, gravid adults were exposed to concentration ranges of $AgNO_3$ and the two different Ag ENPs at 18°C in the dark for a total exposure period of

72 h. Nematodes were exposed in 6 well plates in 2 mL pore water, one individual per well, and the *E. coli* strain OP50 added at an optical density of 0.35 prior to exposure as food source [20]. Ag ENPs exposure concentrations ranged 2 to 32 mg Ag/L for OM ranges, and 1 to16 mg Ag/L for pH ranges; and $AgNO_3$ (as ionic control): 0.04 to 2.54 mg Ag/L for OM ranges, 0.04 to 0.635 mg Ag/L for pH ranges. Exposures were fully randomised and six replicates used for each tested concentration for all exposures.

Immediately at the end of the exposure period, adults and produced offspring in each well were stained by adding 2 mL of 0.5 g/l Bengal Red B (Sigma Aldrich, dissolved in water) to the pore waters and incubated at room temperature for 40 min. All nematodes were then killed by heating to 55°C for 1 h. This prevented further reproduction and immobilised the animals to allow more accurate enumeration. The presence of the adult was confirmed and the total number of offspring was recorded for each replicate, by placing the plates onto a grid and counting all eggs and juveniles under a Nikon SMZ800 dissection microscope. Randomly selected wells were re-counted to confirm accuracy.

2.5 Characterisation of ENP fate in exposure media

Characterisation was carried out in the pore waters in the presence of the natural soil bacterial community.

a) Concentration validation and ENP dissolution measurement

Immediately after preparation of the exposure media, 750 µl of each concentration (including controls) in 3 replicates, was acidified with 600 µL *aqua regia* (450 µL 36 % HCl + 150 µL 69 % HNO_3) and stored at 4°C in the dark until analysis for concentration validation. Silver content of the samples was determined by graphite furnace atomic absorbance spectroscopy (GF-AAS, Perkin Elmer 1100B).

Nanoparticle dissolution was measured at 5 mg Ag/L after 72 h in 4 replicates following a method by Diez et al 2015 [24]. At the end of the exposure period each sample was split and half ultrafiltered with 10 kDa ultrafiltration membranes (Amicon Ultra-15 Filters, Millipore) by centrifugation at 4000 g for 30 min; particulate silver >1 nm was retained in the filters. Prior to filtration membranes were soaked in 0.1 M $Cu(SO_4)_2.5H_2O$ to occupy binding sites and then washed to prevent any adsorption of silver to the filtration membranes thus decreasing the measured concentration. The concentration of dissolved silver was measured in the filtrate and total silver in the corresponding unfiltered sample volume after digestion. Prior to digestion porewater samples containing Ag ENP suspensions were bath sonicated over melting ice for 15 minutes. Then, 1 ml sample aliquots were pipetted into PFA microwave vessels followed by the addition of 1 ml nitric acid (69%, Baker, Ultrex II reagent) and 3 ml of hydrochloric acid (38%, Baker Instra-analyzed reagent). The microwave vessels were capped and the acidified pore waters digested at 180 °C for 30 minutes using a CEM Mars Xpress microwave digestion system (CEM Corporation, Matthews, USA). Once the digestion procedure was complete the vessels were allowed to cool at room temperature and then the digests were made up to a final volume of 50 ml with 0.1 M hydrochloric acid. Total Ag concentrations were determined using Inductively Coupled Plasma Mass Spectrometry (ICPMS; Perkin Elmer Nexion 300D instrument). After a tenfold dilution with 1M hydrochloric acid ICPMS measurements were made against calibration standards in range 0-10 µg/l. ^{115}In was used as internal standard to compensate for instrument drift and effects due to differing matrix between samples

b) UV-vis spectroscopy tracking stability

ENP agglomeration/aggregation dynamics (“clustering”) in the pore waters over the 72 h duration of the nematode toxicity tests were monitored using UV-vis spectroscopy (Shimadzu UV-2400 spectrophotometer) at regular intervals (0, 1, 3, 6, 24, 48, and 72 h). Measurement scans (350 – 750 nm) were performed at in 1 mL 5 mg Ag/L Ag ENP in standard 2 mL quartz

cuvettes. Prior to analysis the instrument was calibrated with pore water extracts without Ag ENPs.

c) Asymmetrical flow field flow fractionation (AF4)

Additionally, the size distribution of the ENPs in the different pore waters after 48 h, as intermediate time point, at 10 mg Ag/L was determined by AF4 analysis (Wyatt Technologies, Eclipse 3 AF4 with a UV-vis and ICP-MS detector (Agilent 1200 series and Agilent 7500cx)). The retention time of a particle is positively correlated with its hydrodynamic diameter. ENP size distributions were calculated from ICP-MS fractograms using ISIS Chromatogram Prediction and Simulation Software (version 1.2.0, Wyatt Technology). Concentrations for the AF4 analysis were validated by open vessel acid microwave digestion and ICP-MS analysis (Agilent 7500cx). A detailed description of the applied method, running conditions and results are reported in the Supplementary Information (SI) in Tables S1 and S2.

