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3 **Determination of the bioaccessible fraction of cupric oxide nanoparticles in**
4 **soils using an *in vitro* human digestibility simulation.**

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24   **Abstract**

25   This study investigated the bioaccessible fractions (BAFs) of Cu from copper-based

26   nanomaterials present in soil to humans using an *in vitro* artificial simulation of the stomach;

27   followed by the simulation of the small intestinal environment. The work compared the

28   behaviour of coated and uncoated cupric oxide nanoparticles (CuO NPs) with $CuSO_4$ and the

29   equivalent bulk CuO, and earthworms as a potential surrogate of human bioaccessibility. The

30   calculated BAFs for the BGS 102 reference soil and the LUFA 2.2 soil (no added Cu) were $\leq$

31   40%. In contrast, the LUFA 2.2 Cu-dosed soils measured statistically significant higher mean

32   BAFs (ANOVA, $p < 0.05$); in general all above 60%. The calculated BAFs in the gastric phase

33   did not differ statistically amongst the materials tested, both at low and high Cu dosing

34   (ANOVA, $p > 0.05$). In the gastro-intestinal conditions, at the 200 mg Cu kg^{-1} soil concentration,

35   the calculated BAFs for $CuSO_4$, bulk and nano CuO were 76.6%, 72.7% and 83.4%,

36   respectively, and also did not differ statistically (ANOVA, $p > 0.05$). At the 1000 mg Cu kg^{-1} soil

37   concentration, only the coated CuO NPs measured BAFs > 80%; with the COOH- and PEG-

38   coated CuO NPs significantly more bioaccessible (ANOVA, $p < 0.05$) than all the other Cu-

39   based materials. In terms of human health risks from ingested soil, this study did not show

40   significant differences between soluble and particulate forms of Cu, but Cu concentrations from

41   the gastro-intestinal phase digestion of soil were predicted by earthworm Cu concentrations.

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45   Keywords: engineered nanomaterials, earthworms, copper, soil, BARGE

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Introduction

Engineered nanomaterials (ENMs) acquire some of their novel properties from a typically high surface area to volume ratio, which influences both the physical and chemical behaviours of the materials.[1] Their unique properties at the nanoscale, including quantum chemistry and enhanced reactivity, are underling innovations in nanotechnology; with a continual increase in the manufacturing volumes of ENMs. Nanomaterials have found applications in new electronics, industrial coatings, textiles, building materials, medicines, cosmetics, food packaging and in chemical/biological remediation. In particular, copper-containing ENMs have been proposed as additives in animal feeds[2] and as components of antifungal biocides for agriculture use.[3]

Inevitably, ENMs will enter the environment and the predicted concentrations in surface waters are in the low $\mu g \ l^{-1}$ range or less, depending on the type of material.[4] However, an important final sink for ENMs is the soil environment.[5] ENMs may find their way into soils directly through the application of nano-enhanced biocides or fertilisers, from atmospheric deposition, leaching from streams, and also accidental releases. However, a main concern for the fate of ENMs is the application of sewage sludge to agricultural soils; where environmental concentrations of ENMs in sludge-amended soil are expected to be around the $\mu g \ kg^{-1}$ range.[6] Worse case predictions in the $mg \ kg^{-1}$ range have also been reported for soils.[7] Unfortunately, the quantification of ENM release into the environment, especially in complex matrices such as soil, is very challenging[8] and there is a dearth of field measurements from natural soils to confirm any predictions.

Soil quality is important to the health of terrestrial ecosystems, for agriculture, and for human health with respect to food safety and the incidental ingestion of soil. Consequently, there are guideline values for allowable total metal concentrations in soils. Some countries have set guideline values for total copper (Cu) in soils (e.g., Canada, 63 - 91 $mg \ Cu \ kg^{-1}$, CCME[9]; Finland, 100 – 200 $mg \ Cu \ kg^{-1}$, MEF[10]); but as yet there are no guideline values for nano forms of Cu, despite some predicted concentrations (e.g., $CuCO_3$ ENMs, 32 – 100 $\mu g \ kg^{-1}$, Gottschalk $et \ al.$[11]). For Cu and other metals, the hazard to wildlife and human health from ingested soil is not from the total metal content of the soil, but the bioavailable fraction that may be taken up internally by the organism. From an environmental chemistry perspective, the dissolved metal in the pore water and any labile metal easily removed from the soil grains might be regarded as

81 bioavailable. For ingested soil, the bioaccessible fraction is also considered. The precise

82 distinction between 'bioavailable' and 'bioaccessible' fractions of contaminants is debated (e.g.,

83 Semple *et al.*[12]), but the bioaccessible fraction can be defined as the fraction released in the gut

84 lumen during digestion that has the potential to be taken up by the organism.[13] In the context of

85 human exposure to ingested soil, the bioaccessible fraction represents the maximum amount of

86 contaminant that is available for intestinal absorption. Regardless of the definitions, these

87 concepts were developed with the dissolved metal paradigm in mind, and as yet, it remains

88 unclear if these notions can also be applied to ENMs.

89 It is estimated that children ingest 100 mg of soil a day[14], and this is a concern for human

90 health risk assessments. Thus for the predictions of 100 μg kg^{-1} of Cu ENMs in soil above, this

91 might represent a daily ingestion of 0.01 μg in the nano form. Copper is an essential nutrient,

92 with humans requiring 1 - 2 mg of Cu day^{-1}, and under these normal circumstances the

93 bioavailability of Cu salts is around 30 - 40% of the dose.[15] However, the gut is a protective

94 barrier, and absorption declines exponentially with dose, so that only a few percent is

95 bioavailable across the gut in potentially toxic situations.[15,16] Whether or not nano forms of Cu

96 behave in this way is unclear, but for TiO_2 particles at least, the metal uptake rates across the

97 vertebrate intestine are consistent with a bioavailable fraction of a few percent of the dose.[17]

98 Of course, it is not possible to conduct human oral exposure studies on contaminants, and

99 for risk assessment purposes data on uptake of ENMs has been collected using oral gavage

100 studies in rodents;[18] or *in vitro* models such as Caco-2 cells[19] and perfused intestines.[17] Animal

101 studies should be limited in keeping with the ethical considerations of the 3Rs, but even the latter

102 *in vitro* approaches require considerable technical expertise and these methods have not yet been

103 standardised for regulatory toxicology. Alternatively, *in chemico* approaches that simulate the

104 digestive processes in the lumen of the human gut have been available for many years and

105 standardised with regulatory use in mind. The approach uses artificial saliva, gastric and

106 intestinal juices to mimic the human digestive system from the oral cavity through to the small

107 intestines; with adjustments of pH and additions of enzymes as appropriate for each region of the

108 gut.[20] The large intestine is not simulated in these models, as it is assumed that most of the

109 contaminant would be absorbed earlier in the digestive tract (an assumption not yet proven for

110 nano). Nonetheless, this approach also known as '*in vitro* digestibility' by the nutrition discipline

111 has been used to study metal releases from food,[21] and contaminated soils[22] so that the

112 bioaccessible fractions can be estimated. However, the simulated human digestion of soil has

113 not been established for ENMs.

114 The aim of the present study was to determine the bioaccessible fraction of Cu from

115 copper sulfate ($CuSO_4$) compared to pristine cupric oxide (CuO) ENMs and a bulk CuO powder

116 in soil. In addition, the effect of surface coatings on the ENMs was investigated using a range of

117 coatings on the common CuO core to represent anionic (carboxylate, COOH), cationic

118 (ammonium, NH_4^+) and neutral ligands (polyethylene glycol, PEG). To add some environmental

119 realism, a natural soil was used that had been subject to bioturbation by earthworms prior to the

120 determination of the bioaccessible fractions in the soil. The unified bioaccessibility research

121 group of Europe (BARGE) method[23] was selected for this work and adapted for ENMs. The

122 method involved a two phase *in chemico* digestion process to simulate, (i) the mouth conditions

123 and the low acidic environment of the human stomach, and (ii) the ensuing human upper

124 intestinal with very mild acidic conditions.

