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

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25 **Abstract**

26 The bioaccumulation potential and toxic effects of engineered nanomaterials (ENMs)
27 on earthworms are poorly understood and there are concerns that the hazards will
28 change with the type of coating and soil ageing. Two studies were conducted following
29 OECD TG 222 with minor modifications and additional endpoints to assess the effects
30 of CdTe QDs with different coatings on earthworms (*Eisenia fetida*); and to determine
31 whether bioaccumulation or toxicity changed after soil ageing. Earthworms were
32 exposed to carboxylate (COOH), ammonium (NH₄⁺), or polyethylene glycol (PEG)
33 coated CdTe QDs, or a micron scale (bulk) CdTe material, at nominal concentrations
34 of 50, 500 and 2000 mg CdTe QD kg⁻¹ dry weight (dw) for 28 days in Lufa 2.2 soil. The
35 endpoints included survival, body weight, reproduction, metal accumulation, and
36 markers of oxidative or ionoregulatory stress. In the fresh soil study, earthworms
37 accumulated similar amounts of Cd and Te in the CdTe-bulk exposures, while the
38 accumulation of Cd was higher than Te during the exposures to CdTe QDs. However,
39 neither the total Cd nor Te concentrations in the earthworms were easily explained by
40 the extractable metal fractions in the soil or particle dissolution, indicating other particle
41 metrics altered bioavailability. There were no effects on survival, but some retardation
42 of growth was observed at the higher doses. Inhibition of Na⁺K⁺-ATPase activity with
43 disturbances to tissue electrolytes, as well as tissue Cu and Mn were observed, but
44 without depletion of total glutathione in the fresh soil experiment. Additionally, juvenile
45 production was the most sensitive endpoint, with estimated nominal EC₅₀ of >2000,
46 108, 65, 96 mg CdTe kg⁻¹ for bulk, PEG-, COOH- and NH₄⁺-coated CdTe QDs,
47 respectively. In the aged soil study, the accumulation of Cd and Te was higher than in
48 the fresh soil study in all CdTe QD exposures. Survival of the adult worms was reduced
49 in the top CdTe-COOH and -NH₄⁺ QD exposures by 55 ± 5 and 60 ± 25 %, respectively;
50 and with decreases in growth. The nominal EC₅₀ values for juvenile
51 production in the aged soil were 165, 88, 78 and 63 mg CdTe kg⁻¹ for bulk, PEG-,
52 COOH- and NH₄⁺-coated CdTe QDs, respectively. In conclusion, exposure to
53 nanoscale CdTe QDs, regardless of coating, caused more severe toxic effects than the
54 CdTe bulk material and the toxicity increased after soil ageing. There was a coating-
55 mediated effect in some cases that was not explained by the coating itself, but due to
56 subsequent differences in the metal content and behaviour of the materials.

57

58 1. Introduction

59 Quantum dot (QD) technology is a growing market within the nanotechnology
60 industry. The applications include bio-imaging and medical diagnostics (Kairdolf et al.,
61 2013), light-emitting diode (LED) technologies (Hardman, 2005), and photovoltaics
62 that offer alternative energy solutions (Sinha et al., 2012). Inevitably, the manufacture
63 of QDs and their use in industrial processing or products will likely cause some
64 releases to the environment, as with other engineered nanomaterials [ENMS, (Lead
65 et al., 2018)]. The disposal of QDs in electronics and the subsequent burning of e-
66 waste by developing countries is a particular concern for metal releases from CdTe
67 QDs (Luo et al., 2011). Once in the environment, CdTe QDs may also slowly degrade
68 and release their hazardous dissolved metals (Navarro et al., 2008).

69 Cadmium is a non-essential metal and known to bioaccumulate in earthworms
70 (Hopkin, 1989). The background concentrations of Cd in soil are usually around 1 mg
71 kg^{-1} dry weight (dw) or less (Keshavarz Jamshidian et al., 2017), but contaminated
72 soils can have tens of mg of Cd (Spurgeon et al., 1994). Dissolved cadmium toxicity
73 is well established in earthworms; with a 28-day lethal concentration (LC_{50}) of 588 mg
74 Cd kg^{-1} dw in artificial soil (Van Gestel et al., 1991), and 56-day EC_{50} for impairment
75 of reproduction of 46.3 mg Cd kg^{-1} dw in artificial soil (Spurgeon et al., 1994). In
76 contrast to Cd, the ecotoxicity of tellurium is poorly understood. It has no known
77 biological functions in eukaryotic cells (Ba et al., 2010) and not much is known about
78 its toxicity to soil organisms. So far, the only known biological function of tellurium has
79 been found in fungi, that can use tellurite instead of sulphur for production of amino
80 acids when sulphur is limited or absent (Ramadan et al., 1989). Due to its
81 biogeochemistry, Te is a difficult metalloid to measure in complex environmental
82 matrices; nonetheless a few measurements of Te in soil and sediments are emerging,
83 suggesting that it is naturally present at low $\mu\text{g kg}^{-1}$ concentrations (Belzile and Chen,
84 2015).

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

91 biosynthesise CdTe QDs from dissolved metals (Stürzenbaum et al., 2013). There are
92 some studies on neurotoxicity (Wu et al., 2015) and oxidative stress (Srivastava et al.,
93 2016) in the nematode worm, *Caenorhabditis elegans*, with CdTe QDs; and at least
94 one study on marine polychaete worms with CdS (Buffet et al., 2014). However, there
95 is sparse information on the toxicity of additions of CdTe QDs to the soil for
96 earthworms.

97 This experiment aimed to provide a 'baseline dataset' for the hazard of CdTe
98 QDs in freshly prepared soil. The approach used the Organisation for Economic
99 Cooperation and Development (OECD) technical guidance (TG) 222 method (OECD,
100 2004), but with modification and additional endpoints to give a detailed mechanistic
101 understanding of the data. Recently, we also reported a coating related effect on the
102 toxicity of CuO ENMs to earthworms (Tatsi et al., 2018). However, coating-mediated
103 effects on the toxicity of CdTe QDs are unknown in earthworms, and exactly the same
104 coatings were used here for the CdTe QDs ENMs. The study design therefore included
105 a control soil, commercially available micron-sized (bulk) CdTe powder, and CdTe
106 QDs made with organic surface coatings with terminal residues of carboxylate
107 (COOH), ammonium (NH₄⁺), or polyethylene glycol (PEG); to represent negative,
108 positive and neutral surface charges respectively. The potentially toxic metal
109 component(s) of the composite QDs were explored by determining the extractable
110 metal fractions from soil compared to the observed accumulation of Cd and Te in the
111 earthworm tissues. Also to enable some comparison with our previous experiments
112 on CuO ENMs (Tatsi et al., 2018), the survival and growth of the earthworms in freshly
113 dosed soils were determined. Biochemical measurements were also made in the fresh
114 soil experiment to assess known mechanisms of metal toxicity including; effects on
115 ionic regulation (tissue metal concentrations, Na⁺/K⁺-ATPase activity) and oxidative
116 stress (total glutathione). Having established the response in fresh soil, a second
117 experiment including similar endpoints was carried out with newly exposed
118 earthworms after a six-month period of ageing the soils in order to understand the
119 persistence of any hazard from CdTe QD materials in the soil.

120

121 **2. Methodology**

122 Two experiments were carried out using a low (50), medium (500) and high
123 (2000 mg as CdTe kg⁻¹ dw) nominal concentration of CdTe QDs in a quadruplicate
124 test design. Unexposed controls and an equivalent CdTe micron-sized powder was

