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Contact CEH NORA team at
noraceh@ceh.ac.uk

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

Pauline L. Waalewijn-Kool[a], Svenja Rupp[b], Stephen Lofts[c], Claus Svendsen[d], Cornelis A.M. van Gestel[a]*

[a] Department of Ecological Science, Faculty of Earth and Life Sciences, VU University, Amsterdam, The Netherlands

[b] Aquatic Ecology, Faculty of Biology, University Duisburg-Essen, Essen, Germany

[c] Centre for Ecology and Hydrology, Lancaster Environment Centre, Lancaster, United Kingdom

[d] Centre for Ecology and Hydrology, Wallingford, Oxfordshire, United Kingdom

* Corresponding authors

Dr. C.A.M. (Kees) van Gestel

Department of Ecological Science

Faculty of Earth and Life Sciences

VU University

De Boelelaan 1085

1081 HV Amsterdam

Phone: +31-20-5987079 / 7004

Fax: +31-20-5987123

E-mail: kees.van.gestel@vu.nl

32 **ABSTRACT**

33

34 Organic matter (OM) and pH may influence nanoparticle fate and effects in soil. This study

35 investigated the influence of soil organic matter content and pH on the toxicity of ZnO-NP

36 and $ZnCl_2$ to *Folsomia candida* in four natural soils, having between 2.37 and 14.7% OM and

37 pH_{CaCl2} levels between 5.0 and 6.8. Porewater Zn concentrations were much lower in ZnO-

38 NP than in $ZnCl_2$ spiked soils, resulting in higher Freundlich sorption constants for ZnO-NP.

39 For $ZnCl_2$ the porewater Zn concentrations were significantly higher in less organic soils,

40 while for ZnO-NP the highest soluble Zn level (23 mg Zn/l) was measured in the most

41 organic soil, which had the lowest pH. Free Zn^{2+} ion concentrations were higher for $ZnCl_2$

42 than for ZnO-NP and were greatly dependent on pH (pH_{pw}) and dissolved organic carbon

43 content of the pore water. The 28-d EC_{50} values for the effect of $ZnCl_2$ on the reproduction of

44 *F. candida* increased with increasing OM content from 356 to 1592 mg Zn/kg d.w. For ZnO-

45 NP no correlation between EC_{50} values and OM content was found and EC50 values ranged

46 from 1695 in the most organic soil to 4446 mg Zn/kg d.w. in the higher pH soil. When based

47 on porewater and free Zn^{2+} concentrations, EC_{50} values were higher for $ZnCl_2$ than for ZnO-

48 NP, and consistently decreased with increasing pH_{pw}. This study shows that ZnO-NP toxicity

49 is dependent on soil properties, but is mainly driven by soil pH.

50

51

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

53 bioavailability

54

1. Introduction

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

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

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

89 and increases the colloidal stability (Quik et al., 2010). Several metal oxide nanoparticles are

90 stabilized in aqueous solutions by the adsorption of NOM, due to increased electrostatic

91 repulsion (Domingos et al., 2009; Yang et al., 2009; Zhang et al., 2009). It has been

92 demonstrated that NOM is able to coat nanoparticles (Lead and Wilkinson, 2006), resulting

93 in a nanoscale coating of the particles (Lowry et al., 2012). The thickness of the coating

94 increases with increasing humic acid concentration (Baalousha et al., 2008). Such a natural

95 coating causes charge neutralization and colloidal stability, as the surface of metal-based

96 nanoparticles is mostly positively charged at circum-neutral pH and humic acid molecules are

97 partially deprotonated (Baalousha et al., 2008; Bian et al., 2011; Tourinho et al., 2012). This

98 coating by humic acids could imply that the release of the toxic metal ions is diminished by

99 blocking the nanoparticle surface, but so far this has not been studied in much detail.

100 Understanding the interactions of manufactured nanoparticles in soils is difficult,

101 because complex interactions occur with the solid phase and the pore water. It is already

102 known that a long time is needed to reach equilibrium for soil systems spiked with ZnO-NP

103 (Scheckel et al., 2010; Waalewijn-Kool et al., 2013a). Complex interactions with the soil

104 matrix could diminish the exposure of soil organisms to pristine ZnO-NP, but could lead to

105 more complex exposures to coated NP, released Zn ions and mixtures of ZnO-NP and

106 released ions at varying ratios (Tourinho et al., 2012). Unfortunately, appropriate

107 characterization techniques for nanoparticles in soil do not exist and most techniques start

108 with pre-treatment of the soil by preparing a suspension of soil extracts (Lead and Wilkinson,

109 2006). Similar to aquatic solutions, NOM is likely to affect the transformations and

110 speciation of ZnO-NP in soils. It is hypothesized that with increasing OM content, ZnO-NP

111 interacts stronger with OM by forming OM-ZnO clusters, resulting in a decreased

112 bioavailability, dissolution and toxicity. Other soil properties, such as pH and cation

113 exchange capacity (CEC) may also affect the bioavailability and toxicity of ZnO-NP. In a

114 previous study, the effect of soil pH has been studied in amended field soils. Increased

115 toxicity of ZnO-NP with decreasing pH was demonstrated for earthworms (Heggelund et al.,

116 2013) and springtails (Waalewijn-Kool et al., 2013b), but pH effects were less consistent for

117 the toxicity of ZnO-NP to isopods (Tourinho et al., 2013).