125

126 **Methodology**

127

128 *Soil preparation*

129

130 The exposure of the soils was conducted as part of an earthworm acute toxicity test using an

131 adaptation of OECD TG 207 for ENMs. The results of the earthworm ecotoxicity tests are

132 reported elsewhere,[24] and the focus here is on the soil chemistry. Briefly, the experimental design

133 included a control soil (no added Cu or ENMs), a metal salt control of Cu as $CuSO_4$ at 200 mg

134 Cu kg^{-1} dry soil weight, the uncoated CuO ENM, and those coated with $-NH_4^+$, $-COOH$ or $-PEG$

135 respectively. The precise details of how the coatings were synthesised and attached to the ENM

136 core is commercially sensitive information of the suppliers, but for clarity we use the term '–

137 NH_4^+' to mean an $-NH_3$ terminal ligand that has been ionised with H^+ ions to achieve positive

138 charge. The CuO ENMs were provided by PlasmaChem as part of the Nanosolutions EU project.

139 The microscale (bulk) CuO material was obtained from BDH Chemicals Ltd, UK, and the metal

140 salt ($CuSO_4.5H_2O$) from Sigma-Aldrich. The hypothesis was that the bioaccessibility potential of

141 the copper dosed in the soil may be affected by the material type (e.g., nano *versus* bulk material

142 or metal salt) and also coating-effects in case of the nanomaterials. For the ENMs, two test

143 concentrations were selected; a lower concentration of 200 mg Cu kg^{-1} soil representing around

144 three times the expected background concentration of total Cu in European soils (the latter, ~ 60

145 mg Cu kg^{-1}, Heijerick *et al.*[25]). An upper concentration of 1000 mg Cu kg^{-1} equivalent to that

146 suggested in the limit test for soil organisms according to OECD[26] was also used.

147   A standard sandy loam LUFA 2.2 (LUFA Speyer, Germany) soil was used with the

148 following composition (supplier's information, mean ± SD, dry soil, n = not specified): pH of 5.5

149 ± 0.2 (measured in 0.01 M CaCl$_2$ solution); organic carbon, 1.8 ± 0.2%; nitrogen content at 0.17

150 ± 0.02%; and cation exchange capacity, 10.1 ± 0.2 meq 100 g^{-1}). The water-holding capacity of

151 the soil was measured in-house and was 41.3 ± 3.0 g 100 g^{-1} dry weight. The soil used for the

152 earthworm tests was sieved through a 2 mm mesh and air dried at 25 °C. The soil pH was

153 measured prior to the start and at the end of the experiment (in a 1:1 soil: water slurry, using a

154 glass combination electrode, Corning 420), in addition to the metal composition (see below).

155   The ENMs, bulk CuO and CuSO$_4$ were mixed into the soil as dry powders by hand to

156 ensure the test substance was evenly distributed, and the soils were then wetted to 50 - 55%

157 water holding capacity (WHC) with ultrapure Milli-Q water (18.2 Ω). Four replicate boxes of

158 soil *per* treatment were prepared and the soil was left to equilibrate with the moisture for one day

159 prior to adding the earthworms. Adult *Eisenia fetida* (Savigny, 1826) with a typical mean starting

160 wet weight of 5.5 ± 0.1 g (mean ± S.E.M, for a subsample of 12 of the initial earthworms) were

161 exposed in 4 replicates (n = 12 earthworms *per* box, n = 48 earthworms *per* treatment) at 20 ± 1

162 °C at 12:8 light:dark cycle.

163   After 14 days in the earthworm tests, approximately 30 g of wet soil was collected from

164 each box and weighed (Sartorius BP 210) into previously acid washed (5% (*v/v*) nitric acid,

165 Fisher, Primer Plus Trace Metals Analysis Grade) and deionised ceramic drying boats. The soil

166 samples were then dried to constant mass at 85 °C (Gallenkamp OV-160), allowed to cool to

167 room temperature, and sieved to < 250 μm. The particle size fraction was chosen to represent the

168 upper limit that is likely to stick to infants' hands.[27]

169   In addition to the natural soils from the earthworm tests, BGS Guidance Material 102,[28]

170 which is an ironstone soil from Lincolnshire, England was also used to validate the analytical

171 chemistry. This reference soil had not been used in the earthworm tests. In order to ensure

172 complete soil re-homogenisation, the BGS 102 soil sample bottle was shaken manually for a few

173 minutes before it was opened. The soil reference samples were digested and chemically analysed

174   strictly using the same approach applied to the LUFA 2.2 soil samples, but without any additions

175   of Cu materials.

176

177   ***Characterisation of Nanomaterials***

178

179   The characterisation included measurements of the primary particles sizes, the dispersion of the

180   particles in ultrapure water and dialysis experiments to assess any dissolution of dissolved Cu

181   from the particles. The CuO NPs were first examined using transmission electron microscopy

182   (TEM, JEOL-1200EX II) for the primary particle size. Fresh stock suspensions, at 100 mg l^{-1}

183   nominal concentration, were prepared in Milli-Q water and sub-samples were examined visually

184   with $n = 60$ measurements of particle diameter *per* sample (conducted manually using ImageJ).

185   The particle size distribution of the ENMs in the stock dispersions were also measured by

186   nanoparticle tracking analysis (NTA) using a Nanosight LM 10 (Malvern Instruments, UK).

187   Three sub-samples from each of the fresh stock suspensions were vortexed for 10 s immediately

188   before analysis by NTA (Table 1).

189   Dialysis experiments were conducted in Milli-Q water at room temperature to measure

190   the degree of copper metal ion dissolution from all the ENMs. Dialysis bags were filled with 8

191   ml of the appropriate test suspension at 100 mg l^{-1} nominal concentration, and suspended in a

192   600 ml beaker containing 492 ml of Milli-Q water (in triplicate beakers). Samples of 1 ml were

193   taken from the external compartment of the beaker at time zero, 30 min, 1, 2, 3, 4, 6, 12 and 24 h

194   for total Cu determination by ICP-OES or ICP-MS as appropriate. The data were subsequently

195   fitted to a rectangular hyperbola (using SigmaPlot 13), and the maximum initial dissolution rate

196   calculated from the maximum slope.

197

198   ***Aqua regia acid digestion of the soils, earthworms and nanomaterials***

199

200   This was performed so that the total copper concentrations in the soil samples could be

201   determined in order to facilitate calculations of the percentage of bioaccessible fractions. Briefly,

202   a*qua regia* was prepared by adding 1 volume of $> 68\%$ concentrated nitric acid to 3 volumes of

203   concentrated ~37% hydrochloric acid; both acids were of trace metal analysis grade (Fisher).

204   This acidic mixture was allowed for a few minutes to develop into a golden coloured solution.

205 Then 10.0 ml of the *aqua regia* mixture was gently added into each 50 ml polypropylene tube

206 containing 0.3 g of accurately weighed dried, sieved soil ($n = 2$ technical replicates, in

207 accordance to INERIS[23]) from each box ($n = 4$ boxes) *per* treatment or control exposure. Two

208 blank samples (without any soil) were analysed with every set of unknown samples. The tubes

209 were heated with gentle mixing for 15 hours in a water bath set at 50 °C. At the end of the

210 heating time, each tube was mixed and its contents were allowed to cool down. Afterwards, 1 ml

211 samples were taken from the clear upper part of each tube and diluted with 4 ml of 0.1 M nitric

212 acid.

213   Acid digestion of the earthworms following day 7 and day 14 soil exposure is described

214 elsewhere.[24] In addition, the original ENMs, bulk CuO and the equivalent metal salt, as dry

215 powders, were also acid digested to verify their metal content. A known amount of powder ($n =$

216 3) was accurately weighed into a 20 ml polypropylene tube; three additional empty tubes with no

217 material added to them were included as blanks. To each tube, 10.0 ml of the *aqua regia* mixture

218 was gently added, followed by the same acid digestion method (see above) used for the soil

219 samples.