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

129

130 *2.1 Engineered quantum dots and characterisation*

131 The ENMs used in the experiments were provided by PlasmaChem as part of
132 a European Commission Framework 7 project (www.nanosolutionsfp7.com). The
133 details of the coatings and their synthesis are commercially sensitive information.
134 However, the different coatings were polyethylene glycol (PEG), carboxylate (COOH)
135 and ammonium (NH_4^+) to represent neutral, negative and positive coatings
136 respectively. For clarity, we use the term ‘ $-\text{NH}_4^+$ ’ to mean an $-\text{NH}_3$ terminal ligand that
137 has been ionised with H^+ ions to achieve positive charge. An appropriate micron-sized
138 CdTe powder (size < 250 μm , Sigma-Aldrich, UK, CAS No. 1306-25-8) was used as a
139 bulk material control (refer to hereafter as ‘CdTe-bulk’). The materials were extensively
140 characterised as part of the NANOSOLUTIONS project [e.g., (Vassallo et al., 2018)]
141 and the characterisation of the batches of the materials used here is summarised in
142 Table 1. This includes the nominal primary particle size and chemical composition of
143 each nanoscale QD based on the manufacturer’s information, as well as
144 measurements of primary particle diameter, hydrodynamic diameter and dissolution
145 of Cd and Te made in Plymouth (Table 1; Fig. S1). The QD powders supplied were
146 dark red (NH_4^+ -coated) or bright red (PEG-, COOH-coated) in colour and no impurities
147 were identified by the supplier. The CdTe bulk powder was black in colour and no
148 impurities were noted by Sigma-Aldrich. All other chemicals used were analytical
149 grade and purchased from Sigma-Aldrich unless stated otherwise. The
150 thermogravimetric analysis (TGA) of the materials (Table 1) tentatively indicated that
151 the combustible organic components of the materials contributed roughly 8, 23 and
152 50 % of the mass of the CdTe- NH_4^+ , -COOH and -PEG coated QDs, respectively.
153 However, this would include any ‘unknown’ organic impurities and potentially residual
154 water molecules in between the polymers of the coatings. At the time of the study (and
155 currently) it was not technically feasible to reliably quantify the molecular weight of the
156 coatings *in situ* on the particles, or the precise stoichiometry or coverage of each
157 coating in the overall material in order to spike the soils solely based on Cd or Te
158 concentrations in the core. Therefore, pragmatically, the same approach was taken as

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

163

164 2.2 Stock animals and test soil

165 Adult *Eisenia fetida* originating from a commercial supplier (Blades Biological,
166 Kent, UK) were used from an internal age synchronised laboratory breeding culture
167 held at University of Plymouth for both experiments. The test species were kept in an
168 artificial medium that comprised of bark chippings (1/3), Irish Moss Peat (1/3) and
169 loamy sand topsoil (1/3) with surplus horse manure (from un-medicated horses) as
170 feed at a temperature of 20 ± 1 °C. Adult earthworms between two to four months old,
171 and with visible clitellum, were hand-selected for the experiments. The earthworms
172 were acclimatised to the test soil (Lufa 2.2) with feed one week prior to the experiment.
173 A standard sandy loam Lufa 2.2 (LUF A Speyer, Germany) soil was used with the
174 following composition (supplier's information, mean ± SD, dry soil, *n* = not specified):
175 pH of 5.5 ± 0.2 (measured in 0.01 M CaCl₂ solution); organic carbon, 1.8 ± 0.2 %;
176 nitrogen content at 0.17 ± 0.02 %; cation exchange capacity, 10.1 ± 0.2 meq 100 g⁻¹.
177 The water-holding capacity of the soil was measured in-house and was (mean ± SD,
178 *n* = 3): 41.3 ± 3.0 g 100 g⁻¹ dw. The soil used in the experiments was sieved through
179 a 2 mm mesh and was air dried at 25 °C for 2 days. Soil pH was measured (see
180 Supplementary Information, Table S1) prior to the start and at the end of the
181 experiment in a 1:1 soil: deionised water slurry, using a glass combination electrode
182 (Corning 420).

183

184 2.3 Experimental designs and spiking of the test soil

185 2.3.1 Fresh soil experiment

186 The experiment was conducted using the standard OECD test guideline (TG)
187 222 for the earthworm reproduction test (OECD, 2004), but with a reduced number of
188 earthworms and additional sub-lethal endpoints. The study design included an
189 unexposed control soil, bulk-CdTe and the three variations of coated CdTe QDs: PEG,
190 COOH, NH₄⁺ at nominal concentrations of 50, 500 and 2000 mg CdTe kg⁻¹ dw. The
191 50 and 500 mg CdTe kg⁻¹ dw concentrations were selected on the basis of the known
192 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 $1.86 \pm$
210 0.02 g for 5 earthworms (mean \pm SEM, $n = 52$, individual earthworm weight ~ 0.36 g)
211 were exposed in 4 replicates boxes of soil at $20 \pm 1^\circ\text{C}$ 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 °C) 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

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

229

230 *2.3.2 Aged soil experiment*

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

247 The same study design and methods were used in the aged soil experiment as
248 described in the fresh soil experiment. Five earthworms with a mean weight of $1.89 \pm$
249 0.03 g (mean \pm SEM, $n = 52$, individual weight ~ 0.38 g) were used in the experiment.
250 On days 0, 14 and 28, endpoints such as survival, biomass and appearance were
251 recorded. Reproduction was assessed after 56 days of exposure (described above).
252 In addition, the total concentration of Cd, Te and other electrolytes and trace metals in
253 earthworm tissues were measured on day 28. Due to a technical fault with a freezer,
254 earthworm samples collected for biochemical analyses (total glutathione and Na^+/K^+ -
255 ATPase) were defrosted twice. These defrosted samples were analysed, but the
256 results were considered unreliable and therefore not used (data not shown). In
257 addition, juvenile weight was not assessed in the aged soil study due to the difficulty
258 of obtaining the alive and intact juveniles from the soil. This was due to the presence
259 of plant roots in the soil that severely hampered the collection of juveniles. The roots
260 were inevitably very tangled in the soil, making it difficult to manually find and remove

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

264

265 *2.4 Total and extractable metal analysis in samples*

266 Total metal analysis in the soils and the earthworms was conducted to confirm
267 the exposure and the bioavailable fraction from the QDs, respectively, exactly as
268 previously described (Tatsi et al., 2018). Total *aqua regia* extractable Cd and Te
269 concentrations were measured in triplicate at the beginning of both the fresh and aged
270 soil experiments ($n = 12$ per treatment). Additionally, to provide insight to the mobile
271 fractions of Cd and Te, water (deionised water) and 0.1 M HCl extractable fractions
272 were determined in duplicate in the nominal 2000 mg CdTe kg⁻¹ dw exposures only in
273 the fresh soil experiment ($n = 8$ per treatment), aged soils were not analysed.
274 Earthworms collected at the end of the fresh and aged soil experiments ($n = 8$ per
275 treatment) were allowed to depurate for 24 h on moist filter paper (the latter was
276 changed following 12 h to avoid coprophagy) according to (Arnold and Hodson, 2007).
277 Then the worms were washed, freeze-dried (for 48 h), weighed and digested in 1 ml
278 concentrated HNO₃. All acid digested samples were allowed to cool, diluted with 7 ml
279 ultrapure deionised water (18.2 Ω) and stored in the dark. The total concentration of
280 Cd and Te was measured in the soil and earthworm samples. In earthworms, also
281 other essential elements (Ca, Fe, K, Mg, Mn, Na, Zn, Cu) were measured to assess
282 changes in the trace element and electrolyte composition of the earthworms by
283 inductively coupled plasma optical emission spectrophotometry (ICP-OES, iCAP 700,
284 Thermo Fisher) or inductively coupled plasma mass spectrometry (ICP-MS, Thermo
285 Scientific X Series 2) as appropriate. Prior to analysis, the samples were sonicated for
286 15 minutes (at 0.05 kva, 30 kHz, Ultrawave Ltd), vortexed for 10 s, then hand shaken
287 immediately prior to analysis to ensure good mixing. All samples were analysed
288 against matrix-matched standards where possible. The certified reference materials
289 for total Cd metal reported close to the expected values and were $100 \pm 4 \%$ ($n = 3$,
290 EnviroMAT contaminated soil, SS-1) and $102 \pm 7 \%$ ($n = 3$, TORT-2, contaminated
291 lobster hepatopancreas). Spike recovery tests of earthworm and soil digests using
292 CdTe-NH₄⁺ QDs were 78 and 73 %, respectively (values were calculated based on the
293 sum of [Cd] and [Te] and normalised to expected coating mass which was $8.8 \pm 0.5 \%$
294 for NH₄⁺). Using CdCl₂ and Te standard for ICP-MS and -OES (TraceCERT by Sigma),

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

300

301 *2.5 Biochemical analyses*

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

319

320 *2.6 Statistical analyses and data presentation*

321 Statistical analyses were performed using R studio software (version 2.1) and
322 graphs were drawn using SigmaPlot (version 13.0 or 14.0). Data were checked for
323 normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test). Non-parametric
324 data were transformed (log₁₀) and reanalysed as appropriate. The student's *t*-test (two-
325 tailed, un-/paired) or Mann Whitney test were used for comparing two samples sets as
326 appropriate. Due to a few individual significant differences in pH between treatments
327 in the fresh or aged soil experiments, analysis of covariance (ANCOVA) was carried
328 out for the interactive effects of pH on all endpoints. When no interactions were found,

329 pH was omitted from the model to allow the performance of a post-hoc test. Treatment
330 effects were assessed by one-way ANOVA followed by the Tukey-Kramer (due to
331 unequal sample size) post hoc test. Changes in biomass were analysed with repeated
332 measures ANOVA followed by Tukey's honest significance difference (HSD) test to
333 identify the differences. The non-parametric Kruskal-Wallis test was used followed by
334 a distribution free multiple comparisons test, Dunn's test, where data transformation
335 was unsuccessful. To evaluate the biological associations known for metal toxicity,
336 the Spearman's rank, r_s , correlations were carried out on all of the data, regardless of
337 treatment, for a specified endpoint within each experiment. The reproduction results
338 were normalised to the control values and the 50 % effect concentration (EC₅₀) values
339 and its 95 % confidence intervals were estimated by logistic nonlinear regression
340 analysis (sigmoid) on raw data using the log-transformed nominal concentrations in
341 SigmaPlot v 14.0. To estimate the nominal effect value, the log-transformed value was
342 reversed. The statistical significance level (α) for all tests was set at 0.05.