2.6 Statistical analysis

Results of the reproductive toxicity tests (total number of offspring per individual) were analysed for concentration-response relationships in Sigmaplot 12.0 (Systat Software Inc, USA) fitting a 3 parameter logistic regression and estimating upper asymptote, EC_{50} and slope parameters for each of pore water media separately. These coefficients were then compared across pore waters by z-test (*z*-scores and *p*-values reported in SI Tables S1 and S2) and differences between concentration-response curves established using the *F*-test [25] (*F*- and *p*-values reported in SI Table S3).

3. RESULTS

3.1 Nematode toxicity assays

a) Comparison of relative toxicity of Ag ions and Ag ENPs

Overall nematode reproduction (total number of offspring) was unaffected by the type of soil pore water used (Fig 1). Across the toxicity tests ionic silver had a significantly higher toxicity than both tested Ag ENPs (p<0.01, Table S1, S2), with EC_{50} values (Table 2) being at least an order of magnitude lower for Ag ions compared to the two different Ag ENPs across all soil extracts. Overall, the two Ag ENPs showed similar toxicity; their EC_{50} values in each of the tested media were not significantly different with the exception of lowOM (z=3.91, p<0.01, Table S1). Such similarity indicates that the differences between the two tested nanomaterials did not strongly influence their resulting toxicity despite differing ENP fate (see Fig 2, 3, S1, section 3.2b,c) with the tested media mediating silver toxicity.

b) Influence of dissolve organic matter content on toxicity

$AgNO_3$ reproductive toxicity responses were not significantly different in the in the pore waters of lowOM and highOM (F=1.64, p=0.18) producing identical EC_{50} values of 0.19 mg Ag/l (Table 2). Whereas the toxicity of Ag 3-8Unf was significantly increased in the highOM compared to the lowOM soil as indicated by significant difference in the concentrations response curves (F=7.30, p<0.001) and lower EC_{50}: lowOM 7.80 ± 0.82 mg Ag/L and highOM 3.48 ± 0.97 mg Ag/L (z=3.39, p<0.01). Exposure of *C. elegans* to Ag 52PVP in the pore waters with different OM contents also produced significantly different concentration response curves between the two OM pore waters (F=5.82, p=0.002), yet with similar EC_{50} values (lowOM: 4.47 ± 0.23 mg Ag/L, highOM: 4.07 ± 0.69 mg Ag/L). For both nanoparticles the lower slope for highOM soil indicated a more gradual onset of toxicity in the pore water extracts of this soil, with observable toxicity occurring at lower concentrations than in the lowOM soil (Fig 1). The steeper concentration response relationship for lowOM, on the other hand, indicated a much narrower

range of concentrations between no effect on nematode reproduction and full inhibition for exposures in this pore water.

c) Influence of pH on toxicity

Soil pore water pH had a significant effect on toxicity to *C. elegans* for all silver exposures. In all cases, toxicity was increased at lower pH (Tables S2, S3). In $AgNO_3$ a 3 fold decrease in EC_{50} values from pH 7.2 and 6.1 to pH 4.8 was observed, and response curve shape was significantly different (F=33.18, p<0.001 and F=43.10, p<0.001 respectively). However, there was no difference in toxicity between the two higher soil pH extracts. For both ENPs EC_{50} values were found to be over a magnitude lower at pH 4.8 compared to either of the two higher pH conditions (Table 2).

3.2 Characterisation of ENP fate in exposure media

a) Concentration validation and dissolution

Total Ag concentrations were within 15% of nominal concentrations ($AgNO_3$: 86%, Ag 3-8Unf: 110%, Ag 52PVP: 85%) indicating the validity of the dispersion and dosing protocols used for media preparation. As there was no evidence of systematic deviation, all treatments are referred to as nominal concentrations for clarity.

Ag ENPs dissolution of 5 mg Ag/l was below the detection limit (40 µg Ag/l) or no higher than the control measurements across all tested pore waters. Since the detection limit was 3 fold lower than concentrations of Ag ions found to cause overt toxicity in the $AgNO_3$ studies, this data suggests that the particulate form of the silver exposure in the medium drove the observed toxicity in the ENP exposures under the tested conditions. However, a contribution of dissolved silver to effects occurring at concentrations greater than 5 mg Ag/l cannot be excluded.

b) UV-vis spectroscopy tracking stability

Despite calibration with the respective pore waters interference from dissolved organic matter was found for wavelength below 350-400 nm which can be seen as random spikes in Fig 2 F, 3 A and D.

