The biological effects of engineered nanomaterials on soil organisms: surface coating and age matter

by

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Abstract

Engineered nanomaterials (ENMs) have been increasingly used in various applications. Often, the ENMs are functionalised with a surface coating to enhance their properties. Decades of research has provided information on mostly pristine and unmodified ENMs, while ecotoxicity of coated ENMs and how their hazard changes with age in soils is still uncertain. The thesis aimed to determine the toxic effects and bioaccumulation potential of CuO ENMs and CdTe quantum dots (QDs) with different chemical coatings (carboxylate, COOH; polyethylene glycol, PEG; ammonium, NH_4^+) on the earthworm (*Eisenia fetida*), and compare the effects to their metal salt ($CuSO_4$) or micron-sized counterpart. Then, to determine if any observed toxicity was altered after ageing the soils for up to one year. Incidental plant growth was studied in the exposure soils to maximise the scientific value of the earthworm tests. Toxic effects of CuO ENMs were also assessed in *Caenorhabditis elegans* exposed in liquid and soil media to understand effects of the media and method of dosing on ENM toxicity. CuO ENMs were equally toxic to earthworms, or less toxic to plants than the dissolved Cu; whereas CdTe QD ENMs were more toxic than the micron-sized CdTe QDs. There was a coating effect in both, CuO and CdTe QD ENM experiments, the -COOH coated ENMs were most toxic in the fresh soil study, while -NH_4^+ coated ENMs were most toxic in the aged soil study. Despite the similarities in the toxicity ranking, the biological effects exerted were different between CuO and CdTe QD ENMs. In *C. elegans* exposures, the ENMs were more hazardous than dissolved Cu, but ranking of ENMs depended on the media and method of dosing. The results suggest the coating effect is determined by the reactivity of the coating in a given media, and it also depends on the core of the ENMs. As such, coating and ageing effects should be considered in the risk assessment of ENMs.

Table of Contents

List of Tables

List of Figures

List of abbreviations and acronyms

(US) EPA	The United States Environmental Protection Agency
ATP	Adenosine triphosphate
CdTe	Cadmium-telluride
CEC	Cation exchange capacity
COOH	Carboxylate
CuO	Copper (II) oxide
dw	Dry weight
ECHA	European Chemicals Agency
ENM	Engineered nanomaterial
ERA	Environmental Risk Assessment
EU	European Union
GSH	Glutathione
ISO	International Standardisation Organisation
Na^+/K^+ ATPase	Sodium/potassium adenosine triphosphatase / Sodium pump
NH_4^+	Ammonia
NP	Nanoparticle
NTA	Nanoparticle tracking analysis
OECD	Organisation for Economic Co-operation and Development
PEG	Polyethylene glycol
QD	Quantum dot
SOD	Superoxide dismutase
TEM	Transmission electron microscopy

Chapter 1. General Introduction and Literature Review

"*A mind which has once imbibed a taste for scientific enquiry, and has learnt the habit of applying its principles readily to the cases which occur, has within itself an inexhaustible source of pure and exciting contemplations.*"

-Sir John Herschel (1831)

1.1 Introduction

Soil is a highly complex living media that is essential for all life forms. Soil has a pivotal role in numerous ecosystem services and it provides a habitat to a diversity of organisms. The soil environment, as a whole, is involved in the nutrient cycles that partly control the climate (Lavelle et al., 2008), used in drug discovery of antibiotics for human health (from fungal and microbial species in soil), agriculture, and even waste management (e.g., landfills). Soils face many stresses, related to natural or anthropogenic sources. These include salinity, drought, extreme temperatures, flooding and, ever increasingly, man-made chemical toxicity. Decades of unsustainable use of soils, population growth and increasing contamination world-wide has resulted in a decrease in biodiversity and a negative impact on climate change (Abrahams, 2002). The ongoing advancements in technology in already developed areas and increased industrialisation of developing countries has led to higher inputs of contaminants to soil, e.g. metals, organic contaminants and novel engineered contaminants such as produced by nanotechnology. After decades of research, there are still areas that require urgent attention, e.g. environmental fate (specifically in soil) and the biological effects of ENMs on soil organisms to prevent issues the society has faced with contaminants such as asbestos (Gottschall, 2010) and persistent organic pollutants (EC, 2004).

1.2 Nanotechnology and types of nanomaterials

The existence of very small particles has been discussed since the early 19th century in terms of the Brownian motion (random movement of colloidal particles in liquid or gas caused by collision with surrounding molecules), most groundbreakingly by

Albert Einstein in 1905, and experimentally proven by Jean Perrin in 1908 (Newbergh et al., 2006). Decades later, Richard Feyman (1960) suggested the idea of nanotechnology, which is the use of engineered nanomaterials (ENMs) in products and processes (Roco, 2003).

Globally it is believed nanotechnologies have the potential to change the modern world with its numerous applications, e.g., consumer products including clothing and personal care (Buzea et al., 2007), electronics, various materials and coatings (insulation, self-cleaning surfaces), environmental remediation, medicine and more (several applications summarised in Roco et al., 2011). According Vance et al. (2015) over 1000 nanoproducts were on the market (in 2014), including food and cosmetics, although, this is likely to be an underestimation since many companies do not reveal all details due to trademark issues. Since 2001 more than 60 countries have followed the US and started nanotechnology initiatives (Clunan et al., 2014). It is expected the global value of nanomaterials and associated nano-enabled products will reach around $ 4.4 trillion by 2018 (Lux Research, 2014) since hundreds of companies make products that either contain nanomaterials or utilise nanotechnology.

Nanotechnology is not only about the size, but the unique physiochemical, optical and biological properties at this scale that are novel when compared to the micron counterparts of the ENMs (Falkner and Jaspers, 2012). In colloid chemistry, the scale has been regarded as the range from 1 – 1000 nm (Lead and Wilkinson, 2006). While the 1 – 100 nm is being utilised by several international regulators and institutions as the range for nanomaterials (Table 1.1), there is still a lot of debate around the arbitrary cut off size (Handy et al., 2008b). The JRC definition of the nanomaterial for regulation purposes (Table 1.1) brings in the cut off as a

percentage, which is another debatable point, since several chemical powders, that have not been intended as nanomaterials, have a fraction in the nano-scale. Furthermore, the most appropriate definition should fit the purpose of the product, i.e. different application need a different context for the definition that is applicable to the legal framework of the product type as well as relevant to it.

Table 1.1 The definition of 'engineered nanomaterial' in different countries and organisations (extracted from the report by JRC, 2010).

Organisation or Country	Definition	Reference
JRC*	A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 – 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 0 % may be replaced by a threshold between 1 and 50 %	JRC, 2010
ISO-CEN (draft)	Material with any external dimension in the nanoscale or having internal or surface structure in the nanoscale	ISO, 2010
OECD	Material which is either a nano-object or is nanostructured	OECD, 2010
EU SCENIHR	Any form of a material composed of discrete functional parts, many of which have one or more dimensions in the nanoscale.	SCENIHR, 2007
EU SCCP	Material with one or more external dimensions, or an internal structure, in the nanoscale, which could exhibit novel characteristics compared to the same material without nanoscale features	SCCP, 2007
EU (Cosmetic Products)	An insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, in the nanoscale.	EC, 2009
EU (Novel Foods)	Any intentionally produced material in the nanoscale, or is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions in the nanoscale.	EC, 2008
ACC	An Engineered Nanomaterial is any intentionally produced material in the nanoscale.	ACC, 2008

Australia (NICNAS)	Industrial materials intentionally produced, manufactured or engineered to have specific properties or specific composition, in the nanoscale.	NICNAS, 2009
Canada	Manufactured material at or within the nanoscale in at least one spatial dimension, or is smaller or larger than the nanoscale in all spatial dimensions and exhibits one or more nanoscale phenomena.	Health Canada, 2010
Denmark	Materials having structures in the nanoscale.	in JRC (2010)
The UK	Materials having structured components in the nanoscale.	The Royal Society and The Royal Academy of Engineering, 2004
The UK (DEFRA)	Materials in the nanoscale and are deliberately engineered i.e. not natural or unintentional by-products of other processes, and are 'free' within any environmental media at any stage in a product's life-cycle.	DEFRA, 2006
US-EPA	Engineered nanoscale material is any particle, substance, or material that has been engineered to have one or more dimensions in the nanoscale.	US EPA, 2006
USA NNI	Man-made materials with at least one dimension smaller than 100 nm	NNI, 2000

* a definition for "nanomaterial"

The importance of natural nanosized particles has been acknowledge for decades, however these particles have been referred as environmental colloids, and they include both organic and inorganic substances (Buffle and Leeuwen, 1992; Lead and Wilkinson, 2007). Natural sources of nanoparticles (NPs) include volcanic dust, most natural waters, soils and sediments (Hochella et al., 2008). They are generated by several geological and biological processes (e.g., weathering of minerals). Examples of natural nanomaterials include, iron (as hematite) and silicate particles, as well as other nano-scaled objects such as organic matter, clay minerals, polysaccharides, proteins, ash as bismuth oxide-and cristonalite-NPs, and silicone dioxide nanocrystals (Biswas and Wang, 2012), fullerenes (Buseck et al., 1992), and

photonic crystals in beetles (Kim et al., 2017). Certain naturally occurring nanoparticles, such as fullerenes and graphene, are also being engineered (Becker et al., 2000). Although what sets the natural nanoparticles apart from man-made (engineered) particles, is that they have not been specifically modified (size, shape, surface functionalization) and are not novel, i.e. organisms have been continuously been exposed to such particles.

There are also incidental sources of nanomaterials from anthropogenic activity. These include combustion processes (resulting in soot), weathering of roads, car tyres, fuel combustion and mining activities which release metal particles as well as dissolved metals (Buzea et al., 2007). In addition to this, cleaning (oxidation of d-limonene, Karlberg et al., 1995), cooking, smoking and many more can lead to nano-scale particles (comprehensive overview in Buzea et al., 2007). In addition to activities, spontaneous production of particles from objects containing, e.g., Cu and Ag has been shown (Glover et al., (2011).

Engineered nanomaterials (ENMs) and -particles (ENPs) are different to the natural and incidental particles, since they have been specifically produced and modified to achieve specific purposes. For example, ENMs can have quantum properties (quantum dots, QDs, < 10 nm) and can be used in technologies for more efficient energy harvesting (solar panels with QDs) and energy storage (carbon storage), medical applications such as imaging (QDs) and drug delivery (Au NPs) and antimicrobial/fungal applications (Ag, Cu NPs) *etc.* (comprehensive overview of uses and benefits in Roco et al., 2011). Currently, the most widely used ENMs are TiO_2 ENMs used in food and personal care products, Ag ENMs used in healthcare, CeO_2 ENMs used in fuel additives and carbon based ENMs used in durability enhancement of consumer products (Hendren et al., 2011; Clunan et al., 2014).

There are a multitude of types of ENMs, depending on their shape and structure (e.g., spheres, fibres, tubes, plates, wires *etc*.) and surface modification (e.g. organic, inorganic or charged coatings) as well as approaches how to classify the nanomaterials (JRC, 2010). The notion in classification is to fit the ENMs into the current classification system of chemicals (i.e. grouped by chemical type), however, this is still highly controversial, since it is not straightforward to fit the shape, size and composites (including coated ENMs) into a simple chemical substance scheme (Handy et al., 2008a; Stone et al., 2010). An option for classification of nano-enabled products may be by the intended use that are then subject to specific legislation and effects testing. For example, if a nano-enabled product is intended as a biocide, all the regulatory testing to register it as a biocide must be done (Handy et al., 2008a, b).

1.3 Regulatory testing and model organisms in soil ecotoxicology

The strategy of environmental effects testing has been traditionally based on a hierarchical (or tiered) system (Forbes and Forbes, 1994). The first tier consists of acute screening of standard species (that have been used in regulatory ecotoxicology). If the risks established in the first stage were unacceptable, the second tier of tests is carried out. These include a series of predictive tests that enable a more accurate estimation of risks to be made, and they usually entail testing different species and life-stages, and effects of varying abiotic factors. The third tier involves validating results from stage two in field or simulated field conditions. The fourth tier is based on environmental monitoring of the specific chemical (Forbes and Forbes, 1994). Within this tiered system, a set of standardised testing guidelines, usually by OECD, ISO or a governmental agency, such as EPA,

have been used for years in regulatory testing. Under EU regulations such as: Registration, Evaluation and Assessment of Chemicals (REACH), the Biocidal Products Regulations (BPR), Plant Protection Products (PPPs) and human and veterinary pharmaceuticals regulations, a variety of standardised soil tests are required (ECHA, 2016). These tests also include studies with soil invertebrates. Several organisms have been used to represent a variety of species, e.g., mites, collembola, molluscs, annelids, roundworms (van Gestel, 2012). While it is important to establish effects in a multitude of species for ecological relevance, when novel chemicals, e.g., ENMs are being assessed, the most studied organisms may provide a more useful starting point, since effects can be compared between other chemicals and metals.