343

344 **3. Results**

345 *3.1 Soil pH*

346 In the beginning of the fresh soil experiment the soil pH ranged from 5.36 to
347 5.89, and soil pH differed between QD treatments and the control soils. However, by
348 the end of the exposure (day 28) there were no differences in pH between the QD-
349 treated soils and the control soils, with the pH values ranging from 5.39 to 5.78 (Table
350 S1). In the aged soil experiment, the pH ranged from 5.38 to 5.65, both at the
351 beginning and the end of the experiment, and was not statistically different from the
352 corresponding pH measurements in the fresh soil experiment ($P > 0.05$, Student's t -
353 test). Soil pH was included as a cofactor in all the endpoints analysed in both
354 experiments, but no significant interactions were found ($P > 0.05$, ANCOVA). Soil pH
355 was therefore not a factor in any of the biological measurements.

356

357 *3.2 Total and extractable Cd and Te concentrations in soil*

358 To enable the interpretation of the results, the total *aqua regia* extractable
359 concentration of Cd and Te was measured at the beginning of both the fresh and aged
360 soil experiments. In the former experiment, the control soil contained a background
361 Cd concentration of 0.61 ± 0.05 mg Cd kg⁻¹ dw (mean \pm SEM, $n = 8$), of this total Cd,
362 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, $< 0.01 \text{ mg Te kg}^{-1} \text{ dw}$. 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: $\text{COOH} \leq$
378 $\text{PEG} < \text{NH}_4^+ < \text{CdTe-bulk}$. 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 $\text{PEG} > \text{COOH} > \text{NH}_4^+$ (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-NH₄⁺
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

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

402

403 *3.3 Total Cd and Te in earthworm tissue*

404 The total Cd or Te concentrations in the earthworms are shown in Fig. 3. The
405 control earthworms in the both the fresh and aged experiments had some background
406 Cd in the tissues (4.23 ± 0.34 mg Cd kg⁻¹ dw, mean \pm SEM, $n = 8$), while no Te was
407 detected. In the fresh soil experiment, an exposure concentration-dependent increase
408 in the total body burden of Cd was seen in all the CdTe-bulk and all the CdTe QD
409 exposures (Fig. 3). The accumulation of Cd was significantly lower in the CdTe-bulk
410 when compared to the QD exposures in fresh soil at each exposure concentration.
411 However, within the CdTe QDs, there was no significant differences between the total
412 concentrations of Cd in the earthworm tissues by the type of coating of the CdTe QDs
413 at each nominal test concentration (Fig. 3A, $P > 0.05$, ANOVA). The total
414 concentration of Te in the earthworms during the fresh soil experiment was also
415 measured (Fig. 3B). Here, the tissue Te concentration increased in a concentration-
416 dependent manner in the CdTe-bulk exposures, but not in the CdTe QD exposures
417 where the Te remained uniformly low. Within the QDs there was also no clear effect
418 on Te accumulation by the type of surface coating on the ENM in the fresh soil
419 experiment (Fig. 3B). Together these observations suggest some Te accumulation for
420 the bulk material, but very limited Te accumulation for CdTe QDs in fresh soil. The
421 accumulation of Cd and Te were strongly correlated within each treatment group (r_s =
422 0.9, 0.8, 0.8 and 0.9 for CdTe-bulk, -PEG, -COOH and -NH₄⁺ exposures, $P < 0.05$,
423 Spearman) and this is consistent with the notion of being exposed to a composite
424 material containing both metals. The total Cd or Te in the soil was also significantly
425 correlated to the total Cd or Te in earthworms when analysing all the data together (r_s
426 = 0.8 and 0.7 for Cd and Te, $P < 0.05$, Spearman); and within the animals in each
427 treatment. However, this contrasts with the absence of any clear relationship between
428 the water- or acid-extractable fractions and accumulation of Cd or Te by the
429 earthworms noted in Fig. 2.

430 In the aged soil experiment, the accumulation of Cd and Te by earthworms was,
431 overall, higher than in the fresh soil experiment. In the CdTe-bulk exposures the Cd
432 concentrations in the earthworms were significantly higher than those in the fresh soil
433 study (Fig. 3). In the nominal 50 mg CdTe kg⁻¹ dw test concentration there were no
434 significant differences in the concentration of Cd in the earthworms by the type of
435 coating on the CdTe QDs in the aged soil (Fig 3C). In the nominal 500 or 2000 mg
436 CdTe kg⁻¹ dw test concentrations in aged soil, there were no statistically significant
437 differences between the tissue concentrations of Cd between doses, but there was a
438 trend of higher tissue Cd concentration in earthworms exposed to the differently
439 coated CdTe QDs when compared to the CdTe-bulk, although the trend was not
440 statistically significant. In the aged soil experiment, the concentration of Te in the
441 earthworms followed a similar pattern of accumulation to the fresh soil experiment,
442 where the highest amount of Te was found in earthworms exposed to CdTe-bulk
443 material in all test concentrations. In the nominal 50 mg CdTe kg⁻¹ dw test
444 concentration, there were no statistically significant differences in Te accumulation by
445 the type of coating on the CdTe QDs. However, some differences in the total Te
446 accumulation emerged in the 500 mg CdTe kg⁻¹ dw test concentration, where the
447 concentration was highest in the CdTe-NH₄⁺ QD exposure, compared to the other
448 materials, and the Te accumulation was similar in the CdTe-PEG and -COOH QD
449 exposures. In the nominal 2000 mg CdTe kg⁻¹ dw test concentration, the accumulation
450 of Te was not statistically significantly different between the different CdTe QD
451 exposures, although there was a non-significant trend of higher Te in the CdTe-NH₄⁺
452 groups.

453

454 *3.4 Survival, biomass and appearance of earthworms*

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

464 observed in the nominal 50 mg CdTe kg⁻¹ dw for the QD exposures by day 28, but the
465 2000 mg CdTe kg⁻¹ dw exposure concentration of the QDs caused a uniform decrease
466 in biomass of around 50% by day 28, regardless of the type of coating on the ENMs
467 (Table 2). There were no obvious behavioural or changes in appearance of the
468 earthworms exposed to the CdTe-bulk or the nominal 50 and 500 mg CdTe kg⁻¹ dw of
469 the CdTe QDs in the fresh soil experiment. However, earthworms from all the CdTe
470 QD exposures (excluding CdTe-bulk) at the nominal 2000 mg CdTe kg⁻¹ dw test
471 concentration were sluggish and were not actively feeding (evidence of left-over
472 manure), although they were still moving in the soil. Furthermore, morphological
473 changes were evident in earthworms from the 2000 mg CdTe kg⁻¹ dw CdTe QD ENM
474 exposures. The earthworms were visibly smaller and showed evidence of shedding of
475 the posterior segments (the last segments were clearly lighter coloured with signs of
476 regeneration). A strong negative correlation was found between tissue Cd or Te and
477 biomass for the fresh soil experiment when analysing all the data from all treatments
478 together ($r_s = 0.6$ and 0.5 for Cd and Te respectively, $P < 0.05$, Spearman).