The Ag 3-8Unf showed a high stability in the two OM range field soil extracts (Fig 2 B, C). This indicated an overall stabilisation, with only slight clustering/sedimentation occurring in the highOM extract after 72 h and minimal aggregation indicated in the lowOM soil media. For the Ag 52PVP, the decrease in the peak maxima over time together with a broadening of the peaks and shift of the absorbance maximum to higher wavelengths suggested a settling of the ENPs caused by the clustering of the particles in both pore waters. However, in the highOM extract these characteristics were slightly less pronounced indicating that agglomeration and sedimentation was slowed compared to the lowOM (Fig 2 E, F). The settling process could have been enhanced at a higher concentration of ENPs or decrease at lower concentrations due to a change in the ENP:OM ratios.

In the different soil pH extracts, the Ag 3-8Unf the showed only minor change in peak height over 72 h as compared to the differing behaviour found between the different soil OM extracts (Fig 3 A-C). For the pH treatments, the greatest reduction in peak height was observed for pH 6.1 and pH 7.2 of ca. 20%, while at pH 4.8 ENPs peak characteristics remained stable over the 72 h period. Ag 52PVP measurements indicated that sedimentation was positively related to extract pH (Fig 3 D-F). Thus, in pore water extracts from the pH 4.8 soil, Ag 52PVP stayed stable in suspension over 72 h, while at pH 6.1 the peak height decreased to about half after this time and at pH 7.2 the majority of ENPs had settled out from suspension.

c) Asymmetrical flow field flow fractionation (AF4)

AF4 analysis of the Ag 3-8Unf ENPs in Milli-Q water indicated the presence of differently sized agglomerates in the stock dispersions, as mentioned in the methods, which here were determined to be 20-30 nm and 90-110 nm in diameter at retention times of t_r ~20 min and ~35 min, respectively. Size measurements were based on retention time and calibrated against the Ag 52PVP ENPs stocks in Milli-Q water previously determined as 80 nm using this technique.

Ag 3-8Unf ENPs in the two OM range pore waters showed similar fractograms to the stock particle distribution, and the slight decrease and broadening of the first peak indicated a potential disagglomeration of the smaller agglomerates (Fig S1a). Ag 52PVP exposure in the lowOM medium resulted in an increased ENP hydrodynamic diameter (estimated from the increased retention time) compared to the primary stock ENPs. In highOM pore waters the retention time of the peak maximum was unchanged, yet much broader. This meant while the size of the primary particles remained the same a greater range of particles with a smaller/larger hydrodynamic diameter were additionally detected potentially caused by the loss of the PVP coating and the adsorption of organic molecules, respectively (Fig S1b).

In the pH range pore waters, for Ag 3-8Unf the primary peak in all media matched that of the larger aggregates in the stocks (90-110 nm, approximately at t_r 35 min). Fractograms in both pH 4.8 and pH 6.1 pore waters showed a suppression of the peak of the smaller stock agglomerates. While this was also the case for pH 7.2, here another peak at an earlier retention time formed, which may have been the 3-8 nm primary particles (Fig S1c). AF4 analysis of Ag 52PVP in pore waters with different pHs found a broadening of the primary particle size peak and a shift of the peak maximum retention time (t_r 34 min) in all media compared to the ENP stock. However, the estimated hydrodynamic diameter increased for pH

4.8, decreased for pH 6.1 and increased only slightly for pH 7.2 from the stock ENP size, thus not revealing any clear pattern in the agglomeration/aggregation dynamics (Fig S1d).

The recoveries of the AF4 separations for Ag 3-8Unf were around 90% and for Ag 52PVP only around 64%, indicating some loss during measurements potentially due to ENPs adhering to the tubing or channel.

4. DISCUSSION

The ecotoxicological assessment of ENPs has to date largely been carried out under artificial settings (high concentrations, synthetic media), with effects under more realistic environmental conditions only recently receiving attention. Published studies conducted to date have suggested that soil properties can influence ENP behaviour and effect in soils [4, 6, 26, 27]. However, systematic studies of the influences of soils and soil pore waters with varying properties such pH and OM contents on the fate and toxicity of ENPs with different starting characteristics are needed to support the development of robust models that can be used to describe the fate and toxicity of ENPs in natural systems.

Effect of dissolved organic matter on toxicity

OM has often been found to have protective effects in aquatic toxicity test systems with Ag and other metal ions [28-30], as well as for ENP toxicity in *C. elegans* in standard laboratory test media [31, 32]. The mechanism of protection is largely attributed to the complexation of free metal with the dissolved OM, thereby reducing free ion concentrations able to interact with organism surfaces [33, 34]. Conversely, dissolution of sulfidised Ag ENPs in *C. elegans* test media was found to be increased in the presence of Pony lake fulvic acid, yet induced mortality of *C. elegans* was still entirely rescued [35]. This suggests additional protective mechanisms such as particle surface passivation or the reduction of oxidative stress by acting as scavenger for free radicals [34]. However, in this study the increased organic matter content