118 This study investigated how organic matter content, in addition to pH, influenced the

119 bioavailability and toxicity of ZnO-NP and $ZnCl_2$ to the springtail *Folsomia candida*.

120 Springtails are abundant in most natural soils and *F. candida* represents the collombolans in

121 ecotoxicological tests (Fountain and Hopkin, 2005). Four natural soils were tested that

122 provide a range of organic matter content (2.37 - 14.7%) and slightly different pH_{CaCl2} levels

123 (5.0 - 6.8). This study does not differentiate between types of NOM (quality), but just
124 considers the total content of organic matter in soils. For both Zn forms, the 28-d EC_{50} values
125 were expressed on the basis of total Zn, porewater Zn and free Zn^{2+} ion concentrations.

126

127 **2.   Material and methods**

128

129 *2.1. Soil properties*

130

131   Four natural soils were collected from different countries within Europe: from Coimbra
132 in Portugal, Speyer in Germany (Lufa standard soil 2.2, Sp 2121), De Kwakel in The
133 Netherlands (grassland, soccer field) and from Rhydtalog in North-Wales, United Kingdom,
134 hereafter called soils 1, 2, 3 and 4, respectively. These soils, which represent different organic
135 matter contents and pH levels, were homogenized, sieved through a 5 mm mesh and air dried
136 before spiking and toxicity testing.
137   The organic matter content was determined as loss on ignition at 500 °C in an ashing
138 oven. Since previous studies showed little change (no more than 0.2 units) of soil pH during
139 28-day toxicity tests on ZnO-NP with *F. candida*, the pH_{CaCl2} of the soils was measured in the
140 middle of the toxicity test, after two weeks exposure, in two replicate samples of each
141 treatment. Soils were shaken with 0.01 M $CaCl_2$ solution (1:5) for 2 hours at 200 rpm. After
142 settlement of the particles, the pH of the soil solution was recorded using a Consort P907
143 meter. The Water Holding Capacity (WHC) was determined following ISO (1999). The
144 cation exchange capacity (CEC) was determined by the Silver Thiourea Method (Dohrmann,
145 2006). Approx. 2 g dry soil was shaken with 25 mL 0.01 M silver thiourea complex cation
146 (AgTU) solution for 3 hours at 200 rpm to achieve a complete exchange of all cations. Four
147 blanks without soil were included. Ag was measured in the supernatant solution by flame
148 Atomic Absorption Spectrometry (AAS) (Perkin Elmer AAnalyst 100). The decrease in Ag
149 concentration is a measure for the CEC of the soil.

150

151 *2.2. Test compounds*

152

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

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

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

167

168 *2.3. Soil and porewater analysis*

169

170   Two samples per test concentration ($\pm$ 100 mg dried soil) were taken from the spiked
171 soils and digested in a mixture of Milli-Q water, concentrated HCl and concentrated HNO_3
172 (1:1:4 by vol.) using an oven (CEM MDS 81-D). After digestion for 7 hours at 140 °C,
173 solutions were analysed for total zinc concentrations by flame AAS (Perkin Elmer AAnalyst
174 100). Certified reference material (ISE sample 989 of River Clay from Wageningen, The
175 Netherlands) was used to ensure the accuracy of the analytical procedure. Detection limit of
176 the analysis was 0.003 mg Zn/L.

177   Pore water was collected at the beginning of each toxicity test by centrifugation. For
178 that purpose, 30 g soil portions were sampled, saturated with Milli-Q water and incubated for
179 another three days equilibration. Then the soils were centrifuged for 45 min. (Centrifuge
180 Falcon 6/300 series, CFC Free) with a relative force of 2000 g over two round filters (S&S
181 597 Ø 47 mm, pore size 11 μm) and a 0.45 μm membrane filter (S&S Ø 47 mm), placed
182 inside the tubes (method cf. Hobbelen et al., 2004). Approximately 7 mL pore water per
183 sample was collected for Zn analysis by flame AAS. Since we expected the grassland soil to
184 be limed, we also measured Ca concentrations in the porewater samples from the controls of
185 soil 3 by flame AAS. For the sake of comparison, Ca concentration was also measured in the
186 porewater of the control Lufa 2.2 soil (soil 2), which we always use as our reference soil. The
187 dissolved organic carbon (DOC) concentration in the pore water was measured by high
188 temperature oxidation at 850°C - 900°C and detection of the formed CO_2 (liquiTOC). The pH
189 of the pore water (pH_{pw}) was measured using a Consort P907 meter.

190   The Zn^{2+} ion concentrations in the pore water from the four soils spiked with ZnO-NP

191   and $ZnCl_2$ were calculated with the speciation model WHAM7 using H^+, Ca, Zn and DOC

192   concentrations (mg/L) of the pore water and soil pH.

193

194   *2.4. Toxicity tests*

195

196   The springtail *F. candida* (Berlin strain; VU University Amsterdam) was cultured in

197   pots with a base of moist plaster of Paris mixed with charcoal at 20 ± 1 °C at a light/dark

198   regime of 12/12 h. The experiments were initiated with juveniles of the same age (10 - 12

199   days) that were obtained by synchronising the egg laying of the culture animals, fed with

200   dried baker's yeast (Dr. Oetker).

