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Copper accumulation and toxicity in earthworms exposed to CuO nanomaterials: Effects of particle coating and soil ageing.

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Abstract

Engineered nanomaterials (ENMs) may be functionalised with a surface coating to enhance their properties, but the ecotoxicity of the coatings and how hazard changes with ageing in soil is poorly understood. This study determined the toxic effect of CuO ENMs with different chemical coatings on the earthworm (*Eisenia fetida*) in fresh soil, and then after one year in aged soil. In both experiments, earthworms were exposed for 14 days to the CuO materials at nominal concentrations of 200 and 1000 mg Cu kg^{-1} dry weight and compared to $CuSO_4$. In the fresh soil experiment, CuO-COOH was found to be the most acutely toxic of the nanomaterials (survival, 20 ± 50 %), with tenfold increase of total Cu in the earthworms compared to controls. Sodium pump activity was reduced in most CuO ENM treatments, although not in the $CuSO_4$ control. There was no evidence of glutathione depletion or the induction of superoxide dismutase (SOD) activity in any treatment. Histology showed a mild hypoplasia of mucous cells in the epidermis with some nanomaterials. In the aged soil, the CuO-NH_4^+ was the most acutely toxic ENM (survival 45 ± 3 %) and Cu accumulation was lower in the earthworms than in the fresh soil study. Depletion of tissue Mn and Zn concentrations were seen in earthworms in aged soil, while no significant effects on sodium pump or total glutathione were observed. Overall, the study showed some coating-dependent differences in ENM toxicity to earthworms which also changed after a year of ageing the soil.

1. Introduction

Copper-based engineered nanomaterials (ENMs) are finding many applications including use as, catalysts in the manufacture of electronics (Gawande et al., 2016), wood-preservatives (Evans et al., 2008), anti-fouling paints (Anyaogu et al., 2008), antimicrobials (Bogdanovic et al., 2015), and as fungicides for agriculture (Tegenaw et al., 2016). It is therefore likely that Cu-based ENMs will be released into the environment. For metals, soil quality is an important consideration for the health of terrestrial ecosystems and ecosystem services such as agriculture, as well as human health. Consequently, there are guideline values for allowable metal concentrations in soils in many countries. For Cu, there is no overall soil guideline value that has been agreed with the European Union, or internationally. However, a few countries have developed their own guidance. For example, in Canada the soil quality guideline is 63 mg Cu kg^{-1} dry weight (dw) for an agricultural soil (CCME, 1999). The total Cu measurements in soils comprise naturally occurring Cu minerals and anthropogenic sources of Cu. The contribution of nanoforms of Cu to the overall contamination of soil is not well understood. Environmental fate modelling predicts that soil will be one of the sinks for metallic ENMs (Gottschalk et al., 2015), with modelled concentrations of $CuCO_3$ ENMs in sludge-treated soils ranging from 32 – 100 μg kg^{-1} dw for best to worst case scenarios respectively.

The effects of elevated metal concentrations in soils on terrestrial organisms is well-known from decades of research on naturally occurring Cu and anthropogenic inputs of dissolved Cu (Nahmani et al., 2007). Copper can be acutely toxic to earthworms with an estimated 14-day LC_{50} of 683 mg Cu kg^{-1} dw soil in laboratory-spiked artificial soil (Spurgeon et al., 1994). The background copper concentration in earthworms from unpolluted soils is around 8 – 12 mg Cu kg^{-1} dw (Streit, 1984) and this can increase to 58 mg Cu kg dw at pollutes sites, e.g., near mine smelters (in *Lumbriculus terrestris*, Ma, 2005). The EC_{50} for cocoon production in *E. fetida* was 210 mg Cu kg^{-1} dw in a laboratory-spiked natural sandy clay soil (Scott-Fordsmand et al., 2000).

The ecotoxicity of Cu-containing ENMs on earthworms has received less attention. Studies on metallic ENMs have shown that nanoparticulate forms of metals are generally less acutely toxic to soil organisms than their dissolved metal counterparts (ZnO, Heggelund

et al., 2014; Ag, Velicogna et al., 2016). Heckmann et al. (2011) reported that Cu nanoparticles were not acutely toxic at a nominal concentration of 1000 mg kg^{-1} dw, although a 10 % decrease in cocoon production was observed. However, there are concerns that ENMs may dissolve in the pore water of soil or release metal ions by dissolution. Direct contact toxicity of metal particles on the surface of the earthworm, or via ingestion, are also possible routes for delivering toxic metal ions.

Sub-lethal effects of ENMs include declines in reproduction as measured by cocoon production and the number surviving offspring (e.g., Ag, Heckmann et al., 2011; ZnO, Heggelund et al., 2014), and avoidance of ENM contaminated soil (Ag, Velicogna et al., 2016). Earthworms show total metal accumulation from exposure to metal-containing ENMs in soil (e.g., Ag, Diez-Ortiz et al., 2015), but whether or not the metal remains in the nanoform inside the organism is not yet clear. Earthworms exposed to metal-containing ENMs show similar modes of toxicity to those well-known for dissolved metals, including oxidative stress and increased metallothionein (Ag NPs, Gomes et al., 2015). However, there is less information on the sub-lethal effects of CuO ENMs on earthworms, and the available studies have mainly used pristine or unmodified versions of the ENMs (Unrine et al., 2010). There are concerns that surface coatings may alter the toxicity of ENMs, additionally, very little is known of the toxicity of aged Cu-containing ENMs in soil. Silver ENMs were found to increase in toxicity after one year of aging (earthworms, Diez-Ortiz et al., 2015), while ZnO ENMs were found to decrease in toxicity (springtails, Waalewijn-Kool et al., 2014). These studies high-light the importance of time scales in ecotoxicological tests for ENMs.

The aims of the current study were to determine the sub-lethal toxicity and accumulation of Cu in earthworms from CuO contaminated soils compared to the relevant metal salt controls. The study design incorporated a range of coatings on a common CuO core to represent anionic, carboxyl group (COOH), cationic, ammonium (NH_4^+) and organic ligands, polyethylene glycol (PEG), on the surface of the particles. In addition to survival and growth in freshly dosed soils, biochemical measurements were made to assess known mechanisms of Cu toxicity including effects on ionic regulation (tissue metal concentrations, Na^+/K^+-ATPase activity) and oxidative stress (total glutathione, superoxide dismutase activity), as well as evidence for histopathology in the tissues to aid interpretation of the

data. Having established the response to fresh soil, the soils were aged for one year, and then a second experiment conducted with selected endpoints to determine the effect of aged soil containing the aged CuO ENMs on newly exposed earthworms.

2. Methodology

Two experiments were conducted. The first with a freshly spiked soil and the second using the same soil after one year of ageing; referred to hereafter as fresh and aged soil experiments respectively. The experiments were conducted with a well-known Lufa 2.2 soil in a quadruplicate design at two nominal Cu concentrations (see below). Controls included unexposed animals (no added Cu or ENMs), and a metal salt control ($CuSO_4$ exposure).

2.2 Engineered nanomaterials and characterisation

The ENMs used in the experiments were provided by PlasmaChem as part of the Nanosolutions EU project (www.nanosolutionsfp7.com). Briefly, the ENMs were supplied as dry powders and with no significant chemical impurities reported by the supplier.
The functional groups that enabled the negative, neutral and positive surface coatings on the ENMs were necessarily of different molecular weight and hydrophobicity.
The precise details of how the coatings were synthesised and attached to the ENM core is commercially sensitive information of the suppliers. However, for clarity, we use the term '–NH_4^+' to mean an –NH_3 terminal ligand that has been ionised with H^+ ions to achieve positive charge. The primary particle size and chemical composition of each material are reported in Table 1 from the manufacturer's information. Further characterisation was done at Plymouth University (Table 1, supplementary Figure S1). Stock dispersions of the ENMs were freshly prepared in ultrapure deionised water (MilliQ water, Elga, 18.2 Ω) for characterisation purposes only at University of Plymouth using a standardised protocol (35 kHz frequency, Fisherbrand FB 11010, Germany) for 2 h to disperse the materials, following this they were subject to nanoparticle tracking analysis (NTA, Nanosight LM10) to determine hydrodynamic diameters. Dissolution of dissolved metals was determined in ultrapure water by dialysis using the method exactly according to Besinis et al. (2014) (Table 1). Some preliminary thermogravimetric analyses (TGA) indicated, as expected, that the mass of

coating varied according to the type of the coating. At the time of the study, it was not technically feasible to precisely determine the proportion of the molecular mass of the entire particle due to presence of the coating with enough certainty for normalising the dosimetry for the soil experiments. Instead, pragmatically, the experiments were prepared on a Cu mass basis, accounting for the known molecular weight of oxygen in the CuO. The treatments containing ENMs are therefore named to indicate the nominal mass concentration of Cu metal in that treatment (reported as mg Cu kg^{-1} dw from CuO ENMs hereafter). The analytical grade $CuSO_4$ used as the metal salt control (CAS 7758-98-7) is also reported as nominal total metal concentration. Unless otherwise stated, all the reagents used in the experiments were purchased from Sigma-Aldrich.

2.3 Stock animals

Adult *E. fetida* were used from an internal synchronous laboratory breeding culture held at University of Plymouth for both experiments. The test species were originally purchased from a commercial supplier (Blades Biological, Kent, UK) and kept in an artificial medium that comprises of bark chippings (1/3), Irish Moss Peat (1/3) and loamy sand topsoil (1/3) with surplus horse manure (from un-medicated horses) as feed. Animals were kept at a temperature of 20 ± 1 °C. Earthworms of between two and four months old (with a fully developed clitellum) were hand selected for the experiments. Earthworms were placed in the test soil (Lufa 2.2) one week prior to the experiment to acclimatise to the conditions and fed with clean horse manure.