The model species *Eisenia fetida* (*E. fetida*) (Savingy, 1826) is one the most commonly used earthworm in metal ecotoxicology (e.g. in uptake studies, reviewed by Nahmani et al., 2007) and it is also a recommended species in the OECD and ISO TGs along with *E. andrei* (van Gestel, 2012). Compared to the other commonly used species, e.g. *Lumbricus* spp., *E. fetida* have a faster life-cyle (Edwards and Bohlen, 1996). Furthermore, E. *fetida* are known to be robust and able to tolerate a range of environmental conditions, e.g. fluctuation in soil pH, moisture, soil type and temperature (Kaplan et al., 1980). *E. fetida* is a compost earthworm, living in areas of rich organic matter in the wild. In the laboratory earthworms thrive in compost rich mixtures of soil and are often fed with oats or manure (Frund et al., 2010).

Nematodes are the most abundant multicellular organisms (Platt, 1994). The soil nematode *Caenorhabditis elegans* (Maupas, 1900) is the most studied nematode and has been also extensively and more increasingly used in eco-/toxicology (Hunt, 2016). Set guidelines have been established for testing (e.g., ISO, 2009) as well as

numerous protocols exist as published papers (e.g., Dhawan et al., 1999). In the wild, *C. elegans* are free-living organisms that feed on bacteria which grow on rotting vegetable matter and compost heaps (Felix and Braendle, 2010). While in the laboratory, *C. elegans* is grown in axenic conditions and fed with an uracil deficient (to ensure the bacteria can only grow on the NGM agar) strain of *Escherichia coli* (Brenner, 1974).

The main concern about these standardised toxicity tests with earthworms and nematodes is the selection of media, test duration, availability of methods for ENM characterization (in soil) as well as application of ENMs into the soil (Handy et al., 2008b). It is important to establish the potential effects of novel chemicals in their pristine form. Adding the dry powders in with soil has been shown to give a good overall dispersion of the ENMs (Waalewijn-Kool et al., 2012). However, it is also important to understand the effects under more realistic exposure scenarios, therefore ageing of the exposure soils may be beneficial.

1.4 Exposure of ENMs in soil

Many chemicals contained in consumer products end up in solid waste or wastewater. Therefore, it is likely that ENMs from such sources are released into wastewater and solid waste (Cornelis et al., 2014). For example, clothing items containing Ag ENMs have been shown to release the particles after a few washes, it is then when the ENMs will enter the wastewater treatment plant (WWTP) (Mitrano et al., 2016a). In addition, it has been shown that Ag ENMs would mostly accumulate in biosolids (Levard et al., 2012). Importantly, however, soils are not exposed to ENMs in their pristine state, but either sulphidised (Ag ENMs, Levard et al., 2012;

Lombi et al., 2013), oxidised (Cu ENMs, Gomes et al., 2015 and QDs, Blickley, 2010) or another way transformed during the nano-enabled product use (Mittrano et al., 2015 and 2016). One of the most likely exposure route of such transformed ENMs to soil is the application of treated sewage sludge (biosolids) to agricultural land (Figure 1.1). This has been generally accepted as a sustainable waste management method that also increases soil fertility (US EPA, 1993; EC, 2010), though it is also banned in certain countries, e.g., in Switzerland since 2005 (Smith, 2009). In the UK, approximately 1,000,000 tonnes of sewage sludge is applied to land each year (DEFRA, 2012). However, ENMs designed to be used in soils, such as fertilisers (DeRosa et al., 2010) and biocides (Kookana et al., 2014) would be exposed in their pristine form (Figure 1.1). In addition, exposure to soil can occur by atmospheric deposition from, e.g., incineration of waste (Garner et al., 2017), accidental release (although not yet reported in the UK for ENMs) and via landfill leachates (Mitrano et al., 2017). It has been suggested ENMs may be able to penetrate the clay liners of landfills, although no conclusive evidence of this yet exists (OECD, 2015).

1.5 Fate and behaviour of ENMs in soils

To understand the fate and behaviour of ENMs in soil the principles of colloid chemistry can be applied since most of the soil surface is linked to colloid-sized particles and soil colloids have been studied for more than a century (e.g. a paper by Cameron, 1915). Nanoparticle stability as described as colloid stability can be defined by a continuum scale theory of forces between particles or the DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory, however this theory is an over simplification and does not consider short-range solvation forces (Goldberg et al.,

2011). Furthermore, Goldberg et al. (2011) discuss the DLVO theory applied to soil also lacks the consideration of organic and mineral interaction. In traditional colloid chemistry, the colloids have been found to be mobilised by clay dispersion, changes in porewater chemistry, water film expansion and air-water interface during periodic wetting events, precipitation impact on soil surface and increased shear forces associated with transient hydrodynamic events (Goldberg et al., 2011).

Similarly to colloids, ENM fate in soil has been found to be influenced by such soil properties (Figure 1.1; Lowry et al., 2012). Fine texture (clay rich soils) can increase retention of ENMs, while larger particles enable ENM movement (Cornelis et al., 2014). Higher soil organic matter can decrease the mobility of ENMs in soil (Ag ENMs Coutris et al., 2012b). Lower soil pH can increase ZnO ENM mobility and release of Zn into porewater (pH ≤ 5 Heggelund et al., 2014; Romero-Freire et al., 2016). Soil porewater that is high in ionic strength may increase ENM aggregation and deposition (hypothesis based on sediment work discussed in Petersen et al., 2011). In addition, CuO ENMs have been found to form CuS and Cu-goethite in simulated soil-rice systems (Peng et al., 2017). Chemical transformation by dissolution of soluble metal species from metal containing-ENMs is possible (Cornelis et al., 2012) and it has also been evidenced in freshwater mesocosms in environmentally relevant conditions (CuO ENMs, Vencalek et al., 2016). ENMs can undergo mechanical transformation, e.g. weathering (Goldberg et al., 2011). Presence of organisms, such as earthworms may influence ENM fate. Earthworms excrete mucus which has been found to reduce metal mobility (Sizmur et al., 2010), this may also influence metal based ENM fate (Figure 1.1).

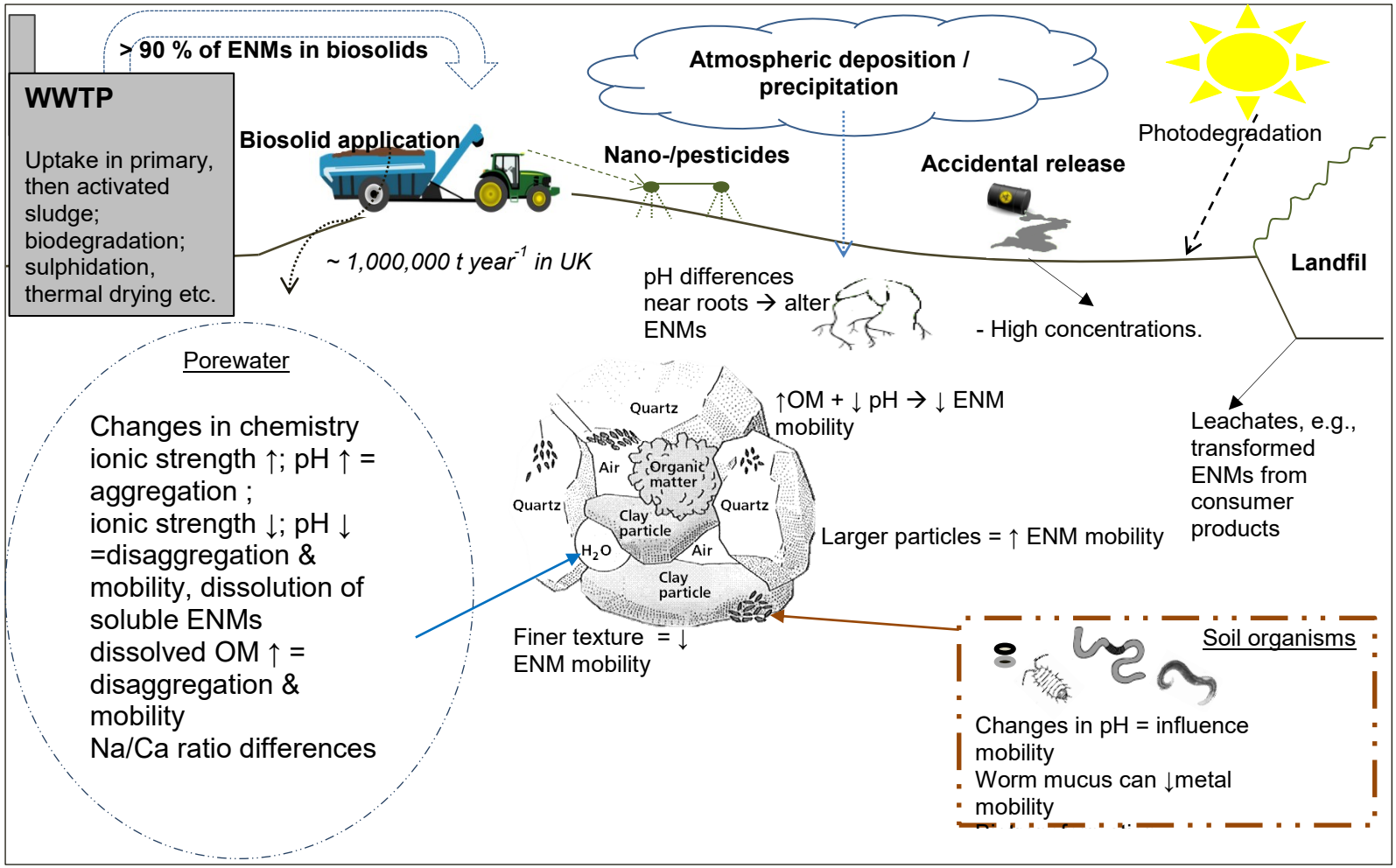

Figure 1.1 Exposure of ENMs to soil (in bold) and factors influencing fate (regular) of ENMs in soil. ↑ = increase; ↓ = decrease; OM – organic matter.

Effects of surface coating on ENM fate

In addition to soil and soil porewater properties, as well as presence of organisms, ENM specific characteristics, such as surface modification can influence their fate and behaviour in soil. Negative surface charge can reduce aggregation, as clay particles are negatively charged, similarly charged ENMs would repulse and thus increase mobility and reduce aggregation with the clay particles (Levard et al., 2012; Kango et al., 2013). Recently, Nickel et al. (2017) found TiO_2 ENMs regardless of coating to be mostly saturated in the surface columns of soils in the OECD TG 312 leaching experiment, thus indicating low mobility of the ENMs. Although the mobility was low, TiO_2 ENMs coated with a mixture of 6 % Al_2O_3 and 2 % dimethicone ($(C_2H_6OSi)_n$) were found to move down the soil column slightly more than uncoated TiO_2 ENMs (Nickel et al., 2017). The coating may impact dissolution of the metal from the particle (PVP coated Ag ENMs in soil solution release more Ag that uncoated Ag ENMs, Cornelis et al., 2012). Furthermore, organic surface coatings might be degraded by microbes (polymer coatings, Kirschling et al., 2011). Additionally, poly(ethyl oxide) has been found to hydrolyse in water as well as undergo photodegradation (Hassouna et al., 2007). This latter may apply to such polymer coated ENMs leading them to be photodegraded if they were on soil surface, and hydrolyzation may occur in soil porewater. In addition, PEG coated ENMs are likely to go through irreversible agglomeration (Kawasaki et al., 2007). Coating of the ENMs may also influence their stability (e.g. by increasing or reducing their reactivity) and as such pose further challenges in measuring ENMs in the environment.

1.6 Environmental concentrations of ENMs.

There are several issues with measuring ENMs in soil, e.g., how to effectively measure the intact particles in soil against a vast number of background colloids in soil (Handy et al., 2012), although methods based on single-particle (sp) ICP-MS have been emerging (CuO ENMs, Navratilova et al., 2015). Also, methods have been developed in measuring nanoparticles in earthworm tissues (Makama et al., 2015). These methods are promising tools, but, also come with limitations. For example, for the spICP-MS information on particle geometry, stoichiometry and density is needed to be able to convert the results (analyte mass) into particle size and only one isotope can be analysed at once (Navratilova et al., 2015). Due to the lack of robust methods for measuring ENMs in the environment, concentrations are currently still being estimated based on modelling of theoretical releases as well as product life cycle parameters (such as described above, Figure 1.1). These predictions have estimated the environmental concentrations of ENMs (TiO_2, ZnO, to approach the low milligram per kilogram level (e.g., 0.5 mg kg^{-1} dw soil for TiO_2) in soil treated with sludge from WWTP (Gottschalk et al., 2009). Recently, Gottschalk et al. (2015) have modelled the concentration of $CuCO_3$ ENMs in sludge treated soils to be in the range of 32-70 µg kg^{-1} dw (based on worst case scenario up to 100 µg kg^{-1} dw).