201   Each soil was tested in a separate toxicity test, including the two Zn forms, control soil

202   without added Zn and Lufa 2.2 soil as a control for springtail performance. The ISO guideline

203   11267 for testing for chemical effects on the reproduction of springtails was followed (ISO,

204   1999). Tests were conducted in 100 mL glass jars containing 30 g moist soil with five

205   replicates for each treatment. At the start of the test, ten synchronised animals were

206   transferred into each test jar. The jars were filled randomly and before introduction the

207   animals were checked under the microscope for a healthy appearance. The animals were fed a

208   few grains of dried baker's yeast (Dr. Oetker). The jars were incubated in a climate room at

209   20 ± 1 °C at a light/dark regime of 12/12 h. Once a week, the moisture content of test soils

210   was checked by weighing the jars, and moisture was replenished with Milli-Q water when

211   necessary. The jars were also aerated by this procedure.

212   After four weeks, the jars were sacrificed for determination of springtail survival and

213   reproduction. Each jar was emptied into a 200 mL beaker glass and 100 mL tap water was

214   added. The mixture was stirred carefully to let all the animals float to the surface. The

215   number of surviving adults and juveniles produced were counted manually after taking a

216   picture of the water surface using a digital camera (Olympus, C-5060).

217

218   *2.5. Data analysis*

219

220   Using the soil and porewater concentrations, sorption of zinc to the test soil was

221   described by a Freundlich isotherm:

222

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

224

225   where,

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

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

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

229   n = shape parameter of the Freundlich isotherm

230

231   EC_{50} values for the effect on reproduction were estimated applying the logistic model

232   of Haanstra et al. (1985) and were determined based on total Zn concentrations in soil and

233   dissolved Zn and free Zn^{2+} concentrations in the pore water. A generalized likelihood ratio

234   test (Sokal and Rohlf, 1995) was applied to compare EC_{50} values based on total Zn

235   concentration obtained for both Zn forms and for each soil. Calculations were performed in

236   SPSS Statistics 20.

237

238   **3.   Results**

239

240   *3.1.   Soil properties*

241

242   Table 1 summarizes the properties of the four test soils used in this study. The organic

243   matter content measured in the different soils increased from 2.37% in soil 1 up to 14.7% in

244   soil 4. The DOC concentrations in the pore water increased with increasing OM content from

245   approx. 64 mg/L in soils 1 and 2 up to 3605 mg/L in soil 4. Soils 1 and 2 had nearly the same

246   pH_{CaCl2} (around 5.7 - 5.8), while the pH_{CaCl2} of soil 3 was slightly higher (6.8) and soil 4 was

247   more acidic (pH_{CaCl2} 5.0). This was probably due to liming of soil 3 in the field, because more

248   than a 3-fold higher Ca level was measured in the pore water of this soil (221 mg/L)

249   compared to Lufa 2.2 (64.3 mg/L). Clay contents ranged between 4.2 and 13% and was

250   highest in the most organic soil 4. CEC increased with increasing OM content for soils 1, 2

251   and 3, but was lower in the more acidic soil 4 (11.8 mval/100g) compared to soil 3 (20.0

252   mval/100g).

253

254   *3.2.   Total Zn analysis and soil pH*

255

256   Total Zn analyses of the four test soils showed recoveries of Zn ranging from 66.9 to

257   127% (Supplementary Data, Tables S1-S4). Zinc concentrations in the soil were corrected for

258   the zinc measured in the controls, which may explain the variability in the Zn recovery. Total

259   Zn concentrations in the untreated soil were 75.6, 17.1, 39.4 and 71.5 mg Zn/kg d.w. for soils

260   1, 2, 3 and 4, respectively. As the measured zinc concentrations in the reference material

261   were within 6% of the certified concentrations, the measured Zn concentrations were

262   considered reliable. All calculations used measured Zn concentrations.

263   Soil pH_{CaCl2} and porewater pH_{pw} increased with increasing ZnO-NP concentrations, and

264   decreased with increasing concentrations of $ZnCl_2$ (Supplementary Data, Tables S5-S8).

265

266   *3.3. Porewater Zn concentration and sorption*

267

268   The Zn concentrations in the pore water of the four test soils are presented in Table S7

269   for ZnO-NP and in Table S8 for $ZnCl_2$. In soils 2, 3 and 4 the Zn concentrations in the pore

270   water increased with increasing ZnO-NP concentrations, up to a maximum of 2.83, 3.52 and

271   23.8 mg Zn/L, respectively. So, the highest porewater concentration was measured in soil 4

272   having the highest organic matter content and the lowest pH_{CaCl2}. For soil 1, the maximum

273   porewater concentration of 3.09 mg Zn/L was measured at an intermediate nominal

274   concentration of 800 mg Zn/kg d.w. The porewater Zn concentrations for $ZnCl_2$ were much

275   higher than for ZnO-NP spiked soils. For the $ZnCl_2$-spiked soils 1, 2, 3 and 4 the porewater

276   Zn levels increased with increasing soil concentrations up to 1021, 720, 10.4 and 63.7 mg

277   Zn/L, respectively. The highest porewater concentrations were found for the soils 1 and 2

278   with the lowest OM content, while for the more organic soils 3 and 4 higher Zn porewater

279   concentration was found in the more acidic soil 4.