2.4 Experimental designs and dosing of the test soil

Fresh Soil Experiment

This experiment was conducted using an approach similar to the standard OECD TG 207 the Earthworm Acute Toxicity (OECD, 1984) with some adaptions and additional endpoints. The experiment included a test soil control (no added Cu or ENMs), a metal salt control of Cu as $CuSO_4$ at 200 mg Cu kg^{-1} dry weight (dw), the uncoated CuO ENM, and those coated with $–NH_4^+$, –COOH or –PEG. For the ENMs, two test concentrations were selected based on the known toxicity of Cu and Cu ENMs to earthworms (Spurgeon et al., 1994;

157 Heckmann et al., 2011). The lower concentration of 200 mg Cu kg^{-1} dw was chosen to be
158 sub-lethal and around three times the expected background concentration of total Cu in
159 European soils (the latter, ~ 60 mg Cu kg^{-1} dw, Heijerick et al., 2006). The upper
160 concentration of 1000 mg Cu kg^{-1} dw was equivalent to that suggested in the limit test
161 according to OCED (2004).
162 A standard sandy loam Lufa 2.2 (LUFA Speyer, Germany) soil was used with the
163 following composition (supplier's information, mean ± SD, dry soil, n = not specified): pH of
164 5.5 ± 0.2 (measured in 0.01 M $CaCl_2$ solution); organic carbon, 1.8 ± 0.2 %; nitrogen content
165 at 0.17 ± 0.02 %; cation exchange capacity, 10.1 ± 0.2 meq 100 g^{-1}). The water-holding
166 capacity of the soil was measured in-house and was 41.3 ± 3.0 g 100 g^{-1} dw. The soil used in
167 the experiments was sieved through a 2 mm mesh and air dried at 25˚C for 2 days. Soil pH
168 was measured (see Table S1) prior to the start and at the end of the experiment (in a 1:1
169 soil: water slurry, using a glass combination electrode, Corning 420), in addition to the metal
170 composition (see below).
171 The ENMs and $CuSO_4$ were mixed into the soil as dry powder and soils were then
172 wetted to 50 – 60 % water holding capacity (WHC) with ultrapure Milli-Q water (18.2 Ω)
173 according to OECD (1984). Dry dosing has been found to be a suitable approach for ENM
174 additions to soils (see Handy et al., 2012). A single batch of soil was dosed, followed by
175 dividing it into replicates. Briefly, the required amount of ENM powder or $CuSO_4$ required to
176 dose each 4 replicates was weighed into 50 g soil, thoroughly mixed by hand for 10 minutes.
177 This 50 g of soil was then added to the remaining amount of soil (2350 g) and mixed by hand
178 to make sure the ENM powder was evenly distributed in the soil. The soil was left to
179 equilibrate with the moisture for one day to minimise the risk of the ENMs changing before
180 the worms were added.
181 Adult *E. fetida* with a typical mean starting wet weight of 5.5 ± 0.1 g (mean ± SEM,
182 for a subsample of 12 of the initial earthworms) were exposed in 4 replicates (n = 12
183 earthworms per container, n = 48 earthworms per treatment) at 20 ± 1 °C at 12:12 light:dark
184 cycle. Survival and body weight of the earthworms were recorded at day 0 (start), 7 and 14
185 of the experiment. Behavioural changes such as avoidance of soil and avoidance of
186 burrowing into the soil were observed manually once a day in the morning in all treatments

(not quantified). Earthworms were collected at day 7 and 14 for Cu determination and at the end of the experiment for biochemistry and histology (see below).

Aged soil experiment

The soil used in this experiment was the same as used in the initial fresh soil experiment. The soil was kept for one year after the first experiment in the original test containers with pierced lids that ensured air-flow, under the same exposure conditions defined above (20 ± 1 °C, 12:12 dark:light cycle, without any further moisture or disturbance). During this time, plants had grown in the soil (from seeds already present in the natural soil and likely from the added horse manure). One week prior to the experiment the plant material (excluding roots) was removed by cutting and the soil moisture adjusted to 50 - 60 %, then soil pH measured as described above. In this more selective experiment, fewer earthworms were used (5 in each replicate, 20 per treatment) with a mean wet weight of 1.3 ± 0.03 g of per exposure replicate (mean ± SEM, *n* = 40 treatments). Endpoints such as survival and biomass were recorded as in the fresh soil experiment. Other selected endpoints, including tissue Cu concentrations and biochemistry were measured at the end of the experiment (day 14).

2.5 Metal analysis in soils and earthworms

To confirm the exposure, the total concentration of Cu in the soil and the earthworms was measured. In addition, the water and acid extractable fractions of Cu in the soil were determined to inform on the extractability of Cu from soils. Total metals were determined in soil samples collected at days 0 (samples collected on the first day of exposing the earthworms) and 14. Briefly, approximately 1 g of the moist soil was collected and oven-dried (24 h at 80 ^{o}C). A sub-sample of approximately 200 mg (in triplicate for each soil container, *n* = 12 sub-samples/treatment) was subject to acid digestion using 10 ml of *aqua regia* (3:1 mixture of 37 % HCl and 70 % HNO_3) in covered Pyrex beakers at 80 ^{o}C for 1 h in a fume hood (based on a method in Chen and Ma, 2001). The digests were diluted with ultrapure water prior to analysis and stored in the dark. For the total metal concentration in the earthworms, n = 8 earthworms were sampled at random from all the test containers (2

animals/container) after 7 and 14 days (fresh soil experiment), and 14 days (aged soil experiment). The earthworms were washed in deionised water, and allowed to void their gut content on moist filter paper for 24 h. The filter paper was changed every 12 h to avoid coprophagy. After 24 h, the earthworms were rinsed, dried, and frozen at 20 °C until further analysis. . Subsequently, the earthworms were individually freeze-dried for 48 h, the dry weight recorded, followed by acid digestion at 70 °C in analytical grade 70 % HNO_3 for 1 h. The digests were allowed to cool, then diluted with ultrapure deionised water (18.2 Ω) and stored in the dark. Finally, the total concentration of copper was measured along with other essential elements (Ca, Fe, K, Mg, Mn, Na, Zn) to look for changes in the trace element and electrolyte composition of the earthworms by ICP-OES (iCAP 700) or ICP-MS (Thermo Scientific X Series 2) as appropriate. The samples were sonicated for 15 minutes (at 0.05 kva, 50-60 Hz, 30 kHz, Ultrawave Ltd.), vortexed for 10 s, then hand shaken immediately prior to analysis to ensure good mixing. All samples were analysed against matrix-matched standards. The certified reference materials for total Cu metal reported close to the expected values and were 94 ± 1.3 % (n = 3, EnviroMAT contaminated soil, SS-1) and 93 ± 4.2 % (n = 3, TORT-2, contaminated lobster hepatopancreas). Spike recovery tests of earthworm digests reported 81 ± 1.4 % of the expected value for the core CuO ENM, and 94 ± 1.8 % for $CuSO_4$. The limit of detection (LOD) of the ICP-OES and ICP-MS for Cu was equivalent to 1 mg kg^{-1} and 0.001 mg kg^{-1} (both soil and earthworm tissue), respectively.

To complement the data on total copper concentration in earthworm tissues, the extractable Cu fractions from the soils were also determined by a two-step sequential extraction of Cu from the soil samples from all treatments at day 0, based on an optimised method from Black (1965). The first extraction was with ultrapure water to reveal freely soluble Cu, followed by 0.1 M HCl (37%) for Cu bound to organic matter (Black, 1965); both at ratios of 1:10 (soil:solution) in 15 ml centrifuge tubes. The tubes were rotated for 1 h (Grant bio, PTR-60; orbital rpm 100, 250; reciprocal degree 82), followed by centrifugation at 6000 x *g*, both for 10 minutes. Each solution was then decanted, filtered (Whatman, 0.22 µm), then acidified with 2 % HNO_3 and stored at room temperature in the dark until analysis by ICP-MS or -OES the same day.

2.6 Biochemistry

Biochemistry was performed on whole earthworm tissues from samples collected at the end of the experiments (*n* = 8 per treatment), based on Boyle et al. (2014). Two earthworms from each test container were snap frozen in liquid nitrogen and stored at -80 °C until required. The tissues of whole earthworms were diluted (1:5 ratio, weight: volume) to a final volume of 2.5 ml in ice-cold isotonic buffer (150 mM sucrose, 50 mM HEPES, 1 mM EDTA, pH 7.3) and then homogenised on ice (3 × 10 s with 2 min rests at 17,500 rpm, Cat X520D with a T6 shaft, medium speed, Bennett and Co., Weston-super-Mare). Homogenates were centrifuged at 6000 rpm for 3.5 min to remove debris and the supernatants stored at −80 °C until required. Subsequently, aliquots of the crude homogenates were further diluted in the cold isotonic buffer (recipe above, 1:10 dilution, i.e., an overall 15 fold dilution of the original tissue) due to the high protein concentration in the earthworms. The diluted homogenates were assayed in triplicate for total glutathione (GSH) (Owens and Belcher, 1995), superoxide dismutase (commercial SOD assay kit, Sigma Aldrich), and Na^+/K^+-ATPase activity (McCormick 1993) as described in the Supplementary Methods S1 using the VersaMax plate reader (Molecular Devices, UK). The concentrations of GSH, SOD and Na^+/K^+-ATPase activity were normalised to total protein (Pierce BCA kit, Thermo Scientific, UK). Data are expressed as nmol GSH per mg protein, µmol min^{-1} (IU) SOD per mg protein, and µmol ADP mg^{-1} $protein^{-1}$, for total GSH, SOD and Na^+/K^+-ATPase activity, respectively.

2.7 Histology

Histology was conducted as previously described (van der Ploeg et al., 2014 and references therein). Six earthworms were randomly collected from each treatment, and depuration for 24 h, washed in deionised water, and then anesthetised in 60 % carbonated water. Three earthworms from each treatment were used for routine histology. Briefly, transverse sections of earthworm were cut at three segments beneath the clitellum and three above it; then fixed in buffered formal saline and processed into wax blocks following Handy et al. (2002a). Transverse sections (7 µm) were cut from each earthworm and stained with haematoxylin and eosin. Specimens were stained in batches with all treatments to avoid staining artefacts. Slides were examined using a digital micro-imaging device Leica

DMD 108 (Leica, UK) and analysed using Image J (version 1.50) to measure the relative thickness of the cuticle, dermis, longitudinal and circular muscles (see van der Ploeg et al., 2014).

2.8 Statistical Analyses

Statistical analyses were performed and graphs drawn using R studio software (version 2.1) and SigmaPlot (version 14.0). Data were checked for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test for several groups and F-test for two groups). Non-parametric data were transformed (log_{10}) and reanalysed. Student's t-test (two-tailed, un-/paired) or Mann Whitney test were used for comparing two samples sets as appropriate. There were a few significant differences in pH between treatments in fresh and aged soil experiments, therefore an analysis of covariance (ANCOVA) was carried out for the interactive effects of pH on all endpoints. When no interactions were found, pH was omitted from the model to allow the performance of a post-hoc test. Treatment and time effects were determined using two-way-ANOVA and treatment only effects by one-way-ANOVA where appropriate, followed by the Tukey-Kramer (unequal sample size) post hoc test. Changes in biomass were analysed with repeated measures ANOVA followed by Tukey's honest significance difference (HSD) test to identify the differences. Where data transformation deemed unsuccessful, the non-parametric Kruskal-Wallis test was used followed by Dunn's test. To explore the expected biological associations known for metal toxicity (e.g., association between Cu concentration in the tissue and sodium pump inhibition), the Spearman's rank, r_s, correlations were carried out on all of the data, regardless of treatment, for a specified endpoint within each experiment. The statistical significance level (α) for all tests was set at 0.05.