1.7 Bioavailability of metal-based ENMs in soil

A metal is considered bioavailable if it is in an available form to an organism. The bioavailability of metals in soils has been extensively studied (reviews in Anderson et al., 2013; Ardestani et al., 2014). Metal bioavailability is determined by soil chemistry and properties such as described earlier in the environmental fate section (section

1.5), e.g., texture, pH, CEC, presence of organic matter, ionic strength in porewater etc. Since porewater is considered as the main route of uptake of metals in many soil organisms, including earthworms (Vijver et al., 2005), the speciation of the metal in porewater also influences its bioavailability, e.g. usually the free ion is more bioavailable and thus more toxic (Rieuwerts et al., 1998).

The idea that the dissolved metal fraction in the porewater is bioavailable via solute transporters in the dermis of earthworms, might not apply to ENMs since they are not solutes. Therefore, the bioavailability of ENMs is likely to be via different routes. The gut has been shown as a major route of uptake in exposure to ENMs (ZnO ENMs, Laycock et al., 2015; Ag ENMs, Diez-Ortiz et al., 2015). Despite the different uptake routes, similar soil properties and chemistry would influence the bioavailability of ENMs, as metals, e.g. soil pH, soil texture, organic matter *etc*. However, since the pH in the earthworm gut is likely to be different from the surrounding soils (earthworm gut pH ~ 7, Oste et al., 2001; average pH ~ 5 in European soils, Bohner et al., 2008) the bioavailability in the gut may be different. In addition, the secretion of $CaCO_3$ (from calciferous glands) and ammonia in the gut (Laverick, 1963) may further impact the bioavailability of ENMs, e.g. by increasing aggregation. Overall, the studies done to date suggest ENMs can be bioavailable, although most researchers have not been able to quantify the number of intact particles rather than total metal concentrations in the earthworm tissues (references below in Table 1.2).

1.8 Acute and sublethal effects of metal-based ENMs on earthworms

Acute toxicity in earthworms has been mostly focused on Ag, Zn and TiO_2 ENMs (Table 1.2). The general consensus is that ENMs are not acutely toxic in soil, even at

very high concentrations (e.g., 1000 mg kg^{-1} Cu, Ag, Zn, TiO_2 ENMs in Heckmann et al., 2010; more examples in Table 1.2). Effects on survival are evident only when earthworms are exposed on filter paper since that is a pure contact test (Zn ENMs Canas, et al., 2011; Li et al., 2011), and it is likely to due dissolution of the ENMs resulting in higher bioavailability of the dissolved metal. In contrast to survival, reproduction has been shown a more sensitive endpoint in response to ENMs exposed in soil (complete inhibition of reproduction Ag ENMs at 1000 mg kg^{-1}, Heckmann et al., 2010). Although reproduction has been found a more sensitive endpoint, the ENMs have not been found more hazardous than their dissolved metal counterpart (summarised studies in Table 1.2). Effects on biomass have been shown in response to Ag ENMs and although as with reproduction, the effects have not been found more significant than dissolved metal (Table 1.2). Other sublethal effects, such as oxidative protein damage (Ag PVP ENMs, Tsyusko et al., 2012a) and molecular stress (Ag ENMs, Hayashi et al., 2013a) have been found in earthworms. Upregulation of metallothionein (metal binding protein) has been seen in response to Cu ENMs (Unrine et al., 2010b). However, such effects have been shown in response to dissolved Ag and Cu (Tsyusko et al., 2012a and Unrine et al., 2010b, respectively). Less reported sublethal effects include mechanical damage to internal structures analysed by histopathology (C_{60} in van der Ploeg et al., 2010, Ce-$_2$O in Lahive et al., 2014). In some studies, however, ENMs have been shown to induce different effects to their dissolved metal counterpart, e.g. with Ag ENMs where oxidative stress markers were triggered earlier than in exposure to dissolved Ag (Gomes et al., 2015). Hayashi et al., (2013b) have shown the ENMs to attain a “protein corona” that facilitates the cellular uptake of Ag ENMs in *E. fetida* leading to greater bioaccumulation. While the studies with *Eisenia* species have mostly

measured the total metal concentrations, thus confirming total metal accumulation only, it is possible intact ENMs are accumulated. In some studies, the critical body residues (~ 20 mg Ag kg^{-1} dw) have been exceeded yet earthworms are still surviving / reproducing (Ag ENMs, Diez-Ortiz et al., 2015) and evidence of Ag ENMs in earthworm tissue was shown (Ag ENMs, Diez-Ortiz et al., 2015)

In general, most of the studies have been based on pristine and uncoated ENMs and there is a knowledge gap on coating effects as well as ageing effects on ENM toxicity in earthworms (Table 1.2). Although studies on the effects of ageing of ENMs in soil have more recently emerged, the data is conflicting and tends to indicate the results are nanomaterial specific. Diez-Ortiz et al. (2015) showed Ag ENMs to significantly increase in reproductive toxicity after one year (56-day EC_{50} changed from 1420 to 34 mg Ag kg^{-1}), while $AgNO_3$ reproductive toxicity decreased (56-day EC_{50} changed from 49 to 104 mg Ag kg^{-1}). Coutris et al. (2012) showed uncoated Ag ENMs can potentially provide a continuous bioavailable source of Ag over time, compared to citrate coated Ag ENMs or $AgNO_3$. In contrast, the toxicity of uncoated ZnO ENMs to springtails reduced after 3 months of ageing, while toxicity of coated ZnO ENM reduced only after 12 months of ageing (Waalewjin-Kool et al., 2013). Furthermore, a more realistic ENM exposure scenario (Ag and ZnO ENMs applied via sewage sludge) has shown a reduced reproductive toxicity when compared to the pristine ENMs (Lahive et al., 2017). These studies highlight the potentially significant effects on results based on soil ageing, presence of coating as well as exposure route which require more research to fully understand the risks posed by ENMs in soils.

Table 1.2 Acute and reproductive effects and biological effects in the earthworm *Eisenia fetida* (1) / *Eisenia andrei* (2) exposed in soil (filter paper and liquid tests excluded).

ENM	Surface modification and primary particle size[1]	Positive control	Soil type and pH	Test guideline, test duration	Nominal concentration range	Endpoints analysed	Toxicity, biological effects, key notes	Reference
1 Ag	None, Size: 49 ± 8 nm	$AgNO_3$	OECD artificial soil; pH: 6 ± 0.5	NA, 4 and 28 days	$AgNO_3$: 0-200 mg Ag kg^{-1} dw Ag-NPs: 0-1500 mg Ag kg^{-1} dw.	Oxidative stress biomarkers	AgNP effects on total glutathione (increase) and catalase (increase) after 4 days; effects on lipid peroxidation after 28 days (selected concentrations). In summary, different effects on biomarkers in AgNP and $AgNO_3$ exposures.	Gomes et al., 2015
1 Ag	PVP and OA; Size: 10 and 30 -50nm	$AgNO_3$	OECD artificial soil; pH:7. Natural sandy loam, pH: 5.17.	OECD TG 222, 56 days total	Ag NP: 10,100,1000 mg kg^{-1} dw $AgNO_3$:10, 100 mg Ag kg^{-1} dw.	Growth, mortality, bioaccumulation	No gross effects on growth, reproduction, mortality. Some effect on survival in sandy loam soil (85 % in Ag NPs 10 nm 9 mg Ag kg^{-1} dw) No difference in toxicity or bioaccumulation between coatings.	Shoults-Wilson et al., 2011a
1 Ag	PVP and OA; Size: 10 and 30 -50nm	$AgNO_3$	OECD artificial soil; pH: not reported.	OECD TG 222, 56 days	Ag NP: 10,100,1000 mg kg^{-1} dw $AgNO_3$:10, 100 mg Ag kg^{-1} dw.	Growth, mortality, bioaccumulation reproduction.	No effects on survival, growth. Effects on reproduction seen in earthworms exposed to Ag NPs with either coating (727.6 mg kg^{-1} for OA and 773.3 mg kg^{-1} for PVP) and $AgNO_3$, (94.21 mg kg^{-1} dw). Bioaccumulation factor higher in $AgNO_3$ exposures than in NPs.	Shoults-Wilson et al., 2011b
1 Ag	PVP and OA; Size: 10 and 30 -50nm	$AgNO_3$	ISO artificial soil; pH: 7 Low calcium artificial soil; pH: not reported Natural sandy loam; pH 5.17	ISO 17512-1, 48 hours	0.3-54 mg kg^{-1} Dw.	Avoidance	Avoidance of $AgNO_3$ immediate, while avoidance of NPs over 48 h. Avoidance not related to dissolution.	Shoults-Wilson et al., 2011c

1	Ag	PVP, Size:10, 50-50nm	$AgNO_3$	Natural sandy loam; pH: 5.17.	OECD TG 222, 56 days total	100, 500 mg kg^{-1} dw.	Gene expression, oxidative damage to proteins	Gene response and oxidative stress similar by Ag NPs and Ag ions. Catalase and heat shock protein (HSP70) was correlated to Ag concentration in soil.	Tsyusko et al., 2012a
1	Ag	None, Size: 10 nm	$AgNO_3$	OECD artificial soil, pH: 7.3	OECD TG 222, 5 months	Ag NPs, 0.77 ± 0.10 mg g^{-1} dw; Ag ions, 0.55 ± 0.15 mg g^{-1} dw	Dietary uptake, excretion and biodistribution	Ag ions and Ag NPs in earthworms were excreted rapidly. Ag ions and particularly Ag NPs were more inert in the solution. Sequential extraction revealed most of the AgNPs in nitric acid extractable fraction.	Coutris et al., 2012
1	Ag	None Size: 30-50nm	$AgNO_3$	Natural sandy loam, pH: 5.8	no specific TG, 14 days	500 mg kg^{-1} dw.	Molecular stress: days 1, 2, 7, 14	AgNPs induced oxidative stress genes at day 2, but with a temporal pattern shift to immune genes at day 14 following metabolic upregulation at day 7. $AgNO_3$ induced the genes and enzymes related to oxidative stress at day 1, after which markers of energy metabolism were all suppressed at day 2.	Hayashi et al., 2014
1	Ag	None, Size: ~ 50 nm	$AgNO_3$	Lufa 2.2, natural sandy loam, pH: 5.5 ± 0.1 Aged for: 1, 9, 30 52 weeks.	OECD TG 222, 56 days total	Ag NP: 0 – 4395 mg Ag kg^{-1} dw. $AgNO_3$: 0 – 1758 mg Ag kg^{-1} dw.	Survival, reproduction, accumulation	AgNP 1-30 week aged LC_{50} > 4395; 52 week aged LC_{50} > 4504 mg Ag kg^{-1} dw. $AgNO_3$ in general more toxic, however, toxicity decreases with time. Reproduction more sensitive, 1-30 week aged EC_{50} = 142 – 142, 52 week aged EC_{50} = 34 mg Ag kg^{-1} dw. Accumulation of Ag reduces with ageing in AgNP exposures.	Diez-Ortiz et al., 2015
2	Ag	None* Size: 20 nm PVP, Size: 40 nm *dispersed with humic acid	$AgNO_3$	Sandy loam, pH: not reported Biosolids, pH: 7.9	OECD TG 317, 21 days' uptake, 21 days' excretion	Ag NP: 1.9 mg Ag kg^{-1} dw $AgNO_3$: 3.9 mg Ag kg^{-1} dw.	Bioaccumulation kinetics	Initial accumulation very high in all exposures which plateaus within days, followed by slower excretion in sandy loam. In biosolids excretion is rapid. Ag NP accumulation higher than predicted bioavailability, TEM images showed internalised Ag NPs.	Velicogna et al., 2017

1	Ag	PVP, nm Size: 30-50 nm	$AgNO_3$	Sandy loam, pH: 5.8	Limit test design, 21 mortality; and following 21 days for reproductio n 42 days total	1000 mg kg^{-1} dw.	Growth, mortality, reproduction	AgNPs: survival ~100 %, $AgNO_3$: survival ~2 %. Reproduction in both AgNP and Ag-salt: 0 %. AgNPs induced a ~ 30 % loss in biomass.	Heckmann et al., 2011
1	Ag	PVP, Size: 50 nm	$AgNO_3$ and Ag-bulk			Measured: AgNP: ~ 51, 94 mg Ag kg^{-1} dw. $AgNO_3$: ~ 71, 111 mg Ag kg^{-1} dw.	Mortality, reproduction, accumulation	Pristine ENMs more toxic than those added via sewage sludge. Pristine ENM EC_{50}= 97 mg Ag kg^{-1} dw.	Lahive et al., 2017
1	ZnO	None, Size: 30 nm	$ZnSO_4$ and Ag bulk	Sandy loam amended with sewage sludge contaminated with specific ENMs, pH: not reported Sandy loam with added "pristine" ENMs, pH: 5.5	OECD TG 222, 56 days	Measured: ZnO NP: ~ 853, 1360 mg Zn kg^{-1} dw. $ZnSO_4$: ~ 985, 1600 mg Zn kg^{-1} dw.	Mortality, reproduction, accumulation	Pristine ENMs more toxic than those added via sewage sludge. Pristine ENM EC_{50}= 780 mg Zn kg^{-1} dw.	Lahive et al., 2017
1	ZnO	None, Size: 30 – 200 nm	$ZnCl_2$	Natural sandy loam, pH: 4; adjusted to 5.9 and 7.2	OECD TG 222, 56 days total	0, 238, 381, 610, 976, 1520, 2500 mg kg^{-1} dw.	Mortality (21 days) LC_{50}, reproduction (42 days) EC_{50} bioavailability	LC_{50} = >>1669; >>2094; >>2689* *EC_{50} = 918; 901; 2874** pH affected toxicity. *for low, medium, high pH respectively in mg kg^{-1} dw.	Heggelund et al., 2014

