Consequences of surface coatings and soil ageing on the toxicity of cadmium telluride quantum dots to the earthworm *Eisenia fetida*

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Abstract

The bioaccumulation potential and toxic effects of engineered nanomaterials (ENMs) on earthworms are poorly understood and there are concerns that the hazards will change with the type of coating and soil ageing. Two studies were conducted following OECD TG 222 with minor modifications and additional endpoints to assess the effects of CdTe QDs with different coatings on earthworms (*Eisenia fetida*); and to determine whether bioaccumulation or toxicity changed after soil ageing. Earthworms were exposed to carboxylate (COOH), ammonium (NH_4^+), or polyethylene glycol (PEG) coated CdTe QDs, or a micron scale (bulk) CdTe material, at nominal concentrations of 50, 500 and 2000 mg CdTe QD kg^{-1} dry weight (dw) for 28 days in Lufa 2.2 soil. The endpoints included survival, body weight, reproduction, metal accumulation, and markers of oxidative or ionoregulatory stress. In the fresh soil study, earthworms accumulated similar amounts of Cd and Te in the CdTe-bulk exposures, while the accumulation of Cd was higher than Te during the exposures to CdTe QDs. However, neither the total Cd nor Te concentrations in the earthworms were easily explained by the extractable metal fractions in the soil or particle dissolution, indicating other particle metrics altered bioavailability. There were on effects on survival, but some retardation of growth was observed at the higher doses. Inhibition of Na^+K^+-ATPase activity with disturbances to tissue electrolytes, as well as tissue Cu and Mn were observed, but without depletion of total glutathione in the fresh soil experiment. Additionally, juvenile production was the most sensitive endpoint, with estimated nominal EC_{50} of >2000, 108, 65, 96 mg CdTe kg^{-1} for bulk, PEG-, COOH- and NH_4^+-coated CdTe QDs, respectively. In the aged soil study, the accumulation of Cd and Te was higher than in the fresh soil study in all CdTe QD exposures. Survival of the adult worms was reduced in the top CdTe-COOH and -NH_4^+ QD exposures by 55 ± 5 and 60 ± 25 %, respectively; and with decreases in growth. The nominal EC_{50} values for juvenile production in the aged soil were 165, 88, 78 and 63 mg CdTe kg^{-1} for bulk, PEG-, COOH- and NH_4^+-coated CdTe QDs, respectively. In conclusion, exposure to nanoscale CdTe QDs, regardless of coating, caused more severe toxic effects that the CdTe bulk material and the toxicity increased after soil ageing. There was a coating-mediated effect in some cases that was not explained by the coating itself, but due to subsequent differences in the metal content and behaviour of the materials.

1. Introduction

Quantum dot (QD) technology is a growing market within the nanotechnology industry. The applications include bio-imaging and medical diagnostics (Kairdolf et al., 2013), light-emitting diode (LED) technologies (Hardman, 2005), and photovoltaics that offer alternative energy solutions (Sinha et al., 2012). Inevitably, the manufacture of QDs and their use in industrial processing or products will likely cause some releases to the environment, as with other engineered nanomaterials [ENMS, (Lead et al., 2018)]. The disposal of QDs in electronics and the subsequent burning of e-waste by developing countries is a particular concern for metal releases from CdTe QDs (Luo et al., 2011). Once in the environment, CdTe QDs may also slowly degrade and release their hazardous dissolved metals (Navarro et al., 2008).

Cadmium is a non-essential metal and known to bioaccumulate in earthworms (Hopkin, 1989). The background concentrations of Cd in soil are usually around 1 mg kg^{-1} dry weight (dw) or less (Keshavarz Jamshidian et al., 2017), but contaminated soils can have tens of mg of Cd (Spurgeon et al., 1994). Dissolved cadmium toxicity is well established in earthworms; with a 28-day lethal concentration (LC_{50}) of 588 mg Cd kg^{-1} dw in artificial soil (Van Gestel et al., 1991), and 56-day EC_{50} for impairment of reproduction of 46.3 mg Cd kg^{-1} dw in artificial soil (Spurgeon et al., 1994). In contrast to Cd, the ecotoxicity of tellurium is poorly understood. It has no known biological functions in eukaryotic cells (Ba et al., 2010) and not much is known about its toxicity to soil organisms. So far, the only known biological function of tellurium has been found in fungi, that can use tellurite instead of sulphur for production of amino acids when sulphur is limited or absent (Ramadan et al., 1989). Due to its biogeochemistry, Te is a difficult metalloid to measure in complex environmental matrices; nonetheless a few measurements of Te in soil and sediments are emerging, suggesting that it is naturally present at low μg kg^{-1} concentrations (Belzile and Chen, 2015).

Studies on the environmental toxicology of QDs have shown biological effects that relate to the reactive chemical properties of CdTe QD; such as their potential to generate reactive oxygen species, but also toxicity related to Cd dissolution from the materials in various organisms (Rocha et al., 2017). Earthworms, like some other soil invertebrates, have the ability to sequester metals such as Cd, and to form metal storage granules in the tissue (Brown, 1982). Earthworms also have the ability to

biosynthesise CdTe QDs from dissolved metals (Stürzenbaum et al., 2013). There are some studies on neurotoxicity (Wu et al., 2015) and oxidative stress (Srivastava et al., 2016) in the nematode worm, *Caenorhabditis elegans*, with CdTe QDs; and at least one study on marine polychaete worms with CdS (Buffet et al., 2014). However, there is sparse information on the toxicity of additions of CdTe QDs to the soil for earthworms.

This experiment aimed to provide a 'baseline dataset' for the hazard of CdTe QDs in freshly prepared soil. The approach used the Organisation for Economic Cooperation and Development (OECD) technical guidance (TG) 222 method (OECD, 2004), but with modification and additional endpoints to give a detailed mechanistic understanding of the data. Recently, we also reported a coating related effect on the toxicity of CuO ENMs to earthworms (Tatsi et al., 2018). However, coating-mediated effects on the toxicity of CdTe QDs are unknown in earthworms, and exactly the same coatings were used here for the CdTe QDs ENMs. The study design therefore included a control soil, commercially available micron-sized (bulk) CdTe powder, and CdTe QDs made with organic surface coatings with terminal residues of carboxylate (COOH), ammonium (NH_4^+), or polyethylene glycol (PEG); to represent negative, positive and neutral surface charges respectively. The potentially toxic metal component(s) of the composite QDs were explored by determining the extractable metal fractions from soil compared to the observed accumulation of Cd and Te in the earthworm tissues. Also to enable some comparison with our previous experiments on CuO ENMs (Tatsi et al., 2018), the survival and growth of the earthworms in freshly dosed soils were determined. Biochemical measurements were also made in the fresh soil experiment to assess known mechanisms of metal toxicity including; effects on ionic regulation (tissue metal concentrations, Na^+/K^+-ATPase activity) and oxidative stress (total glutathione). Having established the response in fresh soil, a second experiment including similar endpoints was carried out with newly exposed earthworms after a six-month period of ageing the soils in order to understand the persistence of any hazard from CdTe QD materials in the soil.

2. Methodology

Two experiments were carried out using a low (50), medium (500) and high (2000 mg as CdTe kg^{-1} dw) nominal concentration of CdTe QDs in a quadruplicate test design. Unexposed controls and an equivalent CdTe micron-sized powder was

used as a bulk material control. The first experiment was conducted with these substances freshly spiked into the soil, and the second experiment used the same soils after six months of ageing. Hereafter referred to as 'fresh' and 'aged' soil experiments, respectively.

2.1 Engineered quantum dots and characterisation

The ENMs used in the experiments were provided by PlasmaChem as part of a European Commission Framework 7 project (www.nanosolutionsfp7.com). The details of the coatings and their synthesis are commercially sensitive information. However, the different coatings were polyethylene glycol (PEG), carboxylate (COOH) and ammonium (NH_4^+) to represent neutral, negative and positive coatings respectively. 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. An appropriate micron-sized CdTe powder (size < 250 µm, Sigma-Aldrich, UK, CAS No. 1306-25-8) was used as a bulk material control (refer to hereafter as 'CdTe-bulk'). The materials were extensively characterised as part of the NANOSOLUTIONS project [e.g., (Vassallo et al., 2018)] and the characterisation of the batches of the materials used here is summarised in Table 1. This includes the nominal primary particle size and chemical composition of each nanoscale QD based on the manufacturer's information, as well as measurements of primary particle diameter, hydrodynamic diameter and dissolution of Cd and Te made in Plymouth (Table 1; Fig. S1). The QD powders supplied were dark red (NH_4^+-coated) or bright red (PEG-, COOH-coated) in colour and no impurities were identified by the supplier. The CdTe bulk powder was black in colour and no impurities were noted by Sigma-Aldrich. All other chemicals used were analytical grade and purchased from Sigma-Aldrich unless stated otherwise. The thermogravimetric analysis (TGA) of the materials (Table 1) tentatively indicated that the combustible organic components of the materials contributed roughly 8, 23 and 50 % of the mass of the CdTe–NH_4^+, -COOH and –PEG coated QDs, respectively. However, this would include any 'unknown' organic impurities and potentially residual water molecules in between the polymers of the coatings. At the time of the study (and currently) it was not technically feasible to reliably quantify the molecular weight of the coatings *in situ* on the particles, or the precise stoichiometry or coverage of each coating in the overall material in order to spike the soils solely based on Cd or Te concentrations in the core. Therefore, pragmatically, the same approach was taken as

we had done previously with CuO materials of the same coatings (Tatsi et al., 2018), where the mass concentration of the whole material as CdTe QDs was used for dosing and reported as mg CdTe kg^{-1} dw for each soil treatment, and with measurements of the metal concentrations in the soils and organisms to confirm the exposure.

2.2 Stock animals and test soil

Adult *Eisenia fetida* originating from a commercial supplier (Blades Biological, Kent, UK) were used from an internal age synchronised laboratory breeding culture held at University of Plymouth for both experiments. The test species were kept in an artificial medium that comprised 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 at a temperature of 20 ± 1 °C. Adult earthworms between two to four months old, and with visible clitellum, were hand-selected for the experiments. The earthworms were acclimatised to the test soil (Lufa 2.2) with feed one week prior to the experiment. A standard sandy loam Lufa 2.2 (LUFA Speyer, Germany) soil was used with the following composition (supplier's information, mean ± SD, dry soil, *n* = not specified): pH of 5.5 ± 0.2 (measured in 0.01 M $CaCl_2$ solution); organic carbon, 1.8 ± 0.2 %; nitrogen content at 0.17 ± 0.02 %; cation exchange capacity, 10.1 ± 0.2 meq 100 g^{-1}. The water-holding capacity of the soil was measured in-house and was (mean ± SD, *n* = 3): 41.3 ± 3.0 g 100 g^{-1} dw. The soil used in the experiments was sieved through a 2 mm mesh and was air dried at 25℃ for 2 days. Soil pH was measured (see Supplementary Information, Table S1) prior to the start and at the end of the experiment in a 1:1 soil: deionised water slurry, using a glass combination electrode (Corning 420).