220

221 ***Preparation of the synthetic gastro-intestinal digestive fluids***

222

223 All the reagents used for the copper bioaccessibility determination in soil were of analytical

224 grade, and are listed in Supplementary Table S1 for each type of synthetic fluid. The pH meter

225 (Thermo Scientific Orion 2-Star Plus meter fitted with a Russell combination electrode) was

226 precisely calibrated to pH 4.0, 7.0 and 10.0 at the start of each experiment. The synthetic

227 digestive fluids (saliva, gastric, duodenal, bile) were prepared using sterile glass distilled water

228 (distilled from ion-free ultrapure water, 18 MΩ resistance). The different fluid components

229 (inorganic and organic, respectively) were placed separately on a magnetic stirring (IKA-

230 WERKE R015) set at speed 3 for at least 3 h to ensure adequate mixing of each solution. Then,

231 each digestive fluid was prepared by combining 250 ml of the inorganic and 250 ml of the

232 organic components solutions, and with the addition of enzymatic components. Once the

233 digestive fluid components were all mixed together, the fluids were allowed to acclimatise and

234 stir for an hour at 37 °C before use. Fig. S1 shows the serial additions of each type of fluid in

235 relation to the steps in the digestion method.

236

Gastric phase and gastro-intestinal phase digestion

238

An outline of the adapted *in vitro* gastro-intestinal digestion protocol from INERIS[23] is depicted

in Fig. S1. As for the *aqua regia* acid digestion method (see above), 0.3 g of dried, sieved soil

was used for each bioaccessibility digestion (gastric phase and gastro-intestinal phase,

respectively). The same order of statistical replication (*n* = 2 technical soil replicate samples for

each soil box) was also followed for each digestion phase, including the use of two blanks.

244

Total copper determination

246

The total copper concentration in the samples following *aqua regia* digestion or the adapted

BARGE methods were determined by inductively coupled plasma optical emission

spectrophotometry (ICP-OES, Thermo Scientific, iCAP 7000 Series), or equivalent mass

spectrophotometry (ICP-MS, Thermo Scientific, X Series 2). The instrument detection limit for

the ICP-OES was 0.008 mg l^{-1} Cu and for the ICP-MS was 0.003 mg l^{-1} Cu. Briefly, samples

were acidified, matrix-matched to the ICP-OES/ICP-MS standard metal solutions used for

calibration, with 0.8 mg l^{-1} yttrium as an internal standard. Sample blanks were included every

10 samples in each run of the instruments.

255

Calculation of the bioaccessible fractions

257

The bioaccessible fraction (BAF) was calculated as a percentage of the total metal for each box

from the earthworm study, and for the BGS reference soil using Eq. (1);

260

$$BAF\ [\%] = \frac{\text{Cu}_{bioaccessible}\ [\text{mg Cu kg}^{-1}\ \text{soil}]}{\text{Cu}_{total}\ [\text{mg Cu kg}^{-1}\ \text{soil}]} \times 100 \tag{1}$$

262

where Cu $_{bioaccessible}$ was the mean total copper concentration measured in the soil samples (*n* = 2

technical replicates within each soil sample from each box), following separately either the

gastric phase or the gastro-intestinal phase digestion, and Cu $_{total}$ referred to the mean total

266 copper concentration measured from the *aqua regia* acid digestion ($n = 2$ technical replicates

267 within each soil sample from each box).

268

269 ***Statistical analysis***

270

271 The data are shown as mean ± standard error of the mean (S.E.M). The coefficient of variation

272 (CV) was also calculated to describe the resultant percentage variability amongst the BAF values

273 determined from the separate soil boxes ($n = 4$ boxes *per* treatment). All statistical analyses were

274 carried out using IBM SPSS Statistics 22 and Microsoft Excel 2010. Following descriptive

275 statistics, the Kolmogorov-Smirnov test was used to assess the normality of the distribution of

276 data. Independent student *t*-tests and one-way analysis of variance (ANOVA, Tukey *post hoc*

277 test) were used to check for significant differences amongst responses from within each test

278 material and treatments. In instances where the data was not normally distributed, the non-

279 parametric Mann-Whitney U test was used to assess differences between two independent

280 groups. Likewise, the Kruskal-Wallis test was used as an alternative to a one-way between-

281 groups analysis of variance. Figures were prepared using SigmaPlot 13.

282

283 **Results**

284

285 ***Particle characterisation and the total measured copper content in the test materials***

286

287 The total measured Cu concentration in the different test materials as original powders is

288 presented in Table 1, along with the details of purity, primary particle size and surface area of the

289 materials investigated. The primary particle sizes of the test materials, as measured by

290 transmission electron microscopy (TEM) images, were not found to exceed the manufacturer's

291 reported size range (10 - 20 nm). Following dispersion of the test materials in water, the mean

292 hydrodynamic diameter of the aggregates, as measured by nanoparticle tracking analysis were:

293 41 nm in the uncoated CuO NPs, 46 nm in the ammonium-coated CuO NPs, 121 nm in the

294 COOH-coated CuO NPs and 100 nm in the PEG-coated CuO NPs. The dialysis experiments

295 revealed some Cu dissolution from the different CuO NPs in ultrapure water. The dissolution

296 rate of the uncoated CuO NPs was low (1.68 µg Cu h^{-1}, Table 1), but in comparison, all the

297 coated CuO NPs had higher dissolution rates; greater than 18 μg Cu h^{-1} (Table 1). However, the

298 rates were still micromolar, and even the highest rates would only equate to around 6 – 9% of the

299 total metal being released every hour.

300   On a mass basis of each material, the Cu content of material varied according to the

301 proportion of mass attributed to the coating. As a result of their chemical composition, less total

302 Cu was measured in the coated ENMs relative to the uncoated form (Table 1). For the coated

303 CuO NPs, the NH_4^+-coated NPs were found to contain the highest measured fraction of Cu

304 (0.52), followed by the COOH-coated NPs (0.43) and least Cu in the PEG-coated NPs (0.29).

305 Overall, the total Cu measurements in strong acid digests from the initial ENMs were reliable

306 with low coefficients of variations between replicates. Within-sample precision (triplicate

307 readings from the same sample) produced coefficient of variation values (CVs) ranging from

308 0.6% in $CuSO_4$ to 8.7% in the uncoated CuO NPs. The actual measured concentrations were

309 always a little less than the nominal concentrations. However, the calculated percentages, of the

310 actual measured concentrations relative to the nominal concentrations, for $CuSO_4$, bulk- and

311 nano-CuO were all above 85%.

312

313 ***Total measured copper concentrations in soil***

314

315 The exposure was confirmed by the measured total copper following the *aqua regia* digestion of

316 the soil samples (Fig. 1). The results of precision testing following the *aqua regia* acid digestion

317 in soil are presented in Supplementary Table S2, and the reproducibility of measurements

318 between boxes of soil was good. The unexposed control soils, without any addition of copper

319 (LUFA 2.2 soil and BGS 102 soil reference material), showed low Cu concentrations, as

320 expected (Fig. 1). All the Cu-dosed soils showed an increase in Cu concentration (Fig. 1) that

321 was consistent with the material types presented, and the relative proportions of Cu on a mass

322 basis expected in the different particle forms (Table 1). From correlation analysis (Fig. S2), a

323 positive relationship was also clear between the nominal and actual measured Cu concentrations

324 in soil following *aqua regia*, gastric and gastro-intestinal soil digestion. Furthermore, from all

325 soil extractions (Fig. 1), a statistical significant higher mean concentration of Cu in soil

326 (ANOVA, $p < 0.05$) was consistently measured in the *aqua regia* digests, as compared to the

327 gastric and the gastro-intestinal digests (milder digestion methods).

328   At the lower nominal 200 mg Cu kg^{-1} soil concentration (Fig. 1A), the measured

329   concentrations of Cu in the soil were not statistically different (ANOVA, $p > 0.05$) between the

330   gastric and the gastro-intestinal digests; with the exception of the uncoated CuO NPs dosed soils

331   that measured a higher mean concentration of Cu following the gastro-intestinal phase digestion.

332   At the 1000 mg Cu kg^{-1} soil concentration (Fig. 1B), the measured mean Cu concentrations from

333   bulk CuO and the uncoated CuO NPs in the soil digests, following the gastric phase and the

334   gastro-intestinal phase digestion did not differ (ANOVA, $p > 0.05$). In contrast, all coated CuO

335   NPs digests were found to have higher levels of Cu following the gastro-intestinal phase

336   digestion, in comparison with the gastric phase digestion.