280   The porewater Zn levels were translated into Freundlich sorption constants for ZnO-NP

281   and $ZnCl_2$, indicating zinc availability in the four soils (Table 2). The k_f value for $ZnCl_2$

282   increased with OM content except for soil 4, and with CEC for all soils. The highest k_f value

283   of 458 $(L/kg)^{1/n}$ ($n = 0.572$) was calculated for soil 3 having the highest pH. For ZnO-NP, k_f

284   values did not correlate with OM content. The highest k_f value of 1146 $(L/kg)^{1/n}$ ($n = 1.08$)

285   was calculated for soil 3, while the lowest k_f value of 97.1 $(L/kg)^{1/n}$ ($n = 1.45$) was estimated

286   for the most organic soil (soil 4). No k_f value could be derived for ZnO-NP sorption in soil 1.

287   For the other soils, k_f constants increased with increasing soil pH, suggesting higher

288   availability in the more acidic soil 4. In all soils spiked with ZnO-NP the shape parameter *n*

289   of the Freundlich isotherm was higher than one, suggesting saturation of the pore water or Zn

290   precipitation. For all soils spiked with $ZnCl_2$ the shape parameter *n* was below one,

291   suggesting saturation of the solid phase at high porewater concentrations.

3.4. Free Zn^{2+} ion concentrations

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

3.5. Toxicity

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

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

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

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

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

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

359 soil concentrations making that reduction in reproduction was not consistently related with

360 free Zn^{2+} concentration.

361

362 **4.   Discussion**

363

364   In light of nanoparticle risk assessment, soil properties need to be taken into account to

365 predict the bioavailability and toxicity of ZnO-NP. The most important soil properties

366 determining equilibrium partitioning of metals in soils are the adsorption phases (clay, OM

367 and hydroxides), the amount of available sorption sites (CEC) and pH (Janssen et al., 1997).

368 The aim of this study was to investigate the effect of soil OM content and pH on ZnO-NP

369 dissolution and toxicity to *F. candida* in four natural soils, ranging in OM content from 2.37

370 to 14.7% and in pH_{CaCl2} from 5.04 to 6.78. The EC_{50} values for $ZnCl_2$ based on total soil

371 concentrations increased with increasing OM content, but contrary to our hypothesis no

372 correlation between toxicity and OM content was found for ZnO-NP. Soil pH had a major

373 influence on the EC_{50} values estimated on the basis of porewater Zn concentrations.

374   For $ZnCl_2$, the porewater Zn concentrations were much higher in less organic soils

375 (containing approx. 2.37-3.09% OM) than in soils containing approx. 10.6 to 14.7% OM.

376 This is explained by the amount of binding sites available to bind zinc, because it is known

377 that humic and fulvic acids form chelates with Zn ions (Alloway, 1990). The porewater Zn

378 levels in the more organic soils also showed that the CEC influenced the soluble Zn

379 concentrations. The lowest porewater Zn concentrations were measured in the soil with the

380 highest CEC. For both zinc forms, the lowest dissolved Zn concentrations and the highest

381 sorption constants were estimated for the Dutch grassland soil with the highest pH. This can

382 be explained by the absence of competition with H^+ ions for Zn binding at the negatively

383 charged phases in the soil when pH levels are increasing (Alloway, 1990).

384   The solubility of ZnO-NP, measured as dissolved Zn centrifuged over a 0.45 μm filter,

385 was much lower than for the soluble metal salt $ZnCl_2$ in all four soils. For ZnO-NP, the

386 highest soluble Zn levels (max. 23.8 mg Zn/L) were measured in the most organic soil. It

387 cannot be excluded that the dissolution of ZnO-NP in this soil was stimulated by the lower

388 soil pH. The larger amount of water needed to reach 50%WHC of this soil (320, 255, and 115

389 mL water more than in soils 1, 2 and 3, respectively) may have triggered a greater dissolution

390 of ZnO-NP. Nevertheless, this is the first study that shows that ZnO-NP dissolution results in

391 different available Zn concentrations in soils with different soil properties. The aggregation

392 and dissolution of ZnO-NP in different soils have not been explored much. Aquatic studies

393 demonstrated that OM can have a "masking" effect, either by direct coating of the
394 nanoparticle surface or by minimizing dissolution (Lowry et al., 2012). Once coated and
395 aggregated in either the solid phase or in the pore water, it can be more difficult for the ZnO-
396 NP to release Zn into the solution. In soils, the equilibrium processes of metals between pore
397 water and solid phases are rather complex (Tipping et al., 2003). Although increasing
398 solution pH will decrease metal solubility, it may increase dissolved metal concentrations
399 because of the formation of metal complexes with DOC in the porewater. DOC may coat
400 reactive adsorption sites, inactivating them and therefore indirectly inhibit metal adsorption.
401 The shape parameter n of the Freundlich isotherm was higher than one for ZnO-NP spiked
402 soils, supporting this idea of saturation of the pore water with Zn complexes. In our soils the
403 OM could have enhanced the release of Zn from the ZnO-NP, for example by assisting in
404 breaking up large aggregates (de-agglomeration). Several aquatic studies with ZnO-NP and
405 humic acids or clay minerals support this hypothesis (Bian et al., 2011; Li et al., 2011a; Miao
406 et al., 2010; Scheckel et al., 2010).