2.3 Experimental designs and spiking of the test soil

2.3.1 Fresh soil experiment

The experiment was conducted using the standard OECD test guideline (TG) 222 for the earthworm reproduction test (OECD, 2004), but with a reduced number of earthworms and additional sub-lethal endpoints. The study design included an unexposed control soil, bulk-CdTe and the three variations of coated CdTe QDs: PEG, COOH, NH_4^+ at nominal concentrations of 50, 500 and 2000 mg CdTe kg^{-1} dw. The 50 and 500 mg CdTe kg^{-1} dw concentrations were selected on the basis of the known sub-lethal effects and 28-day lethal concentration for Cd toxicity to earthworms

193 respectively (Spurgeon et al., 1994; Van Gestel et al., 1991); and would also
194 encompass the range of concentrations of Cd at contaminates sites such as metal
195 smelters [e.g., 0.2-102 mg Cd kg^{-1} of soil, (Chlopecka et al., 1996)]. The TG 222
196 protocol allows a high dose of 1000 mg kg^{-1} dw of the test substance in the soil as part
197 of a 'limit test.' Given that the CdTe QDs are a composite of Cd, Te, plus any coating;
198 an upper concentration of 2000 mg kg^{-1} dw was used for the QDs so that the individual
199 metals in the composite might approximate to the limit test values in TG 222. It was
200 also intended as a high concentration to reveal any mechanistic aspects of the toxic
201 effects that might be observed. The QDs were mixed into the soil as powders, because
202 dry mixing has been found a suitable dosing method (Handy et al., 2012). The amount
203 of QD powder required to dose each of 4 replicates was weighed into 50 g dw of soil
204 and carefully mixed by hand for 10 minutes. Then, this aliquot of soil was added to the
205 remaining amount of soil (950 g) and further mixed by hand, followed by wetting of the
206 soil to 50 – 60 % of the WHC with ultrapure Milli-Q water (18.2 Ω). The soil was left to
207 equilibrate with the moisture for one day to minimise the risk of the QDs changing
208 before the worms were added.
209 Adult *E. fetida* (*n* = 5 earthworms/250 g dw soil) with a mean weight of 1.86 ±
210 0.02 g for 5 earthworms (mean ± SEM, *n* = 52, individual earthworm weight ~ 0.36 g)
211 were exposed in 4 replicates boxes of soil at 20 ± 1°C at 12:12 light:dark cycle. The
212 earthworms were fed dried horse manure (1 g/earthworm) wetted to 70 % of its water
213 holding capacity (WHC) with Milli-Q water. Life history traits such as survival and
214 weight of the earthworms were recorded at the beginning of the experiment and on
215 days 14 and 28. Behavioural changes such as avoidance of burrowing into the soil,
216 were recorded following visual assessment in all treatments at the beginning of the
217 fresh soil experiment. Further endpoints such as total concentration of Cd, Te and
218 other elements in earthworm tissue and total glutathione and Na^+/K^+-ATPase activity,
219 were measured on day 28 in randomly selected earthworms (*n* = 8 earthworms for
220 each analysis per treatment; methods described below). After 28 days, when all the
221 adults were removed from soils, additional feed was added (5 g dw, wetted to 70 %
222 WHC with Milli-Q water) and soils were left for a further 28 days to allow juveniles to
223 hatch from cocoons. The juveniles were then counted following the OECD TG 222
224 (OECD, 2004). Briefly, each test vessel was placed in a water bath (60 ºC) and the
225 juveniles that emerged to the surface were collected and counted. After counting, soils
226 were manually checked to ensure no juveniles or cocoons remained in the soil. The

juveniles collected from each treatment were washed in deionised water, blotted dry, and weighed to determine the treatment effect on juvenile growth.

2.3.2 Aged soil experiment

The soil used in the aged soil experiment was the same as that used in the initial fresh soil experiment. At the end of the fresh soil experiment the test containers were left for a total of 27 weeks (6 months) in the same experimental conditions, but without any disturbance or watering. During the ageing period the containers were not open, but airflow occurred via the pierced lids. Incidental plant growth had occurred during the ageing period, from seeds likely present in the natural soils or the manure used as feed. It was not an objective of the study to investigate plant growth in the aged soils, but the opportunity was taken. The plant coverage in each exposure container was quantified one day before starting the aged soil experiment. All the containers were photographed, and their percent cover with plants was determined by directly assessing the boxes (an acetate paper with grids was placed on each box to aid the estimation of percent cover). Once the above ground material was quantified (see supplementary material, Figs. S2 and S3), it was removed by cutting. The roots of the plants were left in the soil to increase the environmental realism of the aged soil study. Soil moisture content was then adjusted to 50 - 60 % and soil pH was measured in a 1:1 soil:water slurry using a glass combination electrode (Corning instruments).

The same study design and methods were used in the aged soil experiment as described in the fresh soil experiment. Five earthworms with a mean weight of 1.89 ± 0.03 g (mean ± SEM, n = 52, individual weight ~0.38 g) were used in the experiment. On days 0, 14 and 28, endpoints such as survival, biomass and appearance were recorded. Reproduction was assessed after 56 days of exposure (described above). In addition, the total concentration of Cd, Te and other electrolytes and trace metals in earthworm tissues were measured on day 28. Due to a technical fault with a freezer, earthworm samples collected for biochemical analyses (total glutathione and Na^+/K^+-ATPase) were defrosted twice. These defrosted samples were analysed, but the results were considered unreliable and therefore not used (data not shown). In addition, juvenile weight was not assessed in the aged soil study due to the difficulty of obtaining the alive and intact juveniles from the soil. This was due to the presence of plant roots in the soil that severely hampered the collection of juveniles. The roots were inevitably very tangled in the soil, making it difficult to manually find and remove

the juvenile worms that were extremely delicate. Several individuals stuck in the soil for too long and, unfortunately, they did not survive the handling stress of the collection procedure.

2.4 Total and extractable metal analysis in samples

Total metal analysis in the soils and the earthworms was conducted to confirm the exposure and the bioavailable fraction from the QDs, respectively, exactly as previously described (Tatsi et al., 2018). Total *aqua regia* extractable Cd and Te concentrations were measured in triplicate at the beginning of both the fresh and aged soil experiments (n = 12 per treatment). Additionally, to provide insight to the mobile fractions of Cd and Te, water (deionised water) and 0.1 M HCl extractable fractions were determined in duplicate in the nominal 2000 mg CdTe kg^{-1} dw exposures only in the fresh soil experiment (n = 8 per treatment), aged soils were not analysed. Earthworms collected at the end of the fresh and aged soil experiments (n = 8 per treatment) were allowed to depurate for 24 h on moist filter paper (the latter was changed following 12 h to avoid coprophagy) according to (Arnold and Hodson, 2007). Then the worms were washed, freeze-dried (for 48 h), weighed and digested in 1 ml concentrated HNO_3. All acid digested samples were allowed to cool, diluted with 7 ml ultrapure deionised water (18.2 Ω) and stored in the dark. The total concentration of Cd and Te was measured in the soil and earthworm samples. In earthworms, also other essential elements (Ca, Fe, K, Mg, Mn, Na, Zn, Cu) were measured to assess changes in the trace element and electrolyte composition of the earthworms by inductively coupled plasma optical emission spectrophotometry (ICP-OES, iCAP 700, Thermo Fisher) or inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific X Series 2) as appropriate. Prior to analysis, the samples were sonicated for 15 minutes (at 0.05 kva, 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 where possible. The certified reference materials for total Cd metal reported close to the expected values and were 100 ± 4 % (n = 3, EnviroMAT contaminated soil, SS-1) and 102 ± 7 % (n = 3, TORT-2, contaminated lobster hepatopancreas). Spike recovery tests of earthworm and soil digests using CdTe-NH_4^+ QDs were 78 and 73 %, respectively (values were calculated based on the sum of [Cd] and [Te] and normalised to expected coating mass which was 8.8 ± 0.5 % for NH_4^+). Using $CdCl_2$ and Te standard for ICP-MS and -OES (TraceCERT by Sigma),

the recoveries for earthworm and soil digests were > 90 %. The calculated limit of detection (LOD) for Cd (analysed by ICP-OES) was 1 mg kg^{-1} dw and 0.001 mg kg^{-1} for Cd in soil and earthworm tissue, respectively. While the calculated LOD for Te (analysed by ICP-MS) was to around 0.01 mg kg^{-1} dw and 0.1 mg kg^{-1} dw in soil and earthworm tissue, respectively.

2.5 Biochemical analyses

The biochemical methods had been used previously for ENMs and earthworms in our laboratory (Tatsi et al., 2018). Biochemistry was performed on whole earthworm tissues collected at the end of fresh soil experiments (n = 8 per treatment). Earthworms from each test container were snap frozen in liquid nitrogen and stored at -80 °C until homogenised in ice cold isotonic buffer (1:5 ratio, weight: volume) exactly as described in Tatsi et al. (2018). The homogenates were further diluted prior to analysis due to high protein concentration (15 fold dilution of the original tissue, n = 8 per treatment) and were then were assayed in triplicate for total protein, total glutathione (GSH) and Na^+/K^+-ATPase activity exactly as described in Tatsi et al. (2018) and using a VersaMax plate reader (Molecular Devices, UK). Briefly, GSH was quantified in 20 µl of the diluted homogenate according to (Owens and Belcher, 1965). Na^+/K^+-ATPase activity was determined in 10 µl of the diluted homogenate based on a modification of (McCormick, 1993). Total protein was determined in 25 µl of the diluted homogenate using the Pierce BCA kit (#RE232674, Thermo Scientific, UK). The concentrations of GSH and Na^+/K^+-ATPase activity were normalised to total protein in the sample and data are expressed as nmol GSH per mg protein and µmol ADP per mg protein per hour, respectively.

2.6 Statistical analyses and data presentation

Statistical analyses were performed using R studio software (version 2.1) and graphs were drawn using SigmaPlot (version 13.0 or 14.0). Data were checked for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test). Non-parametric data were transformed (log_{10}) and reanalysed as appropriate. The student's t-test (two-tailed, un-/paired) or Mann Whitney test were used for comparing two samples sets as appropriate. Due to a few individual significant differences in pH between treatments in the fresh or aged soil experiments, 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 effects were assessed by one-way ANOVA followed by the Tukey-Kramer (due to 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. The non-parametric Kruskal-Wallis test was used followed by a distribution free multiple comparisons test, Dunn's test, where data transformation was unsuccessful. To evaluate the biological associations known for metal toxicity, 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 reproduction results were normalised to the control values and the 50 % effect concentration (EC_{50}) values and its 95 % confidence intervals were estimated by logistic nonlinear regression analysis (sigmoid) on raw data using the log-transformed nominal concentrations in SigmaPlot v 14.0. To estimate the nominal effect value, the log-transformed value was reversed. The statistical significance level (α) for all tests was set at 0.05.

3. Results

3.1 Soil pH

In the beginning of the fresh soil experiment the soil pH ranged from 5.36 to 5.89, and soil pH differed between QD treatments and the control soils. However, by the end of the exposure (day 28) there were no differences in pH between the QD-treated soils and the control soils, with the pH values ranging from 5.39 to 5.78 (Table S1). In the aged soil experiment, the pH ranged from 5.38 to 5.65, both at the beginning and the end of the experiment, and was not statistically different from the corresponding pH measurements in the fresh soil experiment ($P > 0.05$, Student's *t*-test). Soil pH was included as a cofactor in all the endpoints analysed in both experiments, but no significant interactions were found ($P > 0.05$, ANCOVA). Soil pH was therefore not a factor in any of the biological measurments.