337

338   *Calculated bioaccessible fractions*

339

340   A relatively high Cu concentration measurement in soil was not found to necessarily represent a

341   greater bioaccessibility potential for that metal in soil, as evident from the gastro-intestinal

342   digestions (Fig. 2). At both the 200 and 1000 mg Cu kg^{-1} soil concentration, higher overall BAF

343   values were calculated from the gastro-intestinal phase relative to the gastric phase digestion

344   (Fig. 2, Table S3). However, in terms of the individual Cu-materials tested (Fig. 2) there were a

345   few statistical differences between the calculated gastric and gastro-intestinal BAFs (*t*-test, $p <$

346   0.05). Mean BAFs greater than 80% were only recorded in the gastro-intestinal phase digestion

347   (Fig. 2, Table S3) for the uncoated CuO NPs (low dose exposure) and in the COOH-, PEG- and

348   NH$_4^+$-coated CuO NPs (high dose exposure).

349

350   *Gastric phase digestion BAFs*

351

352   Following the gastric phase digestion, the calculated mean percentage BAFs for the control BGS

353   102 soil and the LUFA 2.2 soil (with no added copper) were 35.3% and 38.8% respectively

354   (Table S3, Fig. 2). These two mean BAF values were not significantly different (ANOVA, $p >$

355   0.05); whereas all LUFA 2.2 soils dosed with Cu were found to have much higher percentage

356   BAF values, ranging from a minimum of 53.5%, a maximum of 98.1% and a median value of

357   69.3%. Irrespective of the initial nominal soil input Cu concentration (low or high), all soil

358 treatments with CuO NPs, or with bulk CuO or copper sulfate in the gastric phase digestion (Fig.
359 2) did not differ significantly in their calculated percentage BAF (ANOVA, $p > 0.05$).
360
361 ***Gastro-intestinal phase digestion BAFs***
362
363 The calculated Cu BAF values in soil following the gastro-intestinal phase digestion are in
364 general comparable to the outcome following the gastric phase digestion (Figs. 2A and B).
365 However, a greater distribution of the percentage BAF values was evident from the gastro-
366 intestinal phase digestion. Percentage BAFs ranged from a minimum of 38.7%, a maximum of
367 96.7% and a median of 73.4%. The mean Cu percentage BAFs determined for bulk CuO and the
368 uncoated CuO NPs in the gastro-intestinal phase digestion, did not differ statistically between the
369 low and high Cu dosage (ANOVA, $p > 0.05$); but higher mean BAF values were calculated at the
370 200 mg Cu kg^{-1} soil concentration (Figure 2A). The opposite was true for all coated CuO NPs,
371 where higher mean percentage BAFs resulted at the 1000 mg Cu kg^{-1} soil concentration (Fig.
372 2B).
373
374 ***Calculated BAFs relative to metal dissolution and uptake in earthworms***
375
376 In Fig. 3, the concentration of total Cu in the soil (Fig. 3A) and the percentage of bioaccessible
377 fraction (Fig. 3B) for the gastro-intestinal phase were plotted against the maximum dissolution
378 rates of copper following dialysis experiments of the different test materials in Milli-Q water.
379 Dissolution rate was inversely related to gastro-intestinal phase Cu concentration, with the trend
380 most evident at the soil dose of 1000 mg Cu kg^{-1} soil (Fig. 3A). However, there was no clear
381 relationship between the percent of BAF and metal dissolution at the 200 mg Cu kg^{-1} soil
382 concentration (Fig. 3B). At the higher soil exposure dose, an increase in metal dissolution rate
383 coincided with higher measurements of gastro-intestinal BAFs, in the order of: uncoated CuO
384 NPs, NH$_4^+$-coated CuO NPs, PEG-coated CuO NPs and COOH-coated CuO NPs (highest).
385   In contrast to the dissolution rate data, there was a clear correlation between the gastro-
386 intestinal phase Cu concentration and the Cu concentration in the earthworms (Fig. 3C), with an
387 r^2 value of 0.94 for all the data, regardless of exposure concentration. However, this relationship
388 was lost when the data were presented as the calculated gastro-intestinal BAFs and Cu

389 concentrations in the earthworms following 14 days of exposure (Fig. 3D). At the 200 mg Cu kg$^-$

390 ^{1} soil concentration, no clear pattern was evident between the calculated BAFs and earthworm

391 copper concentration. However, at the higher exposure dose in the soil (1000 mg Cu kg^{-1} soil

392 concentration). The measured BAF values for the COOH-, NH$_4^+$-coated CuO NPs and the

393 uncoated CuO NPs were however inversely proportional to the measured Cu content in

394 earthworms. The order of measured metal concentration in the earthworms by coating also

395 coincided with the relative amount of copper in the different test materials (see Table 1).

396

397

398 **Discussion**

399

400 This study reports the BAF of Cu from cupric oxide nanoparticles with different surface

401 coatings, compared to the metal salt and bulk powder controls, using the *in vitro* human gastro-

402 intestinal BARGE method. Overall, the data shows that there is a bioaccessible fraction of Cu

403 (form unknown) from all the materials tested, and this broadly follows the notion of dose-

404 response with more total metal available at the higher nominal concentrations in the soil.

405 Crucially, there was a material-type effect that was also dependent on the phase of digestion. In

406 the gastric phase, the BAF were similar at around 70% of the total metal, regardless of the

407 material tested. However, in the gastro-intestinal phase some material-type effects were revealed;

408 with the CuO NPs sometimes having higher BAFs than the metal salt. While the BAF values

409 correlated well with the original total measured Cu concentrations in the soil, they were not

410 easily explained by the dissolved metal paradigm. For the nanomaterials, there was no

411 correlation between the BAF and the dissolution rate of the particles. Moreover, the

412 bioaccumulation pattern in earthworms from the same soils did not correlate easily with BAF

413 values either. Only when the absolute Cu concentration from the gastro-intestinal phase was

414 plotted against the Cu concentration in the earthworms was a correlation revealed; indicating that

415 metal concentration in earthworms might be a possible surrogate of the human health risks from

416 ingested soil for these nanomaterial.

417

418 ***Validation of the unified BARGE method for ENMs***

419

420 The unified BARGE method is a relatively well-standardised and validated method for

421 determining the bioaccessible fractions of metals in soils. The BARGE method was originally

422 devised with the concern for metal exposure associated with incidental soil ingestion in humans,

423 especially children, in mind.[22] It has since been used to test a variety of soils for bioaccessible

424 metals.[29-31] However, it has not been specifically validated for ENMs. The current investigation

425 attempted to validate the BARGE method using several approaches including: (i) measuring the

426 BAF for Cu metal in a BGS 102 soil reference; (ii) determining the BAF for $CuSO_4$ in a well-

427 known LUFA 2.2 soil, and then, (iii) exploring the within sample and between sample

428 reproducibility of the BARGE method for $CuSO_4$ compared to the ENMs.

429   The performance of the BGS 102 soil reference material was considered first. This soil

430 already contains some naturally occurring Cu, and so no additional Cu amendments were

431 necessary. The measured total Cu in this soil was 17 mg kg^{-1} in comparison to 26 mg kg^{-1} from

432 Wragg[28] (see Table S2). In addition, the BAF values were 35% in the gastric phase and 40% in

433 the gastro-intestinal phase for Cu (Table S3). These BAF values are entirely consistent with

434 previous findings from Hamilton *et al.*[32], with a mean reported Cu BAF of 33%. Furthermore,

435 measurements of the BGS 102 reference soil were within acceptable limits for a standard

436 method, with coefficients of variation being 10 % or much less (Table S3).