479 In the aged soil study, the control earthworms were healthy in appearance and
480 with good survival, however, a trend of biomass loss (8.1 ± 5.6 %, Table 2) was noted,
481 although the change was not statistically significant. The earthworms survived in all
482 the CdTe-bulk exposures with only minor mortalities < 10 % at the nominal 2000 mg
483 CdTe kg⁻¹ dw exposures in the aged soil experiment. In the CdTe-bulk exposures,
484 earthworms lost biomass only at the nominal 2000 mg CdTe kg⁻¹ dw test concentration
485 (Table 2). At the nominal 2000 mg CdTe kg⁻¹ dw test concentration for the CdTe QDs,
486 the CdTe-COOH QDs were the most toxic to earthworms in the aged soil experiment;
487 where survival was reduced to 55 ± 5 , 60 ± 25 , and 75 ± 10 % for the -COOH, -NH₄⁺
488 and -PEG-coated QDs respectively by day 28 (Table 2). In addition, earthworms from
489 these treatments showed statistically significant weight loss compared to controls.
490 There were no obvious behavioural or appearance changes in the CdTe-bulk
491 exposures or the nominal 50 mg CdTe kg⁻¹ dw CdTe QD exposures in the aged soil
492 experiment. Some earthworms appeared lethargic and non-responsive in the 500 and
493 2000 mg CdTe kg⁻¹ dw CdTe QD ENM exposures, and similar to the fresh soil study,
494 there was evidence of shedding of the posterior segments and lack of feeding
495 (presence of surplus feed).

496

497 3.5 Reproduction

498 The reproductive success of the animals is reported in Table 3, and with dose-
499 response curves for juvenile production shown in Fig. S4. The test results on
500 reproduction in the controls of both fresh and aged experiments met the validity criteria
501 of the OECD TG 222. In both the experiment with fresh and aged soil, the control
502 earthworms produced a healthy number of juveniles per earthworm (Table 3), and with
503 a coefficient of variation (CV %) of less than < 30 % in keeping with the OECD test.
504 Reproduction was not significantly reduced in any of the CdTe-bulk test
505 concentrations, or in the nominal 50 mg CdTe kg⁻¹ dw CdTe QD exposures in the fresh
506 soil experiment. However, it was significantly reduced in the CdTe QD treatments at
507 the nominal 500 mg CdTe kg⁻¹ dw test concentration, and completely inhibited at the
508 2000 mg CdTe kg⁻¹ dw test concentration for the CdTe QD exposures, compared to
509 controls in the fresh soil experiment. The 50% effect concentrations (EC₅₀) for
510 reproductive success (i.e., juvenile production) for the different materials are
511 presented in Table 3. The estimated EC₅₀ values were calculated using the nominal
512 CdTe QD concentrations (response curves presented in Fig. S4). For the fresh soil
513 experiment, the EC₅₀ values decreased in the following order: CdTe-COOH < CdTe-
514 NH₄⁺ < CdTe-PEG. For the CdTe bulk material in fresh soil there was no inhibition of
515 juvenile production and so an EC₅₀ value could not be estimated. The fresh wet weight
516 of the juveniles was also measured after they were rinsed in deionised water and
517 padded dry (Table 3). There were some statistically significant differences between
518 treatments in the biomass of the juveniles in the fresh soil experiment, but the
519 differences followed the same order as the total number of juveniles produced. That
520 is, where only a few juveniles were produced, those juveniles also tended to be small
521 (i.e., poor quality offspring).

522 In the aged soil experiment (Table 3), the controls produced more juveniles
523 than in the fresh soil experiment. Unlike the situation in fresh soil, the number of
524 juveniles produced in the CdTe-bulk exposures declined, and with a complete
525 inhibition of reproduction at the nominal 2000 mg CdTe kg⁻¹ dw test concentration in
526 aged soil. The effects of the CdTe QD exposures on juvenile production were more
527 pronounced in the aged soil experiment, with lower EC₅₀ values for the CdTe-PEG
528 and CdTe-NH₄⁺ treatments in the aged soil compared to those in fresh soil (Table 3).
529 The number of juveniles produced was significantly reduced or completely abolished
530 in all the CdTe QD exposures at 500 and 2000 mg CdTe kg⁻¹ dw test concentrations

531 compared to the unexposed controls in aged soil. The EC₅₀ values for reproductive
532 success decreased in the following order in aged soil: CdTe-NH₄⁺ < CdTe-COOH <
533 CdTe-PEG < CdTe-bulk. However, while the ranking of the EC₅₀ values are correct,
534 all of the curve fits (Fig. S4), will have some uncertainty in the precise EC₅₀ value due
535 to the number of data points and their confidence intervals in the dose-response plot.

536

537 *3.6 Sodium pump activity and tissue elemental composition*

538 In the experiment with the fresh soil the control earthworms showed a normal
539 Na⁺/K⁺-ATPase (sodium pump) activity 6 – 8 μmol ADP mg⁻¹ protein h⁻¹ (Fig. 4A). In
540 the fresh soil experiment, there was an overall trend of lower sodium pump activity in
541 all the exposure concentrations of the CdTe bulk material, with significantly reduced
542 activities in the nominal 500 and 2000 mg CdTe kg⁻¹ dw test concentration (Fig. 4).
543 There was also some inhibition of the Na⁺/K⁺-ATPase in the CdTe QD treatments in
544 the fresh soil experiment, but with no clear dose and coating-dependent trend in the
545 data (Fig. 4A). The substrates for the sodium pump include Na⁺ and K⁺ and the tissue
546 electrolytes of the earthworms are shown in Table S2. There was no clear effect on
547 the concentration of Na⁺ in earthworm tissues, however, the concentration of K⁺ was
548 significantly lower in the nominal 500 and 2000 mg CdTe kg⁻¹ dw CdTe-NH₄⁺
549 exposures compared to controls (*P* < 0.05, ANOVA; Table S2). In the aged soil study,
550 unfortunately failure of a deep freeze prevented any reliable determination of enzyme
551 activity in those samples (data not shown). However, there was no depletion or
552 significant increase in the tissue concentration of Na⁺ or K⁺ between the test
553 concentrations and the control for any treatment (*P* > 0.05, ANOVA; Table S2).

554 A range of other electrolytes and essential trace elements in the tissues of the
555 earthworms are shown in Table S2. Overall for the control earthworms, in both the
556 fresh and aged soil studies, the electrolytes and trace elements remained in the
557 expected range for a healthy earthworm population (Table S2). The earthworms in the
558 fresh soil study exposed to the CdTe-bulk showed no significant treatment-dependent
559 changes in electrolytes or trace metals (Table S2). In contrast, the CdTe QD
560 exposures at 500 and 2000 mg CdTe kg⁻¹ dw test concentrations showed statistically
561 significant decreases in essential and trace metals in the earthworms in fresh soil
562 (Table S2). The concentration of Ca, Mg and Fe were significantly reduced in the
563 nominal 500 and 2000 mg CdTe kg⁻¹ dw CdTe-NH₄⁺ exposures compared to
564 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 NH₄⁺ 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-NH₄⁺ and
572 nominal 2000 mg CdTe kg⁻¹ dw CdTe-PEG, COOH and NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ 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-NH₄⁺ 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-NH₄⁺ 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.

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

679 The situation was somewhat different for the ENMs in soil compared to the bulk
680 material (Fig. 2), with all the CdTe QDs showing a water- and acid-extractable fraction
681 of Cd in the soil, but not much extractable Te. The dialysis experiment also showed
682 more dissolution of Cd than Te from the ENMs (Fig. S1); which together implies the
683 Cd was more bioaccessible than the Te in CdTe QDs. Furthermore, the Cd
684 accumulation in the earthworms was at least an order of magnitude higher than Te
685 (Figs. 2 and 3), despite no such differences in the exposure concentration of the metals
686 in the soil from the ENMs (Fig. 1). This observation can only be explained by the
687 differential uptake and/or retention of Cd compared to Te in the tissues, and therefore
688 that the CdTe QDs do not remain intact. It has been suggested that CdTe QD likely
689 degrade in the soil to release dissolved Cd (Navarro et al., 2008). In aqueous media
690 for algae, the dissolution of Cd from CdTe-containing QDs and subsequent free metal
691 ion uptake can explain most of the Cd accumulation by the organisms
692 [*Chlamydomonas reinhardtii*, (Domingos et al., 2011)]. Differential accumulation of Cd
693 and Se have been noted in earthworms exposed to CdSe QDs [9-20 nm, (Stewart et
694 al., 2013)]. Fluorescence imaging has shown what appears to be intact CdTe QDs
695 (assumed from the fluorescence signal itself) in the gut lumen of the nematode, *C.*
696 *elegans*, (Qu et al., 2011) and the gastrointestinal tract of larvae of the leaf roller moth
697 (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 NH₄⁺-coated
731 QDs compared to the others (Fig. 2). In theory, the chemical bond anchoring the

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

740 Regardless of the mechanism(s), this coating-mediated effect on the
741 dissolution (Fig. S1), and extractable metal fractions in the soil (Fig. 1), was not
742 observable in the mean values for total metal accumulation of Cd or Te in the
743 earthworms (Fig. 3). For example, the extractable Cd in soil from the CdTe-NH₄⁺ QDs
744 was relatively high (Fig. 2), but this did not lead to the highest Cd accumulation in the
745 earthworms (Fig. 3). In short, the running order of any coating-mediated effect on the
746 physico-chemical properties of the QDs was not in the same order as the subsequent
747 total metal accumulation in the earthworms. This was not resolved by normalising the
748 total metal bioaccumulation in the earthworms against the measured total metal
749 concentration in the soil (i.e., the transfer factor: the total metal concentration in the
750 earthworm divided by that in the soil, data not shown). Therefore, while there are some
751 coating-mediated effects on the accumulation of Cd and Te (Fig. 3), they are not
752 explained by free metal ions released from the ENMs, although they are related to the
753 total metal in the soil during the exposure. From the viewpoint of environmental
754 protection, threshold levels for metals in soil have been suggested for dissolved metals
755 on the basis of free ion activity (Lofts et al., 2004), and while ligand models have also
756 been applied to ENMs in soil (Judy and Bertsch, 2014), the assumption that free metal
757 ions lead to toxicity or accumulation is not proven for CdTe QDs here, and needs to
758 be verified for many other ENMs in soil.