407   Our experimental data showed that both soil pH and DOC concentrations influenced
408 ZnO-NP dissolution. The measured porewater Zn concentrations reflected to some extent the
409 effect of pH on ZnO-NP solubility. The pH levels in all soils increased with increasing total
410 Zn concentration, confirming that spiking ZnO into the soils did cause an increased soil pH.
411 It should be noted that, to enable the creation of full dose-response curves and compare
412 toxicity among different soils, we had to test rather high ZnO-NP concentration, by far
413 exceeding levels expected to occur in the environment (Gottschalk et al., 2009). Porewater Zn
414 concentrations generally increased with increasing total Zn concentration in the soils, but
415 peaked at intermediate soil concentrations for soil 1. The pH where the net surface charge of
416 ZnO-NP is zero and highest stability of the particles is expected (point of zero charge, pzc),
417 ranges between 7.5 and 9.8 in water (Keller et al., 2010; Kosmulski, 2004) and above 8 in
418 soil (Collins et al., 2012). The pH_{CaCl2} values of our test soils were below this pzc, suggesting
419 low stability of the particles. The pH values of the pore water of soils 1, 2, and 3, however,
420 were closer to this pzc (in between 7 and 8). In theory, an increase in stability could lead to
421 an increase in Zn release from the nanoparticles due to a larger surface area of the individual
422 particles compared to aggregated nanoparticles. The peak in porewater and free Zn^{2+}
423 concentrations for soils 1, 2 and 4 suggested a reduction of Zn release above a pH_{pw} of 7.
424 Such a peak has been observed before in Lufa 2.2 soil for ZnO-NP as well as for non-nano
425 ZnO in aged soils (Waalewijn-Kool et al., 2013a). The present study showed that also in
426 freshly spiked soil ZnO-NP dissolution may be reduced at high exposure concentrations. This

427 phenomenon was however, restricted to soil 1, which seemed strange as in soils 2 and 3 pH_{pw}

428 reached higher levels. When plotting porewater Zn levels, measured at the same total Zn

429 concentrations (Table S7) against pH_{pw} in the four soils, a linear decrease with increasing

430 pH_{pw} was seen for all ZnO-NP treatment levels. This seems to confirm that the Zn release

431 from ZnO-NP decreased with increasing pH_{pw}. At all exposure levels, Zn concentrations in

432 the pore water however, also linearly increased with increasing DOC content (Table 1). This

433 shows that organic matter content also played a role in the dissolution of ZnO-NP, probably

434 by binding (part of) the released Zn, and making that not all Zn in the pore water was freely

435 available. The latter was accounted for when calculating free Zn ion activities.

436 　　　　For ZnO-NP no significant effect (increase or decrease) of soil OM content on the

437 toxicity to springtail reproduction was detected, while the EC_{50} values for $ZnCl_2$ increased

438 with increasing OM content. The EC_{50} for ZnO-NP in Lufa 2.2 soil was 3493 mg Zn/kg d.w.

439 which was comparable to the EC_{50} of 3159 mg Zn/kg d.w. estimated in previous experiment

440 with this type of ZnO-NP (Waalewijn-Kool et al., 2012). The higher pH in the Dutch

441 grassland soil most probably resulted in the lower toxicity observed for ZnO-NP and $ZnCl_2$

442 based on total Zn concentrations. Zinc toxicity has been shown to be related to OM and clay

443 content in freshly contaminated OECD artificial soil with 10% OM (Smit and Van Gestel,

444 1998). The effect of OM on ZnO-NP toxicity is still poorly understood, with many studies

445 producing contradictory results. Li et al. (2011b) found a reduced toxicity of ZnO-NP to

446 bacteria with increasing DOC concentrations in aquatic media, while Blinova et al. (2010)

447 reported that DOC could not decrease the acute toxicity of ZnO-NP to crustaceans in natural

448 water. Li et al. (2011a) exposed *Eisenia fetida* for 96 hours to soil extracts and found that

449 addition of humic acids enhanced ZnO-NP dissolution, but reduced toxicity.

450 　　　　Released Zn may form steady organic complexes, which is likely to occur when more

451 DOC is present in the soil pore water (Fang et al., 2009). From our study, it remained unclear

452 if the effect of DOC concentrations on the EC_{50} values based on porewater concentrations

453 (for both Zn forms) was significant. A protective effect of DOC in the pore water may have

454 occurred in our most organic soils, as could be seen from the increase of porewater Zn

455 concentrations with increasing DOC level in the case of ZnO-NP. But toxicity seemed most

456 affected by pH, as EC_{50}s expressed on the basis of dissolved Zn concentrations in the pore

457 water consistently decreased with increasing pH (see Figure 1). This suggests that at the

458 lower pH_{pw} levels, which especially occurred in the $ZnCl_2$-spiked soils, H^+ ions may have

459 competed with dissolved Zn species resulting in lower toxicity. According to the theory of

460 the terrestrial Biotic Ligand Model the reduction of competition with H^+ ions may lead to

461   higher toxicity data (Thakali et al., 2006). This effect was also observed for the EC_{50} values

462   based on free Zn^{2+} ion concentrations, in particular for $ZnCl_2$, which suggests that the ionic

463   Zn was a good predictor for toxicity.

464   In conclusion, the toxicity of $ZnCl_2$ was related to soil OM content but this was not the

465   case for ZnO-NP, suggesting that ZnO-NP have to dissolve to make Zn bioavailable. Soil pH

466   and DOC play an important role in the dissolution of ZnO-NP and its release into the pore

467   water. Further research is necessary on interactions between ZnO-NP and soil components

468   such as OM, affecting ZnO-NP toxicity, but it cannot be seen separately from the role of

469   other soil properties, such as pH.