437   The LUFA 2.2 soil is also relatively well-known and has been widely used in soil

438 ecotoxicity testing with earthworms. Its natural mean Cu content is low (3 mg Cu kg^{-1} soil, Table

439 S2), in agreement with measured metal concentrations in uncontaminated soils.[33] Criel *et al.*[34]

440 reported a background total Cu concentration in the LUFA 2.2 soil of 6 mg Cu kg^{-1}. In the

441 present work, the measured BAFs in the LUFA 2.2 soil (32 - 39%) were comparable with the

442 calculated BAFs of the soil reference BGS 102 soil (Fig. 2), and other studies from natural

443 uncontaminated soils.[29]

444   Engineered nanomaterials do not behave in the same way as solutes,[35] and as 'difficult to

445 handle' substances, they present a number of challenges to the standard methods used in

446 regulatory testing (reviews, Handy *et al.*[36]; Selck *et al.*[37]). One concern is whether or not the sum

447 of the difficulties in maintaining the exposure, detailing the heterogeneous nature of the materials

448 in biologically-relevant matrices such as soil, and any losses during the analytical procedures for

449 detecting ENMs, etc., cause such high variation between replicates to the extent that the overall

450 attempt at standardisation fails. Of course, the notion of acceptable deviation in a standardised

451   method depends on the context. In this study, the LUFA 2.2 soil was amended with additions of

452    $CuSO_4$, bulk CuO and CuO NPs, respectively. The behaviour of Cu^{2+} ions in soil is relatively

453   well-known and the analytical methods for measuring total Cu in soil samples is established. The

454   between sample deviation reflected this with CVs ranging between 8 - 13% for the calculated

455   BAF values for $CuSO_4$ (Table S3). Furthermore, despite the challenges of handling ENMs, the

456   CVs for the particulate forms of Cu were in the same range (Table S3). The only exceptions were

457   the CuO-PEG NPs which showed variations as high as 26%, and the bulk CuO material with

458   CVs ranging between 7 - 26%. While these latter variations are not as low as one would prefer

459   (ideally, <10 %), they are not beyond acceptability from the view point of standardised protocols

460   for environmental testing. For example, the Organisation for Economic and Cooperation and

461   Development (OECD), allows a 20% deviation in the measured test concentrations in valid acute

462   ecotoxicity tests,[36] even greater deviations in test methods are allowed for 'difficult to handle'

463   substances that are not miscible with water.[38]

464

465   ***The bioaccessibility of copper sulfate in soil***

466

467   There are many studies that use the BARGE method or similar approaches to measure

468   extractable Cu from metal-contaminated soils (review, De Miguel *et al*.[30]). However, to our

469   knowledge, only the present study has specifically assessed bioaccessible fractions of Cu from

470    $CuSO_4$ dosing to the LUFA 2.2 soil using the BARGE method. Soil dosing with $CuSO_4$ at the

471   1000 mg Cu kg^{-1} soil concentration was not undertaken here, as the metal salt at such high doses

472   is known to be toxic to invertebrates, including earthworms.[39] At the 200 mg Cu kg^{-1} soil

473   concentration, as anticipated, the measured total Cu from $CuSO_4$ was close (99%) to the nominal

474   concentration (Table S2). The calculated mean BAF for the metal salt (Table S3, Fig. 2) did not

475   differ (*t*-test, $p > 0.05$) between the gastric (73%) and the gastro-intestinal phase digestion (77%).

476   These values are much higher than found by simpler $CaCl_2$-extractable Cu measurements in

477   contaminated LUFA 2.2 soil where only about 30% or less of the Cu is labile.[40] The fact that the

478   Cu from $CuSO_4$ was predicted as bioaccessible to both the stomach and the intestines is not

479   surprising given the solubility of the metal salt. However, the uptake of dissolved Cu by the gut

480   also depends on the anatomical locations of the necessary Cu transporters in the gut epithelium.

481   Pharmacological studies with gut preparations of vertebrate animals show it is the intestines, not

482 the stomach involved in Cu uptake, and that the uptake mechanisms include a luminal chloride-

483 dependent pathway.[16] Similarly, *in vivo* studies with rodents using radiolabelled Cu show the

484 intestine as the main location for dietary Cu uptake.[41] Thus for Cu, the BAF does not necessarily

485 indicate a hazard, only that the metal may become hazardous if the BAF is present in the

486 intestines where the Cu transporters occur.

487

488 ***Are particulate forms of Cu more bioaccessible than CuSO$_4$?***

489

490 There have been some studies on the dissolution of ENMs in the presence of gastro-intestinal

491 fluids. For example, with Ag NPs,[42,43] silica NPs[44] and CdSe QDs.[45] However, these studies were

492 more focused on the physico-chemical properties and aggregation behaviours of the ENMs in the

493 digestive juices. In the gastric phase, the calculated BAFs from all the test materials, including

494 the metal salt, were not statistically different and remained around 70% (Fig. 2). There was no

495 evidence of any difference between the particles and the metal salt that might infer a particle

496 size-effect, and no particle-coating effects (Fig. 2, Table S3). Arguably, this observation for the

497 stomach could be explained by the strong acid (pH < 1.5) simply dissolved the different

498 materials at similar rates; regardless of their surface areas or aggregate sizes (Fig. 1). The rapid

499 dissolution of Cu NPs at low pH has also been observed in studies with freshwater fish,[46] in acid-

500 extractions of soil during earthworm studies,[24] and at low pH in the physiological salines used

501 for oral gavage in rodents.[47] Thus, *in vivo* the Cu NP are likely transformed into soluble Cu in the

502 stomach, which is then absorbed in the intestine, and then can accumulate in the internal organs

503 and have toxic effects, albeit with some slight delay compared to oral gavage with the metal

504 salt.[47]

505 However, the Cu bioaccessibility from soil was generally was similar for each substance

506 in both the acidic gastric phase and the neutral gastro-intestinal phase (Fig. 2, Table S3). There

507 was no metal salt and or nanomaterial effects, apart from the CuO-PEG material which showed

508 less bioaccessibility in the gastro-intestinal phase at the 200 mg Cu kg^{-1} soil concentration (Fig.

509 2A). The reduction was only a few % change, and one might argue that this is of limited

510 biological importance. However, the mechanism behind this effect for a PEG-coated material is

511 unclear. It might be that the PEG is more stable at neutral pH and vulnerable to some acid

512 degradation in the gastric phase. Regardless, this reduction in BAF is also consistent with the

513   same CuO-PEG material causing only moderate Cu accumulation in earthworms ingesting

514   contaminated soil compared to the other coatings after 14 days, and no appreciable mortality.[24]

515   At the 1000 mg Cu kg^{-1} soil concentration, the BAF values were greater for the coated

516   CuO NPs in the gastro-intestinal phase relative to the gastric phase; and compared to the

517   uncoated CuO and the bulk material (Fig. 2B). This apparent effect of the coated materials to be

518   more accessible in the gatro-intestinal phase needs more investigation, but might be explained by

519   the nanoparticle coatings absorbing (electrostatic attraction in the case of –COOH) or becoming

520   associated with (e.g., by steric hindrance in the case of –PEG) macromolecules present in the

521   gut digestive juices such as proteins. This might, in theory, render their surfaces more

522   bioaccessible than that of the uncoated NPs, through the rapid initiation of a corona.[48] *In vivo*,

523   the CuO-COOH was the most toxic to earthworms in fresh soil,[24] in keeping with it also being

524   one of the more bioaccessible forms here in the soil. Unfortunately, as yet, there are no *in vivo*

525   studies with mammals to confirm if the coating-effects for CuO NPs observed in earthworms

526   might also apply to humans.

527

528   ***Conclusions and implications for human health risk assessment***

529

530   Human health risk assessment from soils considers the total and the bioaccessible fraction. The

531   BARGE approach used here gave the expected findings for reference soils and those spiked with

532   CuSO$_4$. The methodology also performed well for CuO-based NPs. This study has shown that

533   the bioaccessible fractions of Cu are similar for CuSO$_4$, the CuO bulk material and most of the

534   forms of the CuO NPs (Fig. 2); suggesting that the existing human health risk assessment for the

535   ingestion of Cu in soil may also be protective of particulate forms. For the coated CuO NPs, the

536   bioaccessible fraction was greatest at the high exposure concentration; implying that the BAF is

537   not fixed for nanomaterials, but dependent on dosimetry. The greatest hazard was arguably

538   presented in the gastro-intestinal phase at the highest concentrations of the different CuO NPs

539   used, where BAF values were around 70% or more (Fig. 2B). The BARGE method here is a

540   simulated digestion in the human gut without food, and therefore represents a worst case

541   scenario for potential uptake. It is also unfortunate that the greater bioaccessibility in the gastro-

542   intestinal phase is also coincident with the intestinal location of copper transporters involved in

543   absorption across the gut.[49] However, it is clear that the dissolved fraction of the metal from the

544 particles did not correlate easily with the BAF values in the present study. This strongly suggests

545 that the bioaccessible fraction includes a particulate load.   Further work is needed to confirm

546 this and the gastrointestinal locations of any CuO NP uptake *in vivo*. Finally, the BARGE

547 method here is intended for human health risk assessment, and this might therefore be followed

548 by *in vivo* dietary studies on mammals when a concern for bioaccessibility has been identified.