2	ZnO	None, Size: 20-40 nm	$ZnCl_2$	3 types of natural soil: forest(Spain), garden (Netherlands), sandy loam (Germany, Lufa 2.2), pH: 7.6, 5.9, 5.6, respectively.	OECD TG 222, 56 days. Different ageing times 1 – 168 days	Lufa and garden soil: 500, 1000 mg Zn kg^{-1} dw as ZnO NP and $ZnCl^2$ Forest soil: 1250, 2500 mg Zn kg^{-1} dw as ZnO NP and $ZnCl_2$.	Mortality, growth, reproduction, accumulation	No significant effects on mortality (doses chosen were based on known LC_{50}) except in the forest soil dosed with $ZnCl_2$ (500 mg Zn kg^{-1} dw after 56 days of ageing. Differences in weight (Growth) were dependent on soil type, than treatment. Ageing of soils resulted in increased weight loss but did not influence any other endpoints. Reproduction was affected more in $ZnCl_2$ exposures. Bioaccmulation was lowest in forest soils.	Romero-Freire et al., 2016
1	ZnO	None, Size: 20 – 100 nm	NA	Agricultural soil amended with WWTS, pH: 7.2 – 7.9 and with WTS, pH: 7.9 - 8	No specific guideline, multiple species test, 21 days	0 – 1000 mg ZnO NP kg^{-1} dw.	Accumulation, growth, enzyme activity.	Accumulation was evident in all exposure concentration and it was higher in WST treated soils. Growth (weight) was affected by 33 % in amended soils. No effect on enzyme activity.	Fernandez et al., 2014
1	TiO_2	None Size: 21 nm	TiO_2 -bulk	Sandy loam, pH: 5.8	Limit test design, 21 mortality; and following 21 days for reproduction 42 days total	1000 mg kg^{-1}dw. Concentration not confirmed.	Growth, mortality.	ENM no effect on survival; reproduction reduced to 80 %; TiO_2 bulk no effect.	Heckmann et al., 2011
1	TiO_2	None Size: 5,10, 21 nm	TiO_2-bulk	Natural sandy loam, pH:6.7; Artificial soil, pH: 6.7	Both ISO and OECD TGs[2]	200 - 20,200 mg kg^{-1} dw.	Growth, mortality, reproduction, juvenile growth avoidance	No toxic effects; avoidance of soil at 1000 - 5000 mg kg^{-1} dw; suggest earthworms differentiate between bulk and nano TiO_2	McShane et al., 2012
1	Cu	None Size: 20-40; < 100 nm	$CuSO_4$	OECD Artificial soil, pH: 7	OECD TG 222, 56 days total	5, 20, 50 mg kg^{-1} dw.	Growth, mortality, reproduction, gene expression, bioaccumulation	Particle size had no effect; low bioaccumulation observed of Cu NPs and dissolved Cu. Increased regulation of mtl >20 mg Cu kg^{-1} dw as Cu NP; > 10 mg Cu kg^{-1} dw as $CuSO_4$	Unrine et al., 2010b

1	Cu	None, Size: 80 nm	$CuCl_2$	Sandy loam, pH: 5.8	Limit test, 21 mortality; and following 21 days for reproduction 42 days total	1000 mg kg^{-1} dw. Concentration not confirmed.	Growth, mortality, reproduction	ENM had no effect on survival; reproduction 10 % $CuCl_2$ – survival 17 %; reproduction 2 %	Heckmann et al., 2011
1	Ni	None, Size: 20nm	$NiCl_2$					ENM had no effect on survival and reproduction 93 %; $NiCl_2$ 32 % survival; reproduction 2.4 %	Heckmann et al., 2011
1	Al_2O_3	None, Size: 12-14 nm	Al_2O_3 -bulk					ENM had no effect on survival; reproduction 92 %; Al_2O_3 No adverse effect on survival; reproduction;	Heckmann et al., 2011
1	Al_2O_3	None, Size: 11 nm	Al_2O_3 -bulk	Natural sandy loam, pH: 7.0	ASTM E1676-04, 28 days; avoidance test Environment Canada TG (2004) 48 hours.	100-10,000 mg kg^{-1} dw toxicity / 625 - 10,000 mg kg^{-1} dw avoidance	Growth, mortality, bioaccumulation, reproduction and avoidance	Reproduction effects > 3000 mg/kg No EC_{50} calculated More Al in worms exposed to nano Al_2O_3 Nano Al_2O_3 $EC_{50avoid}$ = 4951.25 mg kg^{-1} dw; Bulk Al_2O_3 $EC_{50avoid}$ = 1338.44 mg kg^{-1} dw.	Coleman et al., 2010
1	Au	Citrate Size: 20-55nm	$HAuCl_2$	OECD Artificial soil, pH: 7	OECD TG 222, 56 days total	5, 20, 50 mg kg^{-1} dw	Growth, mortality, reproduction, gene expression, bioaccumulation	Particle size had no effect; low bioaccumulation observed; Au ENMs had no effect on gene expression; ionic Au caused increased regulation of *mtl*	Unrine et al., 2010a
1	Co	None, Size: 5nm	$CoCl_2$	OECD artificial soil, pH: 7.3	OECD TG 222, 5 months	Co NPs, 1.20 ± 0.45 mg g^{-1}; Co ions, 0.72 ± 0.04 ng g^{-1} dw; Nominal concentration not confirmed.	Dietary uptake, excretion and biodistribution	32% of the cobalt accumulated from Co ions and Co NPs were excreted within four months. High accumulation of cobalt was found in blood and in the digestive tract. Dissolution of Co NP was rapid. Most Co NP water extractable.	Coutris et al. 2012a
1	Ce_2O	None, Size: 5-80 nm	$((NH_4)_2$ CeIV $(NO_3)_6$	Sandy loam (Lufa 2.2) loam pH: 5.5	OECD TG 222, 56 days	4.1-10 mg Ce kg^{-1} dw.	Growth, mortality, reproduction,	No effect on survival, reproduction. Ce uptake dose-dependent in CeO_2 ENM treatments. Subtle histological effects.	Lahive et al., 2014

	bioavailability, histopathology

[1] nominal size as reported in articles; [2] See McShane et al. for details; PVP – Polyvinylpyrrolidone; OA- oleic acid; dm - diameter; SOD – superoxide dismutase; WWTS = wastewater treatment sludge, WTS, water treatment sludge. *mtl* - metallothionein. dw – dry weight.

1.9 Acute and sublethal effects of metal-based ENMs on nematodes

ENMs have been shown to be more acutely toxic to *C. elegans* than earthworms (Table 1.3). This is very likely because the exposures with *C. elegans* have been carried out in liquid media, and thus the exposure to ENMs is more direct. Ag ENMs have been shown to be acutely toxic (72 h LC_{50} = 2.8 mg l^{-1} and EC_{50} = 0.7 mg l^{-1}as 28 nm PVP coated Ag ENM, Ellegaard-Jensen et al., 2012). Reproductive toxicity has been shown to decrease, if the ENMs are sulphidised (72 h EC_{50} 0.56 mg l^{-1} as pristine Ag ENMs, 72 h EC_{50} = 4 mg l^{-1} as sulphidised Ag ENMs). In *C. elegans* ENMs have been found do reduce growth (citrate and PVP Ag ENMs, Meyer et al., 2010), induce oxidative stress (uncoated Ag ENMs, Roh et al., 2009), interfere with feeding capability (CuO ENMs, Mashock et al., 2016) and cause endoplasmic reticulum stress (citrate coated Au ENMs, Tsyusko et al., 2012b). The latter may have been a result of Au ENM uptake via endocytosis (Tsyusko et al., 2012b). Further to Au ENMs, Ag ENMs (Meyer et al., 2010) and quantum dots (Qu et al., 2017) have been found to be internalised in *C. elegans*, therefore suggesting that ENMs can potentially bioaccumulate.

The key concern with the current published studies with *C. elegans* is that most studies use different media, either with or without food and cholesterol (Table 1.3). The addition of latter, however, is crucial in longer studies, since *C. elegans* are not able to produce it (Lagido et al., 2009). It is clear the media can influence toxicity (Table 1.3), e.g. by increasing particle aggregation (Cl^- rich K-medium) or providing protective cations such as the moderately hard reconstituted water (MHRW). Furthermore, conflicting results exist with regards to the presence of food. Starnes et al. (2015) showed Ag ENMs were less toxic, while Ellegaard-Jensen et al. (2012) showed Ag ENMs were more toxic, when fed. As with earthworm experiments (Table

1.2), there have not been many studies investigating the effects of coatings on ENM toxicity to *C. elegans* and no published studies exist in soils. Although soil nematodes live in porewater, an exposure in soil with sufficient moisture would provide a more environmentally realistic scenario.

Table 1.3 Acute and reproductive effects (LC_{50} survival and EC_{50} reproduction) and biological effects in *C. elegans*.

ENM	Surface modification and primary particle size[1]	Positive control	Media and pH.	Test Guideline, test duration	Nominal concentration range	Endpoints analysed	Toxicity, biological effects, key notes	Reference
Ag	PVP and sulphidised Ag NPs	$AgNO_3$	MHRW with and without food, pH: not reported.	NS, 24 or 72 h.	Variety dependent on endpoint and type of treatment 0 – 5000 µg Ag l^{-1}	Mortality, growth, reproduction, uptake	Sulphidised Ag NPs were less toxic (both acute and reproductive) to *C. elegans* than $AgNO_3$ and non-modified AgNPs. Ag was found in *C. elegans* tissues from both AgNO3 and Ag NP exposures. ENMs less toxic when fed.	Starnes et al., 2015
Ag	None, Size: < 100 nm	$AgNO_3$	K media, no food pH: not reported	NS, 24 h, 72 h	0.05-0.5 mg l^{-1}	Mortality, growth, reproduction, gene expression	Concentration dependent reduction in reproduction, upregulation of SOD (superoxide dismutase) compared to ionic control *Note:* Organisms incubated without food 20 h prior to exposure	Roh et al., 2009
Ag	Citrate, PVP Size: 7, 21, 75 nm	$AgCl_{2/3}$	K media, pH: 6.5	NS, 72 h	0.05-50 mg l^{-1}	Mortality, growth, gene expression, bioaccumulation	Growth inhibition, toxicity partly due to dissolution; Ag found internalised	Meyer et al., 2010
Ag	PVP and none Size: 28 and 1 nm	None	K media with cholesterol with and without food pH: not reported	NS, 72 h, 96 h	0.6, 1, 1.5, 2, 2.5, 3 mg l^{-1} and 0.5, 1, 3, 5, 7, 10 mg l^{-1}	Mortality, reproduction, toxicity with and without food	72 h LC_{50} and EC_{50} were estimated to 2.8 and 0.7 mg l^{-1} Ag ENM 28 nm ; 72 h LC_{50} and EC_{50} were 13.9 and 2.1 mg l^{-1} Ag ENM 1 nm. Both ENPs toxic; PVP coated more toxic; when fed more toxic.	Ellegaard-Jensen et al., 2012
Ag	Citrate and PVP Size: 26, 83 and 70 nm.	$AgNO_3$	MHRW with food, pH : not reported	NS, 24h – 65 h depending on exposure.	0.01-20 mg l^{-1}	Mortality, locomotion and development, bioaccumulation	Locomotion influenced by surface coating, size and duration of exposure.	Yang et al., 2017
QDs	CdTe-MPA, negatively charged;	NA	NGM agar plates with food	NS, up to 48 h	20, 200 nM for all	Full physiological assessment, including reproduction	All types of QD showed a similar biodistribution. QD uptake	Qu et al., 2017