3. Results

3.1 Soil pH

Soil pH is known to be a factor in metal speciation and the ecotoxicity of soils. Soil pH, varied between treatments at the beginning of the experiment (day 0) in the fresh soil

306 experiment (see Supplementary Table S1), with statistically significant differences in the
307 initial soil pH between $CuSO_4$ treated soils (pH 4.9 ± 0.02, mean ± SEM, n = 4) and the rest of
308 the treatments (pH range 5.3 – 5.8) including the control (pH 5.5 ± 0.1, mean ± SEM). By day
309 14, the pH decreased in most of the treatments by a minimum of 0.2 units (Table S1),
310 however, there was no differences from the control, except in the nominal 200 mg Cu kg^{-1}
311 CuO-COOH treatment (Table S1). In the aged soil experiment, the soil pH on day 0 was
312 generally lower than in the fresh soil experiment (control soil, 5.2 ± 0.02 and $CuSO_4$ or CuO
313 ENM treated soils 5.1 – 5.3; Table S1, $P < 0.05$, *t*-test); but there were no statistically
314 significant differences between treatments ($P > 0.05$ ANOVA, Table S1). Soil pH was not a
315 factor in any of the biological endpoints in either experiments ($P > 0.05$, ANCOVA), and
316 therefore did not alter the responses of the earthworms.

317 ## 3.2 Total and extractable soil copper

318 The exposure was monitored by measuring the total metal concentrations in the soil
319 in both the fresh soil and aged soil experiments. In the former experiment, the control soil
320 contained a background Cu concentration of 1.37 ± 0.2 mg Cu kg^{-1} dw (mean ± SEM, *n* = 4),
321 and of this total Cu, 5 ± 0.6 and 55 ± 5 % were water and 0.1 M HCl-extractable respectively,
322 indicating that at least half of the total Cu was labile. Exposure to $CuSO_4$ caused the
323 expected increase in total soil Cu concentrations that were close to the nominal values
324 (Figure 1a). The measured Cu in the ENM-dosed soil varied depending on the type of ENM
325 coating (Figure 1a); with the total Cu concentration in the fresh soils in the following order:
326 Core > NH_4^+ > COOH > PEG. Regardless, the total soil Cu concentrations in the ENM
327 treatments were much higher than the unexposed control. Despite differences in measured
328 total soil Cu between the ENM treatments, the extractable fractions remained similar (no
329 statistical differences), regardless of the type of coating on the ENMs (Figure 1a). The total
330 concentration of Cu was confirmed again at the start of the aged soil experiment. The values
331 were slightly lower in all treatments, but only significantly lower in the $CuSO_4$ and in the
332 nominal 1000 mg Cu kg^{-1} dw CuO-COOH and CuO- NH_4^+ treatments, compared to a year
333 earlier (compare Figures 1a and b).
334

335 ### 3.3 Total Cu concentrations in earthworm tissue

336 Total Cu was also measured in whole earthworms following depuration of the gut contents
337 (Figure 2). In the fresh soil experiment, the unexposed control earthworms had Cu
338 concentration of 13.8 ± 2 mg Cu kg^{-1} dw at the end of the experiment (day 14). In contrast,
339 the $CuSO_4$ and the ENM treatments all showed elevated Cu concentrations compared to the
340 control earthworms (Figure 2). There was no nano-effect on Cu accumulation with those in
341 the $CuSO_4$ treatments being similar to the CuO core material in the fresh soil. However,
342 there was a coating-effect within the CuO ENMs, with exposure to the CuO-PEG resulting in
343 less total Cu in the earthworms compared to the other treatments, which was related to
344 lower the amount of total Cu measured in soil (Figure 2). No interaction between time and
345 treatment were observed for total Cu concentration in the earthworms in the fresh soil
346 experiment thus only data for day 14 is shown ($P > 0.05$, two-way-ANOVA). The total Cu
347 concentration in the earthworms, regardless of treatment (i.e., all data for the tissue [Cu])
348 were correlated with the total soil Cu and extractable fractions (r_s values = 0.83-0.84, $P <$
349 0.05, Spearman's).

350 In the aged soil experiment (Figure 3), the control earthworms had an internal Cu
351 concentration of 9.8 ± 0.2 mg Cu kg^{-1} dw. The same final Cu concentration was observed in
352 the earthworms from the $CuSO_4$ and the CuO-Core ENM treatments. The only significant
353 difference was found in the nominal 1000 mg Cu kg^{-1} dw treatments, where tissue Cu
354 concentrations were significantly higher in the CuO-COOH treatments compared to the
355 other ENMs. The correlation between the total body burden and total soil Cu was similar to
356 the fresh soil experiment (r_s values = 0.8, $P < 0.05$, Spearman's). Compared to the fresh soil
357 study, the pattern of Cu accumulation was more uniform across the types of material within
358 exposure concentration. Furthermore, the maximum tissue concentrations achieved were
359 around two times lower in the nominal 1000 mg Cu kg^{-1} dw treatments for the aged
360 compared to fresh soils.
361

362 ### 3.4 Survival, growth and behaviour

363 In the fresh soil experiment, the control worms remained healthy (100 % survival)
364 and with no statistically significant loss of biomass over time (Table 2). Similarly, in the

365 $CuSO_4$ treatment, the exposure was sub-lethal with 90 % or more survival and no
366 differences in biomass compared to controls. For the ENM treatments in fresh soil, the 200
367 mg Cu kg^{-1} dw nominal exposure had negligible effects on survival (survival > 95 %).
368 However, for the ENMs used in the higher Cu dose (1000 mg Cu kg^{-1} dw) some toxicity was
369 observed, with the CuO-COOH and CuO-NH_4^+ treatments showing statistically significant
370 mortality compared to the unexposed controls and other forms of the ENM by the end of
371 the experiment (Table 2). Biomass also declined in all the ENM treatments compared to the
372 controls at the 1000 mg Cu kg^{-1} dw exposure concentration on day 7, and this persisted with
373 often further loss of biomass in the ENM-exposed groups until the end of the experiment
374 (Table 2). The survival, regardless of the type of Cu presented, was also positively correlated
375 (r_s = 0.7, P < 0.05, Spearman's) with the approximate individual wet weights (i.e., total
376 biomass divided by the number of surviving animals) of the earthworms.
377 Some behavioural changes in the earthworms preceded toxicity. At the beginning of
378 the experiment in the nominal 1000 mg Cu kg^{-1} dw CuO-COOH treatment, the earthworms
379 were avoiding the soil, rather than burrowing. While the earthworms in all the other
380 treatments burrowed almost immediately. One day later, other behaviours were observed
381 in the highest test concentrations of ENMs. In all ENM treatments, except for the CuO-core,
382 the earthworms were observed bundled together; potentially to minimise contact with the
383 soil. Furthermore, by day 7, even the earthworms in the higher CuO treatment were
384 showing this behaviour. At the end of the experiment with fresh soils, the earthworms from
385 the ENM treatments (except the CuO-PEG) appeared to have more soil stuck to their
386 epidermis; potentially indicating a change in mucus secretion or hydration state. Notably,
387 most of the earthworms from the higher CuO treatment did not survive when placed on
388 moist filter paper in a Petri dish to depurate their guts overnight, indicating that the
389 earthworms were moribund.
390 The survival of earthworms in the aged soil experiment was broadly similar to those
391 of the experiment with fresh soil (Table 2), with the animals from the control and $CuSO_4$
392 treatments surviving, as well as those from the lower concentrations of the CuO ENMs
393 regardless of coating. In the latter for the CuO ENM, the biomass even increased. However,
394 the higher exposure concentration of the CuO-core and CuO-NH_4^+ ENMs were toxic,

decreasing survival compared to the controls. Biomass also showed a statistically significant decrease in all the higher concentrations of the ENMs, except for the CuO-PEG which also less toxic. Earthworm survival and approximate individual wet weight were positively correlated (r_s = 0.5, P < 0.05, Spearman's). In contrast to the fresh soil experiment, soil avoidance was not observed at the start of the experiment, although on day 14 earthworms in all higher CuO ENM treatments, regardless of coating, were found bundled together or curled up alone as in the fresh soil experiment.

3.5 Sodium pump activity and tissue elemental composition

In the experiment with fresh soil, the control animals showed normal Na^+/K^+-ATPase activity at around 8 µmol ADP per mg^{-1} protein h^{-1} (Figure 4a). The earthworms exposed to $CuSO_4$ showed no change in the activity of the Na^+ pump. However, of those exposed to the 200 mg Cu kg^{-1} dw of the ENMs, only the CuO-core and CuO-PEG treatments showed a statistically significant decrease in enzyme activity compared to unexposed controls or to the $CuSO_4$ treatment. At the test concentrations of 1000 mg Cu kg^{-1} dw of the ENMs, there was an overall trend of lower sodium pump activity compared to the controls, but at the end of the experiment, this was only statistically significant for the CuO-core, CuO-PEG and CuO-NH_4^+ treatments (Figure 4a). Statistically significant negative correlation was found between total Cu in earthworm tissue and Na^+/K^+ ATPase activity regardless of treatment (r_s = - 0.5, P < 0.05, Spearman's, Figure S2). Further individual scatterplots by treatment were explored, and these revealed that exposure to the CuO-PEG ENMs did not follow a clear concentration-dependent pattern, with the Na^+/K^+ ATPase activity being reduced regardless of the measured total Cu concentration in the earthworms; indicating the –PEG form as a potent inhibitor of the sodium pump. The total concentrations of tissue electrolytes (Ca^{2+}, Mg^{2+}, K^+, Na^+) and trace elements (Mn, Fe and Zn) were measured in both the fresh and aged soil experiments (see supplementary Table S2). For the fresh soil experiment (Table S2), electrolyte concentrations showed no clear time or treatment-dependent statistically significant changes.

In contrast to the fresh soil experiment, there was no statistically significant effect of exposure to any of the treatments on the activity of the sodium pump in the aged soil

experiment (Figure 4c). However, in the most toxic treatment, the 1000 mg Cu kg^{-1} dw of $CuO\text{-}NH_4^+$ ENM, the Na^+/K^+-ATPase activity also the lowest measured in the aged soil experiment (3.6 ± 0.5 µmol ADP mg $protein^{-1}$ $hour^{-1}$), although not statistically significant. If the sodium pump activity was compared across the fresh and aged soil experiments by treatment, the 1000 mg Cu kg^{-1} dw CuO-core and CuO-COOH treatments were significantly higher in the latter experiment (Figures 4a and c). Of the electrolytes and trace elements measured in the tissues of the earthworms in the aged soil experiment, only Zn and Mn showed statistically significant treatment-dependent changes ($P < 0.05$, ANOVA, Table S2). For example, the concentration of Zn in earthworm tissue was the lowest in the nominal 1000 mg Cu kg^{-1} dw $CuO\text{-}NH_4^+$ exposure (54 ±4 mg Zn kg^{-1} dw) and the concentration of Mn in earthworm tissue was lowest in the CuO-Core exposure (17.07 ± 2.72 mg Mn kg^{-1} dw).