549 From an animal welfare perspective, dietary toxicity tests on vertebrate animals are to be avoided

550 where possible. The alternative approach of using surrogate soil organisms such as earthworms

551 to predict the dietary bioaccessibility of metal in soil to humans has some merit. Button *et al*. [50]

552 found that BAF values for arsenic in soils correlated with total As accumulation in earthworms.

553 Similarly in the present study, the measured Cu remaining in the soil following the gastro-

554 intestinal phase correlated with the Cu concentrations in the worms (Fig. 3C). However, care

555 must be taken with the choice of data as the Cu accumulation in the earthworms was not

556 predictive of the calculated gastro-intestinal BAF when expressed as percentages (Fig. 3B). Only

557 the measured concentrations should therefore be used in any read across attempt from

558 earthworms to humans for the human health risk assessment for soils.

559

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723 **Table 1** Test materials characterisation from the original powders.

Test material (Supplier)	Manufacturer's information	[1]Measured primary particle size (nm)	[2]Measured hydrodynamic diameter in ultrapure Milli-Q water (nm)	[3]Total measured copper concentration (mg l^{-1})	Between-replicate percentage CV (%)	[4]Percentage of nominal concentration (%)	[5]Measured copper fraction in coated CuO NPs	[6]Metal dissolution rate in water (µg Cu h^{-1})
CuSO$_4$.5H$_2$O, CAS 7758-99-8 (Sigma-Aldrich 31293 , Lot SZBC0170V)	Purity, 99 - 102%	--	--	102.7 ± 0.4	0.6	100.8 ± 0.4	--	---
CuO Bulk, CAS 1317-38-0 (British Drug Houses Ltd)	Analar grade	---	---	285.0 ± 13.4	8.1	89.1 ± 4.2	--	---
#CuO NPs uncoated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF1309121)	99% purity; diameter, 10 - 20 nm; $surface area 42 ± 2 m^2 g^{-1}	12.00 ± 0.37	41 ± 28	287.1 ± 14.4	8.7	89.7 ± 4.5	--	1.68
#CuO NPs COOH-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	99% purity; diameter, 10 - 20 nm; $surface area, 7.4 ± 0.5 m^2 g^{-1}	6.45 ± 0.16	121 ± 91	154.3 ± 6.9	7.7	-	0.43 ± 0.02	69.12
#CuO NPs NH$_4^+$-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	99% purity; diameter, 10 - 20 nm; $surface area, 6.1 ± 0.5 m^2 g^{-1}	9.53 ± 0.22	46 ± 36	185.9 ± 7.2	6.7	-	0.52 ± 0.02	18.6
#CuO NPs PEG-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	99% purity; diameter, 10 - 20 nm	7.46 ± 0.42	100 ± 36	105.0 ± 3.5	5.8	-	0.29 ± 0.01	52.02

#Supplied as dry powders, bespoke design and production of spherical particles for the NANOSOLUTIONS project *via* Alexei Antipov, PlasmaChem GmbH; $Brunauer–Emmett–Teller (BET) surface area values (mean ± one standard deviation, $n = 3$) from NANOSOLUTIONS project; [1]Based on transmission electron microscopy (TEM) images of CuO ENMs from a 100 mg l^{-1} Cu stocks in Milli-Q water where data are mean ± standard error of the mean (S.E.M) with $n = 60$ measurements; [2]Particle size distribution measurements (mean ± one standard deviation, $n = 3$) by Nanoparticle tracking analysis (NTA) on 100 mg l^{-1} Cu ENM stocks in Milli-Q water; [3]Data are means ± S.E.M ($n = 3$ replicates) of total measured copper concentration by ICP-OES following *aqua regia* acid digestion of the dry powders, and after normalisation to an initial 0.02 g weight of material; Cupric oxide nanoparticles (CuO NPs); Coefficient of variation (CV); [4]With a 0.25 fraction of copper by weight in CuSO$_4$.5H$_2$O, and 0.80 fraction of copper in both CuO bulk and uncoated CuO NPs; [5]Relative to the measured copper content in the uncoated CuO NPs; [6]Maximum slope from rectangular hyperbola function of curve fitting used to estimate the maximum rate of dissolution of copper from the dialysis experiments, in triplicate;.- Not possible to calculate from the manufacturer's information on material composition; -- Data not applicable to the test material; --- Not measured.

724

Figure Legends

726 **Fig. 1** Total measured copper concentration (mg Cu kg^{-1}) in dry soil, from the different treatment

727 exposures at day 14 in the earthworm tests, following *aqua regia* acid digestion, gastric phase

728 and the gastro-intestinal phase digestion, respectively; (A) at the 200 mg Cu kg^{-1} soil initial

729 nominal dosing and (B) at the 1000 mg Cu kg^{-1} soil initial nominal dosing. Materials labelled as

730 BGS102 soil and LUFA 2.2 soil, refer to control soils (no added Cu or ENMs). The BGS 102

731 soil was not used in the earthworm tests, but solely included to validate the analytical chemistry.

732 Data are mean ± S.E.M ($n = 8$). Different letters indicate significant differences within each

733 material (ANOVA, $p < 0.05$). Observed differences in soil measured Cu amongst the test

734 materials, with varied initial relative mass proportion of Cu, are not identified with statistical

735 labels.

736 **Fig. 2** Calculated percentage bioaccessible fraction (BAF) from the different treatment exposures

737 at day 14 in the earthworm tests, following the gastric phase and the gastro-intestinal phase

738 digestion, respectively; (A) at the 200 mg Cu kg^{-1} soil initial nominal dosing and (B) at the 1000

739 mg Cu kg^{-1} soil initial nominal dosing. Materials labelled as BGS102 soil and LUFA 2.2 soil,

740 refer to control soils (no added Cu or ENMs). BGS 102 soil was not used in the earthworm tests,

741 but solely included to validate the analytical chemistry. Data are mean ± S.E.M from ($n = 4$)

742 separate soil boxes *per* treatment. Different letters in panel (A) or (B) indicate significant

743 differences amongst the relative tested materials (ANOVA, $p < 0.05$) in gastric or gastro-

744 intestinal phases, respectively. *, in panel (A) or (B) refers to a statistical significant difference in

745 calculated BAF between gastric and gastro-intestinal phases in that relative test material and

746 concentration (*t*-test, $p < 0.05$).

747 **Fig. 3** The relationship between the mean total measured copper concentration in the soil

748 following the gastro-intestinal digestion (left-hand panels), or the mean percentage of the gastro-

749 intestinal bioaccessible fractions (BAFs) of Cu (right-hand panels), plotted against the measured

750 copper dissolution rates of the ENMs in Milli-Q water (panels A and B), or the total mean copper

751 concentration in the earthworms at day 14 (panels C and D). Data on the *y-axis* are from $n = 4$

752 separate soil boxes, at either 200 or 1000 mg Cu kg^{-1} soil concentration, and $n = 8$ earthworms

753 *per* treatment, except for NPs COOH at high dose where $n = 2$ as a result of high animal