in highOM pore waters did not have a protective effect on Ag 3-8Unf toxicity to *C. elegans* compared to lowOM but rather promoted the inhibition of reproduction. This is consistent with findings that the type of organic matter and its elemental composition, rather than simply the total content, is a key factor in nanoparticle fate and dissolution [31, 35, 36]. For example, the complexation of Ag with OM is linked to its sulphur content since Ag has a strong affinity to thiol ligands [37]. The dissolution kinetics of ZnO ENPs also have been related to aromatic carbon content of the tested humic and fulvic acid [36]. Analysis of the fate of the Ag 3-8Unf indicated a general stabilising effect of organic matter on ENPs, as reported previously [9], showing neither the characteristic broadening of the UV-vis absorbance peak and formation of a secondary peak at larger wavelengths associated with ENP agglomeration/aggregation [38, 39]. The comparison of the lowOM and highOM soils both in the UV-vis and AF4 analysis found little difference between the pore waters indicating this stabilisation was already saturated at the lower OM content. This suggested a rapid attachment of the OM molecules to the surface of these particles, and once fully covered increasing OM concentration did not change the agglomeration/aggregation dynamics over time. Further, the bacteria and biomolecules present in the pore waters could have interacted with the ENPs thus influencing their fate [40]. The altered Ag 3-8Unf toxicity to *C. elegans* despite only small changes in its stability, suggests a mechanism of toxicity is not solely dependent on agglomeration/aggregation state. As ENP dissolution in the medium was not measurable it can also be excluded as the main driver of the effects. Additionally, $AgNO_3$ toxicity showed no differences between the lowOM and highOM extracts. Possibly, ENP toxicity was influenced indirectly by ENP interactions with the soil bacteria and sequestered biomolecules that differed between the two soils. Greater ENP attachment to bacteria in the highOM extracts could have increased their uptake by the bacterivorous nematode. The pore water pH was also considered when interpreting the toxicity results. LowOM soil had a pH of 5.9 and highOM of 5.5. Although the pH values for the soils were similar the actual increase in $[H^+]$ in the medium is considerable and falls within the tested pH range that significantly increased Ag ENP toxicity

(i.e. pH 4.8 to 6.1), which could have been linked to the decrease in Ag 3-8Unf EC_{50} from low to high OM.

Unlike Ag 3-8Unf soil OM content did not influence the reproductive toxicity of Ag 52PVP to *C. elegans* when comparing EC_{50} values. However, the concentration response relationships were found to be OM dependent. LowOM exposure caused a significantly steeper concentration response relationship with effects occurring more rapidly once a threshold concentration has been exceeded than in highOM extracts. Ag 52PVP was found to be less stable in the OM soil pore waters than Ag 3-8Unf. In the lowOM extracts the absorbance maximum shifted towards larger wavelengths indicating an increase in agglomerate/aggregate size. In the highOM pore water the kinetics were less pronounced, i.e. precipitation was slowed down compared to lowOM, yet not completely absent. Thus indicating that the critical threshold amount of OM to completely stabilise the ENPs in suspension may not have been reached, or the type of OM present in solution did not fully stabilise this type of Ag ENP [39, 41]. This is supported by the AF4 results where lowOM exposed ENPs were found to be larger than the pristine ENPs while in highOM pore waters average sizes matched the pristine ENPs. For Ag 52PVP particle stability over time was linked to its toxicity. The difference in the concentration response relationship between the particles may have been correlated to the greater presence of individual particles in suspension in highOM pore water over time. Such dispersed ENPs could have been taken up more readily than the aggregates/agglomerates present in the lowOM extracts at earlier time points.

Together findings for both ENPs suggest that the stabilising effect of organic matter may depend on particle size and surface coating, e.g. a more rapid attachment of the organic molecules to the uncoated surface of the smaller particles than for the larger PVP-coated Ag ENPs.

Influence of pH on toxicity

Reproductive toxicity of the ionic and both particulate Ag forms was significantly increased at pH 4.8 compared to pH 6.1 and 7.2. For both ENPs EC_{50} values differed by approximately ca. 10 fold across the pH range. Increased toxicity of ENPs with decreasing pH has previously been reported [10, 26]. The increased toxicity at the lowest pH was not associated with ENP dissolution, as had been found others [37] excluding free Ag^+ in the medium as driver of the increased toxicity. Varying soil pore water pH had only little effect on Ag 3-8Unf stability, thus the aggregation state also did not appear to influence its toxicity. However, at the low pH ENP competition with H^+ for binding sites could have resulted in a greater amount of bioavailable ENPs in the pore water resulting in greater uptake and toxicity. For Ag 52PVP UV-vis analysis revealed increased ENP agglomeration/aggregation with increasing pH. This observed increase in agglomeration/aggregation of Ag 52PVP may have been a by-product of the way the pH of the soil was adjusted, i.e. the addition of $CaCO_3$ to achieve the higher pHs increased the amount of Ca^{2+} in the pore water which has been shown to increase aggregation [42]. This phenomenon is attributed to the non-specific binding of the attached, negatively charged OM molecules that leads to a bridging effect between adjacent molecules by Ca^{2+} and destabilisation of the suspension [8]. Thus, the decrease in toxicity at the higher pHs again may have been linked to a decreased uptake of the larger clusters (potentially being too large to pass the nematode pharynx) compared to the individual, dispersed ENPs at the lowest pH.