3.6 Oxidative stress markers and histological observations

Total glutathione (Figure 4b) and SOD activity were also measured in the earthworms as indicators of oxidative stress. There was no clear exposure concentration-dependent depletion of total GSH within or between treatments. However, total GSH showed statistically significant increases in the 200 mg Cu kg^{-1} dw CuO-PEG, CuO-COOH, and $CuO\text{-}NH_4^+$ treatments; as well as in the 1000 mg Cu kg^{-1} dw CuO-PEG treatments compared to the unexposed or $CuSO_4$ controls. The absence of glutathione depletion in the fresh soil experiment was complemented by the absence of treatment-dependent changes in SOD activity in the earthworms. At the end of the experiment the SOD activity at the highest exposure concentrations was (mean ± SEM, n = 8) 13.3 ± 1.4, 12.2 ± 0.9, 17.7 ± 2.4, 18.7 ± 6 and 18.7 ± 3.7 IU mg $protein^{-1}$ for the unexposed control, CuO-core, CuO-PEG, CuO-COOH and $CuO\text{-}NH_4^+$ respectively. Nonetheless, there was a statistically significant negative correlation between total SOD and survival at the end of the experiment regardless of treatment ($r_s = -0.4$, $P < 0.05$).

In the aged soil experiment, there was no evidence of glutathione depletion, and in fact, the 200 mg Cu kg^{-1} dw, CuO-COOH and $CUO\text{-}NH_4^+$ treatments showed statistically significant increases in total GSH compared to unexposed controls (Figure 4d). However, no induction of total GSH was observed at the higher test concentrations in the aged soil

455 experiment. When the total glutathione response was compared to fresh and aged soils by
456 treatment, some statistical differences are observed at the 1000 mg Cu kg^{-1} dw test
457 concentrations of the ENMs for the CuO-core, CuO-PEG and CuO-COOH treatments; but the
458 direction and magnitude of the changes were not consistent by material coating across the
459 experiments.
460 Histology was conducted at the end of the fresh soil experiment to observe any
461 morphological evidence of inflammation or other tissue injuries that might be indicative of
462 oxidative stress (Figure 5). This end point was restricted to the control, $CuSO_4$ treatment and
463 the 1000 mg Cu kg^{-1} dw of the various ENMs. Generally, earthworms from all treatments
464 showed normal histology. All earthworms showed an intact cuticle. There was no indication
465 of necrosis or reactive hyperplasia in the epidermis. However, for all the types of CuO ENMs
466 (except the PEG-coated), a mild hypoplasia of mucous (goblet) cells was observed in 2/2,
467 2/3, 3/3 animals respectively for the CuO-core, CuO-COOH, and CuO-NH_4^+ treatments
468 compared to the controls (Figure 5). There was no loss of architecture of the circular and
469 longitudinal muscles or other pathologies to these tissues. However, the presence of
470 granular like pigment was observed in the circular muscle, and this was broadly the same
471 across the CuO ENM treatments (Figure 5). There were no statistically significant differences
472 between the thickness of the epidermis, circular or longitudinal muscle. In the absence of
473 organ pathology in the fresh soil experiment, histology was not pursued in the aged soil
474 study.
475
476 ## 4. Discussion
477
478 This study found that CuO ENMs can be toxic to earthworms, but the magnitude and types
479 of effects are broadly similar to that of $CuSO_4$. There were also some differences in the sub-
480 lethal effects that were dependent on the type of coatings, and although the ranking of the
481 coating effect was not consistent across biological endpoints, the CuO-COOH material was
482 the most hazardous overall in fresh soil. Critically, both Cu toxicity and the coating-effects,
483 changed with soil ageing. In the latter experiment, the aged soil was generally less

hazardous than fresh soil, but importantly the CuO-NH_4^+ now had the most effects on the earthworms.

4.1 Copper exposure, extractable Cu fractions from soil and Cu accumulation in earthworms.

Copper exposure was initially confirmed by measuring the total Cu concentrations in the soils (Figure 1). The background total Cu concentration in the Lufa 2.2 soil was as expected (~1.4 mg Cu kg^{-1} dw, Figure 1) and comparable to previous values (e.g., 1.5 mg Cu kg^{-1} dw, Bastos et al., 2015). In the fresh soil experiment, the measured total Cu concentrations were for the $CuSO_4$ and CuO core (uncoated) material were also close to the nominal values (Figure 1). For the coated ENMs, the total Cu varied because of uncertainty of the stoichiometry and the technical challenges in measuring the mass attributed to the coating. Thus it was expected that the measured total Cu concentrations from the soils dosed with the coated-ENMs would be lower than the equivalent CuO core treatment, but still be a Cu exposure that was much higher than the controls (as observed, Figure 1). Interestingly, the thermogravimetric analysis of the batches of the material (Table 1) indicated that the –NH_4^+, -COOH and –PEG coatings contributed roughly 12, 22 and 46 % of the mass of the materials, and this was broadly reflected in the measured total Cu concentrations in the soil with the CuO-NH_4^+ showing the most Cu and the CuO-PEG the least.

Regardless of the measured total Cu concentrations in the soil, the main concern for hazard assessment is the bioavailable fraction of the metal. This is normally addressed by measuring the extractable fractions of metal from the soil (Figure 1). In the fresh soil experiment, the water extractable fraction remained very small (~ 2.3 mg Cu kg^{-1} dw) and identical to previous reports for Cu salts (2 mg Cu kg^{-1} dw, Scott-Fordsmand et al., 2000). However, the dilute acid-extractable fraction dominated, accounting for 50 % or much more of the total Cu, regardless of the test concentration or type of material. For solutes, the acid-extractable fraction represents the loosely bound fraction of cations on the surface of soil grains that can be eluted by simple ion exchange (i.e., exchanging H^+ with Cu^{2+} in this

513 case). As expected for ion competition between solutes (see Handy and Eddy, 2004), more
514 than half of the Cu as $CuSO_4$ was recovered. However, ENMs are not solutes (see Handy et
515 al., 2008), and with moderate rates of Cu dissolution from the particles (Table 1), it is likely
516 that the acid-extractable fraction here also represent a mixture of free Cu ions and intact
517 particles removed from (low energy) agglomerates, or those loosely attached to the soil
518 grains (e.g., by electrostatic attraction). Unfortunately, interferences from colloids already in
519 the soil prevented any useful attempt of determining particle number concentrations in the
520 extract by NTA to confirm this.
521 Copper exposure was mainly confirmed by measuring the total Cu concentrations in
522 the *E. fetida*. The background Cu concentrations in the earthworms from the control soils
523 were low (~8 - 10 mg Cu kg^{-1} dw, Figure 2) and similar to previous reports (e.g., 8 mg Cu kg^{-1}
524 dw, Streit, 1994). Following the dissolved metal paradigm, in fresh soil (Figure 2), Cu from
525 the $CuSO_4$ treatment was also accumulated in the tissues within 7 days and persisted to the
526 end of the experiment. This was no different from the CuO core material at the nominal 200
527 mg Cu kg^{-1} dw exposure in fresh soil; suggesting the bioavailability of Cu from $CuSO_4$ and the
528 uncoated CuO are similar in the conditions here (Figure 2).
529 A key concern is whether the surface coating of ENMs imparts any additional
530 bioaccumulation risk compared to uncoated ENMs. All of the ENM treatments in the fresh
531 soil experiment, regardless of coating, showed concentration-dependent increases in the
532 earthworms that were generally consistent with the notion of concentration-response
533 (Figure 2). Despite the presence of lower total Cu in the soil with the coated ENMs
534 compared to the CuO core (Figure 2), there was generally no difference between the tissue
535 Cu concentration in the earthworms (Figure 2). Thus, in the fresh soil, proportionally more
536 total Cu is transferred from the soil to the earthworms, depending on the type of ENM
537 coating. If the ratio of the measured total Cu in the soil to measured total Cu in the
538 earthworms is considered, the values would be 0.29, 0.35, 0.74, 0.76 and 0.76 for the
539 $CuSO_4$, CuO-core, -PEG, -COOH and $–NH_4^+$ coatings respectively for the nominal 200 mg Cu
540 kg^{-1} dw exposure.
541 In the aged soil study, Cu accumulation in newly exposed earthworms was assessed
542 after ageing the soils for one year. The aged soil contained the remains of any Cu from the

543 original dosing the previous year, and the pattern of total Cu concentrations were consistent
544 with the original measurements in fresh soil (compare Figure 1b with Figure 1a), but a little
545 lower. This is explained by the total metal removed from the soil by the earthworms in the
546 first experiment, plus that lost to plants spontaneously growing in the soil during the year
547 (data not shown). Nonetheless, exposure of new earthworms to the one-year aged soils for
548 14 days did result in elevated total Cu concentrations in the worms (Figure 4), although the
549 metal concentrations achieved in earthworms were generally less than the fresh soil
550 experiment. However, the pattern of accumulation by material-type was generally the same
551 (compare Figures 2 and 3). The ratios for Cu accumulation from the 200 mg Cu kg^{-1} dw
552 treatments in aged soil were 0.31, 0.24, 0.55, 0.54 and 0.46 for the $CuSO_4$, CuO-core, -PEG, -
553 COOH and $–NH_4^+$ coated materials respectively. The apparent accumulation factors are less
554 than the fresh soil experiment, but the ranking has also changed with the CuO core being
555 less available than the $CuSO_4$. Moreover, the –PEG and –COOH coated ENMs are more
556 available than the $–NH_4^+$ form. An interaction between ENM coating and soil ageing has not
557 been previously reported. However, the finding on $CuSO_4$ are consistent with previous
558 results of lower Cu accumulation in earthworms from aged soils (Lock and Janssen, 2003).
559

560 ## 4.2 Survival, growth and behaviour

561 Earthworms were from a healthy population as confirmed by the survival and normal
562 behaviour of the control animals in both experiments. The nominal concentration of 200 mg
563 Cu kg^{-1} dw soil was intended as a sub-lethal exposure and this was the case with 92 %
564 survival in the $CuSO_4$ treatment in the fresh soil experiment, in keeping with previous
565 findings at the same concentration (95 % survival after 8 weeks, Spurgeon et al., 1994). The
566 survival was also good for the animals exposed to 200 mg Cu kg^{-1} dw soil as ENMs (95 % or
567 more, Table 2). However, a coating-effect on survival was revealed at the higher nominal
568 concentration of 1000 mg Cu kg^{-1} dw soil, with the survival ranked in the following order by
569 material in the fresh soil experiment: CuO-PEG > CuO-core > CuO-NH_4^+ > CuO-COOH (Table
570 2). The decreases in survival were also reflected in reduced biomass (declining growth) of
571 the *E. fetida* (Table 2). Some of the mortality and slower growth could be attributed to
572 reduced feeding since the worms avoided burrowing at the high exposure concentrations.

There was also evidence of specific trace element deficiencies (see below). Similar avoidance behaviour and biomass loss has been seen in exposures to diethylene glycol (DEG) coated ZnO ENMs (~ 7.5 g kg^{-1} loading of DEG, Laycock et al., 2017).