759

760 *4.3 Effect of soil ageing on metal accumulation*

761 Soil guidelines are not available for ENMs, and while the individual parameters
762 in colloid theory such as soil pH and ionic strength have been considered (Cornelis et
763 al., 2014), the sum effect of all the possible transformations of an ENM that constitute
764 'ageing' of the material in soil are not yet understood. In the present study, only the
765 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 $< 0.1 \mu\text{g}$
782 h^{-1} for Cd (Table 1), and even at the highest soil dose with around $800 \text{ mg Cd kg}^{-1}$ of
783 soil (Fig.1), in a 250 g box of the soil ($\sim 200 \text{ mg Cd}$ in total), this would equate to a
784 dissolution of $16.8 \mu\text{g}$ of Cd per week, or about $403 \mu\text{g}$ 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 sulphidation 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

800 due to the presence of the coatings. It is possible that organic ligands in the soil, such
801 as the polyanionic charges of humic and fulvic acids, could electrostatically attract the
802 positive coating of the CdTe-NH₄⁺ QDs, repulsion in the case of CdTe-COOH QDs, or
803 show steric entanglement with the CdTe-PEG QDs. Similar ideas have been proposed
804 for the behaviour of QDs in natural water (Rocha et al., 2017). However, such
805 phenomena would demand a clear and consistent coating-effect, but this was not
806 observed with different coating-mediated effects on bioaccumulation in the fresh
807 compared to aged soil experiments, and with exposure dose within each experiment
808 (Fig. 3). Regardless of the mechanisms, the bulk material presents a bioaccumulation
809 concern with soil ageing that is greater than the nano forms.

810

811 *4.4 Effects on survival and body weight*

812 In the fresh soil experiment, the control earthworms survived, gained weight,
813 and did not show any abnormalities, indicating that the animals were healthy.
814 Furthermore, there were no effects on survival in any of CdTe treatments; and in the
815 CdTe-bulk treatment the earthworms also gained weight (Table 2). However, despite
816 good survival, the animals in the CdTe QD treatments lost biomass (Table 2). There
817 was also a negative correlation between the metal concentrations in the tissue and
818 biomass (Fig. S5). The loss of biomass was partly attributed to a lack of feeding
819 coupled with some shedding of the posterior segments. There were no consistent
820 effects of percentage change in body mass by the type of coating on the CdTe QDs in
821 the fresh soil experiment; and by day 28 all the earthworms in the 2000 mg kg⁻¹ CdTe
822 QD treatments had lost around 50% of their body mass (Table 2). The latter suggests
823 the earthworms at the higher doses were just below the threshold for mortality. Indeed,
824 the shedding of posterior segments has been interpreted as a survival strategy (Sims
825 and Gerard, 1985) and possibly an 'excretion' mechanism for metals in earthworms.
826 The survival of the earthworms at the nominal 50 and 500 mg kg⁻¹ CdTe QD
827 concentrations in the fresh soil experiment was expected with respect to Cd toxicity,
828 as earthworms are known to tolerate very high concentrations of dissolved Cd [28 day
829 mortality LC₅₀ = 588 mg Cd kg⁻¹ dw, (Van Gestel et al., 1991)], which are partly
830 chelated by metallothionein in the tissue (Stürzenbaum et al., 2001). Furthermore, the
831 extractable Cd from the fresh soil experiment in most of the QD exposures was far
832 less than the expected lethal concentration of dissolved Cd, except in the nominal
833 2000 mg CdTe kg⁻¹ dw CdTe-NH₄⁺ QD exposures. In earthworms, a critical body

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

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

852 In contrast to the fresh soil experiment, exposure to CdTe QDs in aged soil
853 caused considerable mortality, even at the nominal concentration of 50 mg kg⁻¹ dw of
854 CdTe QDs (Table 2). The CdTe QDs were, therefore, much more toxic after ageing of
855 the soil. In addition, a coating-mediated effect on survival was also apparent in the
856 aged soil experiment, with the toxicity ranking CdTe-PEG < CdTe-NH₄⁺ < CdTe-COOH
857 QDs at the highest exposure concentration (Table 2). In a study using the same
858 coatings on CuO ENMs at a concentration of 1000 mg Cu kg⁻¹, the NH₄⁺-coated CuO
859 ENMs were also acutely toxic following a period of ageing, however, COOH-coated
860 CuO ENMs were not (Tatsi et al., 2018). This implies that, while coating-mediated
861 effects can occur, the precise ranking of the effect also depends on the type of metal
862 core in the ENM. This might be expected, because the stability constants of metals
863 with organic ligands vary according to the charge density of the metal and the type of
864 ligand (Albert, 1950). However, stability constants are not yet available for metals in
865 the solid or crystalline state as found in nanomaterials with ligands of different chain
866 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 $1000 \text{ mg Cu kg}^{-1} \text{ dw}$ 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: $\text{CdTe-COOH} < \text{CdTe-}$
885 $\text{NH}_4^+ < \text{CdTe-PEG}$ (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 $780 \text{ mg Cd kg}^{-1} \text{ dw}$, 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 $46.3 \text{ mg Cd kg}^{-1} \text{ dw}$ 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

901 reports of juvenile production from adult earthworms exposed to ENMs, but like the
902 present study, TiO₂ ENMs also decrease juvenile production in earthworms (Schlich
903 et al., 2012).

904 In the aged soil experiment, the control earthworms produced more juveniles
905 than in the fresh soil study (Table 3). This is likely due to the higher organic matter
906 (i.e., plant roots) in the aged soil that is known to encourage earthworms to produce
907 more offspring (Lahive et al., 2017). Unlike the fresh soil experiment, in aged soil, the
908 CdTe bulk material inhibited juvenile production to the extent that no juveniles were
909 produced at the highest exposure concentration (Table 3) and the Cd concentration in
910 the adult earthworms strongly correlated with this reproductive failure (Fig. S5); again
911 suggesting that mobilisation of Cd from the bulk material during soil ageing caused the
912 toxicity. Furthermore, the reproductive toxicity of the CdTe QDs also increased in the
913 aged soil experiment, but the difference between the coated QDs was less
914 pronounced, although CdTe-NH₄⁺ QDs remained most hazardous (Table 3). The
915 further reduction in reproduction in the aged soil experiment may be explained by the
916 higher accumulation of Cd and Te in the earthworms (Fig. 3), which was also
917 associated with lower biomass in the adult worms. So bioenergetically, like the fresh
918 soil experiment, poor growth or health in the adults likely gave rise to fewer offspring.

919

920 *4.6 Effects on ion regulation*

921 Cadmium is known to cause ion regulatory disturbances in organisms
922 (Stürzenbaum et al., 2004), therefore, the sodium pump activity was assessed. In the
923 fresh soil study, earthworms in the controls sustained sodium pump activity (Fig. 4),
924 as well as an overall concentration of Na⁺ and K⁺ (Table S2), as expected in the
925 earthworms (Tatsi et al., 2018). The sodium pump activity was inhibited the most in
926 the CdTe-bulk treatment and at the highest exposure concentration (Fig. 4). However,
927 exposure to CdTe QDs in fresh soil also inhibited the sodium pump. Of the ENMs, the
928 CdTe-PEG and -NH₄⁺ QDs were most potent in inhibiting the sodium pump activity
929 (Fig. 4). In the nominal 500 and 2000 mg CdTe kg⁻¹ dw exposures to CdTe-NH₄⁺,
930 tissue K⁺ concentrations also decreased (Table S2), being consistent with inhibition of
931 the sodium pump. There appears to be no other reports of the effects of CdTe QDs on
932 osmoregulation in earthworms, and whether the sodium pump inhibition is caused by
933 Cd alone, Te alone, or both, requires further investigation.