The development of site specific risk assessments for trace metals has benefited from the development of deterministic and mechanistic models that are able to incorporate the effects of major soil properties on metal speciation and exposure [43]. Within such models for soil, pH and OM content (although with soil clay content and cation exchange capacity), are common driving variable of differential toxicity between variant soils [44]. Rising releases of nanoparticles to the environment is increasing the need for better understanding of how soil properties affect ENP behaviour and toxicity as a step toward are more mechanistically valid

approach to site specific hazard assessment. Given their key role in determining metal speciation and exposure, the effects of pH and soil OM, are important parameters to consider with respect to their influence on metal nanoparticle fate and toxicity.

5. CONCLUSION

The findings in this study suggest that both OM content and pH did affect the stability of 52 nm PVP-coated ENPs whereas for Ag 3-8Unf only OM, and not pH, influenced stability. Further, the data indicated that toxicity of Ag ENPs is a function of concentration as well as the pH and OM content of the test medium. The observed effects could not be attributed to dissolved ion concentrations since these were too low to be detected, let alone cause the observed toxicity in the presented study. Instead it would seem that effects were related to the particulate nature of the silver exposures. The extent of this effect was related to the degree of agglomeration observed, through a threshold effects of OM (i.e. similar effects above a certain level of OM present in the media) but a fully dependent effect for pH (i.e. increased stability and toxicity at lower pH). The observed patterns of ENP effects were different from those for Ag ions indicating that new fate and hazard modelling approaches are needed for ENPs. In fact, exposure of the earthworm *Eisenia fetida* to ionic and nanoparticulate silver has shown ionic silver to be more toxic in short term toxicity tests, whereas Ag ENPs were found to be more toxic after ENPs were aged in soil prior to exposure [24]. Further, soil properties resulted in nuanced different effects between the two ENPs. Such differences indicate that between the Ag 3-8Unf and Ag 52PVP ENPs, their varying properties subtly influenced on the nature of the relationships between media pH and OM and thus fate and toxicity. For a comprehensive assessment of media effects the nematode Ag tissue concentrations after exposure in a greater number of different pore waters is needed to relate ENP fate to bioavailability and uptake, thus furthering development of mechanistic models that describe the relationships between ENP characteristics, soil pore water chemistry and ultimately toxicity.

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Authors' contributions

CLS and EL designed the soil extraction methods and carried out the pore water analysis. CLS carried out the nematode test and analysed the bioassay data. AL carried out the dissolution measurements. JMU led the AF4 analysis. JMU and VP provided characterisation advice for the UV-Vis. AC provided characterisation advice for the UV-Vis and AF4. CS and DJS designed and coordinated the study and associated with the design of the characterisation assay. All authors were involved with the initial drafting and review of the manuscript.

Data accessibility

Data associated with the research will be made available on request. Please contact corresponding author David Spurgeon (dasp@ceh.ac.uk).

Conflict of interest

The authors confirm that they have no competing financial interests.

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Legends to Figures

Figure 1: Reproductive toxicity of different concentrations of $AgNO_3$, Ag 3-8Unf and Ag 52PVP expressed as total number of offspring produced (n=6, averages ± SE) and fitted 3 parameter log logistic regression curves in pore waters from soils with A-C) different OM (▲ lowOM, ◆ highOM) and D-F) pHs (◆ pH 4.8, ● pH 6.1, ▲ pH 7.2). x-axis: log_2 exposure concentrations [mg Ag/l].

Figure 2: UV- vis spectra of pristine A) Ag 3-8Unf and D) Ag 52PVP stock in MilliQ-water, and time series of B-C) Ag 3-8Unf and E-F) Ag 52PVP exposed to B, E) pore water from low organic matter soil, C, F) pore water from high organic matter soil at t = 0, 1, 3, 6, 24, 48, and 72 h, normalised to the absorbance maximum at t = 0h.

Figure 3: UV-vis time series spectra of A-C) Ag 3-8Unf and D-F) Ag 52PVP in pore waters from a soil adjusted to A, D) pH 4.8, B, E) pH 6.1 and C, F) pH 7.2 at t = 0, 1, 3, 6, 24, 48, and 72 h, normalised to the absorbance maximum at t = 0h.