754 mortality (*x-axis*).
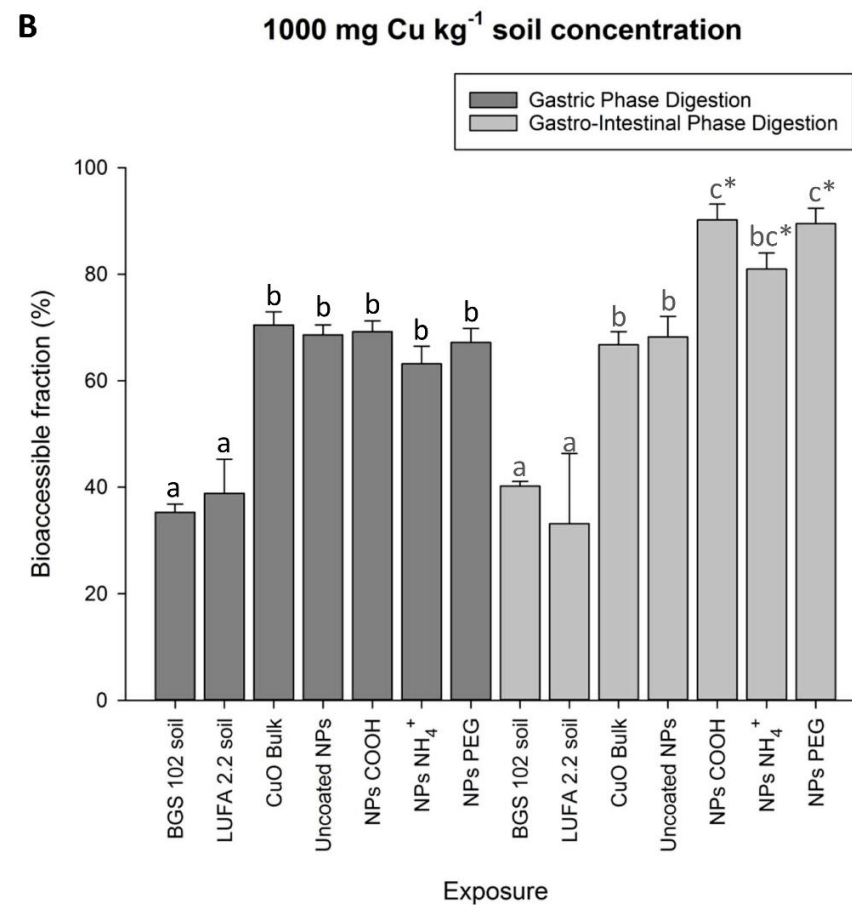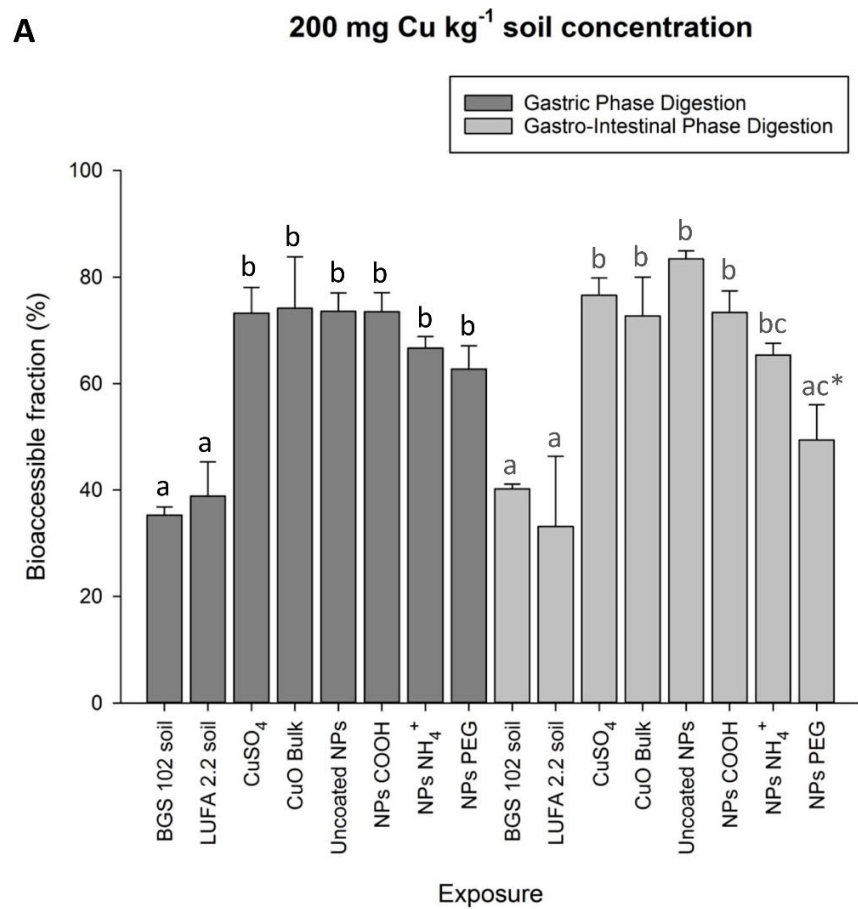
755   **Fig. 1**

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A
200 mg Cu kg⁻¹ soil concentration