3.2 Total and extractable Cd and Te concentrations in soil

To enable the interpretation of the results, the total *aqua regia* extractable concentration of Cd and Te was measured at the beginning of both the fresh and aged soil experiments. In the former experiment, the control soil contained a background Cd concentration of 0.61 ± 0.05 mg Cd kg^{-1} dw (mean ± SEM, $n = 8$), of this total Cd, 9.2 ± 1.4 and 10 ± 1.5 % was water and 0.1 M HCl-extractable respectively; indicating

363 that less than 10 % of the Cd in the soil was mobile. Te was not detected in the control
364 soil, as the results were below the limit of detection, < 0.01 mg Te kg^{-1} dw. Since the
365 CdTe QDs were dosed into the soil on the basis of the mass of the whole material,
366 including both metals present and any coating, it was expected that the measured total
367 Cd and Te concentrations (i.e., measured concentration of each metal) would be a
368 fraction of the total exposure concentration of the whole material. This was indeed the
369 case (Fig. 1). Nonetheless, exposure to CdTe bulk and the differently coated CdTe
370 QDs caused the expected increase in total soil Cd concentrations in the fresh soil
371 experiment (Fig. 1). However, the measured total Cd in the soil did not differ
372 significantly between the types of CdTe QD exposures within each nominal test
373 concentration (Fig. 1, $P > 0.05$, ANOVA). The measured total Te concentrations also
374 increased with the nominal exposure concentration of the whole material in the fresh
375 soil experiment. However, in contrast to Cd, the total tellurium concentration varied
376 significantly between materials in each nominal test concentration (Fig. 1A and B, $P <$
377 0.05, ANOVA); with the amount of Te in the exposures followed the order of: COOH ≤
378 PEG < NH_4^+ < CdTe-bulk. This was not interpreted as a 'coating effect' per se,
379 because it did not follow the order of mass loss of the materials in the TGA
380 measurement of PEG > COOH > NH_4^+ (Table 1) and in any case, the effect was absent
381 in the Cd measurements. Instead, this was likely attributed to the ratio of Cd to Te in
382 the different materials as manufactured.
383 The water- and dilute acid-extractable Cd or Te was measured at the beginning
384 of the fresh soil experiment at the highest nominal exposure concentration and plotted
385 against the total metal concentrations in the earthworms (Fig. 2). The amount of water-
386 and 0.1M HCl-extractable Cd and Te varied between treatments. In the CdTe-bulk
387 exposures, less than 1 % of the Cd and Te were extractable and the Cd or Te metal
388 concentrations in the earthworms were also modest (Fig. 2); suggesting low availability
389 of metal from the bulk material in the soil. For the CdTe QD treatments, regardless of
390 the extraction method or type of coating, only a small proportion of the Te was
391 extractable and with similarly low total Te concentrations in the earthworms. However,
392 the situation was different for the extractable Cd from the QDs (Fig. 2), with a
393 considerable fraction of acid-extractable Cd in the soil. Overall, with the QD
394 treatments, the water available fraction of Cd and Te was the highest from CdTe-NH_4^+
395 QD exposures, although this did not necessarily lead to a concomitant elevation of
396 total Cd or Te in the earthworms according to the type of coating (Fig. 2). The total

concentration of Cd and Te in the soil were measured again prior to beginning of the aged soil experiment. In general, the concentration of Cd and Te in the aged soil followed a very similar pattern to the fresh soil experiment, but with a trend (not statistically significant) of slightly lower values than in the fresh soil experiment. The extractable fractions were not assessed in the aged soils.

3.3 Total Cd and Te in earthworm tissue

The total Cd or Te concentrations in the earthworms are shown in Fig. 3. The control earthworms in the both the fresh and aged experiments had some background Cd in the tissues (4.23 ± 0.34 mg Cd kg^{-1} dw, mean ± SEM, n = 8), while no Te was detected. In the fresh soil experiment, an exposure concentration-dependent increase in the total body burden of Cd was seen in all the CdTe-bulk and all the CdTe QD exposures (Fig. 3). The accumulation of Cd was significantly lower in the CdTe-bulk when compared to the QD exposures in fresh soil at each exposure concentration. However, within the CdTe QDs, there was no significant differences between the total concentrations of Cd in the earthworm tissues by the type of coating of the CdTe QDs at each nominal test concentration (Fig. 3A, P > 0.05, ANOVA). The total concentration of Te in the earthworms during the fresh soil experiment was also measured (Fig. 3B). Here, the tissue Te concentration increased in a concentration-dependent manner in the CdTe-bulk exposures, but not in the CdTe QD exposures where the Te remained uniformly low. Within the QDs there was also no clear effect on Te accumulation by the type of surface coating on the ENM in the fresh soil experiment (Fig. 3B). Together these observations suggest some Te accumulation for the bulk material, but very limited Te accumulation for CdTe QDs in fresh soil. The accumulation of Cd and Te were strongly correlated within each treatment group (r_s= 0.9, 0.8, 0.8 and 0.9 for CdTe-bulk, -PEG, -COOH and -NH_4^+ exposures, P < 0.05, Spearman) and this is consistent with the notion of being exposed to a composite material containing both metals. The total Cd or Te in the soil was also significantly correlated to the total Cd or Te in earthworms when analysing all the data together (r_s = 0.8 and 0.7 for Cd and Te, P < 0.05, Spearman); and within the animals in each treatment. However, this contrasts with the absence of any clear relationship between the water- or acid-extractable fractions and accumulation of Cd or Te by the earthworms noted in Fig. 2.

In the aged soil experiment, the accumulation of Cd and Te by earthworms was, overall, higher than in the fresh soil experiment. In the CdTe-bulk exposures the Cd concentrations in the earthworms were significantly higher than those in the fresh soil study (Fig. 3). In the nominal 50 mg CdTe kg^{-1} dw test concentration there were no significant differences in the concentration of Cd in the earthworms by the type of coating on the CdTe QDs in the aged soil (Fig 3C). In the nominal 500 or 2000 mg CdTe kg^{-1} dw test concentrations in aged soil, there were no statistically significant differences between the tissue concentrations of Cd between doses, but there was a trend of higher tissue Cd concentration in earthworms exposed to the differently coated CdTe QDs when compared to the CdTe-bulk, although the trend was not statistically significant. In the aged soil experiment, the concentration of Te in the earthworms followed a similar pattern of accumulation to the fresh soil experiment, where the highest amount of Te was found in earthworms exposed to CdTe-bulk material in all test concentrations. In the nominal 50 mg CdTe kg^{-1} dw test concentration, there were no statistically significant differences in Te accumulation by the type of coating on the CdTe QDs. However, some differences in the total Te accumulation emerged in the 500 mg CdTe kg^{-1} dw test concentration, where the concentration was highest in the CdTe-NH_4^+ QD exposure, compared to the other materials, and the Te accumulation was similar in the CdTe-PEG and -COOH QD exposures. In the nominal 2000 mg CdTe kg^{-1} dw test concentration, the accumulation of Te was not statistically significantly different between the different CdTe QD exposures, although there was a non-significant trend of higher Te in the CdTe-NH_4^+ groups.

3.4 Survival, biomass and appearance of earthworms

The survival and biomass (growth) of the earthworms are reported in Table 2 for both the fresh and aged soil experiments. First, consider the fresh soil experiment. The control animals were healthy and increased in biomass (19.9 ± 5.1 %, Table 2). In the fresh soil experiment, there were no statistically significant changes in the survival in any of the treatments throughout the experiment, except some minor mortalities < 10 %. The biomass of earthworms in the CdTe-bulk exposures also increased by as much as 22 % by day 28 (Table 2); overall indicating no effects on growth or survival of earthworms exposed to the bulk material compared to the unexposed controls in the fresh soil experiment. An increase in biomass was also

observed in the nominal 50 mg CdTe kg^{-1} dw for the QD exposures by day 28, but the 2000 mg CdTe kg^{-1} dw exposure concentration of the QDs caused a uniform decrease in biomass of around 50% by day 28, regardless of the type of coating on the ENMs (Table 2). There were no obvious behavioural or changes in appearance of the earthworms exposed to the CdTe-bulk or the nominal 50 and 500 mg CdTe kg^{-1} dw of the CdTe QDs in the fresh soil experiment. However, earthworms from all the CdTe QD exposures (excluding CdTe-bulk) at the nominal 2000 mg CdTe kg^{-1} dw test concentration were sluggish and were not actively feeding (evidence of left-over manure), although they were still moving in the soil. Furthermore, morphological changes were evident in earthworms from the 2000 mg CdTe kg^{-1} dw CdTe QD ENM exposures. The earthworms were visibly smaller and showed evidence of shedding of the posterior segments (the last segments were clearly lighter coloured with signs of regeneration). A strong negative correlation was found between tissue Cd or Te and biomass for the fresh soil experiment when analysing all the data from all treatments together (r_s = 0.6 and 0.5 for Cd and Te respectively, $P < 0.05$, Spearman).

In the aged soil study, the control earthworms were healthy in appearance and with good survival, however, a trend of biomass loss (8.1 ± 5.6 %, Table 2) was noted, although the change was not statistically significant. The earthworms survived in all the CdTe-bulk exposures with only minor mortalities < 10 % at the nominal 2000 mg CdTe kg^{-1} dw exposures in the aged soil experiment. In the CdTe-bulk exposures, earthworms lost biomass only at the nominal 2000 mg CdTe kg^{-1} dw test concentration (Table 2). At the nominal 2000 mg CdTe kg^{-1} dw test concentration for the CdTe QDs, the CdTe-COOH QDs were the most toxic to earthworms in the aged soil experiment; where survival was reduced to 55 ± 5, 60 ± 25, and 75 ± 10% for the –COOH, -NH_4^+ and –PEG-coated QDs respectively by day 28 (Table 2). In addition, earthworms from these treatments showed statistically significant weight loss compared to controls. There were no obvious behavioural or appearance changes in the CdTe-bulk exposures or the nominal 50 mg CdTe kg^{-1} dw CdTe QD exposures in the aged soil experiment. Some earthworms appeared lethargic and non-responsive in the 500 and 2000 mg CdTe kg^{-1} dw CdTe QD ENM exposures, and similar to the fresh soil study, there was evidence of shedding of the posterior segments and lack of feeding (presence of surplus feed).

3.5 Reproduction

The reproductive success of the animals is reported in Table 3, and with dose-response curves for juvenile production shown in Fig. S4. The test results on reproduction in the controls of both fresh and aged experiments met the validity criteria of the OECD TG 222. In both the experiment with fresh and aged soil, the control earthworms produced a healthy number of juveniles per earthworm (Table 3), and with a coefficient of variation (CV %) of less than < 30 % in keeping with the OECD test. Reproduction was not significantly reduced in any of the CdTe-bulk test concentrations, or in the nominal 50 mg CdTe kg^{-1} dw CdTe QD exposures in the fresh soil experiment. However, it was significantly reduced in the CdTe QD treatments at the nominal 500 mg CdTe kg^{-1} dw test concentration, and completely inhibited at the 2000 mg CdTe kg^{-1} dw test concentration for the CdTe QD exposures, compared to controls in the fresh soil experiment. The 50% effect concentrations (EC_{50}) for reproductive success (i.e., juvenile production) for the different materials are presented in Table 3. The estimated EC_{50} values were calculated using the nominal CdTe QD concentrations (response curves presented in Fig. S4). For the fresh soil experiment, the EC_{50} values decreased in the following order: CdTe-COOH < CdTe-NH_4^+ < CdTe-PEG. For the CdTe bulk material in fresh soil there was no inhibition of juvenile production and so an EC50 value could not be estimated. The fresh wet weight of the juveniles was also measured after they were rinsed in deionised water and padded dry (Table 3). There were some statistically significant differences between treatments in the biomass of the juveniles in the fresh soil experiment, but the differences followed the same order as the total number of juveniles produced. That is, where only a few juveniles were produced, those juveniles also tended to be small (i.e., poor quality offspring).

In the aged soil experiment (Table 3), the controls produced more juveniles than in the fresh soil experiment. Unlike the situation in fresh soil, the number of juveniles produced in the CdTe-bulk exposures declined, and with a complete inhibition of reproduction at the nominal 2000 mg CdTe kg^{-1} dw test concentration in aged soil. The effects of the CdTe QD exposures on juvenile production were more pronounced in the aged soil experiment, with lower EC_{50} values for the CdTe-PEG and CdTe-NH_4^+ treatments in the aged soil compared to those in fresh soil (Table 3). The number of juveniles produced was significantly reduced or completely abolished in all the CdTe QD exposures at 500 and 2000 mg CdTe kg^{-1} dw test concentrations

compared to the unexposed controls in aged soil. The EC_{50} values for reproductive success decreased in the following order in aged soil: CdTe-NH_4^+ < CdTe-COOH < CdTe-PEG < CdTe-bulk. However, while the ranking of the EC_{50} values are correct, all of the curve fits (Fig. S4), will have some uncertainty in the precise EC_{50} value due to the number of data points and their confidence intervals in the dose-response plot.

3.6 Sodium pump activity and tissue elemental composition

In the experiment with the fresh soil the control earthworms showed a normal Na^+/K^+-ATPase (sodium pump) activity 6 – 8 µmol ADP mg^{-1} protein h^{-1} (Fig. 4A). In the fresh soil experiment, there was an overall trend of lower sodium pump activity in all the exposure concentrations of the CdTe bulk material, with significantly reduced activities in the nominal 500 and 2000 mg CdTe kg^{-1} dw test concentration (Fig. 4). There was also some inhibition of the Na^+/K^+-ATPase in the CdTe QD treatments in the fresh soil experiment, but with no clear dose and coating-dependent trend in the data (Fig. 4A). The substrates for the sodium pump include Na^+ and K^+ and the tissue electrolytes of the earthworms are shown in Table S2. There was no clear effect on the concentration of Na^+ in earthworm tissues, however, the concentration of K^+ was significantly lower in the nominal 500 and 2000 mg CdTe kg^{-1} dw CdTe-NH_4^+ exposures compared to controls ($P < 0.05$, ANOVA; Table S2). In the aged soil study, unfortunately failure of a deep freeze prevented any reliable determination of enzyme activity in those samples (data not shown). However, there was no depletion or significant increase in the tissue concentration of Na^+ or K^+ between the test concentrations and the control for any treatment ($P > 0.05$, ANOVA; Table S2).