934 Several essential metals for earthworm health, were also measured (Table S2).
935 Of these, there were some transient changes in Zn and Fe in the fresh soil experiment,
936 but no clear dose effect, and no effects in the aged soil experiment. However, copper
937 and manganese showed some interesting changes. The control earthworms showed
938 the expected tissue Cu [8 – 10 mg Cu kg⁻¹ dw, (Streit, 1984; Tatsi et al., 2018)] and
939 Mn concentrations [43-54 mg Mn kg⁻¹ dw this study; ~ 50 mg Mn kg⁻¹ dw in *L. rubellus*,
940 (Oste et al., 2001)]. The manganese concentration was reduced by almost 3 times in
941 the CdTe QD ENM exposures, for both the fresh and aged soil experiments, and also
942 for the bulk material exposures in the aged soil experiment (Table S2). The
943 mechanistic cause of this Mn depletion in earthworms requires further investigation,
944 but Cd-dependent inhibition of Mn uptake has been observed in fibroblast cell lines
945 (Yanagiya et al., 2000), possibly mediated by competition for uptake on divalent metal
946 ion transporter 1 (DMT1). Copper depletion was correlated with higher tissue Cd (Fig.
947 S5) in the CdTe-COOH exposures (500 and 2000 mg CdTe kg⁻¹ dw) and CdTe-NH₄⁺
948 exposure (500 mg CdTe kg⁻¹ dw), and with the effects evident in both fresh and aged
949 soil experiments (Table S2). The loss of Cu (and Mn) might arise from a passive non-
950 specific electrolyte leak associated with tissue injury since other essential elements
951 such as K, Ca and Mg concentrations were significantly reduced compared to the
952 controls in the fresh soil (Table S2). However, this seems unlikely in the absence of
953 glutathione depletion (Fig. 4, no clear difference in total GHS pool were observed) and
954 the major electrolytes were undisturbed in the tissues in the aged soil experiment,
955 despite some tissue Cu depletion. Cu is a regulated metal in earthworms (Streit, 1984),
956 and it could simply be that Cd²⁺ is displacing Cu from ligands such as metallothionein
957 and glutathione in the tissue. Regardless of the mechanism, both Cu and Mn were
958 decreased in the tissue of earthworms from both the fresh and aged soil studies, and
959 with the same coated QDs, COOH and NH₄⁺, being most potent for this effect (Table
960 S2).

961

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-NH₄⁺ 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-NH₄⁺ QDs.
995 However, this was not a direct coating effect *per se*, but likely a consequence of other

996 subtle changes in the physico-chemical properties of the material during manufacture,
997 such as the differences in the ratio of the apparent metal concentrations in each
998 material. In keeping with this notion, the toxic effects seen in the study were related to
999 accumulation of Cd in earthworm tissues, however, this was not easily explained by
1000 the measured dissolution rate of Cd from the QDs (Table 1) or the extractability of Cd
1001 from the soils (Fig. 2). The existing environmental risk assessment for dissolved Cd is
1002 likely to be protective of both bulk and nano forms of CdTe materials, although the
1003 type of coating and any subsequent changes in particle properties associated with
1004 adding the coating, should be considered in the environmental risk analysis. Crucially,
1005 the toxicity of the CdTe QDs increased after a period of ageing of the soil, suggesting
1006 that the hazard should be monitored, and any environmental risk assessment revisited
1007 in order to maintain protection of the soil ecosystem. Finally, it would be important to
1008 ascertain the mechanisms of toxicity for Te, since it is widely used in a QD form and
1009 currently there are no environmental risk assessments which give guidance on safe
1010 levels of Te in soils.

1011

1012 **Conflicts of interest**

1013 The authors alone are responsible for the content and writing of the paper. The authors
1014 report no conflicts of interest.

1015

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1026

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1170 **Tables and figures**

1171 **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 ($\mu\text{g h}^{-1}$)	
					Cd	Te
CdTe-Bulk	CASRN 1306-25-8 (Sigma-Aldrich 256544), Lot No. MKBK6448V, $\geq 99.99\%$ purity trace metal basis	< 250 μm	172 \pm 28	NA	<0.1	0.2
CdTe-Polyethylene Glycol	Lot No.YF140402, 99% purity, size 3-5 nm.	< 4	156 \pm 72	50.4 \pm 8.3	6.4	2.3
CdTe- Carboxylate	Lot No.YF140402, 99% purity, size 3-5 nm.	< 4	84 \pm 58	23.4 \pm 4.2	3.9	1.3
CdTe- Ammonium	Lot No. YF140402, 99% purity, size 3-5 nm.	< 4	75 \pm 50	8.8 \pm 0.5	29.0	14.9

1172 ¹ Supplied as dry powders, spherical particles for the Nanosolutions project via Alexei Antipov, PlasmaChem GmbH.

1173 ² It was not possible to detect the QDs using electron microscopy, therefore an estimate is given (Denmark Technical University)

1174 ³ NTA- Nanoparticle Tracking Analysis using NanoSight using 100 mg CdTe l⁻¹ ENM stocks in Milli-Q water at University of
 1175 Plymouth. Data are mean \pm S.D. $n = 3$ samples

1176 ⁴TGA – thermogravimetric analysis. Triplicate measurements (technical replicates) made on the same batch of the dry powders
 1177 using a TGA 4000 (Perkin Elmer) under an N₂ flow of 20 ml min⁻¹ from 25°C to 995°C at a heating rate of 10°C min⁻¹ at the
 1178 University of Manchester. Note, this measurement would include the pyrolysis of the organic coating and any other combustible
 1179 (presumably organic) impurities in/on the materials.

1180 ⁵Maximum slope from rectangular hyperbola function of curve fitting used to estimate the maximum rate of dissolution of Cd and Te
 1181 from dialysis experiments conducted at University of Plymouth, conducted exactly according to (Besinis et al., 2014).

1182 NA – not applicable to the test item.

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1186 **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

1187 Survival is reported as the total number of earthworms per treatment (total) and as percent survival (%). Similarly, the biomass is
 1188 reported as the total biomass of surviving earthworms per treatment (wet weight, g) and the percentage weight increase or
 1189 decrease relative to the animals at the start of the experiment. Data presented as mean ± SEM (*n* = 4 boxes of worms per
 1190 treatment). Treatments that do not share a letter are statistically significantly different within an experiment at the specified time-

1191 point and the hash (#) denotes statistically significant differences between treatments on day 14 and 28 ($P < 0.05$ repeated
1192 measures ANOVA for biomass data or Kruskal-Wallis for survival data). Asterisk (*) denotes statistically significant differences
1193 between treatments in the fresh and aged soil experiments.

1194 Day 0 mean wet weight was 1.86 ± 0.02 g (mean \pm SEM, for a subsample of 5 of the initial earthworms, $n = 52$ treatments) in fresh
1195 soil experiment.

1196 Day 0 mean wet weight of 1.89 ± 0.03 g of per exposure replicate (mean \pm SEM, for a subsample of 5 of the initial earthworms, $n =$
1197 52 treatments) in the aged soil experiment.