470

471 **Supplementary data**

472   Supplementary data include dose-response curves for ZnO-NP (Figure S1) and $ZnCl_2$ (Figure

473   S2) in the four test soils, measured Zn concentrations and pH values in the four test soils

474   (Table S1-S6) and in pore water (Tables S7 and S8) and free Zn^{2+} ion concentrations in the

475   pore water calculated with WHAM7 (Tables S9 and S10).

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484

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Figures:

Figure. 1. Estimated EC_{50} values for the effect of Zn on the reproduction of *Folsomia*

candida based on total Zn concentrations (mg Zn/kg d.w.) and porewater Zn concentrations

(mg Zn/l) in relation to pH_{CaCl2}, pH_{pw}, soil organic matter content and DOC concentrations in

the pore water (see Table S-5 to S-8 for measured pH values and Table 1 for soil properties).

Data used are from 28-d toxicity tests in the four test soils freshly spiked ZnO-NP and $ZnCl_2$

(see Table 3).

Plots (clockwise from top-left):

Top-left plot:
y = 1555x - 7195
R² = 0.787

y = 779x - 3720
R² = 0.380

Y-axis: EC$_{50}$ (mg Zn/kg d.w.)
X-axis: pH$_{CaCl2}$

Top-right plot:
y = 21.1x + 163
R² = 0.846

y = 2.50x + 22.4
R² = 0.527

Y-axis: EC$_{50}$ (mg Zn/L)
X-axis: pH$_{pw}$

Bottom-left plot:
y = -65.4x + 3652
R² = 0.116

y = 107x + 135
R² = 0.967

Y-axis: EC$_{50}$ (mg Zn/kg d.w.)
X-axis: soil OM content (%)

Bottom-right plot:
y = 0.0061x + 32.6
R² = 0.239

y = 0.0012x + 2.75
R² = 0.991
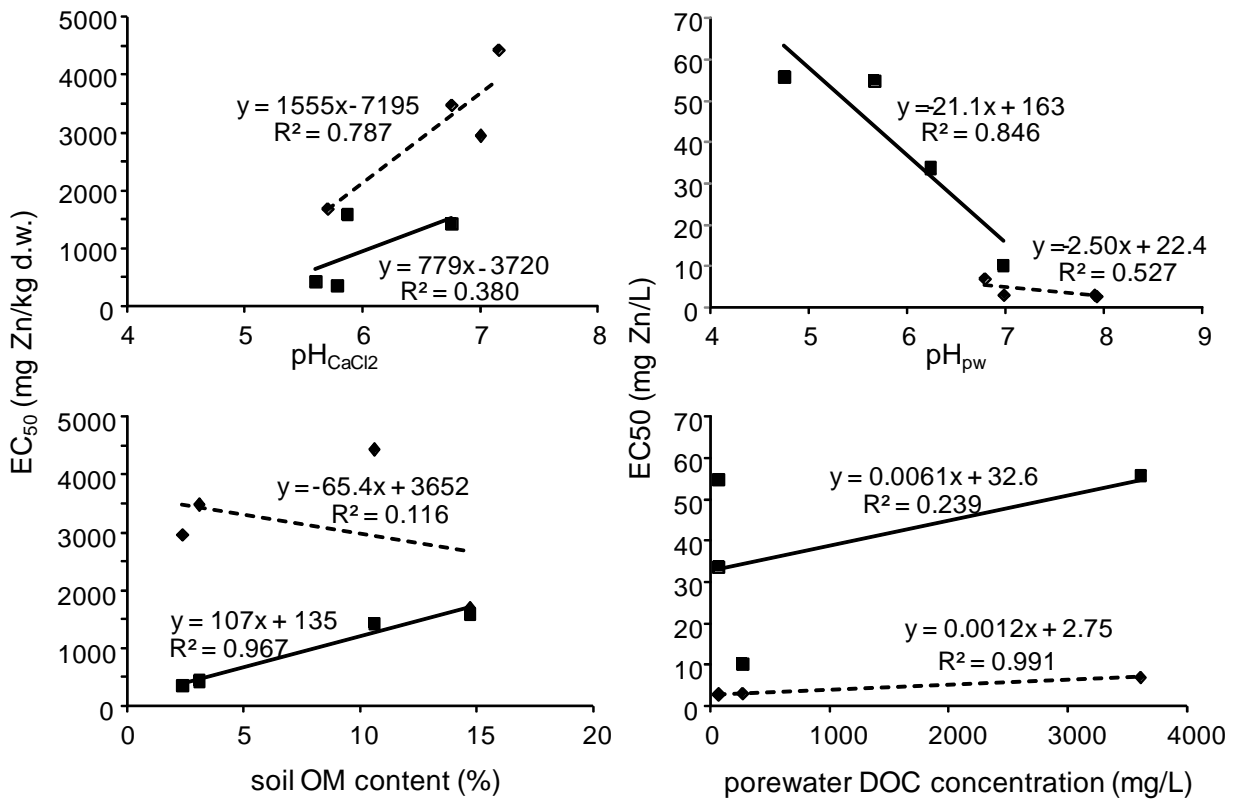
Y-axis: EC50 (mg Zn/L)
X-axis: porewater DOC concentration (mg/L)

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Fig. 1. Estimated EC$_{50}$ values for the effect of Zn on the reproduction of *Folsomia candida* based on total Zn concentrations (mg Zn/kg d.w.) and porewater Zn concentrations (mg Zn/l) in relation to pH$_{CaCl2}$, pH$_{pw}$, soil organic matter content and DOC concentrations in the pore water (see Table S-5 to S-8 for measured pH values and Table 1 for soil properties). Data used are from 28-d toxicity tests in the four test soils freshly spiked ZnO-NP and ZnCl$_2$ (see Table 3).