A range of other electrolytes and essential trace elements in the tissues of the earthworms are shown in Table S2. Overall for the control earthworms, in both the fresh and aged soil studies, the electrolytes and trace elements remained in the expected range for a healthy earthworm population (Table S2). The earthworms in the fresh soil study exposed to the CdTe-bulk showed no significant treatment-dependent changes in electrolytes or trace metals (Table S2). In contrast, the CdTe QD exposures at 500 and 2000 mg CdTe kg^{-1} dw test concentrations showed statistically significant decreases in essential and trace metals in the earthworms in fresh soil (Table S2). The concentration of Ca, Mg and Fe were significantly reduced in the nominal 500 and 2000 mg CdTe kg^{-1} dw CdTe-NH_4^+ exposures compared to unexposed controls in the fresh soil experiment. There was also a significant negative

565 correlation between the tissue concentration of Cd and the influenced metals (r_s = -
566 0.3, -0.4 and -0.3 for Ca, Mg and Fe respectively, $P < 0.05$, Spearman). Earthworm
567 tissue copper concentrations were significantly reduced, ranging from 5 – 6 mg Cu kg^{-1}
568 in nominal 500 mg CdTe kg^{-1} dw CdTe-COOH and NH_4^+ and nominal 2000 mg CdTe
569 kg^{-1} dw CdTe-COOH exposures; compared to an expected concentration of 8 – 10 mg
570 Cu kg^{-1} dw in controls in the fresh soil study. Manganese (Mn) was significantly
571 depleted in earthworms from the nominal 500 mg CdTe kg^{-1} dw CdTe-NH_4^+ and
572 nominal 2000 mg CdTe kg^{-1} dw CdTe-PEG, COOH and NH_4^+ exposures, ranging from
573 14 – 15 mg Mn kg^{-1} dw compared to controls at 43 mg Mn kg^{-1} dw in the fresh soil
574 study. A significant strong negative correlation was found between the tissue Cd and
575 Cu or Mn (r_s = -0.6 for both, $P < 0.05$, Spearman).
576 In the experiment with the aged soil, there were no significant effects on the
577 electrolytes in the earthworms, however trace metals, Cu and Mn, were affected
578 (Table S2). The concentration of Cu was significantly reduced compared to the control
579 in the nominal 50 and 500 mg CdTe kg^{-1} dw CdTe-COOH and NH_4^+ and in all the 2000
580 mg CdTe kg^{-1} dw exposures (ranging from 2 – 5 mg Cu kg^{-1} dw). The concentrations
581 of Mn were significantly reduced compared to the control only in the nominal 2000 mg
582 CdTe kg^{-1} dw CdTe-COOH and NH_4^+ exposures (ranging from 18 – 25 mg Mn kg^{-1}
583 dw). There was also a significantly strong negative correlation between the tissue
584 concentration of Cd and Cu or Mn (r_s = -0.5 for both, $P < 0.05$, Spearman) in the aged
585 soil experiment.
586
587 *3.7 Total glutathione as an oxidative stress marker*
588 Total glutathione (GSH) concentrations were measured as an oxidative stress
589 markers in the earthworms (Fig. 4B). Earthworms from the fresh soil experiment
590 sustained a normal total glutathione concentration (~16 nmol mg^{-1} protein). In the
591 CdTe-bulk exposures there was no significant effect on the total GSH concentration.
592 Also, there was no significant effects in the differently-coated CdTe QD exposures
593 when compared to the controls ($P > 0.05$, ANOVA). The results of the total GSH from
594 the aged soil study were not considered reliable due to thawing of the frozen samples
595 (data not shown).
596
597 *3.8 Quantification of plant material*

598 It was not the aim of this study to assess the effects of CdTe QDs on plants,
599 but there was some incidental plant growth in the soil during the six months of ageing.
600 Prior to the start of the aged soil exposures of the earthworms, the opportunity was
601 taken to measure the plant cover on the exposure soils. The unexposed control soils
602 had an overall cover of > 85 % of plants and ~ 80 % of moss. Overall, effects on plant
603 cover were evident only at the nominal 500 and 2000 mg CdTe kg^{-1} dw exposures
604 (Fig. S2). There was a material size effect with the CdTe QDs being more toxic to plant
605 growth than the CdTe QD bulk counterpart, and there was a coating-mediated effect
606 for the QDs (Fig. S2). At the highest concentration of the CdTe bulk material, the
607 vascular plant and moss cover was significantly reduced to around 20% of that in the
608 controls (Fig. S2). Clear effects on plant cover were also evident in the nominal 500
609 mg CdTe kg^{-1} dw CdTe QD exposures, where the PEG- and COOH-coated QDs
610 showed the least amount of plants and moss, while the CdTe-NH_4^+ exposure was
611 similar to the CdTe-bulk and the controls. However, at the highest QD ENM test
612 concentrations there were no vascular plants or moss growth (Figs. S2 and S3),
613 except in the CdTe-PEG treated soils, which had a very limited (around 10 %) moss
614 cover. The highest CdTe QD treatments with no plant matter had a biofilm-like layer
615 and some mould, and the soil had dried in places (Fig. S4).
616
617
618 **4. Discussion**
619
620 This is the first study to investigate the effects of CdTe QDs with different surface
621 coatings on earthworms compared against a micron-sized (bulk) CdTe material in
622 fresh soil. The hazard to earthworms was also assessed after 6 months of ageing the
623 soils. In the fresh soil experiment, none of the CdTe materials (bulk or ENMs) altered
624 survival, but exposure to CdTe QDs caused a significant reduction in the number of
625 juveniles produced and disturbances to essential metal concentrations in the
626 earthworm tissues. There was also evidence of a coating-mediated effect, which
627 altered the release of dissolved Cd and Te from the materials, rather than an effect
628 due to the type of chemical substance in the coating itself. In the aged soil experiment,
629 unlike the fresh soil study, the CdTe-NH_4^+ QDs reduced the survival of the earthworms.
630 Both the CdTe-bulk and the CdTe QDs caused more pronounced effects on juvenile

631 production in the aged soil experiment compared to the fresh soil study, but the CdTe
632 QDs remained more toxic than CdTe-bulk QDs.
633
634 *4.1 Extractable metal fractions in the soil in relation to Cd and Te accumulation by*
635 *earthworms*
636 The exposures were confirmed by measuring the total metal concentrations of
637 Cd and Te in the soil (Fig. 1), and in the earthworms (Fig. 3). For the unexposed
638 controls, the background concentration of Cd in Lufa 2.2 soils was low (0.6 ± 0.2 mg
639 Cd kg^{-1} dw, Figure 1) and consistent with previous reports for the same soil [e.g., 0.38
640 mg Cd kg^{-1} dw, (Keshavarz Jamshidian et al., 2017)]. In the control soil, less than 10 %
641 of the total background Cd concentration was water and acid extractable, indicating
642 that the background Cd in the control soil was not mobile. There was no Te detected
643 in the control soils, as expected for a rare metalloid with very low background
644 concentrations in unpolluted soil [e.g., values < 5 µg kg^{-1} dw, (Belzile and Chen,
645 2015)]. The background concentration of Cd in the earthworms was also in agreement
646 with previous studies using the same soils [4.23 ± 0.34 mg Cd kg^{-1} dw this study; 5.6
647 mg Cd kg^{-1}, (González et al., 2013)], while tissue Te concentration remained below
648 the detection limit.
649 The soils treated with CdTe QDs had elevated Cd and Te concentrations
650 compared to the unexposed control soils, and as expected, the measured metal
651 concentrations increased with the nominal exposure concentration. This occurred in
652 both the fresh and aged soil experiments (Fig. 1). However, within each soil dose for
653 each type of CdTe QD, there was also some slight variation in the measured
654 concentration of total Cd or Te according to the type of coating on the starting material.
655 This was likely due to the stoichiometry of the materials giving rise to slightly different
656 ratios of Cd or Te to the coating, by mass. The effect was small within each soil dose,
657 and only influenced the Te measurements in the soil for the CdTe-PEG and CdTe-
658 COOH materials (Fig. 1). This was very similar to our previous findings on CuO ENMs
659 that had the same coatings; where the proportion of the overall mass due to the particle
660 core relative to the coating inevitably varied with the type of coating (Tatsi et al., 2018).
661 Nonetheless, in the present study, within each type of coating on the CdTe QDs, the
662 ratios of the measured Cd and Te concentrations in the soil were consistent for that
663 material.

In the fresh soil experiment, attempts were made to determine the bioaccessible fractions of Cd and Te in the soil. The water- and acid-extractable fractions of metals from the soils were plotted against the measured metal concentrations in the earthworms (Fig. 2). For the unexposed controls, the Cd or Te concentrations were very low or at the detection limit, as expected. The CdTe bulk material also revealed very low water- or acid-extractable metal concentrations (e.g., water-extractable of around 0.1 Cd kg^{-1} dw or 0.035 mg Te kg^{-1} dw of soil, Figs. 2C and D), which represented <1% of those metals in the bulk material exposures. The CdTe bulk materials also showed negligible metal dissolution (Fig. S1). Together, this suggests the metals in the CdTe bulk material are not bioaccessible as dissolved metals to the earthworms, and that the observed Cd and Te accumulation in the earthworms was attributed to uptake of the intact CdTe bulk material. The concentration of Cd compared to Te in the earthworm tissue (compare panels A and B in Fig. 3 for fresh soil) was consistent at around 1.7 which also indicates some accumulation of the intact CdTe bulk material.

The situation was somewhat different for the ENMs in soil compared to the bulk material (Fig. 2), with all the CdTe QDs showing a water- and acid-extractable fraction of Cd in the soil, but not much extractable Te. The dialysis experiment also showed more dissolution of Cd than Te from the ENMs (Fig. S1); which together implies the Cd was more bioaccessible than the Te in CdTe QDs. Furthermore, the Cd accumulation in the earthworms was at least an order of magnitude higher than Te (Figs. 2 and 3), despite no such differences in the exposure concentration of the metals in the soil from the ENMs (Fig. 1). This observation can only be explained by the differential uptake and/or retention of Cd compared to Te in the tissues, and therefore that the CdTe QDs do not remain intact. It has been suggested that CdTe QD likely degrade in the soil to release dissolved Cd (Navarro et al., 2008). In aqueous media for algae, the dissolution of Cd from CdTe-containing QDs and subsequent free metal ion uptake can explain most of the Cd accumulation by the organisms [*Chlamydomonas reinhardtii*, (Domingos et al., 2011)]. Differential accumulation of Cd and Se have been noted in earthworms exposed to CdSe QDs [9-20 nm, (Stewart et al., 2013)]. Fluorescence imaging has shown what appears to be intact CdTe QDs (assumed from the fluorescence signal itself) in the gut lumen of the nematode, *C. elegans*, (Qu et al., 2011) and the gastrointestinal tract of larvae of the leaf roller moth (Al-Salim et al., 2011); although neither study demonstrated clear physiological uptake

