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

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ARTICLE INFO

ABSTRACT

Engineered nanomaterials (ENMs) may be functionalised with a surface coating to enhance their properties, but the ecotoxicity of the coatings and how hazard changes with ageing in soil is poorly understood. This study determined the toxic effect of CuO ENMs with different chemical coatings on the earthworm (*Eisenia fetida*) in fresh soil, and then after one year in aged soil. In both experiments, earthworms were exposed for 14 days to the CuO materials at nominal concentrations of 200 and 1000 mg Cu kg^{-1} dry weight and compared to CuSO$_4$. In the fresh soil experiment, CuO-COOH was found to be the most acutely toxic of the nanomaterials (survival, 20 ± 50%), with tenfold increase of total Cu in the earthworms compared to controls. Sodium pump activity was reduced in most CuO ENM treatments, although not in the CuSO$_4$ control. There was no evidence of glutathione depletion or the induction of superoxide dismutase (SOD) activity in any treatment. Histology showed a mild hypoplasia of mucous cells in the epidermis with some nanomaterials. In the aged soil, the CuO-NH$_4^+$ was the most acutely toxic ENM (survival 45 ± 3%) and Cu accumulation was lower in the earthworms than in the fresh soil study. Depletion of tissue Mn and Zn concentrations were seen in earthworms in aged soil, while no significant effects on sodium pump or total glutathione were observed. Overall, the study showed some coating-dependent differences in ENM toxicity to earthworms which also changed after a year of ageing the soil.

1. Introduction

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

well understood. Environmental fate modelling predicts that soil will be one of the sinks for metallic ENMs (Gottschalk et al., 2015), with modelled concentrations of CuCO$_3$ ENMs in sludge-treated soils ranging from 32 to 100 µg kg^{-1} dw for best to worst case scenarios respectively.

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

The ecotoxicity of Cu-containing ENMs on earthworms has received less attention. Studies on metallic ENMs have shown that nanoparticulate forms of metals are generally less acutely toxic to soil organisms than their dissolved metal counterparts (ZnO, Heggelund et al., 2014;

Table 1
Characterisation details of the CuO ENMs used in the experiments.

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

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

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

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

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

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

Ag, Velicogna et al., 2017). Heckmann et al. (2011) reported that Cu nanoparticles were not acutely toxic at a nominal concentration of 1000 mg kg^{-1} dw, although a 10% decrease in cocoon production was observed. However, there are concerns that ENMs may dissolve in the pore water of soil or release metal ions by dissolution. Direct contact toxicity of metal particles on the surface of the earthworm, or via ingestion, are also possible routes for delivering toxic metal ions.

Sub-lethal effects of ENMs include declines in reproduction as measured by cocoon production and the number surviving offspring (e.g., Ag, Heckmann et al., 2011; ZnO, Heggelund et al., 2014), and avoidance of ENM contaminated soil (Ag, Velicogna et al., 2017). Earthworms show total metal accumulation from exposure to metal-containing ENMs in soil (e.g., Ag, Diez-Ortiz et al., 2015), but whether or not the metal remains in the nanoform inside the organism is not yet clear. Earthworms exposed to metal-containing ENMs show similar modes of toxicity to those well-known for dissolved metals, including oxidative stress and increased metallothionein (Ag NPs, Gomes et al., 2015). However, there is less information on the sub-lethal effects of CuO ENMs on earthworms, and the available studies have mainly used pristine or unmodified versions of the ENMs (Unrine et al., 2010). There are concerns that surface coatings may alter the toxicity of ENMs, additionally, very little is known of the toxicity of aged Cu-containing ENMs in soil. Silver ENMs were found to increase in toxicity after one year of aging (earthworms, Diez-Ortiz et al., 2015), while ZnO ENMs were found to decrease in toxicity (springtails, Waalewijn-Kool et al., 2013). These studies high-light the importance of time scales in ecotoxicological tests for ENMs.

The aims of the current study were to determine the sub-lethal toxicity and accumulation of Cu in earthworms from CuO contaminated soils compared to the relevant metal salt controls. The study design incorporated a range of coatings on a common CuO core to represent anionic, carboxyl group (COOH), cationic, ammonium (NH$_4^+$) and organic ligands, polyethylene glycol (PEG), on the surface of the particles. In addition to survival and growth in freshly dosed soils, biochemical measurements were made to assess known mechanisms of Cu toxicity including effects on ionic regulation (tissue metal concentrations, Na$^+$/K$^+$-ATPase activity) and oxidative stress (total glutathione, superoxide dismutase activity), as well as evidence for histopathology in the tissues to aid interpretation of the data. Having established the response to fresh soil, the soils were aged for one year, and then a second experiment conducted with selected endpoints to determine the effect of aged soil containing the aged CuO ENMs on newly exposed earthworms.

2. Methodology

Two experiments were conducted. The first with a freshly spiked soil and the second using the same soil after one year of ageing; referred to hereafter as fresh and aged soil experiments respectively. The experiments were conducted with a well-known Lufa 2.2 soil in a quadruplicate design at two nominal Cu concentrations (see below). Controls included unexposed animals (no added Cu or ENMs), and a metal salt control (CuSO$_4$ exposure).

2.1. Engineered nanomaterials and characterisation

The ENMs used in the experiments were provided by PlasmaChem as part of the Nanosolutions EU project (www.nanosolutionsfp7.com). Briefly, the ENMs were supplied as dry powders and with no significant chemical impurities reported by the supplier. The functional groups that enabled the negative, neutral and positive surface coatings on the ENMs were necessarily of different molecular weight and hydrophobicity. The precise details of how the coatings were synthesised and attached to the ENM core is commercially sensitive information of the suppliers. However, for clarity, we use the term '–NH$_4^+$' to mean an –NH$_3$ terminal ligand that has been ionised with H$^+$ ions to achieve positive charge. The primary particle size and chemical composition of each material are reported in Table 1 from the manufacturer's information. Further characterisation was done at the University of Plymouth (Table 1, Supplementary Fig. S1). Stock dispersions of the ENMs were freshly prepared in ultrapure deionised water (MilliQ water, Elga, 18.2 Ω) for characterisation purposes only at the University of Plymouth, using a standardised protocol (35 kHz frequency, Fisherbrand FB 11010, Germany) for 2 h to disperse the materials; following this they were subject to nanoparticle tracking analysis (NTA, Nanosight LM10) to determine hydrodynamic diameters. Dissolution of dissolved metals was determined in ultrapure water by dialysis using the method exactly according to Besinis et al. (2014) (Table 1). Some preliminary thermogravimetric analyses (TGA) indicated, as expected, that the mass of coating varied according to the type of the coating. At the time of the study, it was not technically feasible to precisely determine the proportion of the molecular mass of the entire particle due to presence of the coating with enough certainty for normalising the dosimetry for the soil experiments. Instead, pragmatically, the experiments were