	CdSe@ZnS core-shell-MPA or MEA for positive or negative charge. Size: ~ 10 nm						follows a different pathway to *E. coli* within the nematode. Positively charged QDs more toxic than negatively charged (juvenile development). While CdTe-MPA QDs more toxic than CdSe@ZnS-MPA.	
CuO	None Size: 28 nm	$CuSO_4$	K media with food, pH: not reported	NS, 96 h	3.8 – 15.9 mg Cu l^{-1}	Mortality, growth, feeding reproduction, neurotoxicity (transgenic strains)	CuO NPs and $CuSO_4$ similar effects on reproduction, while feeding and body length was affected more in the CuO NP exposures. CuO NPs and $CuSO_4$ induce equal amounts of neuron degeneration.	Mashock et al., 2016
ZnO	None Size: 1.5 nm	$ZnCl_2$	Buffered K medium with food. pH: not reported.	NS, 24 h acute, 72 h reproductio n	325-1.625 mg l^{-1}	Mortality, reproduction, movement behaviour, gene expression	LC_{50} = 789; 884 mg Zn l^{-1} ENM and $ZnCl_2$, respectively in buffered medium; LC_{50} = 5173; 5434 mg Zn l^{-1} nano and $ZnCl_2$, in unbuffered medium. $EC_{50\text{-repord}}$ = 46, 59 mg Zn l^{-1} for nano and $ZnCl_2$ (buffered medium) All other endpoints affected but no significant difference between ENM and ionic control	Ma et al., 2009
TiO_2	None Size: 7, 20 nm	TiO_2 bulk	K media, no food, pH: not t	NS, 24 h NS< 24 h, > 40 h for gene expression	1 mg l^{-1}	Mortality, growth, fertility, reproduction, gene expression	7 nm reduced survival, fertility and increased stress response gene expression; size dependent toxicity	Roh et al., 2010
Au	Citrate Size: 4 nm	None	50% K media, pH: not reported	NS, 24 h and 72 h.	2.5,5.5,7,7,15, 30 mg l^{-1}	Mortality, growth, reproduction, gene expression, uptake	LC_{50} = 5.9 mg l^{-1}; 797 genes affected, ENMs bioavailable and potentially induced endoplasmic reticulum stress uptake via endocytosis	Tsyusko et al., 2012b

[1] nominal size as reported in articles; NS – no specific guideline but rather published methods, usually Williams and Dusenbery, 1988, 1990 or Dhawan et al., 1999; MPA 3-mercaptopropionic acid; MEA- 2-mercaptoethylamine

1.10 Environmental risk assessment of ENMs

Environmental risk assessment is a key part of ensuring environmental safety, and is essential part of the regulation of substances worldwide, e.g. in the EU as part of chemical legislation known as REACH, plant protection and biocidal products and pharmaceutical product legislation (ECHA, 2016). The traditional ERA process requires the identification of the hazard, assessment of exposure, assessment of effects or exposure-response (dose-response), risk characterisation and finally risk management (Forbes and Forbes, 1994). The process is based on a paradigm “risk = exposure x hazard”.

Overall the progress has been slow in the ERA of ENMs, as after decades of research there are no robust environmental measurements of ENMs (which are likely to be low in the environment) or measured exposure concentrations (Mitrano and Nowack, 2017). The hazard data on the ENMs is increasing, however, most of the information is based on three ENM types: TiO_2, Ag and carbon nanotubes (CNTs) ENMs (warranted, as these are the most produced ENMs). For a comprehensive risk assessment, species sensitivity distributions (SSDs) have been generally used to determine the value where < 5 % of the species would be negatively influenced. Recently SSDs have been formulated for ENMs (Garner et al., 2015 and Chen et al., 2017), although they come with limitations e.g., data quality, and information still confined to limited species (Chen et al., 2017).

It is not clear if the traditional ERA approach would necessarily apply to ENMs, for example, ENMs do not always follow the classic dose-response scenario (Syberg and Hansen, 2016). There is a need to understand fate and behaviour in soils and in

the organisms in the context of hazard, and identify the most sensitive organisms (including endpoints) and appropriate tests for engineered nanomaterials.

1.11 Hypothesis, aims and objectives

The central hypothesis of this project was that the surface chemistry will affect the biological effects of the ENMs on soil invertebrates. The aims of the project were to determine biological effects and bioaccumulation potential of differently coated and uncoated ENMs on two soil invertebrates (earthworms and nematodes), as well as relate the results to existing metal salts or micron-scale powders of the equivalent chemical substance (Figure 1.2). The ENMs studies were CuO and CdTe quantum dot ENMs. The CuO ENMs included a common CuO-core and while no core material existed for CdTe QDs. The coatings for both CuO and CdTe QDs included organic and hydrophilic polyethylene glycol (PEG), negatively charged carboxylate ($COOH^-$) and positively charged ammonium (NH_4^+). Given the concerns about transformation and fate, the work also aimed to explore the effects of ageing ENMs in soil on selected toxicity endpoints. A further aim was to compare the two most common organisms used in soil ecotoxicology: the earthworm and nematode to assess if the responses in the two organisms are similar. And if there is any potential to use the *C. elegans* test as a screening tool for ENMs, taking into account sensitivity of the organisms, the cost effectiveness of the assays, as well as the reliability and relevance of the test method for ENMs (test media, dosing of the ENMs).

Furthermore, this project was part of a wider research consortium (Nanosolutions, www.nanosolutionsfp7.com), thus data and samples were collected for research partners in other laboratories. The overarching aim of Nanosolutions was to create a

new computational risk assessment tool for the EU, called the 'Nanosafety Classifier' which is based on the effects of surface coatings and particle chemical composition (including size and shape) in multiple species across (e.g. bacteria, soil invertebrates, mussels, crustaceans, fish, rodents).

The principle objectives of this research project were to carry out experiments in earthworms using two metal (Cu and Cd) / metalloid (Te)-based ENMs with a follow up study in nematodes using the most toxic ENM adhering to modified standardised guidelines (OECD, ISO). The methods included substantially more endpoints than in a routine regulatory toxicity test in order to provide a mechanistic understanding of any toxicity. Thus, samples were collected to reflect some of the known mechanisms of metal and/or particle toxicity including ionic composition (known effects of heavy metals on essential and trace metals) and sodium pump activity to reflect any osmoregulatory stress(Cu and Cd are known to interfere with ATPases), biochemical measures of oxidative stress (metals and nanoparticles have been shown to induce oxidative stress), histopathology for evidence of inflammation and/or integrity of biological barriers, and total metal concentrations to confirm exposure or patterns of apparent bioaccumulation.

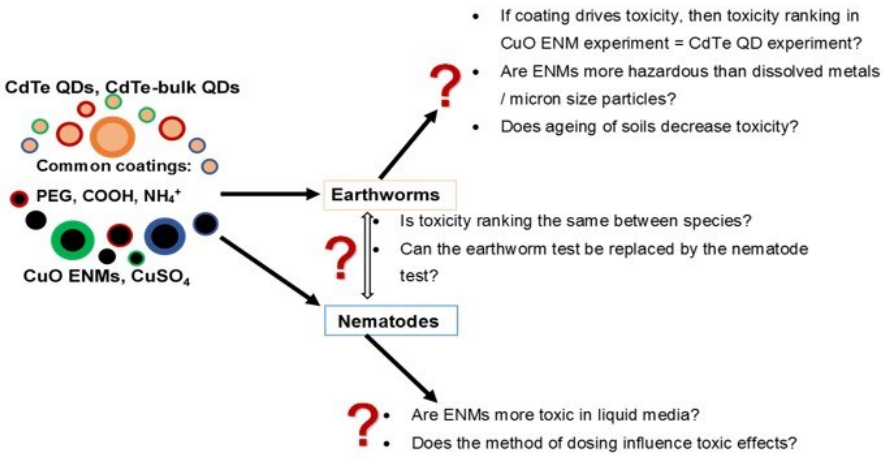

Figure 1.2 The driving questions of the thesis.

Chapter 2. General Methodology

2.1 Common chemicals and laboratory principles

All chemicals used in the laboratory work were analytical grade or above and purchased from Sigma-Aldrich (UK), unless otherwise stated. All glassware used in all experimental work was acid washed (5 % HNO_3) and rinsed in ultrapure Milli-Q (18.2 MΩ-cm) water (referred to as Milli-Q water hereafter) and/or autoclaved where appropriate. The ultrapure water used in all solutions preparation was Milli-Q (18.2 MΩ-cm) unless stated differently. The glassware used were laboratory grade Pyrex glass. All consumables such as plastic ware, Sterilin containers and centrifuge tubes, *etc*., were purchased from Fisher Scientific unless otherwise stated.

2.2 Engineered nanomaterials: characterisation, stock preparation and dosing of the soils

The engineered materials commonly used throughout the chapters were copper oxide or CuO ENMs with different surface modifications, except Chapter 5 which used CdTe QDs with the same surface modifications. The surface modifications included polyethylene glycol (PEG) to represent hydrophilic / neutral coating, carboxylate (COOH) to represent a negatively charged coating and ammonium (NH_4^+) to represent a positively charged coating. The ENMs were obtained from PlasmaChem and extensively characterised as part of the wider Nanosolutions research project (see www.nanosolutionsfp7.com for further details). Briefly, the ENMs were supplied as dry powders, no significant chemical impurities were reported by the supplier (99 % purity, Table 2.1). The characterisation of each material are reported in Table 2.1 from the manufacturer's information. The details of

the coatings and the synthesis are commercially sensitive information, therefore detailed descriptions were not provided. Further characterisation was done at University of Plymouth by colleagues of the research project (Table 2.1, Figure 2.1). The characterisation at University of Plymouth involved nanoparticle tracking analysis (NTA) to determine the hydrodynamic diameter of the particles (Nanosight LM10). Further to this, transmission electron microscope (TEM, TEM, JEOL-1200EX II) was used to image the particles and ImageJ 1.51k software (Rasband W, National Institute of Health, Bethesda, MD, USA) was used to measure the primary particle diameters on at least 60 particles (based on example images in Figure 2.1, image courtesy of Dr Ben Shaw). To assess the release of dissolved metals from the ENMs, dialysis expriments were carried out. For characterisation purposes, stock dispersions of the ENMs were freshly made in Milli-Q water. Dispersions of 100 ml were sonicated using a standardised protocol at room temperature (bath sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 1 h to disperse the materials following which they were characterised by the methods above. All methods, i.e. the NTA, TEM and dialysis experiments were carried out based on Besinis et al., (2014). In addition to the above, thermogravimetric analyses (TGA) were performed at the University of Manchester by partners of the research project to characterise the functionalisation of the surface of the ENMs (Table 2.1). Analytical grade $CuSO_4$ was used as the metal salt control (CAS 7758-98-7) in all CuO ENM experiments. The details of the CdTe QD materials and controls are shown in Chapter 5.

In order to measure the concentration of Cu in the CuO ENMs, stock solutions were prepared and were subject to total metal analyses as described in section 2.6. For this, the dry powders were dispersed in Milli-Q water, followed by sonication (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 1 hour.

There was evident ENM aggregation, as differently shaped aggregates of black ENM powder were present in all stock solutions. Based on visual observation, larger aggregates were present in the CuO-PEG and CuO-COOH stock solutions, while CuO-core and CuO-NH_4^+ solutions had similarly sized aggregates. This was also confirmed by the NTA results presented in Table 2.1.

The TGA analysis of the particles indicated the mass of the coating differs between the ENMs, however, since these results were based on only 1 measurement it was decided the dosing in all the experiments (Chapters 3, 4, 7) would be based on a calculated nominal Cu mass basis. Since there was more uncertainty regarding the CdTe QDs, the dosing was made on a mass CdTe QD basis. The thermogravimetric analysis of the batches of the material (Table 2.1) indicated that the –NH_4^+, -COOH and –PEG coatings contributed roughly 12, 22 and 42 % of the mass of the CuO ENMs. For the CdTe QDs, the -NH4+, -COOH and -PEG coating contributed to approximately 8, 23 and 50 % of the mass of the CdTe QDs (Table 5.1). Following the experimental work, the total metal analysis (section 2.5 in this Chapter) of the ENMs and QD ENMs revealed the amount of metal / metalloid differed significantly between particles (Table 2.4, 2.5). This introduced a complication to the analysis of the results, since the dosing for all experiments using CuO ENMs (Chapter 3, Chapter 4, Chapter 7) had been made under the assumption the amount of Cu is the same in the particles as the exact coating stoichiometry was unknown. When dosing the soils, the amount of oxygen was accounted for, therefore, the intention was to dose on a Cu mass basis, thus the treatments are referred to as mg Cu kg^{-1} dw.

Table 2.1 Characterisation information of CuO ENMs.

ENM variant	[1]Manufacturer's Information	[2]Measured primary particle size (nm)	[3]NTA, hydrodynamic diameter (nm)	[4]TGA, degree of functionalisation (% weight loss)	[5]Maximum rate of dissolution in Milli Q water (µg h^{-1})
CuO-Core	Lot No. YF1309121, 99% purity,10-20 nm.	12 ± 0.37	41 ± 28	9.2	1.68
CuO-Polyethylene Glycol	Lot No. YF140114, 99% purity,10-20 nm.	7.46 ± 0.42	100 ± 36	42.2	52.02
CuO-Carboxylate	Lot No. YF140114, 99% purity,10-20 nm.	6.45 ± 0.16	121 ± 91	22.1	69.12
CuO-Ammonium	Lot No. YF140114, 99% purity,10-20 nm.	9.53 ± 0.22	46 ± 36	11.6	18.6

[1] Supplied as dry powders, bespoke design and production of spherical particles for the Nanosolutions project via Alexei Antipov, PlasmaChem GmbH.
[2] 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)
[3] 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)
[4]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 (n = 1).
[5]Maximum slope from rectangular hyperbola function of curve fitting used to estimate the maximum rate of dissolution of Cu from dialysis experiments conducted at University of Plymouth.