698 inside the cells of the internal organs, it does suggest that CdTe QDs can be presented
699 to the gut of terrestrial organisms. Regardless, the contrasting behaviour of the CdTe
700 bulk material to remain intact with negligible dissolution, compared to the nano forms
701 which show some dissolved Cd, may simply arise from size-dependent effect on the
702 surface to volume ratio of the materials, although further experiments are needed to
703 verify this.
704 In contrast to knowledge on the biology of Cd, there are almost no studies on
705 Te accumulation in earthworms. The Te was not extractable from CdTe QDs (Fig. 2)
706 and so not very bioaccessible. Te was not accumulated as strongly as Cd (compare
707 Fig. 3A with 3B in the fresh soil experiment) and there was also no clear dose-
708 dependent increase in the tissues. For the low concentrations of Te found in the
709 earthworms, this may suggest that either earthworms are effectively excreting Te, or
710 the unidirectional uptake mechanism (currently unknown for Te) is much slower than
711 that for Cd. However, once inside the tissue, it has been suggested that dissolved Cd
712 and Te may together form biogenic CdTe QDs (Stürzenbaum et al., 2013). The
713 mineralisation of biogenic particles in earthworms from dissolved metals is well known
714 (Brown, 1982), so the presence of particles in the tissue may not infer uptake of the
715 intact original material.
716
717 *4.2 Nanomaterial coating-mediated effects on metal accumulation*
718 A central concern for the environmental risk assessment of ENMs is whether or
719 not any particle coating-related effects would make the bioaccumulation potential of
720 materials so different, that each form and type of coating would be required to be
721 treated as a new substance with respect to the regulation of chemicals. Similarly, there
722 are concerns that the transformation of particles in the environment would lead to
723 different hazards compared to the original 'pristine' ENM (Gardea-Torresdey et al.,
724 2014; Lowry et al., 2012). First consider the issue of coating effects on
725 bioaccumulation. The water- and acid-extractable fractions of Cd and Te did cluster
726 by the type of coating with respect to the metal accumulation in the earthworms (Fig.
727 2). However, we do not interpret that as a coating-effect *per se*; but simply as a
728 consequence of the effect of the coating on the dissolution of metal from the materials
729 (Fig. S1). This 'coating-mediated effect' on the extractability of Cd and Te in the soil
730 from the CdTe QD exposures showed that more Cd was extractable from NH_4^+-coated
731 QDs compared to the others (Fig. 2). In theory, the chemical bond anchoring the

coating (i.e., the surface ligand) to the core could add a mechanical stress to the metal atoms on the exterior of the core to promote dissolution [ligand-promoted dissolution, (Louie et al., 2016)]. The exact mechanisms involved in this phenomena remain to be elucidated, but for example, the repulsion of ligands of the same charge might stiffen the coating to add mechanical stress to the anchoring point on the core; hence the coatings designed with surface charge tend to have more water- or acid-extractable Cd in the soil (Fig. 2). Or it may relate to lipid solubility, with the PEG coating forming a barrier to slow the dissolution of solutes.

Regardless of the mechanism(s), this coating-mediated effect on the dissolution (Fig. S1), and extractable metal fractions in the soil (Fig. 1), was not observable in the mean values for total metal accumulation of Cd or Te in the earthworms (Fig. 3). For example, the extractable Cd in soil from the CdTe-NH_4^+ QDs was relatively high (Fig. 2), but this did not lead to the highest Cd accumulation in the earthworms (Fig. 3). In short, the running order of any coating-mediated effect on the physico-chemical properties of the QDs was not in the same order as the subsequent total metal accumulation in the earthworms. This was not resolved by normalising the total metal bioaccumulation in the earthworms against the measured total metal concentration in the soil (i.e., the transfer factor: the total metal concentration in the earthworm divided by that in the soil, data not shown). Therefore, while there are some coating-mediated effects on the accumulation of Cd and Te (Fig. 3), they are not explained by free metal ions released from the ENMs, although they are related to the total metal in the soil during the exposure. From the viewpoint of environmental protection, threshold levels for metals in soil have been suggested for dissolved metals on the basis of free ion activity (Lofts et al., 2004), and while ligand models have also been applied to ENMs in soil (Judy and Bertsch, 2014), the assumption that free metal ions lead to toxicity or accumulation is not proven for CdTe QDs here, and needs to be verified for many other ENMs in soil.

4.3 Effect of soil ageing on metal accumulation

Soil guidelines are not available for ENMs, and while the individual parameters in colloid theory such as soil pH and ionic strength have been considered (Cornelis et al., 2014), the sum effect of all the possible transformations of an ENM that constitute 'ageing' of the material in soil are not yet understood. In the present study, only the bulk material showed a soil ageing-dependent effect on total metal accumulation in

766 the earthworms; with much more metal accumulated in the aged soil experiment
767 (compare Fig 3A and 3B for Cd). This occurred, despite the total concentration of Cd
768 and Te in the soil being slightly lower in the aged compared to fresh soil experiments
769 (Fig. 1); suggesting greater bioavailability and hazard of the CdTe bulk material over
770 time in soil. It was not possible to determine the water- and acid-extractable metals in
771 the aged soil at the start of the aged experiment, as this would have required
772 destructive sampling and disturbance of the soil boxes. However, the slightly lower
773 metal concentrations in the aged soil was somewhat expected, and likely due to
774 previous metal uptake by the earthworms in the fresh soil study, and metal uptake by
775 the plants that had subsequently grown in the exposure containers during soil ageing.
776 Thus the incidental plant growth in the soil (Figs. S2 and S3), may have partly
777 remediated the total metal in the soils. Notably, the ratio of Cd:Te in the tissue of
778 earthworms exposed to the bulk material was different in the aged soil experiment
779 compared to that in fresh soil; suggesting that the bulk material had at least partly
780 dissolved, with then separate uptake mechanisms for Cd and Te by the earthworms
781 (as discussed above). The bulk material had a maximum dissolution rate of < 0.1 μg
782 h^{-1} for Cd (Table 1), and even at the highest soil dose with around 800 mg Cd kg^{-1} of
783 soil (Fig.1), in a 250 g box of the soil (~ 200 mg Cd in total), this would equate to a
784 dissolution of 16.8 μg of Cd per week, or about 403 μg in six months. So, the material
785 form would still remain in excess after six months of ageing, and ignoring
786 transformation processes such as suphidation that might stabilise the materials (Lead
787 et al., 2018). The mechanism causing this soil ageing-dependent change in metal
788 accumulation by earthworms requires further investigation.
789 In contrast to the bulk material, for all the CdTe QDs exposures in aged soil,
790 the accumulation of Cd was similar to that in the fresh soil study, although slightly
791 higher (Fig. 3C). There was also little difference in Te accumulation from the aged
792 compared to fresh soil study (compare Fig 3B and 3D) for the CdTe QDs. It is not clear
793 why soil ageing failed to accelerate the metal accumulation in the earthworms, as it
794 did for the bulk material. The CdTe QDs are smaller and with a faster dissolution rate
795 than the bulk material (Fig. S1, Table 1), and so more uptake of free metal ions might
796 be expected. But this was not the case, suggesting some other particle metric was
797 controlling the bioavailability of the CdTe QDs. Apart from size, the other key
798 difference was that CdTe QDs were all manufactured with a surface coating, when the
799 bulk material was not. So a lack of effect of soil ageing on metal accumulation may be

due to the presence of the coatings. It is possible that organic ligands in the soil, such as the polyanionic charges of humic and fulvic acids, could electrostatically attract the positive coating of the CdTe-NH_4^+ QDs, repulsion in the case of CdTe-COOH QDs, or show steric entanglement with the CdTe-PEG QDs. Similar ideas have been proposed for the behaviour of QDs in natural water (Rocha et al., 2017). However, such phenomena would demand a clear and consistent coating-effect, but this was not observed with different coating-mediated effects on bioaccumulation in the fresh compared to aged soil experiments, and with exposure dose within each experiment (Fig. 3). Regardless of the mechanisms, the bulk material presents a bioaccumulation concern with soil ageing that is greater than the nano forms.

4.4 Effects on survival and body weight

In the fresh soil experiment, the control earthworms survived, gained weight, and did not show any abnormalities, indicating that the animals were healthy. Furthermore, there were no effects on survival in any of CdTe treatments; and in the CdTe-bulk treatment the earthworms also gained weight (Table 2). However, despite good survival, the animals in the CdTe QD treatments lost biomass (Table 2). There was also a negative correlation between the metal concentrations in the tissue and biomass (Fig. S5). The loss of biomass was partly attributed to a lack of feeding coupled with some shedding of the posterior segments. There were no consistent effects of percentage change in body mass by the type of coating on the CdTe QDs in the fresh soil experiment; and by day 28 all the earthworms in the 2000 mg kg^{-1} CdTe QD treatments had lost around 50% of their body mass (Table 2). The latter suggests the earthworms at the higher doses were just below the threshold for mortality. Indeed, the shedding of posterior segments has been interpreted as a survival strategy (Sims and Gerard, 1985) and possibly an ‘excretion’ mechanism for metals in earthworms. The survival of the earthworms at the nominal 50 and 500 mg kg^{-1} CdTe QD concentrations in the fresh soil experiment was expected with respect to Cd toxicity, as earthworms are known to tolerate very high concentrations of dissolved Cd [28 day mortality LC_{50} = 588 mg Cd kg^{-1} dw, (Van Gestel et al., 1991)], which are partly chelated by metallothionein in the tissue (Stürzenbaum et al., 2001). Furthermore, the extractable Cd from the fresh soil experiment in most of the QD exposures was far less than the expected lethal concentration of dissolved Cd, except in the nominal 2000 mg CdTe kg^{-1} dw CdTe-NH_4^+ QD exposures. In earthworms, a critical body

residue (CBR) of around 642 mg Cd kg^{-1} dw is suggested to cause 50 % mortality (Conder and Lanno, 2000). This concentration of Cd was generally exceeded in earthworms exposed to the highest concentrations of CdTe QDs in the fresh (and aged) soil experiments (Fig. 3). Again, supporting the notion that the worms were close to the threshold for mortality at the highest exposure concentration. The absence of effects of the CdTe bulk material on growth and survival (Table 2), and the relatively modest Cd accumulation in those earthworms (Fig. 3), suggests the bulk material is much less hazardous than the nano forms in fresh soil.

In the aged soil experiment, like in the fresh soil study, the control earthworms survived and were healthy without a statistically significant loss of body mass. Similar to the fresh soil experiment, survival was also not affected by the CdTe-bulk exposures. However, unlike the situation in fresh soil, the nominal 2000 mg CdTe kg^{-1} dw test concentration of the CdTe bulk material caused a reduction in biomass (Table 2). Notably, this was accompanied by a much higher total Cd concentration in those earthworms in the aged soil compared to fresh soil (Fig. 3). Together, this suggests that the bulk material becomes more hazardous with soil ageing because the Cd in the material becomes partly dissolved (discussed above) and therefore bioavailable for uptake.

In contrast to the fresh soil experiment, exposure to CdTe QDs in aged soil caused considerable mortality, even at the nominal concentration of 50 mg kg^{-1} dw of CdTe QDs (Table 2). The CdTe QDs were, therefore, much more toxic after ageing of the soil. In addition, a coating-mediated effect on survival was also apparent in the aged soil experiment, with the toxicity ranking CdTe-PEG < CdTe-NH_4^+ < CdTe-COOH QDs at the highest exposure concentration (Table 2). In a study using the same coatings on CuO ENMs at a concentration of 1000 mg Cu kg^{-1}, the NH_4^+-coated CuO ENMs were also acutely toxic following a period of ageing, however, COOH-coated CuO ENMs were not (Tatsi et al., 2018). This implies that, while coating-mediated effects can occur, the precise ranking of the effect also depends on the type of metal core in the ENM. This might be expected, because the stability constants of metals with organic ligands vary according to the charge density of the metal and the type of ligand (Albert, 1950). However, stability constants are not yet available for metals in the solid or crystalline state as found in nanomaterials with ligands of different chain lengths or terminal residues.