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622

623 **Table 1.** Properties of Coimbra (soil 1), Lufa 2.2 (soil 2), Dutch grassland (soil 3) and North

624 Wales (soil 4) test soils used to determine the influence of soil properties on the toxicity of

625 ZnO nanoparticles to *Folsomia candida*. Reported OM, DOC and CEC values are the mean

626 of two measurements.

627

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

628 OM = organic matter; WHC = water holding capacity; CEC = cation exchange capacity;

629 DOC= dissolved organic carbon, measured in the pore water;

630

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

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

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

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

643

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

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

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651 Waalewijn-Kool *et al.*

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653 Supplementary Data

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

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661 Pauline L. Waalewijn-Kool[a], Svenja Rupp[b], Stephen Lofts[c], Claus Svendsen[d], Cornelis A.M.
662 van Gestel[a]*

663

664 [a]Department of Ecological Science, Faculty of Earth and Life Sciences, VU University,
665 Amsterdam, The Netherlands

666 [b]Aquatic Ecology, Faculty of Biology, University Duisburg-Essen, Essen, Germany

667 [c]Centre for Ecology and Hydrology, Lancaster Environment Centre, Lancaster, United
668 Kingdom

669 [d]Centre for Ecology and Hydrology, Wallingford, Oxfordshire, United Kingdom

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673 * To whom correspondence may be addressed

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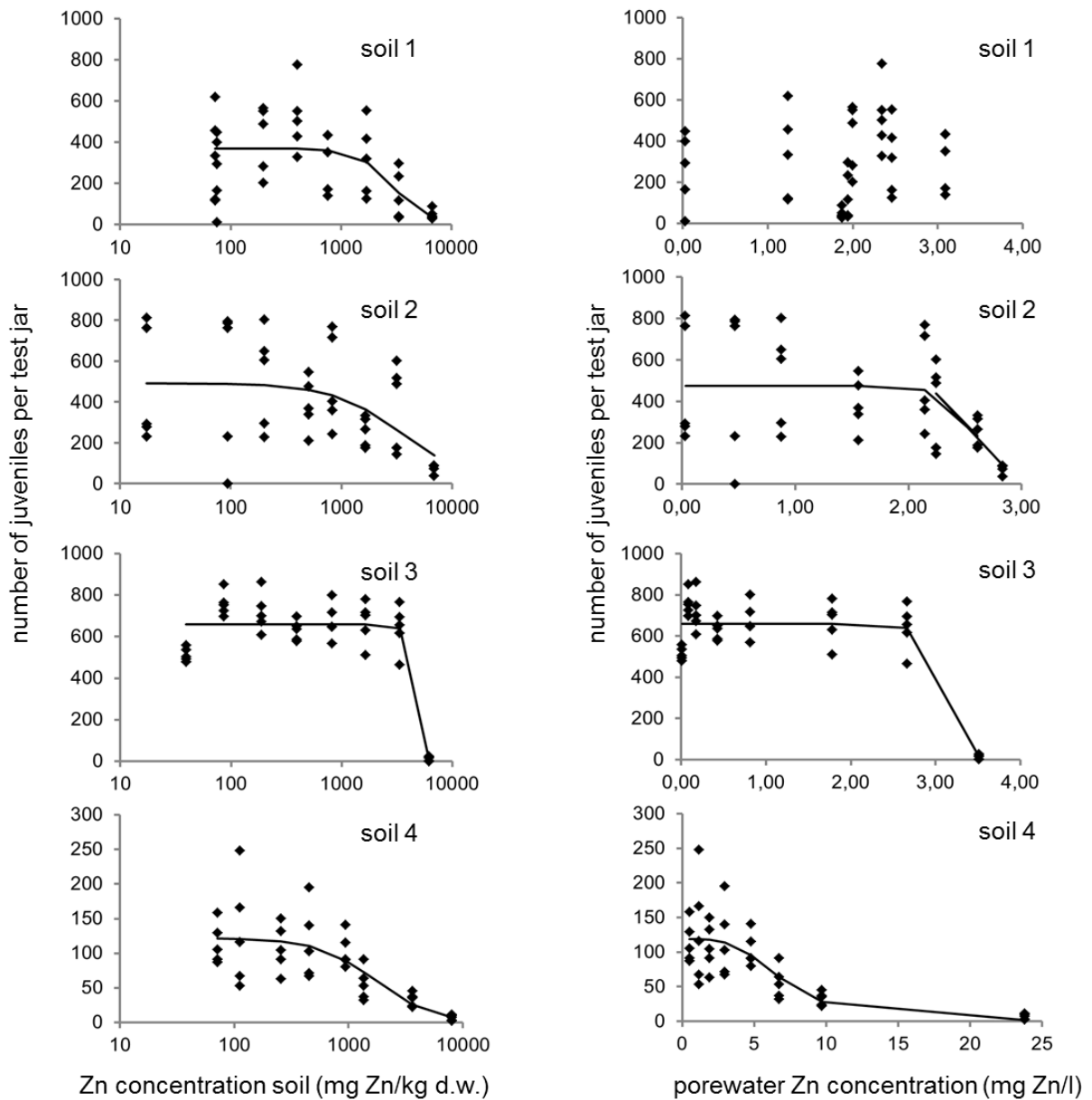
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677 **Fig. S1.** Effect of ZnO-NP on the reproduction (number of juveniles) of *Folsomia candida*
678 after 28 days exposure in four different test soils. See Table 1 for soil properties. Measured
679 concentrations of zinc in the soil (left) and the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