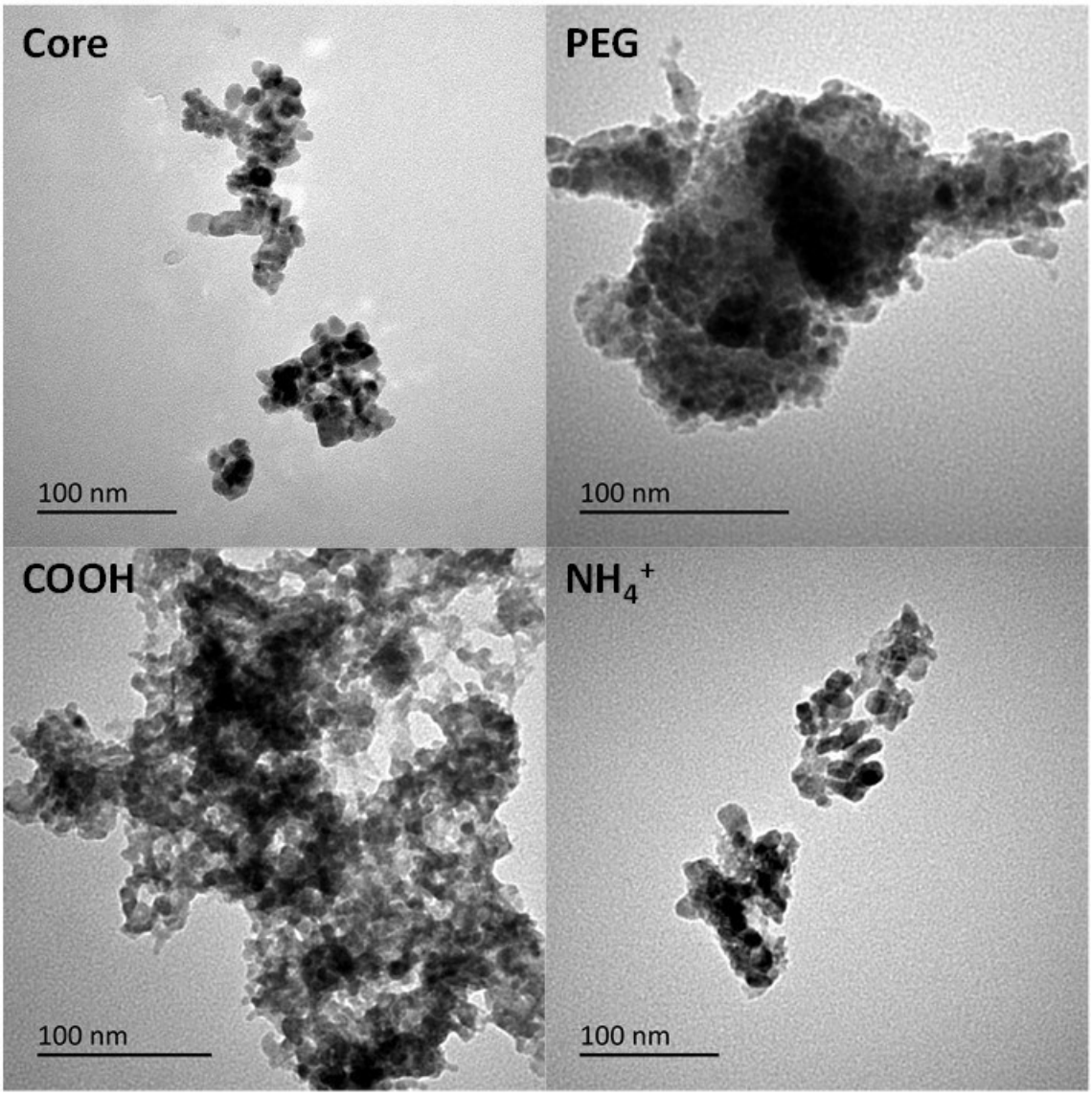

Figure 2.1 Transmission electron microscope (TEM, JEOL-1200EX II) images of CuO engineered nanomaterials (ENMs), obtained from stock dispersions of a nominal 100 mg Cu l^{-1} total Cu, prepared in ultrapure water at University of Plymouth. The images show the different types of the CuO ENMs. Core, the uncoated CuO particles; PEG, particles coated with polyethylene glycol; COOH, carboxylated particles to give a net negative surface charge; and NH_4^+, ammonium coated particles to give a net positive surface charge (see methods for details of particle characterisation). Images: courtesy of Dr Ben Shaw.

The ENMs (either CuO or CdTe QD ENMs) and $CuSO_4$ or another control chemical, e.g. CdTe-bulk material (Chapter 5) were mixed into the soil as dry powder and soils were then wetted to 50-55 % water holding capacity (WHC) with Milli-Q water according to OECD (1984). Dry dosing has been found to be a suitable approach for ENM additions to soils (see discussion in Handy et al., 2012a, b). Dosing of the soil was achieved by dosing a single batch of soil, followed by dividing it into replicates. Briefly the amount of ENM powder required to dose four replicates was added to a single batch of soil (a volume enough for four replicates) and then divided into replicates. For example, 0.480 or 2.4 g (for 200 or 1000 mg Cu kg^{-1} dw, respectively) of ENM powder or 457 mg of $CuSO_4$ required to dose each 4 replicates with a total of 50 g dry soil and then thoroughly mixed by hand for 10 minutes. This 50-g sample of soil was then added to the remaining amount of soil (2350 g) and mixed by hand to make sure the ENM powder was evenly distributed in the soil. The soil was left to equilibrate with the moisture for only one day to minimise the risk of the ENMs changing before the earthworms were added. To confirm the method of dosing, four separate soil samples were collected from the mixed batch of soil at different depths of the CuO-core ENMs. Total copper concentration was analysed by ICP-OES (see section 2.5). All the 4 samples were between 80 - 90 % (842 ± 13.8 mg Cu kg^{-1}, dry weight, mean ± SEM) of the nominal expected concentration of 1000 mg Cu kg^{-1} (analysis described in section 2.5).

2.3 Test species and maintenance

The earthworm species were originally bought from a commercial supplier; Blades Biological (Kent, UK). The earthworms grown in the laboratory and used in all

experiments were identified as *Eisenia fetida* (*E. fetida*) by visual observation using a taxonomic guide. The earthworms were grown in a mixture of equal parts of topsoil (~33 %), peat (~33 %) and bark chippings (33 %), all obtained from Westland's Ltd, UK. The soil was wetted to around 90 % of the water holding capacity with deionised water. The pH of the culturing medium was 5.5 ± 0.5 (mean ± SEM, n = 16 random measurements through-out the culturing process). The earthworms were maintained in a controlled temperature room at 20 ± 2°C in a 12:12 light: dark cycle in 400 lux lighting, but the culturing boxes were covered with opaque lids with pierced lids (OECD, 1997 and OECD, 2004). The earthworms were fed every 2-3 weeks with clean horse manure from unmedicated Dartmoor ponies. The horse manure was frozen after collection, and then thawed as required. The earthworms were transferred into fresh culturing medium every 2 – 3 months. The earthworms used in all the experiments were age-synchronised and approximately two months old. The background concentration of Cu in the culturing soil was 15.8 ± 1.2 mg Cu kg^{-1} dw and in the horse manure 11.8 ± 0.2 mg Cu kg^{-1} dw (mean ± SEM, dry weight, n = 3, analysed by ICP-OES as described in section 2.5).

2.4 Experimental test soil and characterisation

The soil used in all relevant experiments was a standard sandy loam Lufa 2.2 (LUFA Speyer, Germany). This soil offers a natural alternative to the artificial soil (AS), without being significantly different from the AS (van Gestel, 2012) and it is also widely used in earthworm tests. According to the supplier's details (Lufa Speyer, Germany), the soil has a pH of 5.5 ± 0.2 measured in 0.01 M $CaCl_2$, organic carbon and nitrogen content of 1.77 ± 0.2 % and 0.17 ± 0.02 % and a cation exchange

capacity 10.1 ± 0.2 meq 100 g^{-1}. The soil used in the experiments was sieved through a 2-mm mesh and air dried at 25°C. The water holding capacity of the soil was 41.3 ± 3.0 g 100 g^{-1} (measured volumetrically according to Wilke, 2005). Soil pH was measured prior to the start and at the end of the experiment (in a 1:1 soil:water slurry, using a glass combination electrode, Corning 420), in every soil experiment (Chapters 3-7, excluding Chapter 6)

2.5 Total metal analysis in soils and earthworms

In order to confirm the exposure to the metal from the ENMs in both soil and earthworms, the total concentration of copper and/or cadmium and tellurium, were measured along with other essential elements (Ca, Fe, K, Mg, Mn, Na, Zn) to explore changes in the ion concentration in earthworm tissue. Total metals were determined in soil samples by acid digestion using *aqua regia* (3:1 mixture of 37 % HCl and 70 % HNO_3), (broadly based on US EPA, 1986). Briefly, approximately 200 mg oven-dried (24 h at 80 ^oC) soil samples from all treatment replicates were digested in 10 ml of *aqua regia* in Pyrex glass beakers, covered with glass watch glasses, and heated at 80 ^oC on a hot plate for 1 h. The digests were allowed to cool, and then were further diluted with 20 ml ultrapure water, and stored in the dark in Sterilin tubes until further analysis.

Earthworms were sampled at random from each treatment in each experiment, details are given in the relevant chapters (Chapters 3- 4). The earthworms were washed in Milli-Q water and kept on moist filter paper for 24 h in the dark to allow them to void their gut content. The filter paper was changed after 12 h, to avoid coprophagy (Arnold and Hodson, 2007). After depuration, the earthworms were

again washed in Milli-Q water and dried, then frozen at -20 ºC and freeze-dried individually for 48 h (Edwards Modulyo freeze-dryer). The freeze-dried earthworms were then individually weighed and acid digested in 1 ml of 70 % HNO_3 in a water bath at 70°C for 1 h. Samples were allowed to cool and diluted with 7 ml of Milli-Q water and stored in the dark for further analysis.

All samples and extracts were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000, Thermo Scientific) or inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific X Series2) under the operating conditions described in Table 2.2. In order to introduce a well-mixed sample to the instrument, the samples were sonicated for 15 minutes (bath type sonicator, at 0.05 kva, 50-60 Hz, 30 kHz, Ultrawave Ltd.), vortexed for 10 s (Minishaker MS2, Fisherbrand) and hand shaken prior to analysis.

Table 2.2 ICP-MS and ICP-OES instrument specifications.

	ICP MS	**ICP OES**
Name	Thermo Scientific X Series2	iCAP 7000
Forward power	1400 Watts	1150 Watts
Coolant	13 l min^{-1} (argon gas)	12 l min^{-1}
Auxiliary gas flow	0.7 l min^{-1} (argon)	0.5 l min^{-1}
Nebuliser gas	0.74 l min^{-1} (argon)	0.50 l min^{-1}
Nebuliser	V-groove	V-groove
Spray chamber	Sturman - Masters	Sturman - Masters
Dwell time	10 msec. 100 sweeps between samples	
Calibration coefficient	0.99	0.99
Viewing height		8 mm above coil
Reading time	10 sec.	30 sec.

For quality control and assurance a variety of additional samples and standards were included in analysis. Initially, for analytical recovery, matrix-matched standards

(when possible) with ICP-OES grade standards (Aristar) and blanks were used to calibrate the instrument. In addition to this, standard dilution series made with core CuO ENMs (in 2% v/v HNO_3) to ascertain the accuracy of the Cu recovery in the form of ENMs. To assess procedural recovery of Cu or Cd and Te certified reference materials (where available, Table 2.3), spiked samples and procedural blanks were included for quality control (Tables 2.4 and 2.5). Spiked samples included earthworm and soil samples with added ENMs as dry powder and were then subject to analyses as samples from the experiments. The spiked soils were prepared by mixing the dry ENM powder into the soil with a selection of ENMs only, followed by acid digestion as described earlier, to confirm the accuracy of the dosing procedure. The only exception was, that Te ICP-MS standard solution was added to the soils as a liquid, then the soil was dried and processed as described above. Additionally, to assess the total Cu in the ENMs without any addition of soil, the pure ENM powders were digested in *aqua regia* as per method earlier (Table 2.4). Spiked earthworm samples were prepared by the addition of the ENM/metal stock as a liquid into the tube with the dried earthworm and followed by acid digestion. The recoveries of the spiked samples are detailed in Table 2.4 and 2.5 for Cu, and Cd and Te analysis, respectively. Furthermore, due to the coating stoichiometry, the nominal values were normalised taking account of the mass of the coating and the last column of Tables 2.4 and 2.5 presents the percent nominal based on the normalised value.