B
1000 mg Cu kg⁻¹ soil concentration

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758

759　**Fig. 2**

760

A **200 mg Cu kg^{-1} soil concentration**

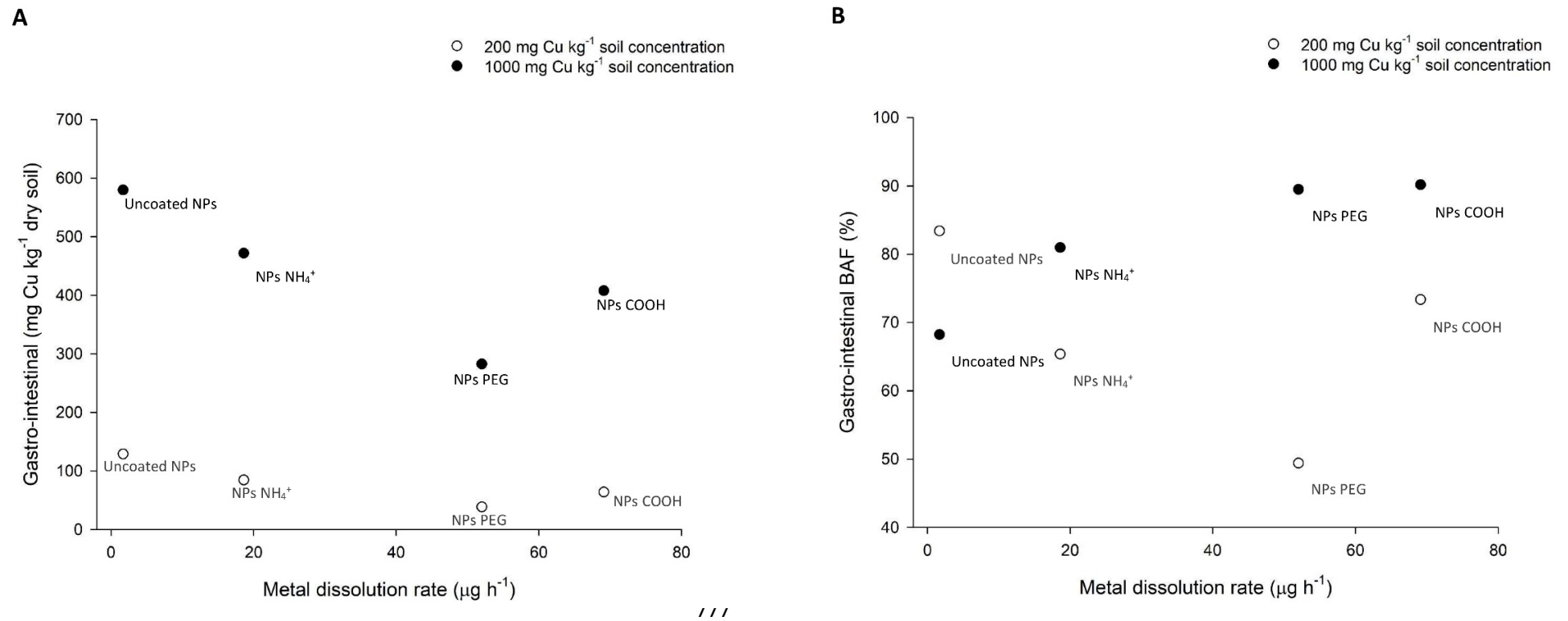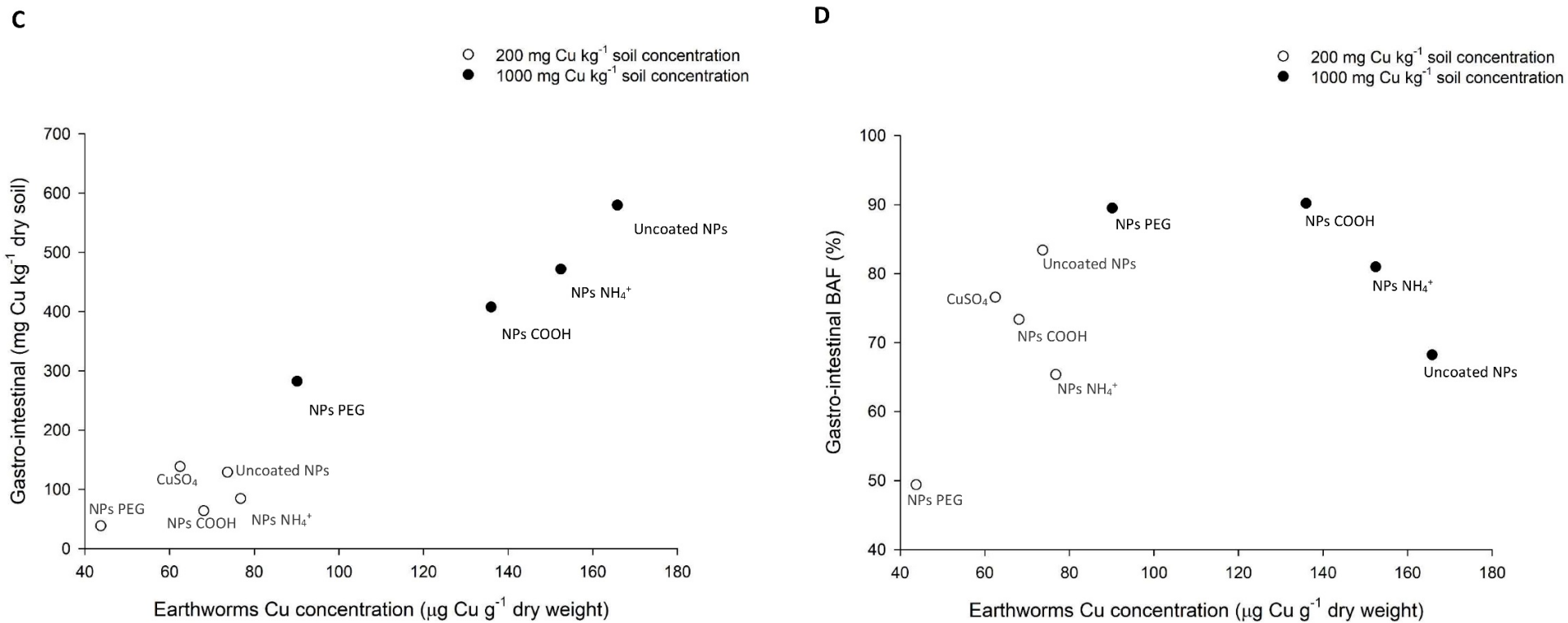B **1000 mg Cu kg^{-1} soil concentration**
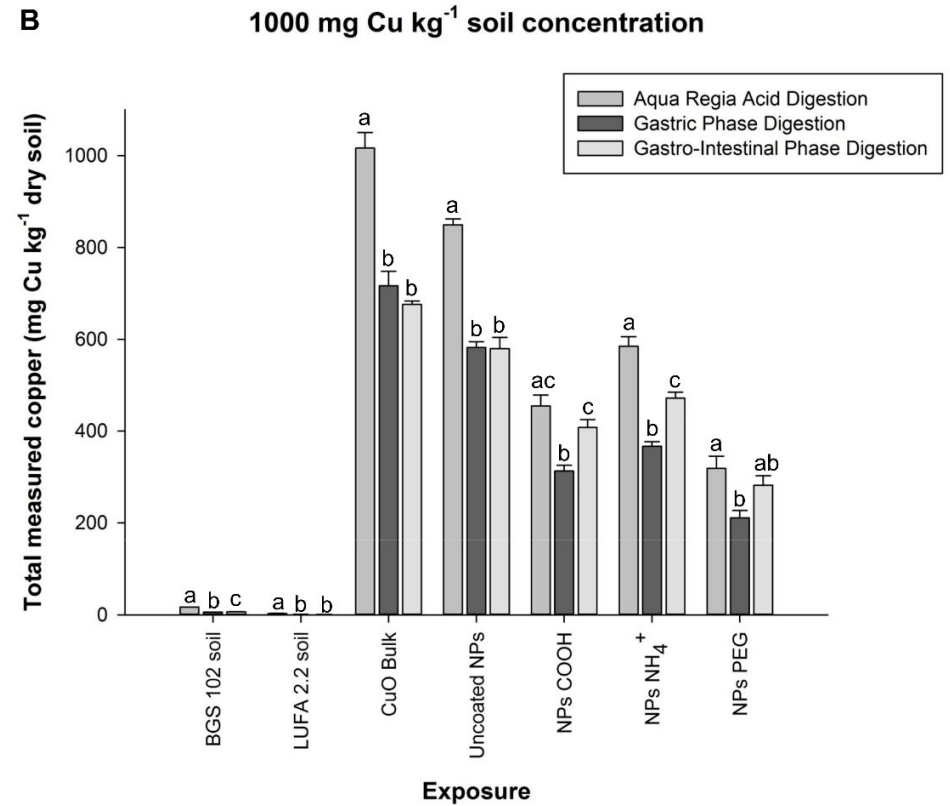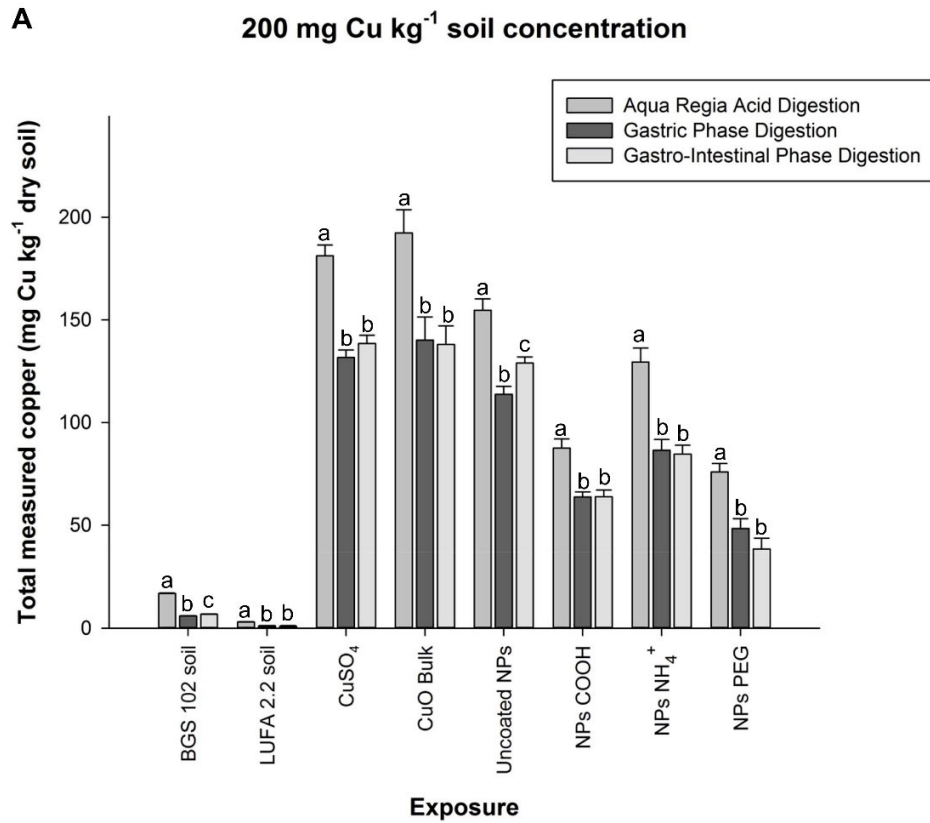
761

762

763

764 **Fig. 3**

765

A

B

778

779

780

781

782 **Fig. 3 cont.**

783

784

C

D

785

786