In the aged soil experiment, the animals all survived at the at the nominal 200 mg Cu kg^{-1} dw, similar to the fresh soil study. However, unlike in fresh soil, the animals gained biomass in the aged soil (Table 2). This may be partly explained by the presence of different food in the soil, as the aged soil experiment had plant matter (roots) that the earthworms could feed on in addition to the added horse manure at the start of the aged soil experiment. However, any benefit was lost at the higher 1000 mg Cu kg^{-1} dw nominal concentration in aged soil. The toxicity ranking by material was also different to fresh soil. In aged soil it was: CuO-PEG = CuO-COOH > CuO core > CuO-NH_4^+; with the CuO-NH_4^+ spiked soil now the most hazardous in aged soils, even though the total Cu accumulation in the earthworms was not the highest (Figure 3). Why the ranking has changed in the aged soil is unclear.

4.3 Effects on ionic regulation

Earthworms actively osmoregulate, but the sodium pump activity and electrolyte composition of earthworms is not often measured in studies on metal toxicity. The crude homogenates of the control earthworms had an Na^+/K^+-ATPase activity of 6 – 8 µmol ADP mg^{-1} h^{-1} (Figure 4) and this was consistent with at least one other report for *E. fetida* (4 – 8 µmol Pi mg h^{-1} in Luo et al., 2012). The tissue K^+ concentrations (8000 – 10 000 mg kg^{-1} Table S2) are also comparable with previous reports (~ 8000 mg kg^{-1}, Janssen et al., 1998).

Dissolved Cu is a well-known inhibitor of the Na^+/K^+-ATPase (Li et al., 1996), and while there was a trend of decreasing Na^+ pump activity, the $CuSO_4$ concentration used here were not sufficient to cause statistically significant inhibition (Figure 4). Nonetheless, some variability of tissue K^+ concentrations were noted in the $CuSO_4$ treatment compared to controls (Table S2) suggesting that the earthworms were on the threshold of osmotic disturbance in the fresh soil experiment. At the same nominal Cu concentration, the CuO-core or CuO-PEG inhibited the Na^+ pump in the fresh soil experiment, and at the highest exposure concentration all the ENMs caused inhibition of the Na^+/K^+-ATPase (Figure 4). The

603 CuO-core and CuO-PEG materials were the most potent, but this potency is not explained by
604 higher Cu body burden in those treatments (Figure S2) or oxidative damage to the Na^+ pump
605 (no depletion of GSH, Figure 4). Notably, this osmoregulatory hazard was not observed in
606 the aged soil experiment with no inhibition of the Na^+ pump (Figure 4) or electrolyte losses
607 (Table S2), presumably because the Cu present was less bioavailable (less Cu in the tissues,
608 Figure 3). The mechanism of coating-dependent inhibition of the Na^+ pump requires further
609 investigation, but the CuO ENMs in fresh soil do present an osmoregulatory hazard that is
610 generally greater than the metal salt.
611 Cu is also known to have some specific interactions with other trace elements,
612 especially Fe and Zn due to shared uptake and/or excretion pathways (see Bury and Handy,
613 2010). No interferences with Fe were observed in either the fresh or aged soil experiments,
614 but there were some losses of manganese and zinc. In the aged soil study, significantly
615 lower tissue manganese was associated with $CuSO_4$ exposure and the nominal 1000 mg Cu
616 kg^{-1} dw ENM exposures only (more than 50 % depletion of Mn, Table S2). This was not a
617 nutritional defect attributed to the indirect effects of Cu on Mn via Fe status (see Andersen
618 et al., 1996), since Fe concentrations did not change (Tables S2). However, taken together,
619 these trace element depletions could partly explain the retarded growth of the earthworms.
620 For example, Zn concentrations in earthworms are regulated to around 80 - 120 mg kg^{-1} and
621 are nutritionally required (Van Gestel et al., 2010). Thus, the values reported here (~40 mg
622 Zn kg^{-1}, Table S2) could represent a Zn deficiency. Any solute transporter explanation of Cu
623 out competing Zn would require the CuO ENMs to dissolve inside the earthworms. The
624 dissolution of micromolar amounts of Cu (Table 1), if it occurred inside the worms, would
625 stimulate these high affinity transporters. Regardless, depletion of Zn and Mn has been
626 observed previously in studies with ENMs (TiO_2 in trout, Federici et al., 2007) and this
627 phenomena requires further investigation.
628

629 ## 4.4 Absence of oxidative stress

630 The sub-lethal effects observed in the present study on growth and osmotic
631 regulation are not explained by oxidative stress because no inflammation pathology was
632 evident in the dermis or muscularis of the earthworms (Figure 5) and no glutathione

depletion was observed (Figure 4). The background total GSH in the earthworms of 16.5 nmol mg^{-1} protein (which equates to ~ 1.2 µg g^{-1} fw) (Figure 4) were similar to previous reports of around 1.3 µg g^{-1} fresh weight (Ribera et al., 2011). However, some induction of total GSH was observed, but only in the coated forms of the ENMs (Figure 4). Increases in total glutathione has been observed previous with *E. fetida* in response to Ag ENMs (Gomes et al., 2015). This might be interpreted as a premature defence to prevent oxidative stress, or an aspect of metal resistance since glutathione is also an intracellular Cu carrier. However, why the response occurs only with the coated materials and not the CuO core or the $CuSO_4$ is unclear.

4.5 Conclusions and implications for risk assessment

A key concern for the risk assessment of ENMs is whether they are more hazardous than the nearest equivalent substance. In the present study the effects of CuO ENMs were broadly of the same magnitude as $CuSO_4$, depending on the endpoint measured and the exposure duration. At environmentally-relevant total concentrations of Cu, the modes of action were also similar; with the ENMs causing ionoregulatory disturbances and Na^+ pump inhibition that are well-known for Cu. The CuO ENMs could cause lethal toxicity, but only at a high concentration of 1000 mg Cu kg^{-1} dw soil, where the effects of surface coatings on the materials was also especially revealed. The hazard ranking of the coatings was not readily explained by a bioavailable fraction of metal according to the dissolved metal paradigm in soils. The ranking also changed with ageing of the soil, and the CuO-NH_4^+ ENMs became more toxic one year on. Thus, the surface coating and soil ageing should be considered in any risk assessment. However, risk assessment should use a weight of evidence approach and with only a handful of studies exploring coating-effects or soil ageing effects on earthworms so far, more data is needed to understand if the current metals risk assessments for soils will also be protective for the nano form.

5. Conflict of interest

The authors declare that they have no conflicts of interest.

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References

Andersen, F., Maage, A. and Julshamn, K. (1996) An estimation of dietary iron requirement of Atlantic salmon, *Salmo salar* L., parr. Aquaculture Nutrition, 2, 41-47.

Anyaogu, K. C., Fedorov, A. V., and Neckers, D. C. (2008) Synthesis, characterization, and antifouling potential of functionalized copper nanoparticles. Langmuir, 24, 4340–4346.

Bastos, A. C., Prodana, M., Oliveira, J. M. M., Calhôa, C. F., Santos, M. J. G., Soares, A. M. V. M., & Loureiro, S. (2014) Water-extractable priority contaminants in LUFA 2.2 soil: back to basics, contextualisation and implications for use as natural standard soil. Ecotoxicology, 23, 1814–1822.

Black, C. A. (1965) Methods of soil analysis. Part 2, chemical and microbiological properties. American Society of Agronomy, New York.

Bogdanovic, U., Vodnik, V., Mitric, M., Dimitrijevic, S., Skapin, S. D., Zunic, V., Budimir, M., Stoiljkovic, M. (2015) Nanomaterial with high antimicrobial efficacy copper/polyaniline nanocomposite. ACS Applied Materials and Interfaces, 7, 1955–1966.

Boyle, D., Fox, J. E., Akerman, J. M., Sloman, K. A., Henry, T. B., & Handy, R. D. (2014) Minimal effects of waterborne exposure to single-walled carbon nanotubes on

behaviour and physiology of juvenile rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology, 146, 154–164.

Canadian Council of Ministers of the Environment (CCME) (1999) Canadian Soil Quality Guidelines for Copper: Environmental and Human Health. Available online at http://ceqg-rcqe.ccme.ca/en/index.html [last accessed: 8 March 2017]

Chen, M., and Ma, L. Q. (2001). Comparison of Three Aqua Regia Digestion Methods for Twenty Florida Soils. Soil Science Society of America Journal, 65(2), 491.

Diez-Ortiz, M., Lahive, E., George, S., Ter Schure, A., Van Gestel, C. A. M., Jurkschat, K., Svendsen, C. and Spurgeon, D. J. (2015) Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; Bioavailability and toxicity of silver ions ($AgNO_3$) and silver nanoparticles to earthworm *Eisenia fetida* in long-term aged soils, Environmental Pollution, 203, 191–198.

Evans, P., Matsunaga, H., Kiguchi, M., (2008) Large-scale application of nanotechnology for wood protection. Nature Nanotechnology, 3, 577.

Federici, G., Shaw, B. J., & Handy, R. D. (2007) Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. Aquatic Toxicology, 84, 415–430.

Fischer, E. and Molnár, L., (1997) Growth and reproduction of *Eisenia fetida* (Oligochaeta, Lumbricidae) in semi-natural soil containing various metal chlorides. Soil Biology and Biochemistry, 29, 667-670.

Gawande, M. B., Goswami, A., Felpin, F.-X., Asefa, T., Huang, X., Silva, R., Zou, X., Zboril, R., Varma, R. S. (2016) Cu and Cu-Based Nanoparticles: Synthesis and Applications in Catalysis. Chemical Reviews, 116, 3722–3811.

Gomes, S. I. L., Hansen, D., Scott-Fordsmand, J. J., Amorim, M. J. B. (2015) Effects of silver nanoparticles to soil invertebrates: Oxidative stress biomarkers in *Eisenia fetida*. Environmental Pollution, 199, 49–55.

Gottschalk, F., Lassen, C., Kjoelholt, J., Christensen, F., and Nowack, B. (2015) Modelling flows and concentrations of nine engineered nanomaterials in the Danish environment. International Journal of Environmental Research and Public Health, 12, 5581–602.