1198 \uparrow Increase in wet weight relative to day 0

1199 \downarrow Decrease in wet weight relative to day 0

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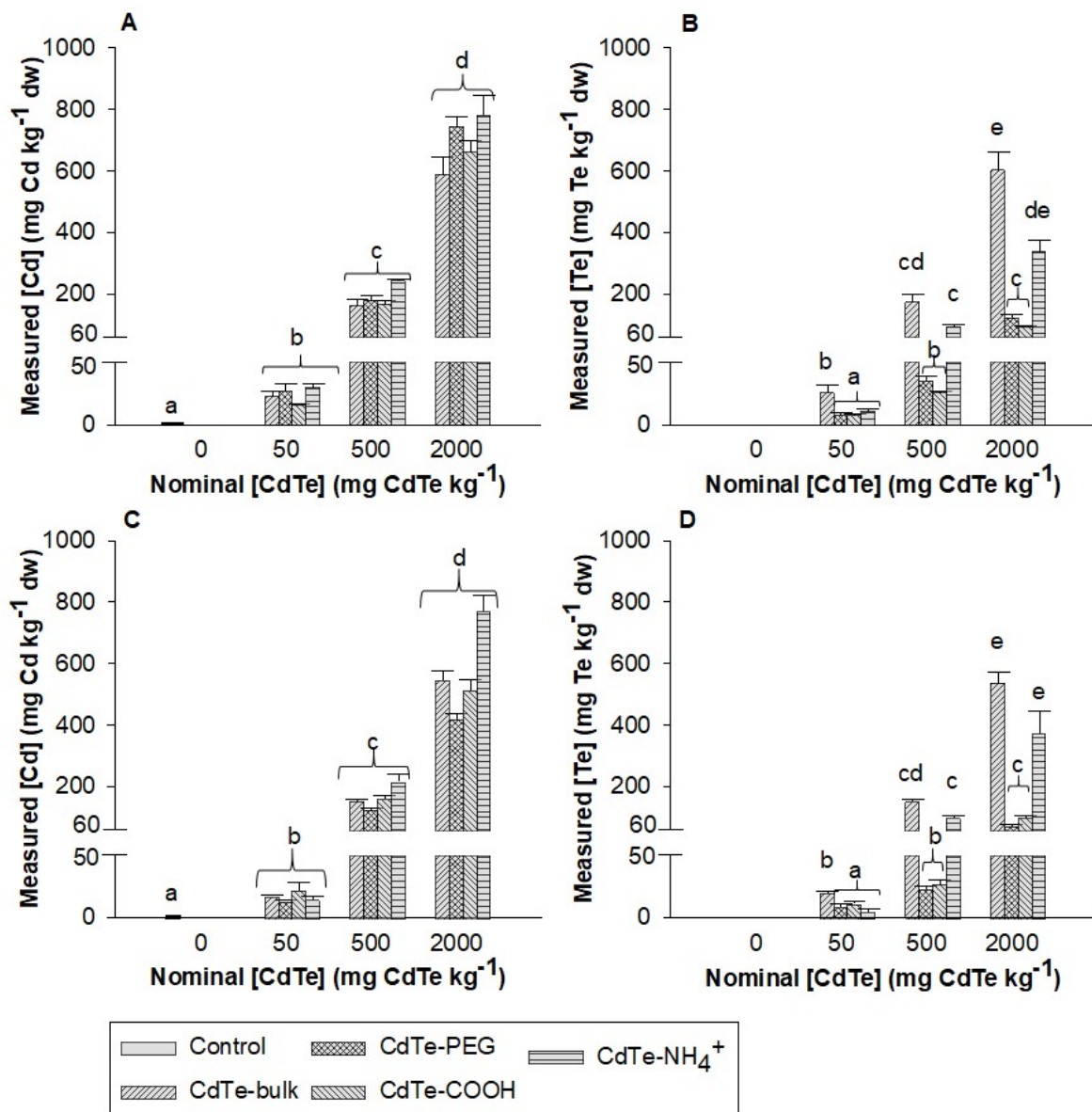
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1209 **Table 3** Total number of juveniles produced and their total fresh weight in the fresh soil experiment and total number of juveniles
 1210 produced in the aged soil experiment. The EC₅₀ and estimated NOEC values in each treatment in the fresh and aged soil
 1211 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	

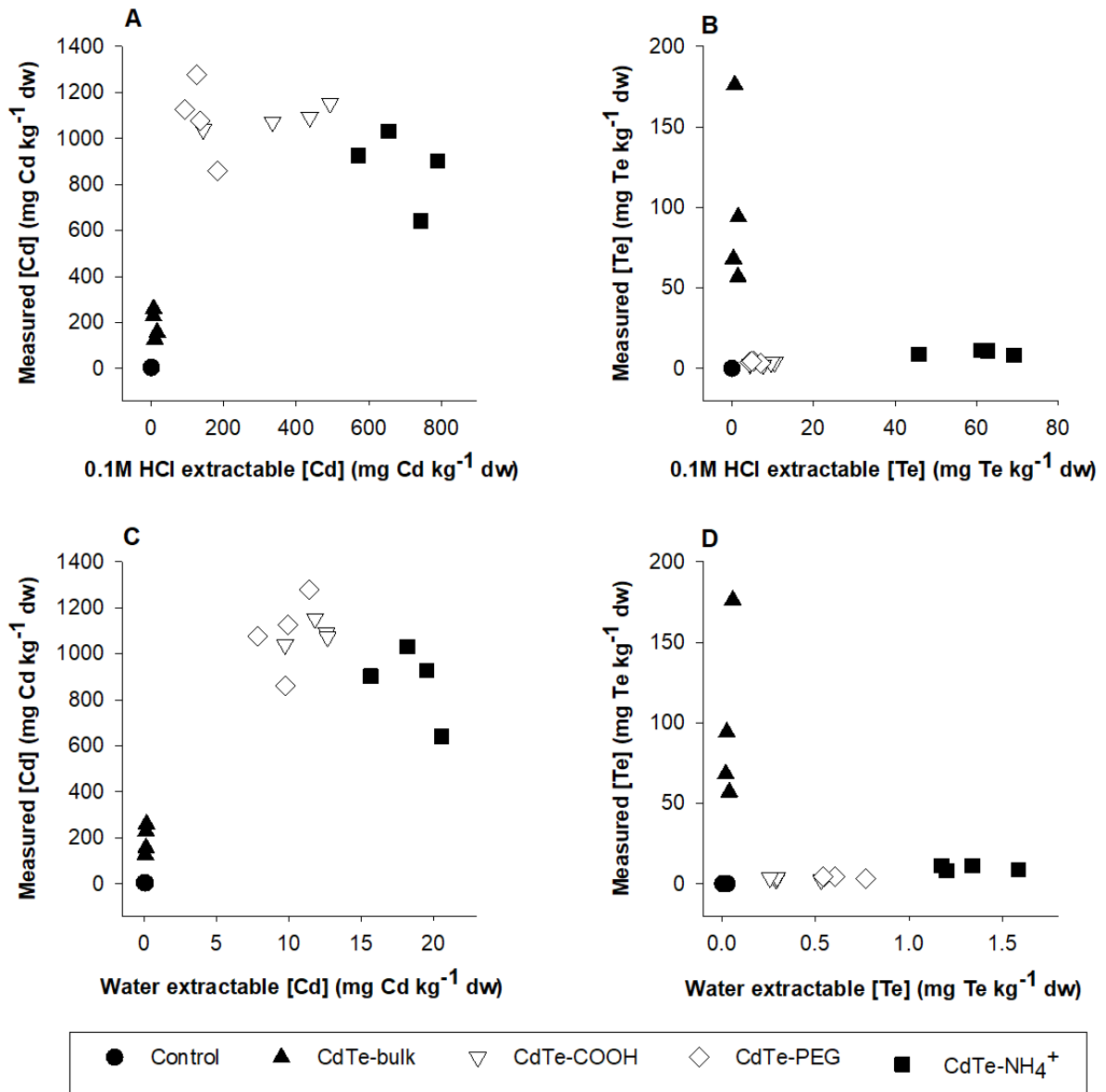
1212 Data presented as mean ± SEM (*n* = 4). Different letters denote statistically significant differences between treatments within an
 1213 experiment (*P* < 0.05, ANOVA). Asterisk (*) denote statistically significant differences between the treatment between fresh and
 1214 aged soil experiment (*P* < 0.05 T-test, unpaired). The biomass is reported as the total biomass all the juveniles produced treatment