Figure 1

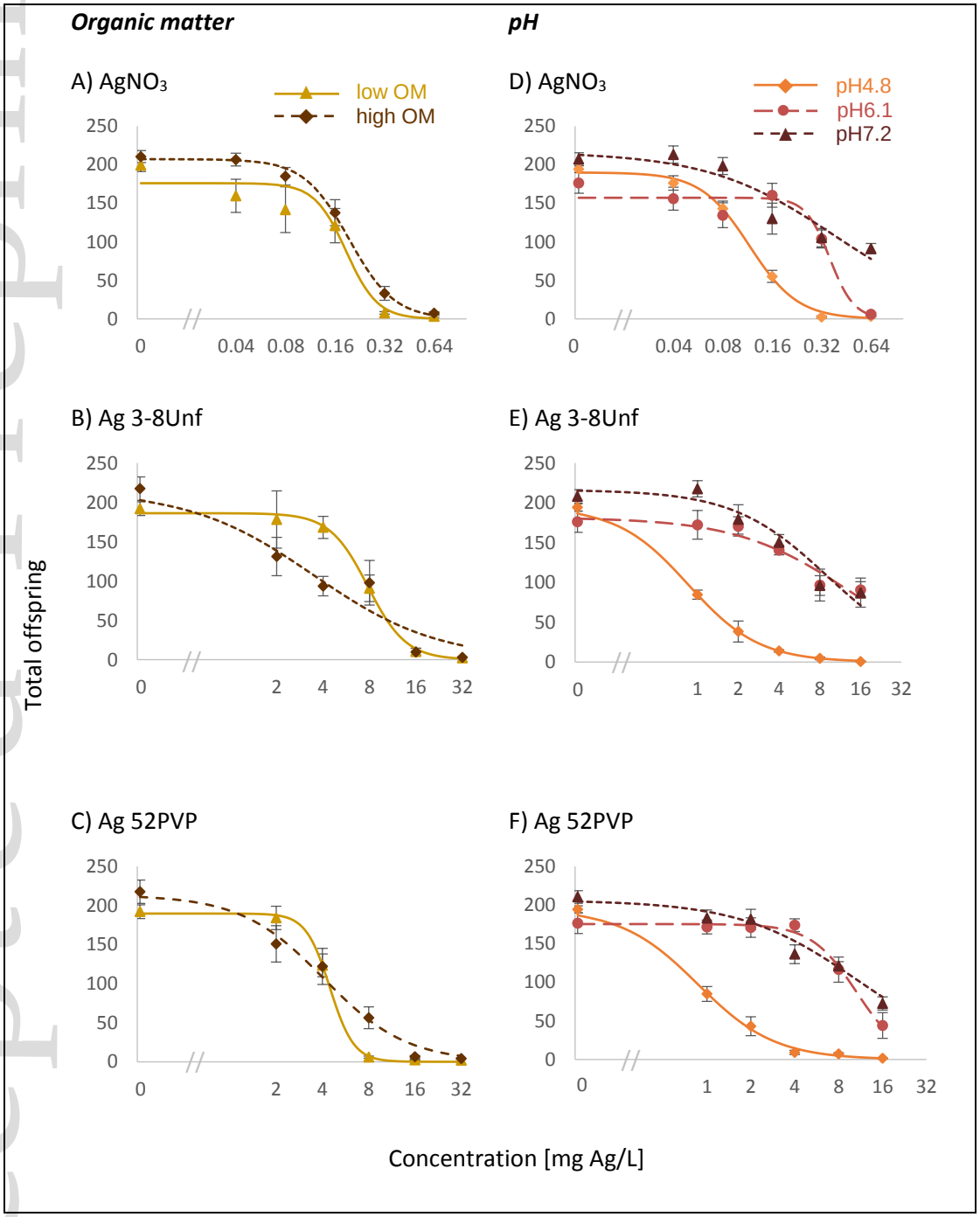

Figure 2

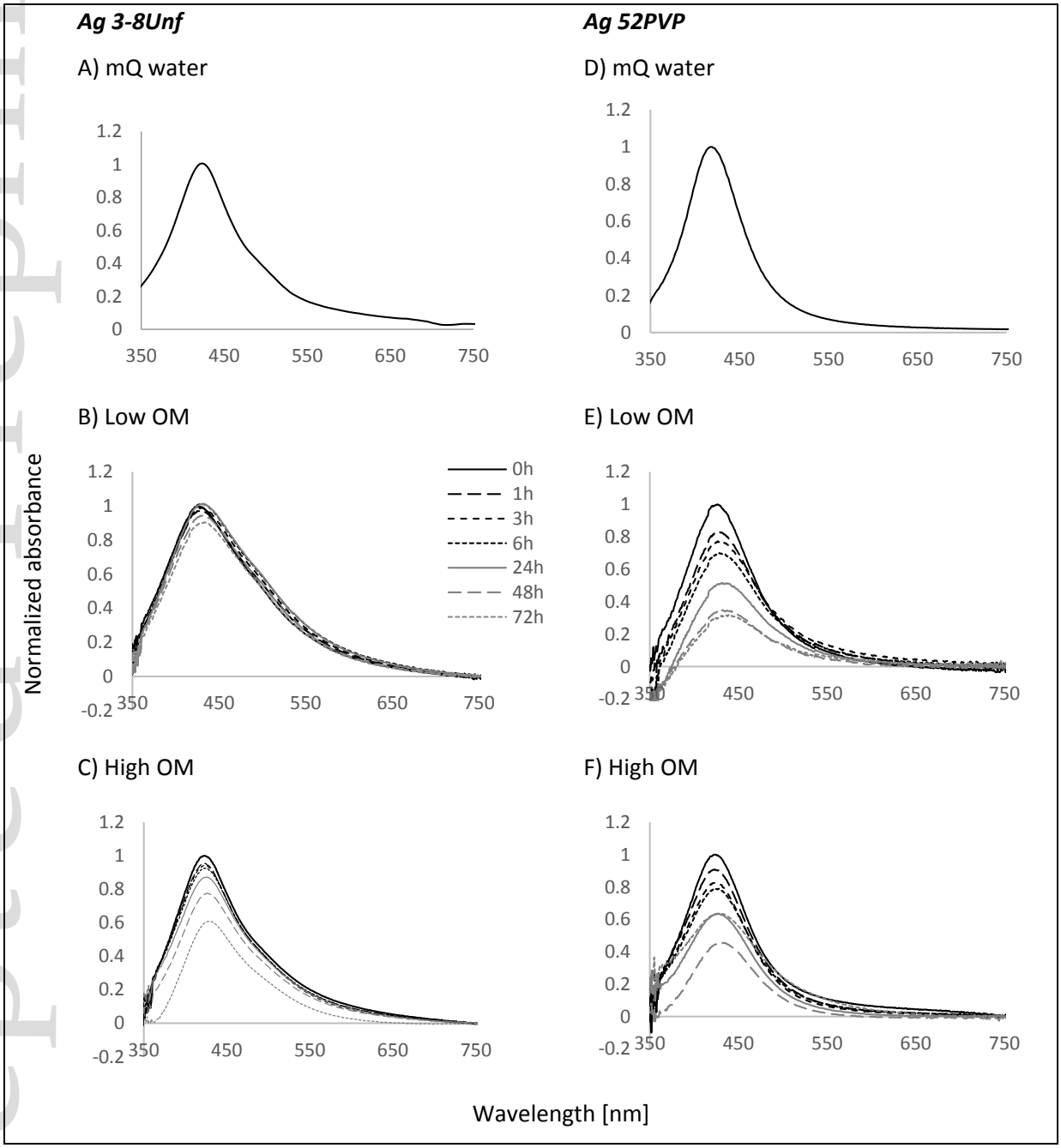

Figure 3

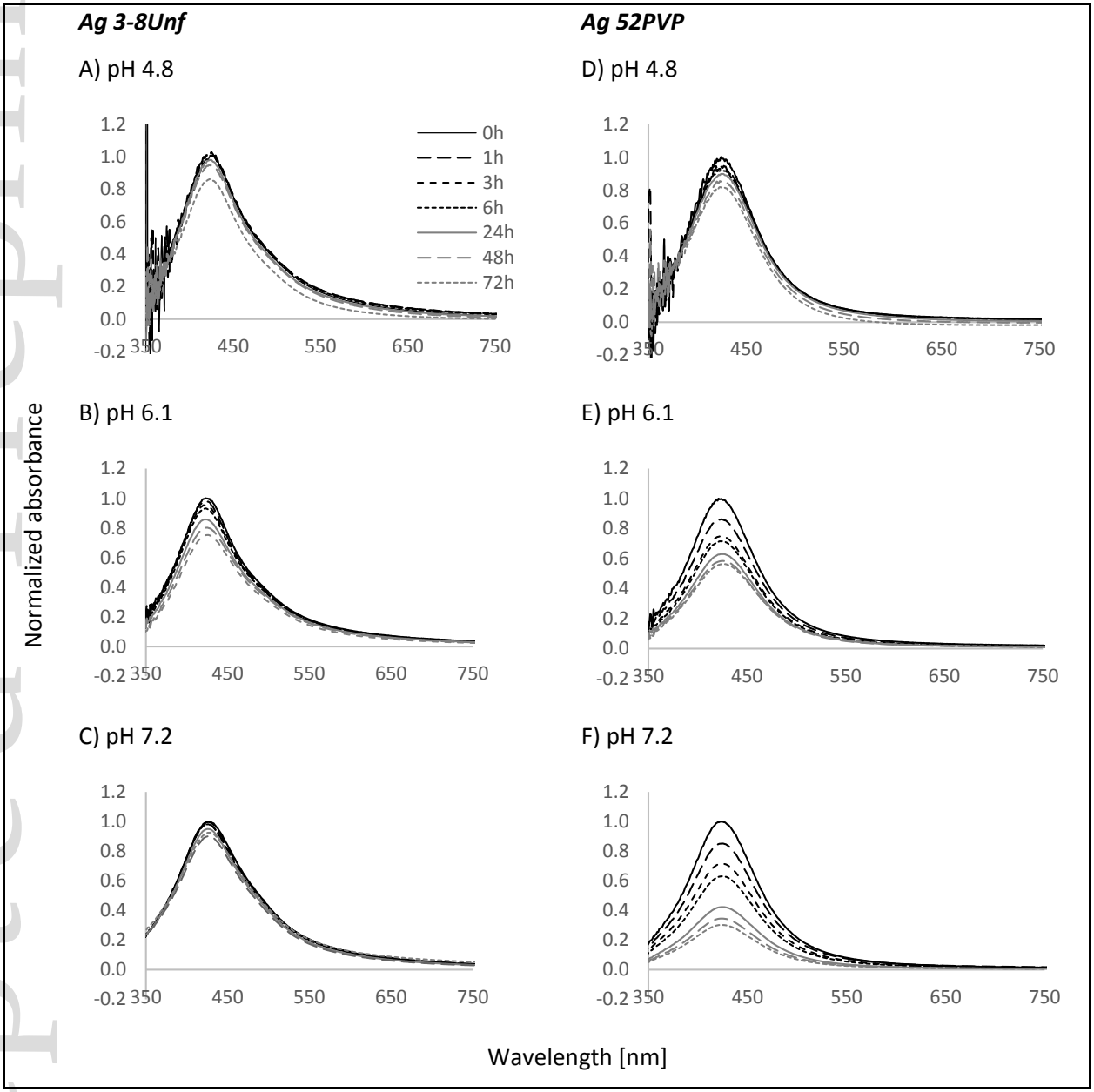

Table 1: Soil properties: Classification, origin, soil texture, 100% water holding capacity (WHC) in mL per 100 g soil (dry weight), soil pH measured in 0.01 M $CaCl_2$ and pore water (PW) pH, organic matter content (OM), and cation exchange capacity (CEC). Data for soils from: LUFA 2.2: http://www.lufa-speyer.de/images/stories/StandardSoil.pdf, North Wales: Waalewijn-Kool et al 2013 [22], Dorset: Heggelund et al 2014 [6].