720 Handy, R. D., Runnalls, T., and Russell, P. M. (2002) Histopathologic biomarkers in three
721 spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in Southern
722 England that meet the freshwater fisheries directive. Ecotoxicology, 11, 467–479.
723 Handy, R. D., Van De Brink, N., Chappell, M., Mühling, M., Behra, R., Dušinská, M.,
724 Riediker, M. (2012) Practical considerations for conducting ecotoxicity test
725 methods with manufactured nanomaterials: What have we learnt so far?
726 Ecotoxicology, 21, 933–972.
727 Handy, R. D., Von Der Kammer, F., Lead, J. R., Hassellöv, M., Owen, R., & Crane, M.
728 (2008) The ecotoxicology and chemistry of manufactured nanoparticles.
729 Ecotoxicology, 17, 287–314.
730 Heckmann, L. H., Hovgaard, M. B., Sutherland, D. S., Autrup, H., Besenbacher, F. and
731 Scott-Fordsmand, J. J. (2011) Limit-test toxicity screening of selected inorganic
732 nanoparticles to the earthworm *Eisenia fetida*. Ecotoxicology, 20, 226-233.
733 Heggelund, L.R., Diez-Ortiz, M., Lofts, S., Lahive, E., Jurkschat, K., Wojnarowswicz, J.,
734 Cedergreen, N., Spurgeon, D., and Svendsen C. (2014) Soil pH effects on the
735 comparative toxicity of dissolved zinc, non-nano and nano ZnO to the earthworm
736 *Eisenia fetida*. Nanotoxicolgy, 8, 559 – 572.
737 Heijerick, D. G., Van Sprang, P. A., and Van Hyfte, A. D. (2006) Ambient copper
738 concentrations in agricultural and natural European soils: an overview.
739 Environmental Toxicology and Chemistry, 25, 858–864.
740 Janssen, M. P. M., Glastra, P., and Lembrechts, J. F. M. M. (1997) The effects of soil
741 chemical characteristics on the ^{134}Cs concentrations in earthworms. Uptake from
742 liquid medium. Journal of Environmental Radioactivity, 35, 313–330.
743 Laycock, A., Romero-Freire, A., Najorka, J., Svendsen, C., van Gestel, C. A. M., &
744 Rehkämper, M. (2017). Novel Multi-Isotope Tracer Approach To Test ZnO
745 Nanoparticle and Soluble Zn Bioavailability in Joint Soil Exposures. Environmental
746 Science & Technology, 51(21), 12756–12763.
747 Li, J., Locka, R. A. C., Klarena, P. H. M., Swartsb, H. G. P., Stekhovenb, F. M. A. H. S.,
748 Bongaa, S. E. W., & Flik, G. (1996) Kinetics of Cu^{2+} inhibition of Na^+/K^+-ATPase,
749 Toxicology Letters, 87, 31–38.

Lock, K. and Janssen, C. R. (2003) Influence of aging on copper bioavailability in soils, Environmental Toxicology and Chemistry, 22, 1162–1166.

Luo, Y. R., San-Hu, W., Li, X. Y., Yun, M. X., Wang, J. J., & Sun, Z. J. (2010) Toxicity of ionic liquids on the growth, reproductive ability, and ATPase activity of earthworm. Ecotoxicology and Environmental Safety, 73, 1046–1050.

Ma, W. C. (2005) Critical body residues (CBRs) for ecotoxicological soil quality assessment: Copper in earthworms. Soil Biology and Biochemistry, 37, 561–568.

McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurement of Na^+/K^+-ATPase activity. Canadian Journal of Fish and Aquatic Science, 50, 656-658.

Nahmani, J., Hodson, M. E. and Black, S. (2007) A review of studies performed to assess metal uptake by earthworms. Environmental Pollution, 145, 402–424.

OECD (1984) OECD guidelines for testing of chemicals: Earthworm, Acute Toxicity Test, TG 207, Organisation for Economic Co-operation and Development, Paris, France.

Owen, R. and Handy, R. (2007) Formulating the problems for environmental risk assessment of nanomaterials. Environmental Science and Technology, 41, 5582–5588.

Ribera, D., Narbonne, J. F., Arnaud, C., and Saint-denis, M. (2001) Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. Soil Biology, 33, 1123–1130.

Scott-Fordsmand, J. J., Weeks, J. M. and Hopkin, S. P. (2000) Importance of contamination history for understanding toxicity of copper to earthworm *Eisenia fetida* (Oligochaeta : Annelida), using neutral-red retention assay. Environmental Toxicology and Chemistry, 19, 1774–1780.

Servin, A. D., and White, J. C. (2016) Nanotechnology in agriculture: Next steps for understanding engineered nanoparticle exposure and risk. NanoImpact, 1, 9–12.

Spurgeon, D. J., Hopkin, S. P., and Jones, D. T. (1994) Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): Assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. Environmental Pollution, 84, 123–130.

779 Streit B. (1984) Effects of high copper concentrations on soil in- vertebrates (earthworms
780 and orbatid mites): Experimental results and a model. Oecologia, 64, 381–388.
781 Tegenaw, A., Tolaymat, T., Al-Abed, S., El Badawy, A., Luxton, T., Sorial, G., and Genaidy,
782 A. (2015). Characterization and potential environmental implications of select Cu-
783 based fungicides and bactericides employed in U.S. markets. Environmental
784 Science and Technology, 49(3), 1294–1302.
785 Unrine, J. M., Tsyusko, O. V., Hunyadi, S. E., Judy, J. D. and Bertsch, P. M. (2010) Effects
786 of particle size on chemical speciation and bioavailability of copper to earthworms
787 (*Eisenia fetida*) exposed to copper nanoparticles. Journal of Environmental Quality.
788 39, 1942-1953.
789 Van der Ploeg, M. J. C., Handy, R. D., Waalewijn-Kool, P. L., van den Berg, J. H. J., Herrera
790 Rivera, Z. E., Bovenschen, J., Molleman, B., Baveco, J. M., Tromp, P., Peters, R. J. B.,
791 Koopmans, G. F., Rietjens, I. M. C. M. and Van den Brink, N. W. (2014) Effects of
792 silver nanoparticles (NM-300K) on *Lumbricus rubellus* earthworms and particle
793 characterization in relevant test matrices including soil, Environmental Toxicology
794 and Chemistry, 33, 743–752.
795 Van Gestel, C. A. M., Kool, P. L. and Ortiz, M. D. (2010) Metal-based nanoparticles in soil:
796 New research themes should not ignore old rules and theories. Comments on the
797 paper by Hu et al. (2010). Toxicological effects of TiO_2 and ZnO nanoparticles in soil
798 on earthworm *Eisenia fetida*. Soil Biology and Biochemistry. 42, 1892–1893.
799 Velicogna, J. R., Schwertfeger, D. M., Jesmer, A. H., Scroggins, R. P., & Princz, J. I. (2017)
800 The bioaccumulation of silver in *Eisenia andrei* exposed to silver nanoparticles and
801 silver nitrate in soil. NanoImpact, 6, 11-18.
802 Waalewijn-Kool, P.L., Ortiz, M.D., van Straalen, N.M., van Gestel, C.A.M., (2013)
803 Sorption, dissolution and pH determine the long-term equilibration and toxicity of
804 coated and uncoated ZnO nanoparticles in soil. Environmental Pollution. 178, 59-
805 64.
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809 **Table 1**. Characterisation details of the CuO ENMs used in the experiments.

ENM variant	[1]Manufacturer's Information	Surface ligand	[2]Measured primary particle size (nm)	[3]NTA, hydrodynamic diameter (nm)	[4]TGA, degree of functionalisation (% weight loss)	[5]Maximum rate of dissolution in Milli Q water (μg h^{-1})
CuO-core	Lot No. YF1309121, 99% purity, size 10-20 nm.	NA	12 ± 0.37	41 ± 28	9.2	1.68
CuO-polyethylene glycol	Lot No. YF140114, 99 % purity, size 10-20 nm.	R-PEG	7.46 ± 0.42	100 ± 36	42.2	52.02
CuO-carboxylate	Lot No. YF140114, 99 % purity, size 10-20 nm.	R-COOH	6.45 ± 0.16	121 ± 91	22.1	69.12
CuO-ammonium	Lot No. YF140114, 99 % purity, size 10-20 nm.	R-NH_3 ionised with H^+	9.53 ± 0.22	46 ± 36	11.6	18.6

810 [1] Supplied as dry powders for the Nanosolutions project via Alexei Antipov, PlasmaChem GmbH.

811 [2] Based on TEM images of CuO ENM from a 100 mg Cu l^{-1} stocks in Milli Q water prepared at Plymouth University. Data are mean ± S.E.M (n = 60
812 measurements)

813 [3] NTA- Nanoparticle Tracking Analysis using NanoSight using 100 mg Cu l^{-1} ENM stocks in Milli Q water at Plymouth University. Data are mean ± S.D. n = 3
814 samples)

815 [4]TGA – thermogravimetric analysis. Single measurements made on dry powders using a TGA 4000 (Perkin Elmer) under an N_2 flow of 20 ml
816 min^{-1} from 25 °C to 995 °C at a heating rate of 10 °C min^{-1} at the University of Manchester.

[5]Maximum slope from rectangular hyperbola function of curve fitting used to estimate the maximum rate of dissolution of Cu from dialysis experiments conducted at ~~Plymouth~~ University of Plymouth.

821 **Table 2**. Survival and biomass of *Eisenia fetida* following 7 and 14 days exposure to controls, CuO ENMs or $CuSO_4$ in the fresh soil and 14 days exposure in
822 the aged soils.

Soil [Cu], nominal mg Cu kg^{-1} dw		Control		$CuSO_4$		CuO-core		CuO-PEG		CuO-COOH		$CuO\text{-}NH_4^+$	
		Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass
Fresh soil, day 7													
200	Total	12^a	5.6 ± 0.3^a	12 ± 0.2^a	3.9 ± 0.1^b	12^a	4.6 ± 0.2^{ab}	12^a	5.1 ± 0.1^a	12^a	5 ± 0.1^a	12^a	5.6 ± 0.2^a
	%	100	↓7.8 ± 3.6	98 ± 0.1	↓17.1 ± 3.5	100	↓14.8 ± 3.8	100	↓6 ± 5	100	↑1.1 ± 1.7	100	↓9 ± 1.3
1000	Total					12 ± 0.2^a	3.4 ± 0.1^b	11 ± 0.4^a	3.5 ± 0.4^b	7 ± 1^b	2.1 ± 0.5^b	11 ± 1^a	3.4 ± 0.1^b
	%					97.9 ± 6	↓34.3 ± 2.9	91.7 ± 10.2	↓24.9 ± 6	56.2 ± 27.7	↓37.4 ± 2.5	87.5 ± 16.1	↓34.7 ± 2
Fresh soil, day 14													
200	Total	10^a	4 ± 0.2^a	9 ± 0.5^a	2.9 ± 0.2^b	10 ± 0.2^a	3.1 ± 0.2^a	10 ± 0.5^a	3.6 ± 0.3^a	10 ± 0.2^a	3.5 ± 0.1^a	10^a	4.1 ± 0.2^a
	%	100	↓21.8 ± 3.3	92 ± 12	↓22.9 ± 3.9	97.5 ± 6.2	↓29.5 ± 3	95 ± 5	↓5.6 ± 5.5	97.5 ± 6.2	↓11.6 ± 4	100	↓20.6 ± 3
1000	Total					7 ± 0.5^b	1.8 ± 0.2^c	9 ± 0.6^a	2.5 ± 0.4^c	2 ± 2.2^c	0.6^d	6 ± 0.8^{bc}	1.5 ± 0.2^c
	%					70 ± 22.6	↓40.5 ± 4.6	85 ± 16.1	↓31.7 ± 1.8	22.5 ± 56.3	↓50.8	57.5 ± 21.3	↓45.4 ± 3.7
Aged soil, day 14													
200	Total	5^a	2 ± 0.1a	5^a	1.3 ± 0.1^a	5 ± 0.2^a	1.4 ± 0.2^b	5^a	1.8 ± 0.04^{ab}	5^a	1.8 ± 0.1^{ab}	5^a	1.6 ± 0.1^{ab}
	%	100	↑53 ± 8.4	100	↑0.1 ± 5.5	95 ± 5		100		100		100	