867 The increased toxicity of the CdTe QDs in aged soil was also reflected in the
868 changes of body mass, with generally greater loss of body mass than that observed
869 in the fresh soil experiment (Table 2). A coating-mediated effect on growth was also
870 apparent, with the most prominent effects in the NH_4^+- and COOH-CdTe QD
871 exposures (Table 2). The same ammonium coating also significantly reduced
872 earthworm biomass in exposures to 1000 mg Cu kg^{-1} dw as CuO-NH_4^+ (Tatsi et al.,
873 2018). Changes in the appearance and feeding behaviour of the worms were also
874 evident at lower concentrations in the aged soil experiment, and consistent with the
875 notion that the aged soil was more hazardous to the health of the earthworms.
876
877 *4.5 Effects on reproduction*
878 Reproduction is regarded as the most sensitive endpoint for metal exposure of
879 earthworms. The control earthworms produced the expected number of juveniles in
880 both, fresh and aged soil experiments, meeting the validity criteria of the OECD TG
881 222 (OECD, 2004). In the fresh soil experiment, the number of juveniles produced was
882 not affected in the CdTe-bulk exposures, while in the CdTe QD exposures the number
883 of juveniles decreased as the exposure concentration elevated (Table 2). A coating-
884 mediated effect was evident, with the EC_{50} values in the order: CdTe-COOH < CdTe-
885 NH_4^+ < CdTe-PEG (Table 2); suggesting the PEG-coated material was the least
886 hazardous with respect to juvenile production. If the measured concentration of total
887 Cd in the exposure soils is considered in the fresh soil experiment (ranging from ~22
888 to 780 mg Cd kg^{-1} dw, Fig. 1), the EC_{50} values of between 65-108 (Table 3) are broadly
889 comparable to the 56 day EC_{50} for cocoon production of 46.3 mg Cd kg^{-1} dw in
890 earthworms exposed to 0-300 mg Cd kg^{-1} soil (Spurgeon et al., 1994). This suggests
891 the reproductive toxicity of the CdTe QDs in the fresh soil study could mostly be
892 explained by Cd exposure, and this was corroborated by a strong negative correlation
893 of juvenile production with the tissue Cd concentration, rather than tissue Te
894 concentration, in the adult worms (Fig. S5). The decrease in reproductive success is
895 also consistent with bioenergetic theory, where the adult earthworms showing the least
896 growth (biomass) are expected to invest less energy in reproduction. Lower adult
897 biomass has been shown to be related to lower reproduction in regulatory tests
898 (OECD, 2004). Crucially, the juvenile earthworms also showed a decrease in biomass,
899 and this followed the same overall pattern as the effects on reproduction in the adults
900 (Table 3), suggesting the exposures also impaired off-spring quality. There are few

reports of juvenile production from adult earthworms exposed to ENMs, but like the present study, TiO_2 ENMs also decrease juvenile production in earthworms (Schlich et al., 2012).

In the aged soil experiment, the control earthworms produced more juveniles than in the fresh soil study (Table 3). This is likely due to the higher organic matter (i.e., plant roots) in the aged soil that is known to encourage earthworms to produce more offspring (Lahive et al., 2017). Unlike the fresh soil experiment, in aged soil, the CdTe bulk material inhibited juvenile production to the extent that no juveniles were produced at the highest exposure concentration (Table 3) and the Cd concentration in the adult earthworms strongly correlated with this reproductive failure (Fig. S5); again suggesting that mobilisation of Cd from the bulk material during soil ageing caused the toxicity. Furthermore, the reproductive toxicity of the CdTe QDs also increased in the aged soil experiment, but the difference between the coated QDs was less pronounced, although CdTe-NH_4^+ QDs remained most hazardous (Table 3). The further reduction in reproduction in the aged soil experiment may be explained by the higher accumulation of Cd and Te in the earthworms (Fig. 3), which was also associated with lower biomass in the adult worms. So bioenergetically, like the fresh soil experiment, poor growth or health in the adults likely gave rise to fewer offspring.

4.6 Effects on ion regulation

Cadmium is known to cause ion regulatory disturbances in organisms (Stürzenbaum et al., 2004), therefore, the sodium pump activity was assessed. In the fresh soil study, earthworms in the controls sustained sodium pump activity (Fig. 4), as well as an overall concentration of Na^+ and K^+ (Table S2), as expected in the earthworms (Tatsi et al., 2018). The sodium pump activity was inhibited the most in the CdTe-bulk treatment and at the highest exposure concentration (Fig. 4). However, exposure to CdTe QDs in fresh soil also inhibited the sodium pump. Of the ENMs, the CdTe-PEG and -NH_4^+ QDs were most potent in inhibiting the sodium pump activity (Fig. 4). In the nominal 500 and 2000 mg CdTe kg^{-1} dw exposures to CdTe-NH_4^+, tissue K^+ concentrations also decreased (Table S2), being consistent with inhibition of the sodium pump. There appears to be no other reports of the effects of CdTe QDs on osmoregulation in earthworms, and whether the sodium pump inhibition is caused by Cd alone, Te alone, or both, requires further investigation.

Several essential metals for earthworm health, were also measured (Table S2). Of these, there were some transient changes in Zn and Fe in the fresh soil experiment, but no clear dose effect, and no effects in the aged soil experiment. However, copper and manganese showed some interesting changes. The control earthworms showed the expected tissue Cu [8 – 10 mg Cu kg^{-1} dw, (Streit, 1984; Tatsi et al., 2018)] and Mn concentrations [43-54 mg Mn kg^{-1} dw this study; ~ 50 mg Mn kg^{-1} dw in *L. rubellus*, (Oste et al., 2001)]. The manganese concentration was reduced by almost 3 times in the CdTe QD ENM exposures, for both the fresh and aged soil experiments, and also for the bulk material exposures in the aged soil experiment (Table S2). The mechanistic cause of this Mn depletion in earthworms requires further investigation, but Cd-dependent inhibition of Mn uptake has been observed in fibroblast cell lines (Yanagiya et al., 2000), possibly mediated by competition for uptake on divalent metal ion transporter 1 (DMT1). Copper depletion was correlated with higher tissue Cd (Fig. S5) in the CdTe-COOH exposures (500 and 2000 mg CdTe kg^{-1} dw) and CdTe-NH_4^+ exposure (500 mg CdTe kg^{-1} dw), and with the effects evident in both fresh and aged soil experiments (Table S2). The loss of Cu (and Mn) might arise from a passive non-specific electrolyte leak associated with tissue injury since other essential elements such as K, Ca and Mg concentrations were significantly reduced compared to the controls in the fresh soil (Table S2). However, this seems unlikely in the absence of glutathione depletion (Fig. 4, no clear difference in total GHS pool were observed) and the major electrolytes were undisturbed in the tissues in the aged soil experiment, despite some tissue Cu depletion. Cu is a regulated metal in earthworms (Streit, 1984), and it could simply be that Cd^{2+} is displacing Cu from ligands such as metallothionein and glutathione in the tissue. Regardless of the mechanism, both Cu and Mn were decreased in the tissue of earthworms from both the fresh and aged soil studies, and with the same coated QDs, COOH and NH_4^+, being most potent for this effect (Table S2).

962 *4.7 Plant growth during soil ageing*

963 It was not the purpose of this study to assess the effects of CdTe materials on
964 plant growth, but some incidental observation on plant coverage were made (Figs. S2
965 and S3). Similar to the earthworms, the QDs were more toxic to the plants and moss
966 than the bulk material and there was a coating-mediated effect on plant coverage; with
967 the CdTe-COOH and CdTe-NH_4^+ QDs abolishing growth at the highest
968 concentrations. In hydroponic media, plant cuttings from poplar trees exposed to
969 Cd/Se-containing QDs with cationic or anionic surface coatings, showed apparently
970 faster uptake of the cationic material; possible because it is electrostatically attracted
971 to the negatively charged surface of the plant (Wang et al., 2014). The effects of
972 organic coatings on CdS QDs have also been recently explored on soya bean seedling
973 (Majumdar et al., 2019); with the amino acid profiles in the plants clustering by
974 material, although the coating-effect was not consistent across a range of endpoints.
975 Currently, there are too many data gaps to offer a clear mechanistic understanding of
976 ENM effects on plants (Schwab et al., 2016), and any apparent coating-mediated
977 effects on vascular plants requires further investigation.

978 The earthworms were inevitably feeding on the plant root material in the aged
979 soil experiment, and so this might be both a source of nutrition and metal exposure
980 from any nanomaterial on the surface of the roots. However, the plant growth or the
981 presence of ENMs in soil might be expected to alter the soil properties. Indeed, QDs
982 may change the porosity and the transport of solutes in soil with subsequent effects
983 on the plants and invertebrates (Al-Salim et al., 2011). In the present study, the
984 moisture content and pH was adjusted in the soils, but changes in the soil structure
985 with soil ageing cannot be excluded as a factor in the metal accumulation of the
986 earthworms in the aged soil experiment.

987

988 *4.8 Conclusions and regulatory perspective*

989 Taken together, the results here compared to the known toxicity of Cd in
990 earthworms indicate the CdTe QD ENMs are of similar acute and reproductive toxicity
991 to earthworms. When comparing the bulk vs nano-scale CdTe QDs, there may be a
992 size-effect, since the nano-scale CdTe QDs were generally more toxic than the CdTe-
993 bulk counterpart. There was also a coating-mediated effect, where the most toxic QD
994 was generally the CdTe-COOH material, closely followed by CdTe-NH_4^+ QDs.
995 However, this was not a direct coating effect ***per se***, but likely a consequence of other

subtle changes in the physico-chemical properties of the material during manufacture, such as the differences in the ratio of the apparent metal concentrations in each material. In keeping with this notion, the toxic effects seen in the study were related to accumulation of Cd in earthworm tissues, however, this was not easily explained by the measured dissolution rate of Cd from the QDs (Table 1) or the extractability of Cd from the soils (Fig. 2). The existing environmental risk assessment for dissolved Cd is likely to be protective of both bulk and nano forms of CdTe materials, although the type of coating and any subsequent changes in particle properties associated with adding the coating, should be considered in the environmental risk analysis. Crucially, the toxicity of the CdTe QDs increased after a period of ageing of the soil, suggesting that the hazard should be monitored, and any environmental risk assessment revisited in order to maintain protection of the soil ecosystem. Finally, it would be important to ascertain the mechanisms of toxicity for Te, since it is widely used in a QD form and currently there are no environmental risk assessments which give guidance on safe levels of Te in soils.

Conflicts of interest

The authors alone are responsible for the content and writing of the paper. The authors report no conflicts of interest.

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Tables and figures

Table 1 Characterisation of the CdTe QDs used in the experiments.

QD variant	[1]Manufacturer's Information	[2]Estimated primary particle size, diameter (nm)	[3]NTA, hydrodynamic diameter (nm)	[4]TGA, (% weight loss)	[5]Maximum rate of dissolution in Milli Q water (μg h^{-1})	
					Cd	Te
CdTe-Bulk	CASRN 1306-25-8 (Sigma-Aldrich 256544), Lot No. MKBK6448V, ≥99.99% purity trace metal basis	< 250 μm	172 ± 28	NA	<0.1	0.2
CdTe-Polyethylene Glycol	Lot No.YF140402, 99% purity, size 3-5 nm.	< 4	156 ± 72	50.4 ± 8.3	6.4	2.3
CdTe- Carboxylate	Lot No.YF140402, 99% purity, size 3-5 nm.	< 4	84 ± 58	23.4 ± 4.2	3.9	1.3
CdTe- Ammonium	Lot No. YF140402, 99% purity, size 3-5 nm.	< 4	75 ± 50	8.8 ± 0.5	29.0	14.9

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

[2] It was not possible to detect the QDs using electron microscopy, therefore an estimate is given (Denmark Technical University)

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

[4]TGA – thermogravimetric analysis. Triplicate measurements (technical replicates) made on the same batch of the dry powders using a TGA 4000 (Perkin Elmer) under an N_2 flow of 20 ml min^{-1} from 25°C to 995°C at a heating rate of 10°C min^{-1} at the University of Manchester. Note, this measurement would include the pyrolysis of the organic coating and any other combustible (presumably organic) impurities in/on the materials.

[5]Maximum slope from rectangular hyperbola function of curve fitting used to estimate the maximum rate of dissolution of Cd and Te from dialysis experiments conducted at University of Plymouth, conducted exactly according to (Besinis et al., 2014).

NA – not applicable to the test item.

Table 2 Survival and biomass of earthworms following 14 and 28 days exposure to CdTe QDs in fresh or 28 days in aged soils.