1215 (wet weight, g). EC₅₀ values for 50 % reduction in reproduction after 4 weeks of exposure in fresh and aged soils with CdTe QDs
1216 are presented with 95 % confidence intervals in the brackets, the concentration response curves are presented in Fig. S4. NA – not
1217 possible to calculate EC₅₀ due to no effect; not possible to calculate confidence intervals due to high effect. Note, because of the
1218 difficulty in removing juveniles from amongst the plant roots that had grown in the soil during ageing, we were not confident in
1219 removing all of the small juveniles intact in order to report an accurate total biomass for juveniles in the aged experiment.
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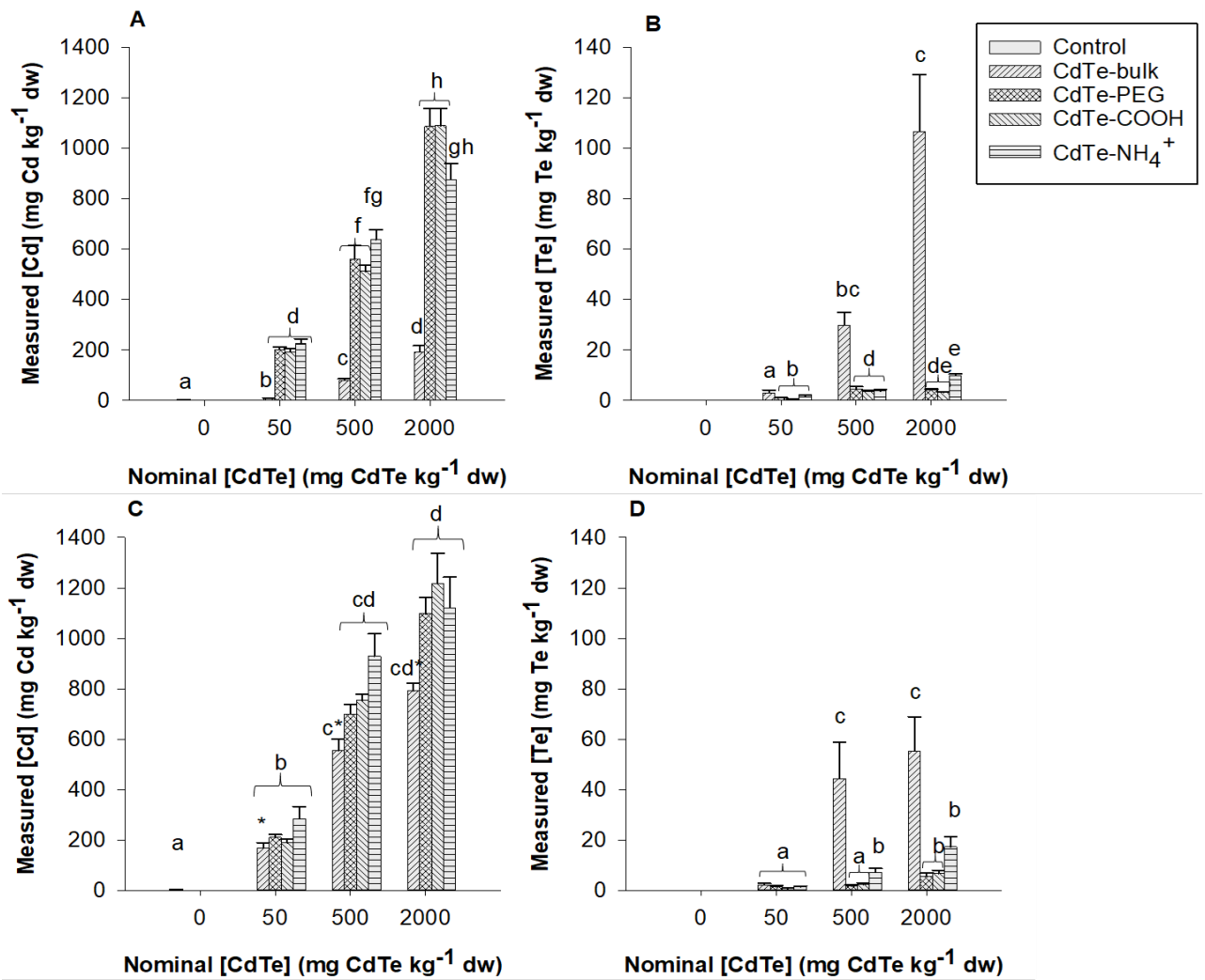
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1222 **Fig. 1** Total measured Cd (A, C) and Te (B, D) concentration in soil (mg kg⁻¹ dw) at
 1223 the beginning of the fresh (upper panels) and aged (lower panels) soil experiment.
 1224 Data expressed as mean ± SEM (*n* = 8). Different letters show statistically significant
 1225 differences between treatments within test concentration (*P* < 0.05, ANOVA). There
 1226 were no statistically significant differences between treatments within a dose in the
 1227 fresh compared to aged exposures (*P* > 0.05, *t*-test, unpaired).



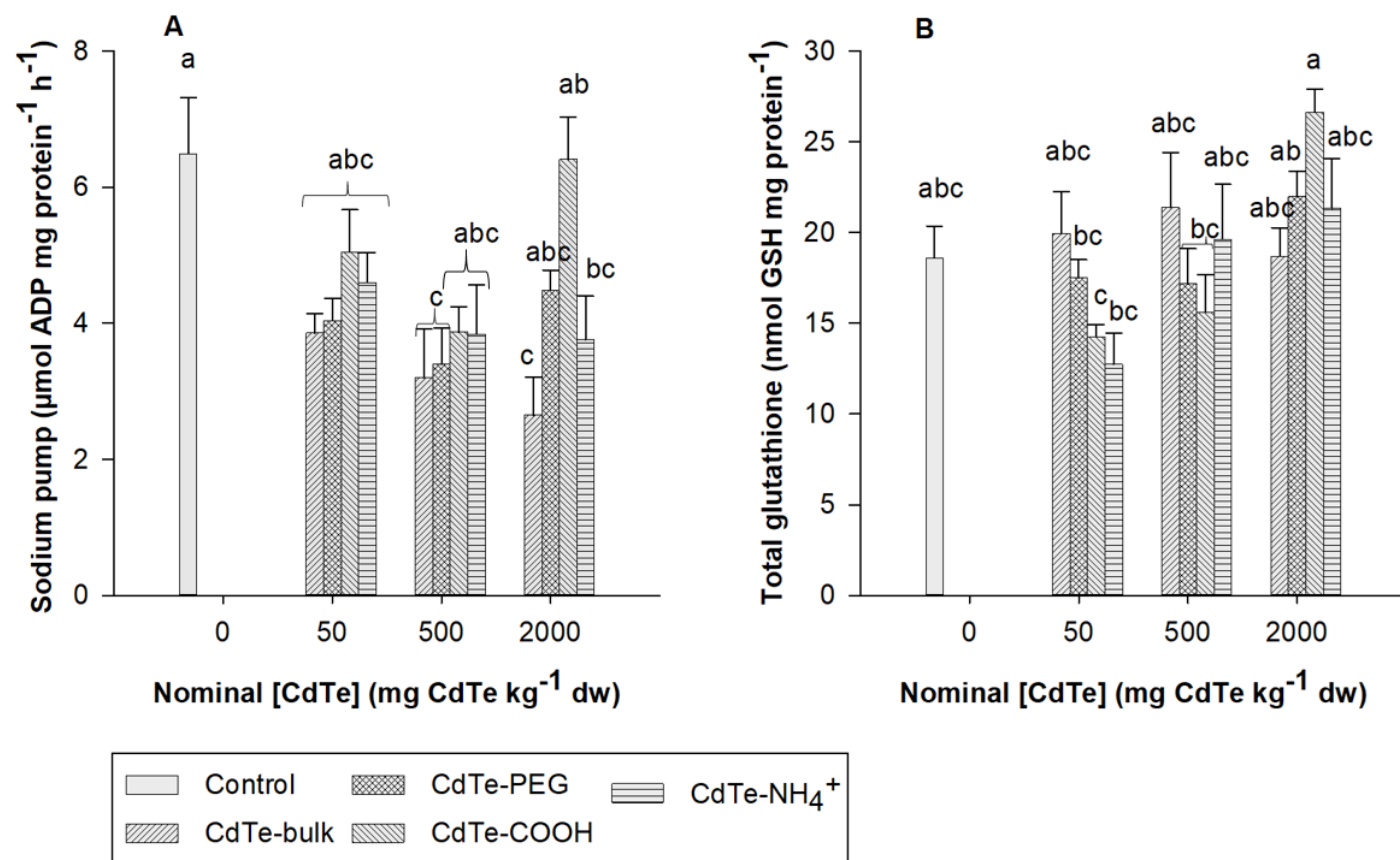
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1229 **Fig. 2** Relationship between the concentration of acid extractable Cd or Te (A or B)
 1230 and water extractable Cd or Te (C or D) and measured total Cd or Te concentration
 1231 in earthworm tissue in the fresh soil experiment. Data shown for nominal 2000 mg
 1232 CdTe kg⁻¹ treatment for each replicate in the fresh soil only ($n = 4$). Data points
 1233 represent mean values ($n = 2$ for soil samples, $n = 4$ for earthworm) for each
 1234 replicate ($n = 4$), error bars excluded for clarity.



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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).



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 1243 **Fig. 4** (A) Na⁺/K⁺-ATPase activity (sodium pump) expressed as ADP μmol released per mg protein per hour in earthworms in
 1244 the fresh soil experiment. (B) Total glutathione nmol per mg protein in the earthworms in the fresh soil experiment. Data is
 1245 presented as mean ± SEM (*n* = 8). Different letters denote statistically significant differences between treatments (*P* < 0.05,
 1246 ANOVA).