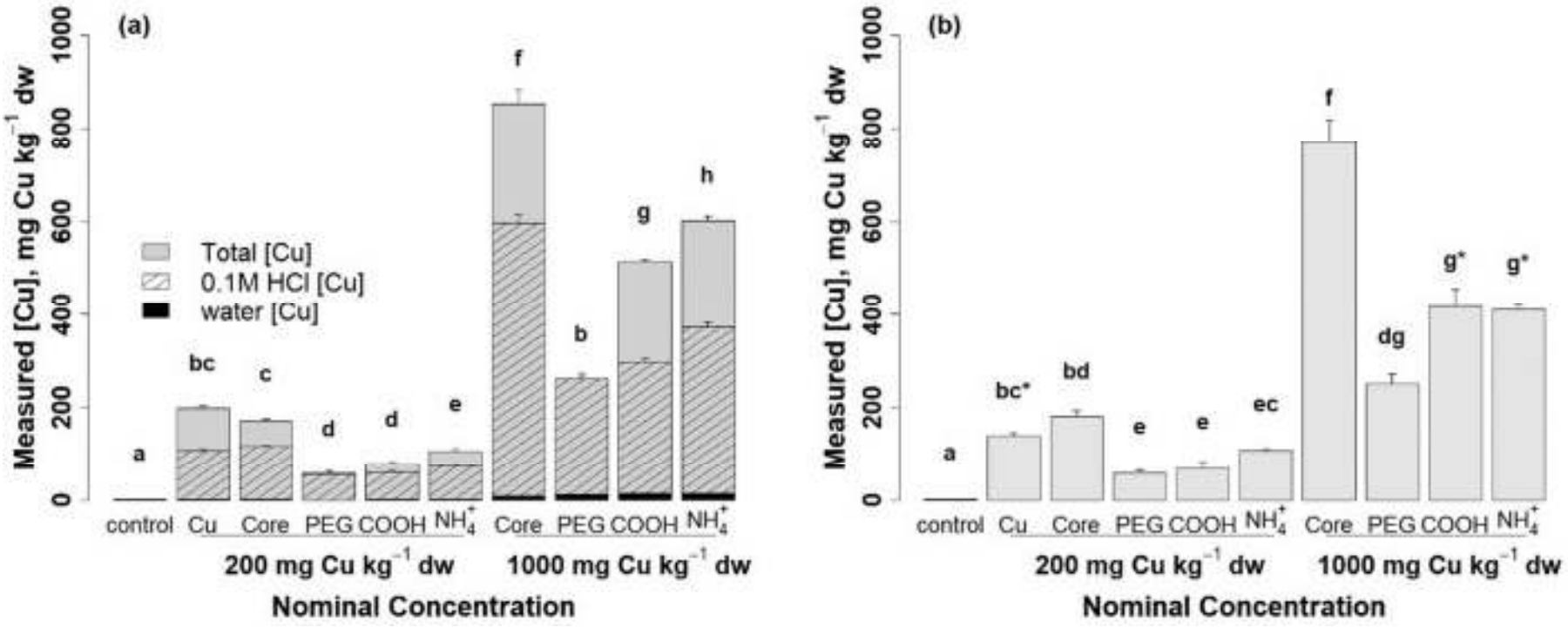
			↑29.4 ± 9.2		↑36.2 ± 8.5		↑45.8 ± 1.1		↑42.1 ± 6.4
1000	Total	4 ± 1[b]	0.7 ± 0.2[c]	5[a]	1.1 ± 0.1[c]	5[a]	1 ± 0.1[c]	2 ± 0.2[b]	0.2 ± 0.04[c]
	%	70 ± 10	↓26.3 ± 2.3	100	↓11.4 ± 9.3	100	↓19.4 ± 9.6	30 ± 5.8	↓37.1 ± 8.1

Survival is reported as the total number of earthworms per treatment (total) and as percent survival (%). Similarly, the biomass is reported as the total biomass of surviving earthworms per treatment (wet weight, mg) and the percentage weight increase or decrease relative to the animals at the start of the experiment. Data presented as mean ± SEM (n = 4 boxes of worms per treatment). Treatments that do not share a letter are statistically significantly different within rows ($P < 0.05$ repeated measures ANOVA for biomass data or Kruskal-Wallis~~, Dunn's test~~ for survival data).

Day 0 mean wet weight was 5.5 ± 0.1 g (mean ± SEM, for a subsample of 12 of the initial earthworms, n = 40 treatments) in fresh soil experiment. Day 0 mean wet weight of 1.3 ± 0.03 g of per exposure replicate (mean ± SEM, for a subsample of 5 of the initial earthworms, *n* = 40 treatments).

↑ Increase in wet weight relative to day 0

↓Decrease in wet weight relative to day 0



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833 **Figure 1** Total Cu concentration in the soil at the beginning of the experiments (a) fresh soil, (b) soils aged for one year (Cu – $CuSO_4$, Core – uncoated CuO
834 ENMs, PEG-, COOH-, NH_4^+ - coated CuO ENMs). Data expressed as mean ± SEM (n = 12, soil samples analysed in triplicate from the fresh soil experiment; n =
835 8, soil samples analysed in duplicate from the aged soil experiment). In panel (a) the water or 0.1M HCl extractable Cu is presented as black or dashed bars,
836 respectively. Treatments that do not share a letter are significantly different from the each other within each experiment (P < 0.05, ANOVA). Asterisks
837 denote a statistically significant difference between the total Cu concentrations in the fresh compared to aged soil experiment (P < 0.05, Mann-Whitney).
838 The horizontal lines indicate which treatment goes with the nominal test concentration.

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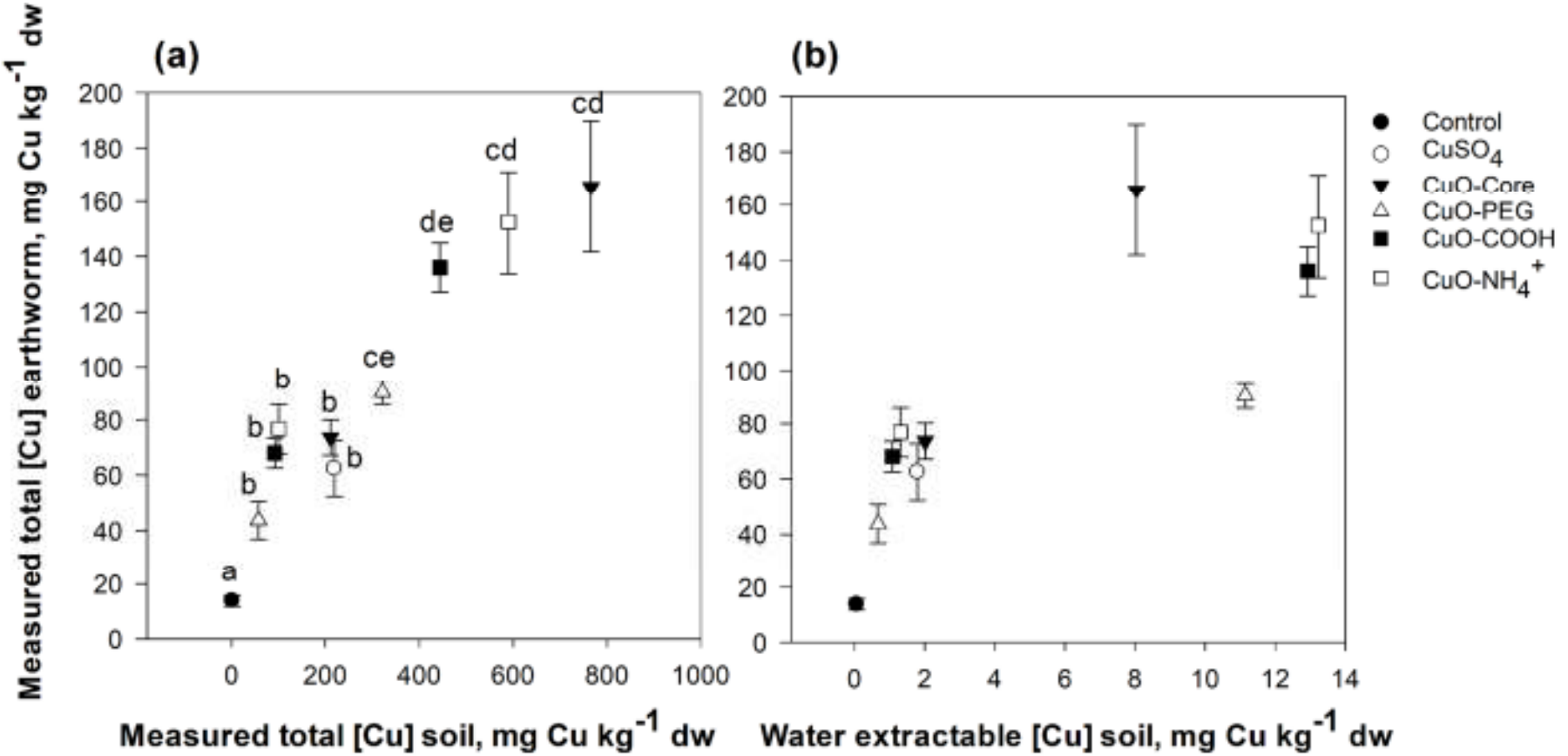


841

842 **Figure 2** Total tissue Cu concentration of adult *Eisenia fetida* exposed for 14 days to $CuSO_4$ and CuO ENMs in the fresh soil experiment in relation to the

843 measured total Cu concentration in soil on day 0 (a), and total water-extractable Cu concentration in soils on day 0 (b). The legend on the side applies to

844 panel (a) and (b). Data are presented as mean ± SEM (n = 8 earthworms from each treatment). Treatments that do not share a letter are statistically

845 significantly different ($P < 0.05$, ANOVA) the same data labels apply to panel (b). Statistical curves were omitted from the figures for clarity.