The limits of detection (LOD) for Cu and Cd on the ICP-OES were 0.01 mg l^{-1} for both, and 0.1 µg l^{-1} for Te on ICP-MS. These LODs correspond to around 1 mg kg^{-1} dw and 0.001 mg kg^{-1} for both, Cu and Cd, in soil and earthworm tissue, respectively. While the LOD of Te equates to around 0.01 mg kg^{-1} dw and 0.1 mg kg^{-1} dw in both soil and earthworm tissue, respectively. The LOD is based on 3σ of

multiple measurements of the lowest standard and the hypothetical per mass LODs are based on 200 mg and 5 mg dry weight soil and earthworm diluted in 25 and 5 ml ultrapure water, respectively.

Table 2.3 Certified reference materials (CRMs) used in ICP-OES / -MS analysis.

	Determined, mg kg^{-1}		**Expected and Certified value, mg kg^{-1}**		**Percent of expected, %**	
Certified Reference Material	[Cu]	[Cd]	[Cu]	[Cd]	[Cu]	[Cd]
EnviroMAT contaminated soil, SS-1	378.8 ± 2.6	3.2 ± 0.4	403 (393 – 413)^{CI}	3.2 (3 – 3.5)^{CI}	94.1 ± 0.6	100 ± 4
DOLT-4, contaminated dogfish liver	33.6 ± 3.1	20.8 ± 1.6	31.2 ± 1.1	24.2 ± 1.4	107 ± 3	85.9 ± 6.5
TORT-2, contaminated lobster hepatopancreas	98.6 ± 3.7	27.4 ± 1.9	106 ± 10	26.7 ± 0.6	93.1 ± 4.3	102.7 ± 7.4
Plant reference hay (Cu only)	8.6 ± 0.6		9.4		91.4 ± 4.9	

^{CI}- confidence intervals. Data presented as mean ± SEM (*n* = 12 for soil SS-1, n = 6 for DOLT-4 and TORT-2, n = 3 for hay) in mg Cd or Cu kg^{-1} dry weight or % of the expected/certified value; NA- not analysed due to human error.

Table 2.4 Recoveries of spiked samples; Cu from CuO ENMs or Cu from $CuSO_4$ in soil and earthworm tissue digests.

Details	**Determined [Cu], mg kg^{-1} or mg l^{-1}**	**Nominal [Cu], mg Cu kg^{-1} or mg Cu l^{-1}**	**[1]Normalised Nominal [Cu] mg Cu kg^{-1} or mg Cu l^{-1}**	**Percent of nominal concentration [Cu], %**	**[1]Percent expected, normalised to coating mass**
CuO-Core ENMs*	1009	1000	908	100.9	111
CuO-PEG ENMs*	343	1000	577	34.3	60
CuO-COOH ENMs*	574	1000	779	57.4	73
CuO-NH_4^+ ENMs*	743	1000	884	74.3	84
Lufa 2.2 spiked with: CuO-Core ENMs	995.7 ± 22	1000		101.3 ± 1.3	
$CuSO_4$	198.4 ± 4.8	200	NA	99.2 ± 2.5	NA
Earthworm (dry) with: CuO-Core ENMs	49.2 ± 0.7	50	45.4	98.4 ± 1.8	107
CuO-PEG ENMs	19.2 ± 1	50	28.85	38.4 ± 1.3	66.5
CuO-COOH ENMs	29.3 ± 1.7	50	38.95	58.6 ± 3.4	70
CuO-NH_4^+ ENMs	31.4 ± 0.9	50	44.2	62.8 ± 0.9	75

$CuSO_4$	37.7 ± 1	40		94.2 ± 1.8	NA

* Pristine ENM powder, n = 1, results in mg l^{-1}; [1]Normalised expected values based on deducting the coating mass (detailed in Table 2.1) from the nominal. Data presented as mean ± SEM (*n* = 6) mg Cu kg^{-1} dry weight. Note, CuO ENMs or $CuSO_4$ were added to the dried earthworm tissue as a liquid stock and then acid digested (results in mg l^{-1}).

Table 2.5 Recoveries of spiked samples; Cd and Te from CdTe QDs or $CdCl_2$ or Te standard (ICP-MS grade) in soil and earthworm tissue digests.

Details	**Determined value [Cd] or [Te], mg kg^{-1} or mg l^{-1}**	**CdTe combined [Cd] + [Te], mg kg^{-1} or mg l^{-1}**	**Expected [CdTe], mg kg^{-1} or mg l^{-1}**	**Percent of expected [Cd], %**	**[1]Percent expected %, normalised to coating mass**
Lufa 2.2 spiked with: CdTe- NH_4^+ QDs	Cd, 526.9 ± 0.8 Te, 180.4 ± 4	704.4	1000	70.7 *	78
CdTe-bulk	Cd, 500 Te,480	1000	1000	98	NA
Lufa 2.2 spiked with: $CdCl_2$	1.05 ± 0.01		1	105.4	NA
Te ICP standard	0.9 ± 0.01		1	90.1	NA
Earthworm tissue (dry) with: CdTe-NH_4^+ QDs	Cd, 0.4 ± 0.03 Te, 0.2 ± 0.02	0.6	1	60	73
CdTe-PEG QDs	Cd, 0.5 ± 0.01 Te, 0.3 ± 0.03	0.8	1	80	66

CdTe-COOH QDs	Cd, 0.4 ± 0.01 Te, 0.1 ± 0.01	0.5	1	50	60
$CdCl_2$	0.79 ± 0.05		0.79	100 ± 2	NA
Te ICP standard	11.8 ± 0.5		10	120 ± 4	NA

Note, pure CdTe QD materials were not digested for analysis. Data presented as mean ± SEM (*n* = 6) mg Cd or Te kg^{-1} dry weight.

Note, CdTe QDs and the salt equivalents were added to the dried earthworm as a liquid stock and then acid digested (mg l^{-1}).

[1]expected values based on deducting the coating mass (detailed in Chapter 5, Table 5.1) from the nominal. Data presented as mean ± SEM (*n* = 6) mg CdTe kg^{-1} dry weight.

2.6 Two-step sequential extraction of soil samples

To offer insight into the accessible Cu or Cd and Te fractions of the specific ENMs, two-step sequential extractions were performed on samples from day 1 from each experiment, based on methods from Black (1965). The first, with water to reveal any freely soluble/mobile metal fraction in the soil, and then a second with 0.1 M HCl (37%) to extract metals held by organic matter in the soil (Black, 1965). The subsequent fractions collected were analysed by ICP-OES to determine copper and cadmium concentration; and ICP-MS to determine Te concentration. Briefly, ultrapure water or 0.1 M HCl was added to soil at ratios of 1:10 (soil: solution) into 15 ml centrifuge tubes. Tubes were rotated for 1 h (Grant bio, PTR-60; orbital rpm 100, 250; reciprocal degree 82), followed by centrifugation (swing out rotor, Harrier 18/80 HSE) at 6000 x g, both for 10 minutes. The solution was then decanted, filtered through a 0.22 µm Whatman filter, acidified with 2 % HNO_3 to prevent metal adhesion and stored for further analysis by ICP-MS/OES (section 2.5).

2.7 Biochemical analyses

All biochemical analyses were optimised for earthworm tissue by, for example, exploring the effects of incubation times on reactions, and the best dilution of the earthworm homogenate samples for each assay. Several runs on non-experimental earthworms preceded each assay, along with checking the linearity and range of standards. The biochemical methods had been used previously for ENMs at University of Plymouth on other organisms (e.g., Boyle et al., 2014 on fish tissues).

2.7.1 Tissue collection and homogenisation

Biochemistry was performed on whole earthworm tissues from samples collected at the end of the soil experiments (usually *n* = 8 per treatment, see chapters 3 and 5 for details). The earthworms were washed and snap frozen in liquid nitrogen and stored at -80°C for further analysis. The tissues of whole earthworms were diluted (1:5 ratio, weight: volume) to a final volume of 2.5 ml in ice-cold isotonic buffer (150 mM sucrose, 50 mM HEPES, 1 mM EDTA, pH 7.3) and then homogenised on ice (3 × 10 s with 2 min rests at 17,500 rpm, Cat X520D with a T6 shaft, medium speed, Bennett and Co., Weston-super-Mare). Homogenates were centrifuged at 6000 rpm for 3.5 min to remove debris. The supernatants were decanted and then stored at −80°C until required for analysis. Subsequently, aliquots of the crude homogenates were further diluted in the cold isotonic buffer (recipe above, 1:10 dilution, i.e., an overall 15-fold dilution of the original tissue) due to the high protein concentration in the earthworms. The diluted homogenates were assayed in triplicate for total protein, total glutathione (GSH), superoxide dismutase and Na^+/K^+-ATPase activity as described below using the VersaMax plate reader (Molecular Devices, UK).

2.7.2 Total glutathione

Total glutathione (GSH) was quantified in triplicate for each sample (n = 8 homogenates per treatment) according to Owens and Belcher (1995). A sub-sample of 20 µl of diluted homogenate, blank or standard was pipetted into a reactions mixture (340 µl) contained 5,5-dithiobis-(2-nitrobenzoicacid) or DTNB (0.58 mmol l^{-1}), ethylenediaminetetraacetic acid or EDTA (3.8 mmol l^{-1}), dihydronicotinamide-adenine dinucleotide phosphate or NADPH (0.2 mmol l^{-1}), phosphate buffer (76.5

mmol l^{-1}, pH 7.3), glutathione reductase (0.1 U ml^{-1}), mixed by pipetting and the change in absorbance measured over 10 minutes at 412 nm. The total glutathione concentration in each homogenate was determined using the standard calibration curve (a dilution series of 200 µmol l^{-1} from a reduced glutathione standard).

2.7.3 Sodium pump (Na^+/K^+-ATPase) activity

Na^+/K^+-ATPase activity was determined using a kinetic coupled enzyme assay based on a modification of McCormick (1993). Briefly, 10 µl of sample (diluted homogenate, blank or standards) was mixed in a buffer containing (which also starts the reaction); lactate dehydrogenase (2.85 U ml^{-1}), pyruvate kinase (3.57 U mL^{-1}), phosphoenolpyruvate (2 mmol l^{-1}), adenine triphosphate or ATP (2.5 mmol l^{-1}), dihydronicotinamide-adenine dinucleotide or NADH (0.28 mmol l^{-1}), HEPES (47.6 mmol l^{-1}), sodium chloride or NaCl (45 mmol l^{-1}), magnesium chloride or $MgCl_2$ (2.63 mmol l^{-1}), potassium chloride KCl (10 mmol l^{-1}) at pH 7.3 to follow the enzymatic hydrolysis of ATP with and without the inhibitor, 0.5 mmol l^{-1} ouabain. The reaction was started with the addition of the buffer and the change in absorbance was measured at 340 nm for 10 minutes. The linear rate of each sample with and without ouabain was determined for both sets of separately and the activity was then calculated using the standard calibration curve of 0-20 nmol ADP assayed with and without ouabain. The final activity of Na^+/K^+-ATPase in each sample was determined by the difference between samples with and without ouabain.

2.7.4 Superoxide dismutase

The concentration of superoxide dismutase (SOD) was determined in the diluted homogenates using an SOD Assay kit-WST purchased from Sigma-Aldrich (Kit #BCBQ2037V) exactly according to the manufacturer's instructions. Briefly, 20 µl of the sample, blank or standard was mixed with 200 µl of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H-tetrazolium, monosodium salt), 20 µl of xanthine oxidase, followed by incubation at 37 ℃ for 20 minutes. Absorbance was measured at 450 nm and converted to enzyme activity using a calibration curve derived from a dilution series of SOD from bovine liver, 0-200 U ml^{-1} (purchased separately from the kit, S8409-3KU, CAS 9054-89-1).

2.7.5 Protein quantification

Total protein in samples was quantified using a colorimetric kit calibrated against a standard curve (dilution series of bovine serum albumin 2 mg ml^{-1} provided with the kit). The concentrations of GSH, SOD and Na^+/K^+-ATPase activity were normalised to total protein in the sample. Total protein was determined using the Pierce BCA kit (#RE232674, Thermo Scientific, UK) using 25 µl of homogenate (in triplicate) and 200 µl of working reagent according to the manufacturer's instructions against bovine serum albumin standards (ranging from 0 – 2 g l^{-1} with linear fit, r^2, exceeding 0.9). Data are expressed as nmol GSH per mg protein, unit (IU) SOD per mg protein, and µmol ADP per mg protein per hour, for total GSH, SOD and Na^+/K^+-ATPase, respectively.

2.8 Statistical analyses and data presentation

All statistical analyses were performed and graphs were drawn using R studio software (version 2.1) with some specific analyses, e.g. curve fitting, carried out in SigmaPlot version 13.0, referred to in the relevant chapters. Data were checked for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test for several groups and F-test for two groups). If data was found to be non-parametric, it was transformed (log_{10}) and reanalysed. The Student's *t*-test (two-tailed, paired or unpaired as appropriate) or Mann-Whitney test was used for comparing two sample sets when the data was parametric or non-parametric, respectively. In experiments with soil, the soil chemistry can be a confounding factor that influences the biological response; and for metals, soil pH is of particular concern. If there were significant differences in pH between treatments, then initially analysis of covariance (ANCOVA) was carried out to analyse for the interactive effects of pH in all endpoint analysis. When no interactions were found, pH was omitted from the model to allow the performance of a relevant posthoc test.