Soil [CdTe], nominal mg CdTe kg^{-1} dw		Control		CdTe-bulk		CdTe-PEG		CdTe-COOH		CdTe- NH_4^+	
		Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass
Fresh soil, day 14											
50	Total	5	2.1 ± 0.1[a]	5	2 ± 0.1[a]	5	2.3 ± 0.1[a]	5	1.8 ± 0.1[a]	5	2.1 ± 0.1[a]
	%	100	↑15.9 ± 6.4	100	↑3.0 ± 3.6	100	↑16.9 ± 3	100	↑22.7 ± 10.4	100	↑25.7 ± 0.9
500	Total			5	2.03 ±0.1[a]	5	1.9 ± 0.06[a]	4.8 ± 0.2	2 ± 0.1[a]	5	1.9 ± 0.1[a]
	%			100	↑12.9 ± 2.6	100	↑10 ± 3.9	95 ± 5	↑11.9 ± 2.4	100	↓4.2 ± 1.5
2000	Total			5	1.9 ± 0.06[a]	5	1.3 ± 0.1[b]	5	1.4 ± 0.1[b]	4.8 ± 0.2	1.23 ± 0.1[b]
	%			100	↑4.2 ± 1.8	100	↓24.9 ± 3.9	100	↓28 ± 2.4*	95 ± 5	↓36.6 ± 4.3
Fresh soil, day 28											
50	Total	5	2.2 ± 0.1[a]	4.8 ± 0.2	1.9 ± 0.1[a]	5	2.4 ± 0.1[a]	5	2.1 ± 0.1[a]	5	2.2 ± 0.1[a]
	%	100	↑19.9 ± 5.1	95 ± 5	↓0.4 ± 4.6	100	↑21.7 ± 6.1	100	↑28 ± 11.9	100	↑31.6 ± 3.1
500	Total			5	2.2 ± 0.1[a]	4.8 ± 0.2	1.9 ± 0.1[b #]	5	1.9 ± 0.1[b #]	5	1.66 ±0.1[b]
	%			100	↑22.7 ± 3.7	95 ± 5	↑8.1 ± 3.3	100	↑8.7 ± 4.2	100	↓15 ± 1.9
2000	Total			4.8 ± 0.2	2.14 ± 0.1[a #]	5	0.91 ± 0.1[c #]	5	1.05 ± 0.1[c #]	4.8 + 0.2	0.95 ± 0.1[c #]
	%			95 ± 5	↑10.9 ± 3.6	100	↓49.4 ± 3.8	100	↓46.6 ± 2.9	95 ± 5	↓50.9 ± 4.4
Aged soil, day 28											
50	Total	5 ± 0.3	1.7 ± 0.1[a *]	5	1.8 ± 0.1[a]	4 ± 0.5	1.4 ± 0.1[a *]	5	1.7 ± 0.1[a*]	5	1.8 ± 0.1[a *]
	%	95 ± 5	↓8.1 ± 5.6	100	↓4.1 ± 7.7	85 ± 10	↓22.4 ± 8	100	↓17.2 ± 5.1	100	↓9.4 ± 3.6
500	Total			5	1.7 ± 0.1[a]	4.8 ± 0.2	1.8 ± 0.2[a *]	5 ± 0.3	1.3 ± 0.1[b *]	4 ± 0.4	1.2 ± 0.1[b *]
	%			100	↓2.9 ± 2.7	95 ± 5	↓17 ± 9.2	90 ± 5	↓28.2 ± 4.9	80 ± 8	↓33.7 ± 3.7
2000	Total			4.8 ± 0.2	1.3 ± 0.1[b]	4 ± 0.5 *	0.7 ± 0.1[c *]	2.8 ± 0.2 *	0.6 ± 0.1[c *]	3 ± 1.1	0.8 ± 0.2[c]
	%			95 ± 5	↓31.4 ± 8.6	75 ± 10	↓59.6 ± 7.4	55 ± 5	↓64.7 ± 5.8	60 ± 25	↓56.8 ± 7.2

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, g) 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 an experiment at the specified time-

point and the hash ($^{\#}$) denotes statistically significant differences between treatments on day 14 and 28 ($P < 0.05$ repeated measures ANOVA for biomass data or Kruskal-Wallis for survival data). Asterisk (*) denotes statistically significant differences between treatments in the fresh and aged soil experiments.

Day 0 mean wet weight was 1.86 ± 0.02 g (mean ± SEM, for a subsample of 5 of the initial earthworms, $n = 52$ treatments) in fresh soil experiment.

Day 0 mean wet weight of 1.89 ± 0.03 g of per exposure replicate (mean ± SEM, for a subsample of 5 of the initial earthworms, $n = 52$ treatments) in the aged soil experiment.

↑ Increase in wet weight relative to day 0

↓Decrease in wet weight relative to day 0

Table 3 Total number of juveniles produced and their total fresh weight in the fresh soil experiment and total number of juveniles produced in the aged soil experiment. The EC_{50} and estimated NOEC values in each treatment in the fresh and aged soil experiment.

Soil [CdTe] nominal	Control		CdTe-bulk		CdTe-PEG		CdTe-COOH		CdTe-NH_4^+	
mg CdTe kg^{-1} dw	No of juveniles	Biomass (g)	No of juveniles	Biomass (g)	No of juveniles	Biomass (g)	No of juveniles	Biomass (g)	No of juveniles	Biomass (g)
Fresh soil										
50	25 ± 5[a]	0.370 ± 0.06[a]	22 ± 6[a]	0.222 ± 0.09[a]	18 ± 1[a]	0.0267 ± 0.057[a]	18.25 ± 4[a]	0.220 ± 0.08[a]	20 ± 3[a]	0.307 ± 0.084[a]
500			18 ± 2[a]	0.250 ± 0.061[a]	3 ± 2[b]	0.033 ± 0.026[b]	3 ± 1[b]	0.005 ± 0.003[b]	1 ± 0[b]	0.006[b]
2000			19 ± 3[a]	0.142 ± 0.024[b]	0[b]	ND	0[b]	ND	0[b]	ND
EC_{50}			NA		108 (72-165)		65 (30-142)		96 (20-610)	
NOEC			>2000		50		50		50	
Aged soil										
50	41 ± 5[a] *		30 ± 3[a]		30 ± 2[a] *		32 ± 2[a] *		35 ± 4[a] *	
500			10 ± 1[b] *		2 ± 1[c]		0[c] *		0[c]	
2000			0[c] *		0[c]		0[c]		0[c]	
EC_{50}			165 (109-243)		88 (62-127)		78 (NA)		63 (NA)	
NOEC			50		50		50		50	

Data presented as mean ± SEM (n = 4). Different letters denote statistically significant differences between treatments within an experiment (P < 0.05, ANOVA). Asterisk (*) denote statistically significant differences between the treatment between fresh and aged soil experiment (P < 0.05 T-test, unpaired). The biomass is reported as the total biomass all the juveniles produced treatment

(wet weight, g). EC_{50} values for 50 % reduction in reproduction after 4 weeks of exposure in fresh and aged soils with CdTe QDs are presented with 95 % confidence intervals in the brackets, the concentration response curves are presented in Fig. S4. NA – not possible to calculate EC_{50} due to no effect; not possible to calculate confidence intervals due to high effect. Note, because of the difficulty in removing juveniles from amongst the plant roots that had grown in the soil during ageing, we were not confident in removing all of the small juveniles intact in order to report an accurate total biomass for juveniles in the aged experiment.

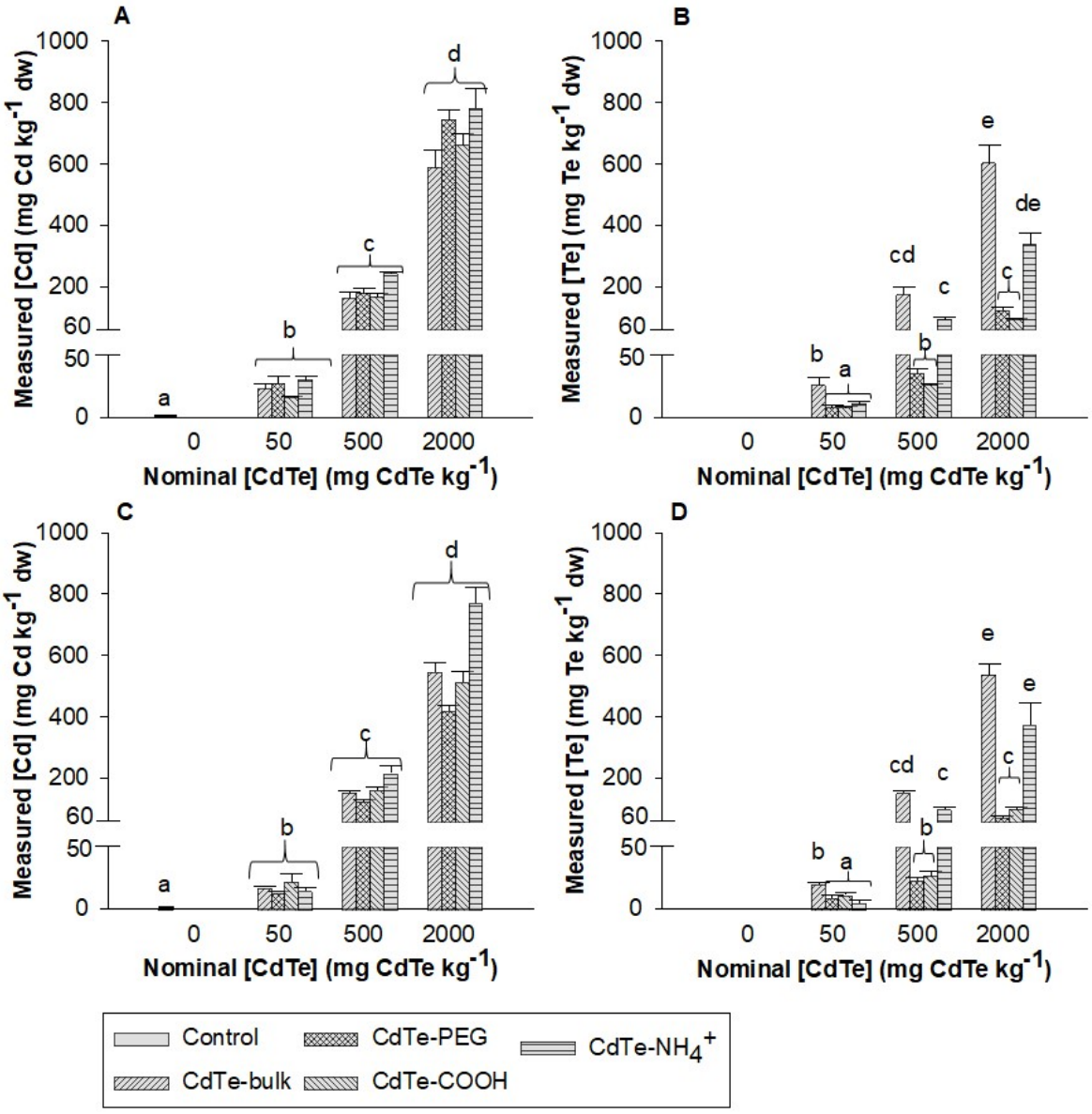


Fig. 1 Total measured Cd (A, C) and Te (B, D) concentration in soil (mg kg^{-1} dw) at the beginning of the fresh (upper panels) and aged (lower panels) soil experiment. Data expressed as mean ± SEM (n = 8). Different letters show statistically significant differences between treatments within test concentration ($P < 0.05$, ANOVA). There were no statistically significant differences between treatments within a dose in the fresh compared to aged exposures ($P > 0.05$, t-test, unpaired).