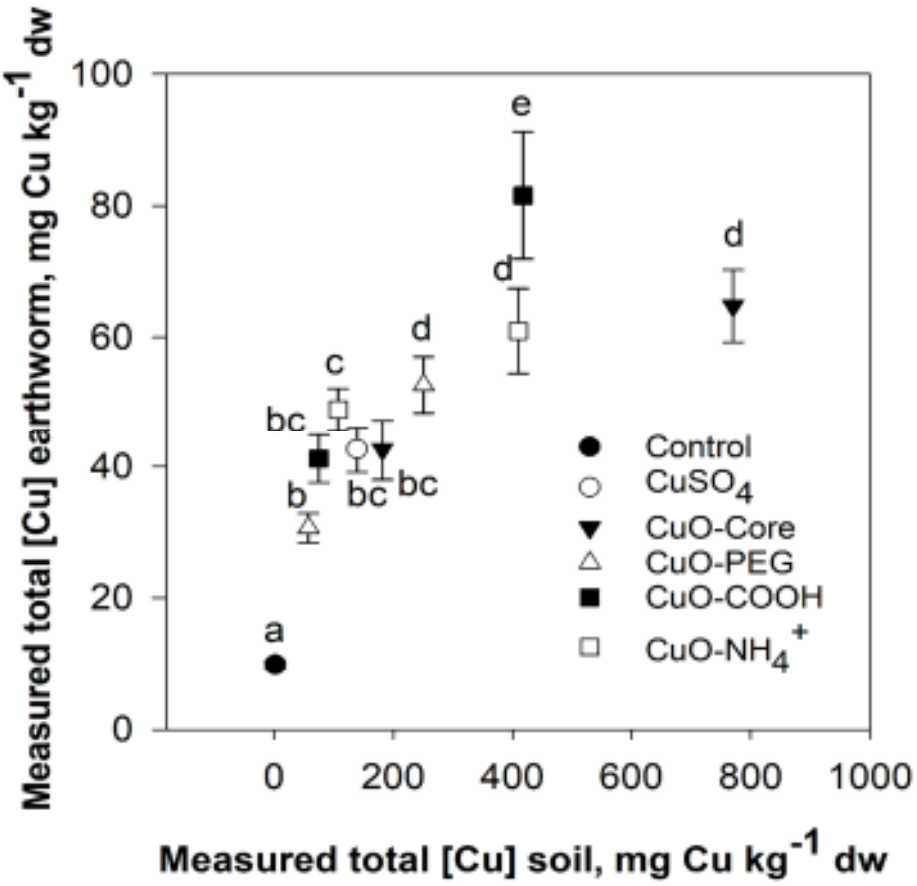
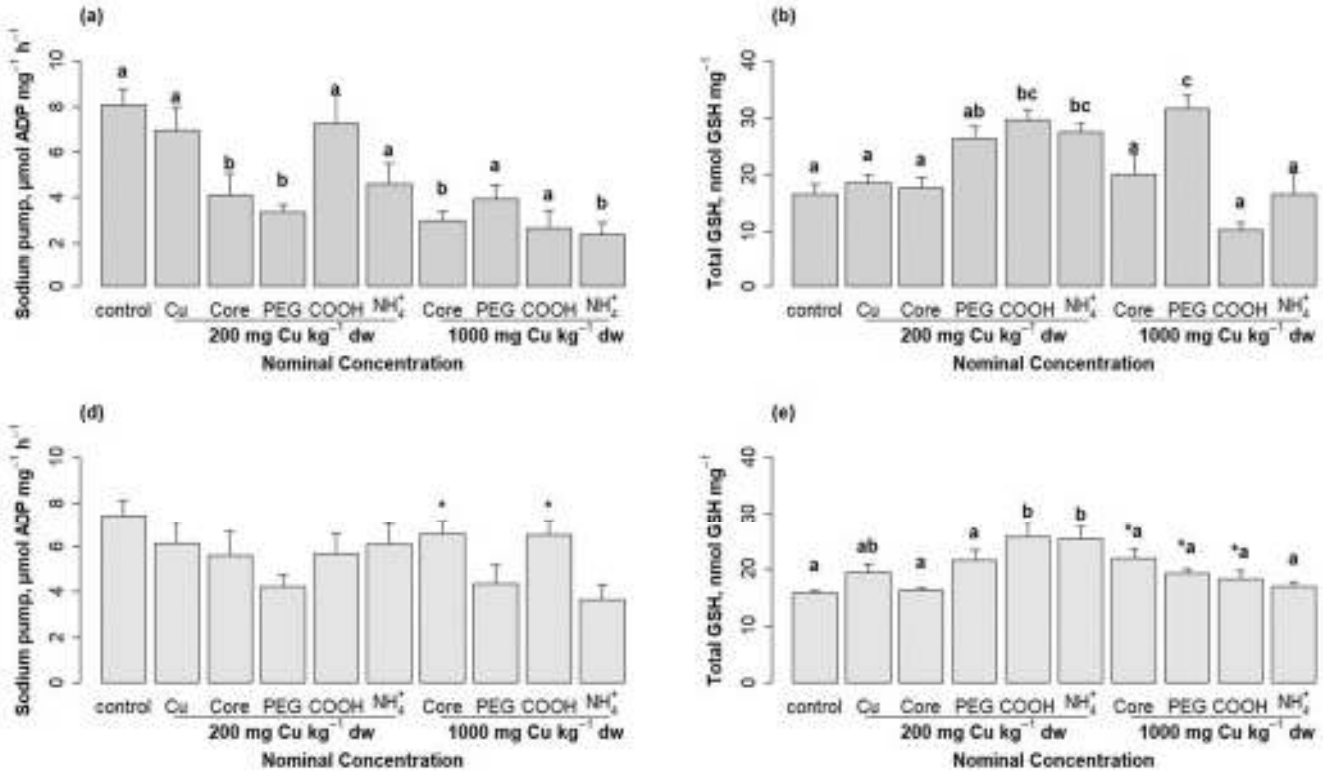


Figure 3 Total tissue Cu concentration of adult *Eisenia fetida* exposed for 14 days to $CuSO_4$ and CuO ENMs in the aged soil (aged for 1 year) experiment in relation to the measured total Cu concentration in soil on day 0. Data are presented as mean ± SEM (*n* = 8 earthworms from each treatment). Treatments that do not share a letter are statistically significantly different ($P < 0.05$, ANOVA). Statistical curves were omitted from the figures for clarity.



857

858 **Figure 4** Biochemical responses of adult *Eisenia fetida* exposed for 14 days to $CuSO_4$ and CuO ENMs in the fresh (panels a and b) and aged soils (panels c and
859 d) (Cu – $CuSO_4$, Core – uncoated CuO ENMs, PEG-, COOH-, NH_4^+ - coated CuO ENMs). Na^+/K^+-ATPase activity is expressed as µmol of ADP released per mg
860 protein per hour. Total glutathione is expressed as nmol per mg protein. Different letters denote statistically significant differences between treatments
861 irrespective of test concentration ($P < 0.05$, one-way ANOVA). Asterisks denotes a statistically significantly difference from their fresh soil counterpart
862 within treatment ($P < 0.05$, Mann-Whitney). Data are means ± SEM (n = 8 earthworms, or less due to high mortality which restricted the number of
863 earthworms for biochemistry at the high exposure concentrations).

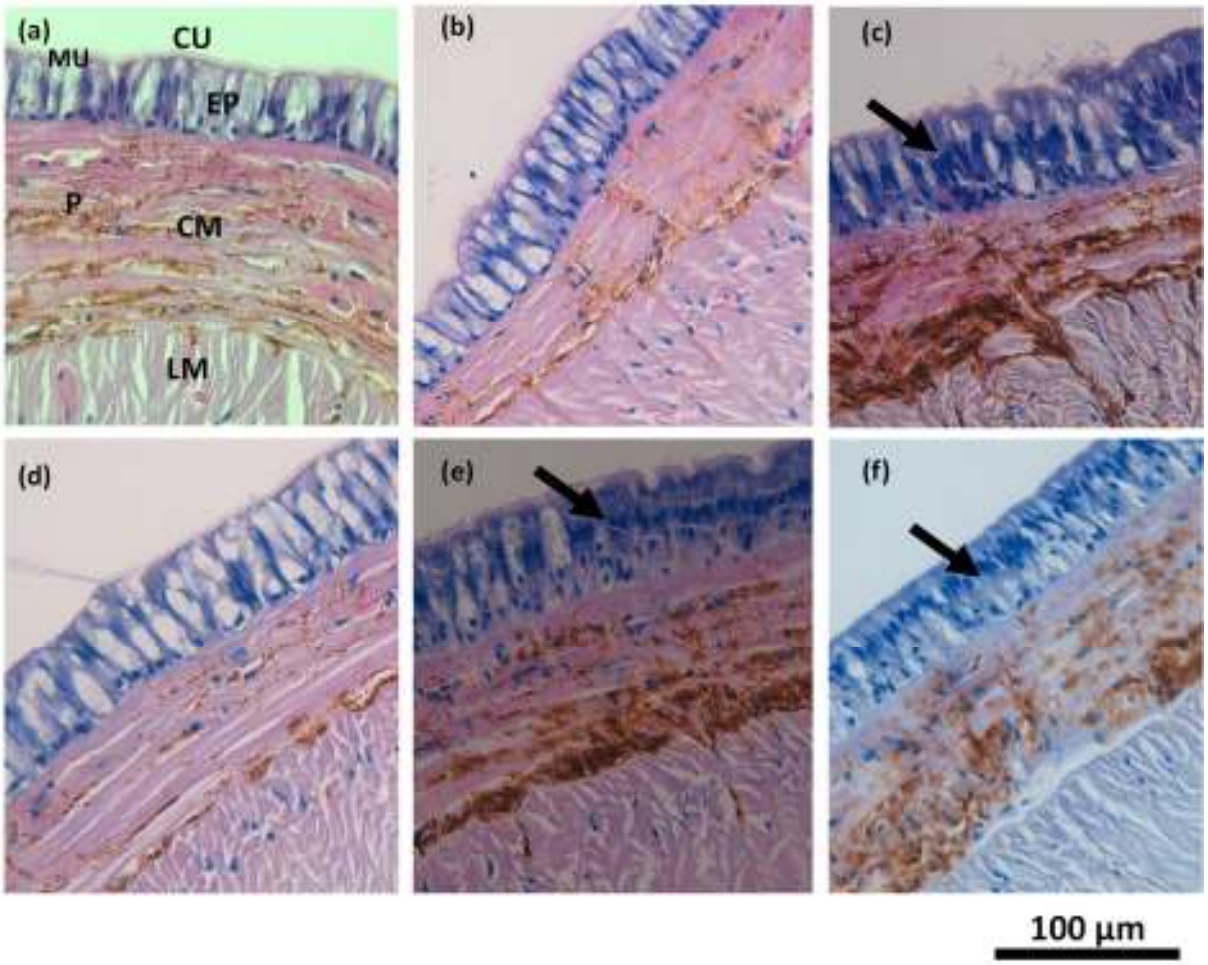


Figure 5 Transverse sections of segments from the anterior region of *Eisenia fetida* exposed for 14 days to $CuSO_4$ and CuO ENMs in the fresh soil experiment: (a) Control, (b) 200 mg Cu kg^{-1} dw $CuSO_4$, (c) 1000 mg Cu kg^{-1} dw CuO-Core, (d) 1000 mg Cu kg^{-1} dw CuO-PEG, (e) 1000 mg Cu kg^{-1} dw CuO-COOH and (f) 1000 mg Cu kg^{-1} dw CuO- NH_4^+ (all nominal values). CU – cuticle, EP – epidermis, CM – circular muscle, LM – longitudinal muscle, P- pigment as granular deposits, most likely porphyrins, MU – mucous cells (clear cylindrical cells with slight deposits). Arrows point to example areas of reduced number of mucous cells.