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684

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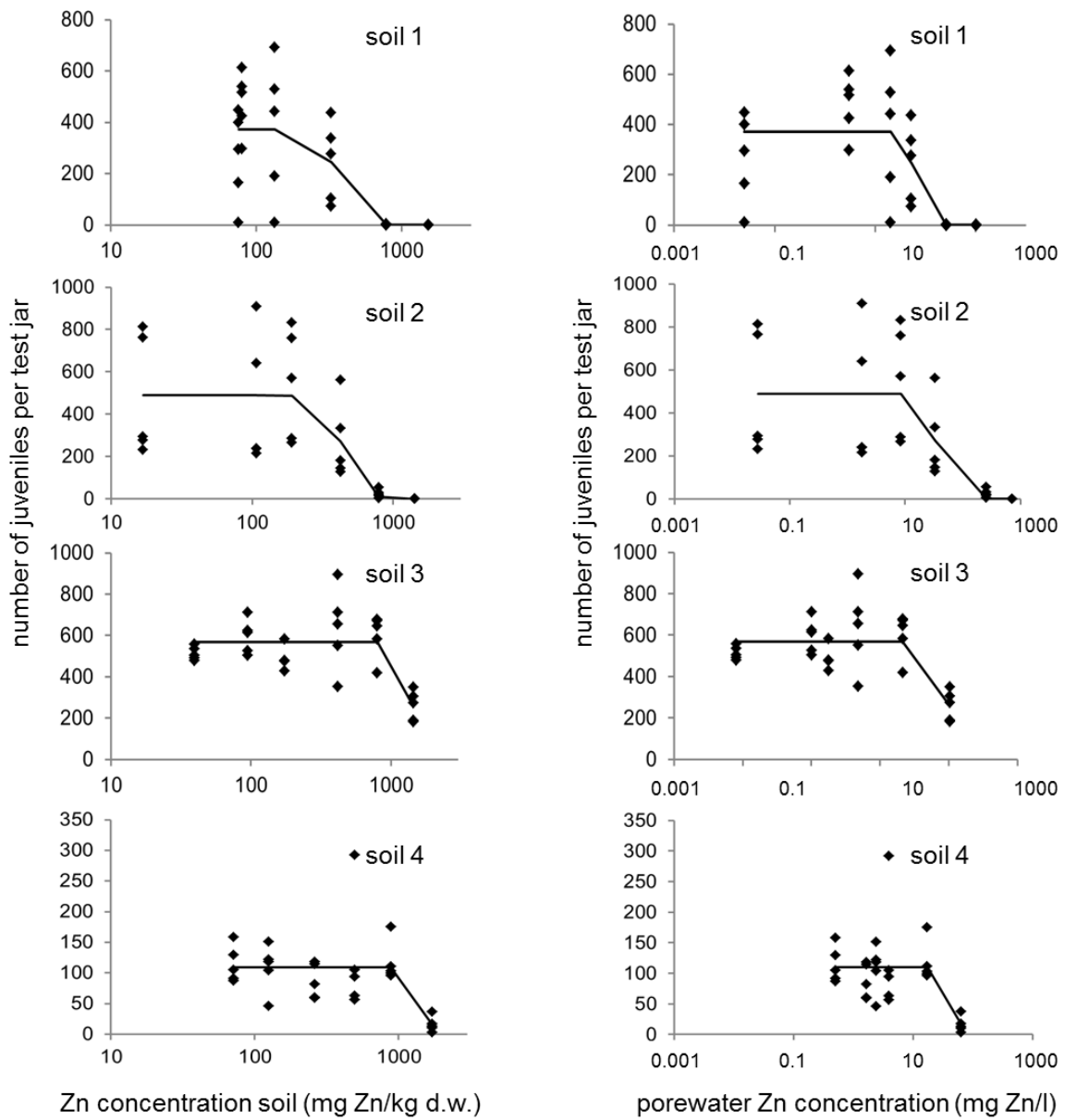686 28 days exposure in four different test soils. See Table 1 for soil properties. Measured
687 concentrations of zinc in the soil (left) and the pore water (right) are provided on the x-axis.
688 Dots represent the results of different replicates. Lines show the fit obtained with a logistic
689 model.

690

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

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

694   - test concentrations not included in this study

695

696

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

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

700   - test concentrations not included in this study

701

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

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

705   - test concentrations not included in this study

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707

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

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

711   - test concentrations not included in this study

712

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

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

715

716

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

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

719

720

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

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

725

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

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

730

731

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

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

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

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