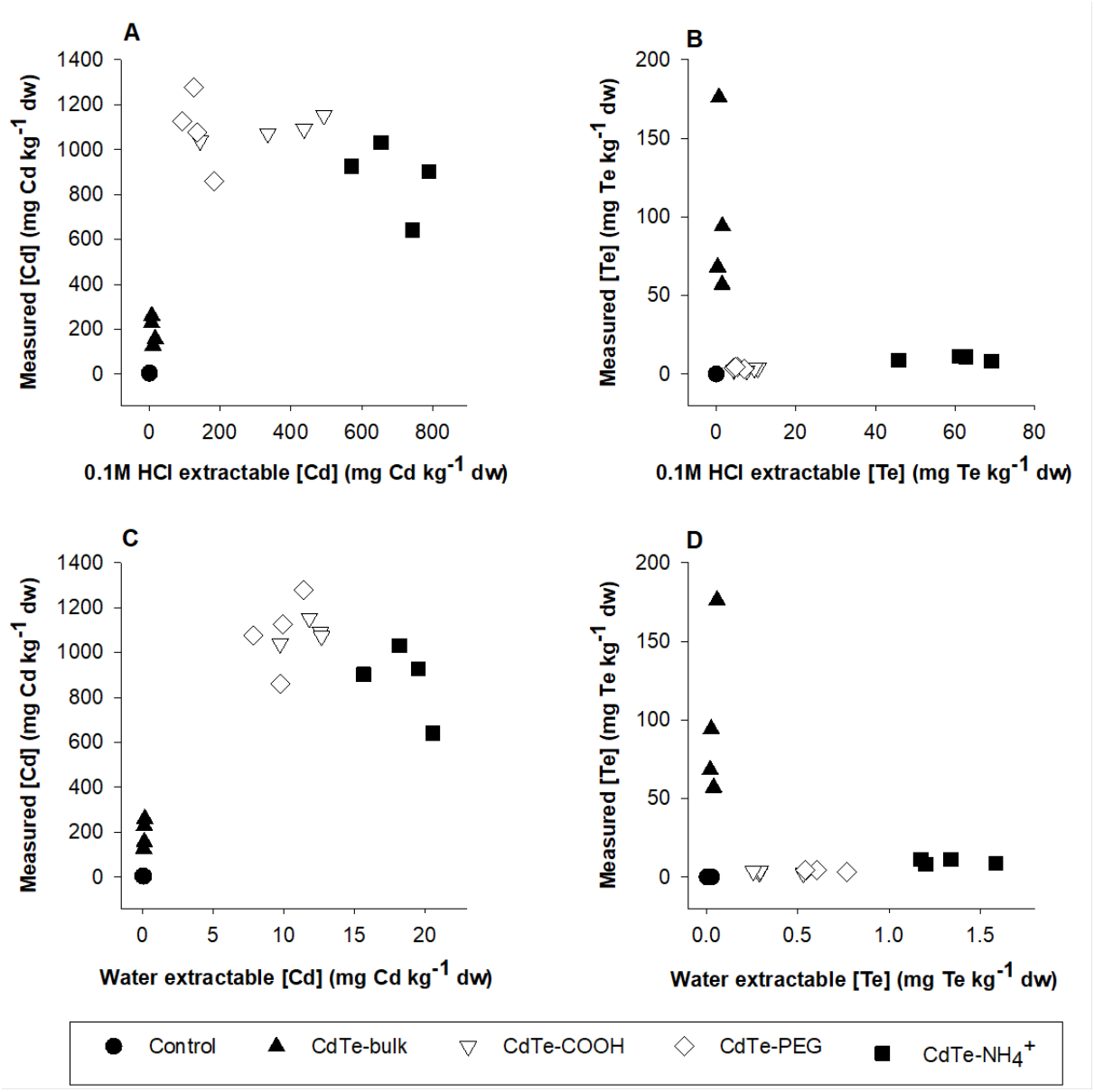
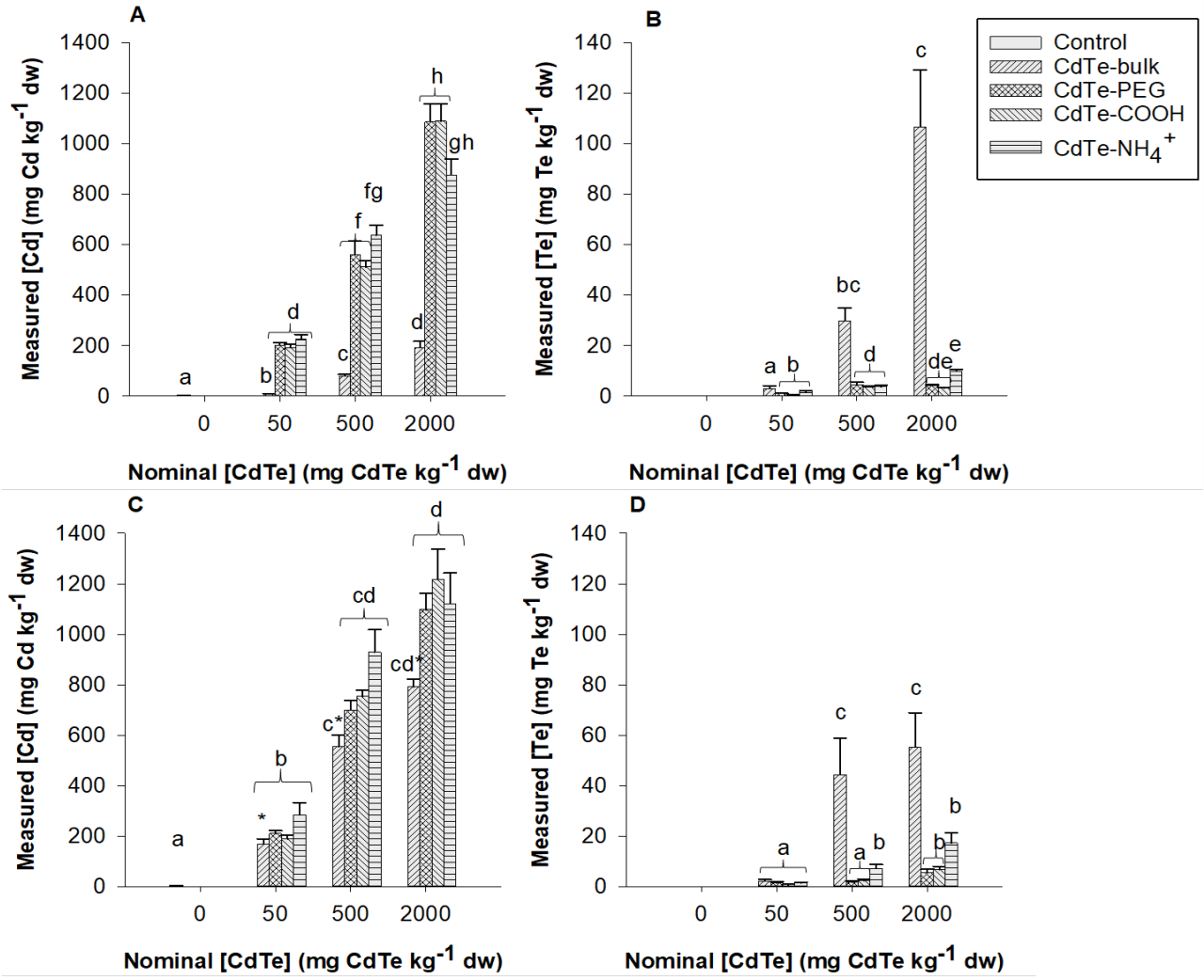


Fig. 2 Relationship between the concentration of acid extractable Cd or Te (A or B) and water extractable Cd or Te (C or D) and measured total Cd or Te concentration in earthworm tissue in the fresh soil experiment. Data shown for nominal 2000 mg CdTe kg^{-1} treatment for each replicate in the fresh soil only (n = 4). Data points represent mean values (n = 2 for soil samples, n = 4 for earthworm) for each replicate (n = 4), error bars excluded for clarity.



1235

1236 **Fig. 3** Total measured Cd (A, C) and Te (B, D) concentration in earthworm tissue
1237 (mg kg^{-1} dw) on day 28 of the fresh (upper panels) and aged (lower panels) soil
1238 experiments. Different letters denote the statistically significant differences between
1239 treatments within test concentration ($P < 0.05$, ANOVA). Asterisks denote statistically
1240 significant differences between treatments within a dose in the fresh compared to
1241 aged exposures ($P < 0.05$, *t*-test, unpaired).

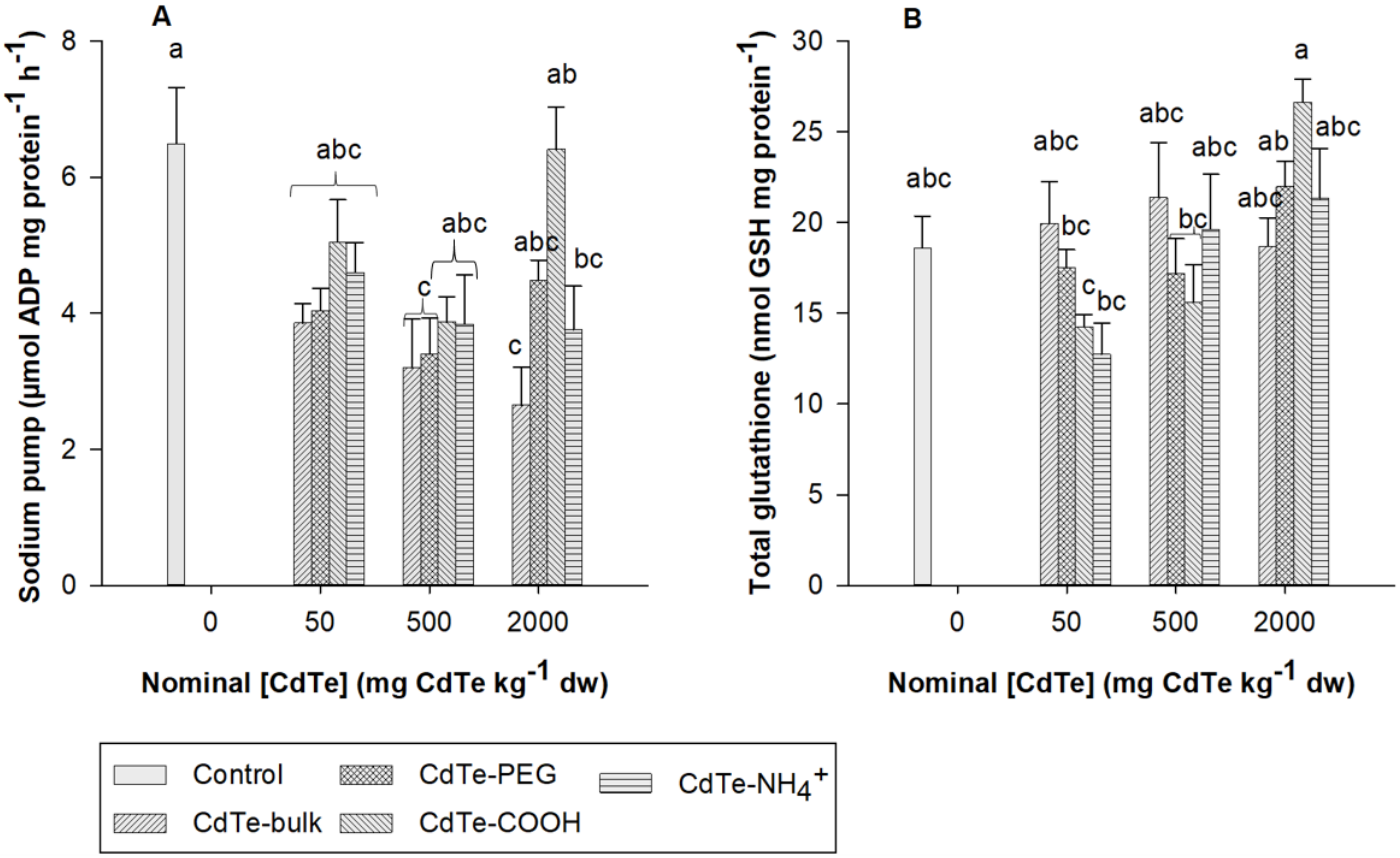


Fig. 4 (A) Na^+/K^+-ATPase activity (sodium pump) expressed as ADP µmol released per mg protein per hour in earthworms in the fresh soil experiment. (B) Total glutathione nmol per mg protein in the earthworms in the fresh soil experiment. Data is presented as mean ± SEM (*n* = 8). Different letters denote statistically significant differences between treatments ($P < 0.05$, ANOVA).