Soil	**Origin**	**Classification**	**Sand %**	**Silt %**	**Clay %**	**100% WHC [mL]**	**Soil pH_{CaCl2}**	**PW pH_{H2O}**	**OM %**	**CEC [mval/100g]**
LUFA 2.2 (lowOM)	Standard soil	Loamy sand	78.9	13.8	7.3	41.8	5.1	5.9	4.18	9.7
North Wales (highOM)	Pasture	Peat loam	57.7	29.7	12.6	96.0	5.0	5.5	16.70	11.8
Dorset (pH range)	Acidic Heath	Sandy	91.7	4.7	3.5	49.2	3.1	4.2	8.00	5.4

Table 2: Regression parameters of 3 parameter log logistic regression of reproductive toxicity data of $AgNO_3$ and Ag ENPs in different pore waters with reproductive EC_{50} concentrations [mg Ag/L], and regression slope averages ± SE and R^2 values.

		$AgNO_3$	**Ag 3-8Unf**	**Ag 52PVP**
lowOM	EC_{50}	0.19 ± 0.03	7.80 ± 0.82	4.47 ± 0.23
LUFA	Slope	4.37 ± 1.68	3.51 ± 1.33	5.56 ± 2.01
	R^2	0.7418	0.8651	0.955
highOM	EC_{50}	0.19 ± 0.02	3.48 ± 0.97	4.07 ± 0.69
North	Slope	3.19 ± 0.46	1.08 ± 0.29	1.61 ± 0.34
Wales	R^2	0.9137	0.7341	0.8621
pH4.8	EC_{50}	0.11 ± 0.01	0.86 ± 0.08	0.87 ± 0.09
Dorset	Slope	2.94 ± 0.34	1.66 ± 0.24	1.65 ± 0.26
	R^2	0.9632	0.9597	0.9515
pH6.1	EC_{50}	0.36 ± 0.05	12.82 ± 3.21	10.57 ± 1.05
Dorset	Slope	5.86 ± 3.90	1.05 ± 0.35	2.80 ± 0.71
	R^2	0.7528	0.5454	0.7295
pH7.2	EC_{50}	0.37 ± 0.11	9.06 ± 1.28	10.67 ± 1.59
Dorset	Slope	1.09 ± 0.26	1.25 ± 0.25	1.08 ± 0.21
	R^2	0.7128	0.7838	0.7876