Treatment and time specific effects were determined using two-way analysis of variance (ANOVA) and treatment only specific effects by one-way ANOVA followed by Tukey honest significance difference HSD test (equal sample size) or Tukey-Kramer (unequal sample size) posthoc test, where relevant. When data was normally distributed, but variance remained unequal, Welch's ANOVA was used followed by Games-Howell posthoc test. Changes in biomass were analysed with repeated measures ANOVA followed by Tukey's HSD test to identify the differences, since the same earthworms were repeatedly measured throughout the exposures (Newman, 2012). Where data transformation deemed unsuccessful, non-parametric Kruskal-Wallis test was used followed by distribution free multiple comparisons test,

Dunn’s test. To analyse underlying potential correlations between biological effects, correlation tests (Spearman’s rank, r_s) were carried out on all data irrespective of treatment. The statistical significance level (α) for all tests was set at 0.05.

All measured and nominal concentrations are based on dry weight unless stated otherwise.

Chapter 3. The biological effects of fresh and aged CuO ENMs on earthworms

3.1 Introduction

Metal-containing engineered nanomaterials (ENMs), especially silver and titanium, are being used in nano-enabled products, but attention is now turning to other metals. Of these, copper-based engineered nanomaterials (ENMs) are being used as catalysts in the manufacture of electronics (Gawande et al., 2016), proposed as wood-preservatives (Evans et al., 2008), in anti-fouling paints (Anyaogu et al., 2008) and as antimicrobials (Bogdanovic et al., 2015). Cu-based ENMs are also finding applications in agriculture, including as biocides (Servin and White, 2016). However, responsible innovation demands that the risks to the environment and human health are understood (Owen and Handy, 2007).

Soil quality is an important consideration for the health of terrestrial ecosystems, as well as ecosystem services such as agriculture. Metal contamination of soil is a concern for the environment as well as the risks to humans via the food chain. Consequently, there are guideline values for allowable metal concentrations in soils in many countries. For Cu, the values range from 63 - 91 mg Cu kg^{-1} dw depending on land use in Canada (CCME, 1999/1997), while no overall value exists in the European Union country-specific guidance have been developed. For example, in Finland values range from 100 – 200 mg Cu kg^{-1} dw (MEF, 2007). These total Cu measurements in soils comprise naturally occurring Cu minerals and anthropogenic sources of Cu. However, 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), as they are for copper metal salts; partly due to the application of sewage sludge to agricultural land.

The effects of elevated metal concentrations in soils on terrestrial organisms is relatively well-known from decades of research on naturally occurring Cu and anthropogenic inputs of dissolved Cu (Neuhauser et al., 1985). 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 dry weight (dw) (Streit, 1984) and this can increase to 58 mg Cu kg^{-1} dw and higher at pollutes sites, e.g., near mine smelters (Ma, 2005). Earthworms, like other animals, require Cu as an essential nutrient and can regulate internal Cu concentrations (Bundy et al., 2008). Consequently, earthworm populations can survive at moderately contaminated sites. However, sub-lethal effects on reproduction and health have been reported. 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). Copper is known to affect volume regulation in animals (via water regulation), and both Cu exposure and the salinity of the soil can influence apparent growth of earthworms (Fischer and Molnár, 1997). Further to this, sublethal concentrations of Cu have been shown to reduce earthworms' ability to survive drought, potentially by interfering with water regulation (Friis et al., 2004). Copper can also promote oxidative stress and interfere with energy metabolism in earthworms (Bundy et al., 2008). These effects raise concerns about the ability of earthworms to survive and provide the important ecosystem service of turning the soil.

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

counterparts (ZnO, Heggelund et al., 2014; Ag, Velicogna et al., 2016). Heckmann et al. (2011) reported acute toxicity limit tests for earthworms, and found Cu nanoparticles were not acutely toxic at a nominal concentration of 1000 mg kg^{-1}, although a 10 % decrease in cocoon production was observed. However, there are concerns that ENMs may dissolve in the porewater of soil or release metal ions by dissolution. Direct contact toxicity of metal particles on the surface of the earthworm, or via ingestion, also cannot be excluded as a way of delivering toxic metal ions. Sub-lethal effects have also been observed with ENMs. These include declines in reproductive success as measured by cocoon production and the number surviving offspring (e.g., Ag NPs at 1000 mg Ag kg^{-1} dw, Heckmann et al., 2011; ZnO NPs at 900 mg Zn kg^{-1}dw, Heggelund et al., 2014), and avoidance of ENM contaminated soil (Ag NPs at 10 mg Ag kg^{-1} dw, Shoults-Wilson et al., 2011). Earthworms do show total metal accumulation from exposure to metal-containing ENMs in soil (e.g., Ag, Diez-Ortiz et al., 2015), but if 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, Hayashi et al., 2013a; Gomes et al., 2015a). 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). The latter study found Cu to accumulate in earthworm tissue, with increased expression of the metal chelator, metallothionein, and therefore no adverse health effects on the earthworms for concentrations up to 65 mg Cu kg^{-1}dw. The effects of surface coatings on Cu-containing ENMs in earthworms remains to be reported. In addition, there are concerns that ENMs as released may be modified in the environment, and yet there

are very few studies of the effect of Cu-containing NPs in aged soils. 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 highlight 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, carboxylate (COOH), cationic, ammonium (NH_4^+) and neutral 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, Cu is a known inhibitor of the latter) 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.

3.2 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).

3.2.1 Test soil, organisms, conditions and engineered nanomaterials

Test soil composition, animals and conditions were exactly as previously described (see Chapter 2.3-2.4 details). Earthworms were placed in Lufa 2.2 soil with feed a week prior to the experiment, to acclimatise to the conditions. Experiments were conducted at 20 ± 2^{o}C and with a 12:12 h light:dark cycle under 400-800 lux lighting. During the experiment the earthworms were fed clean horse manure from unmedicated Dartmoor (Devon, UK) ponies (1 g manure dw / earthworm). The ENMs used in the experiment and the method of dosing with experimental soil preparation is detailed in Chapter 2.2.

3.2.2 Metal analysis in samples

Total Cu and other cations in soils and earthworms were measured. In soils the concentrations were determined in samples from the beginning and end of the fresh soil experiment and in the beginning of aged soil experiment following the methods described in Chapter 2.5. Earthworms (n = 8) were sampled at random after 7 and 14 days (fresh soil experiment) and 14 days (aged soil experiment) from each treatment. The earthworms were processed and analysed following the methods described in Chapter 2.6.

3.2.3 Two-step sequential extraction of soil samples

The accessible Cu fraction was measured in duplicate in samples from each treatment in the beginning of the fresh soil experiment only and analysed as described in Chapter 2.7.

3.2.4 Experimental design

Fresh Soil Experiment

This experiment was conducted using an approach similar to the standard OECD TG 207 the Earthworm Acute Toxicity (OECD, 1984) with some adaptions and additional endpoints. The experiment included a test soil control (no added Cu or ENMs), a metal salt control of Cu as $CuSO_4$ at 200 mg Cu kg^{-1} dw, the uncoated CuO ENM, and those coated with $–NH_4^+$, –COOH or –PEG respectively. For the ENMs, two test concentrations were selected after considering the known toxicity of Cu and Cu ENMs to earthworms (Spurgeon et al., 1994; Heckmann et al., 2011). The lower concentration of 200 mg Cu kg^{-1} dw was chosen to be sub-lethal and around three times the expected background concentration of total Cu in European soils (the latter, ~ 60 mg Cu kg^{-1} dw, Heijerick et al., 2006). The upper concentration of 1000 mg Cu kg^{-1} dw was equivalent to that suggested in the limit test according to OCED (2004).

Adult *E. fetida* with a typical mean starting wet weight of 5.5 ± 0.1 g (mean ± SEM, for a subsample of 12 of the initial earthworms, individual worm weight ~0.45 g) were exposed in 4 replicates (*n* = 12 earthworms per container, *n* = 48 earthworms per treatment) at 20 ± 1 ^oC at 12:12 light:dark cycle. The exposure containers were rectangular (10 x 20 cm) 1 litre food grade polypropylene tubs (Graham Tyson, UK).

Survival and body weight of the earthworms were recorded at day 0 (start), 7 and 14 of the experiment. Behavioural changes, such as avoidance of soil and avoidance of burrowing into the soil, were observed manually once a day in the morning, in all treatments. Earthworms were collected at day 7 and 14 for Cu determination and at the end of the experiment for biochemistry and histology (detailed below).

Aged soil experiment

The soil used in this experiment was the same as used in the initial fresh soil experiment. The soil was kept for one year after the first experiment in the original test containers, with the lids pierced to ensure some airflow in the same exposure conditions defined above. During this time, plants had grown in the soil (from seeds already present in the natural soil and likely from the added horse manure). One week prior to the experiment the plant material (excluding roots) was removed by cutting and the soil moisture adjusted to 50 - 60 %, then soil pH measured as described above. In this more selective experiment, fewer earthworms were used (5 in each replicate, 20 per treatment) with a mean wet weight of 1.3 ± 0.03 g of per exposure replicate (mean ± SEM, *n* = 40 treatments, individual worm weight ~ 320 mg). Endpoints such as survival and biomass were recorded as in the fresh soil experiment. Other selected endpoints, including tissue Cu concentrations and biochemistry were measured at the end of the experiment (day 14).

3.2.4.1 Biochemical analyses

Biochemical analyses were performed on whole earthworm tissues from samples collected at the end (day 14) of both fresh and aged soil experiments (*n* = 8 per treatment). Analyses followed the methods described in Chapter 2.7

3.2.5 Histology

Two earthworms from controls, $CuSO_4$ and high CuO ENM treatments were collected, and after depuration on clean moist filter paper for 24 h the earthworms were washed and anesthetised in 60 % carbonated water (2:1 ratio of carbonated water: deionised water, Shannon et al., 2014) (*n* = 3 earthworms per treatment, or two due to high mortality during depuration). The method of van der Ploeg et al. (2014) was followed to collect the earthworm tissue. Briefly, segments from the earthworms were obtained by cutting the anterior part of the earthworm 3 segments beneath the clitellum using a razor. The sections were then placed in buffered (neutral) formal saline to preserve the samples. The anterior segments adjacent to the clitellum were processed into wax blocks following Handy et al. (2002). Transverse sections of 7 µm were cut from each earthworm, stained with haematoxylin and eosin and coverslipped with DPX. Specimens were stained in batches with all treatments to avoid staining artefacts between treatments. Specimens were imaged using a digital micro-imaging device Leica DMD 108 (Leica, UK) and analysed using Image J (version 1.51k). These sections were analysed blind and measurements of the cuticle, longitudinal and circular muscle were made (van der Ploeg et al., 2014)

3.2.6 Statistical analyses and data presentation

All statistical analyses were performed as described in Chapter 2.9. In addition, to understand potential bioaccumulation a measure was calculated based on the total copper concentration in the earthworms divided by the total concentration of copper soils ([Cu]earthworm / [Cu]soil) and in text it is referred as nBAF or the apparent nano-bioaccumulation factor (further discussion on BAF vs nBAF in Chapter 4). In all figures

"Cu" represents the $CuSO_4$ treatments, 'core' as the uncoated and 'PEG', 'COOH' and 'NH_4^+' to refer the coated CuO ENMs.

3.3 Results

3.3.1 Soil pH

Fresh Soil experiment

Soil pH is known to be a factor in metal speciation and the ecotoxicity of soils. Soil pH varied between treatments at the beginning of the experiment (day 0) in the fresh soil experiment (Table 3.1), with statistically significant differences in the initial soil pH between $CuSO_4$ treated soils and the rest of the treatments including the control. However, by day 14 this was resolved and there were no statistically significant differences in pH between treatments (Table 3.1).

In the aged soil experiment, the soil pH on day zero was generally lower than in the fresh soil experiment (Table 3.1, $P < 0.05$ *t*-test) and there were no statistically significant differences between treatments ($P > 0.05$ ANOVA). No interactive effects were found for soil pH with any of the biological endpoints assessed in either the fresh and aged experiments ($P > 0.05$ ANCOVA), demonstrating that pH was not a factor in the responses of the earthworms.

Table 3.1 Soil pH in the CuO ENM fresh and aged soil experiments on day 0 and 14 of the experiments.

Nominal soil [Cu]		**Treatment**					
mg Cu kg^{-1} dw		**Control**	**$CuSO_4$**	**Core**	**PEG**	**COOH**	**NH_4^+**
Fresh soil	*Day*						
200	0	5.5 ± 0.06^{abc}	4.97 ± 0.02^{d}	5.6 ± 0.03^{b}	5.35 ± 0.02^{c}	5.39 ± 0.02^{bc}	5.30 ± 0.05^{bc}
1000	0			5.80 ± 0.01^{a}	5.17 ± 0.01^{c}	5.31 ± 0.05^{bc}	5.52 ± 0.01^{b}
200	14	5.19 ± 0.04^{ac}	5.08 ± 0.04^{ab}	4.98 ± 0.02^{ab}	4.97 ± 0.02^{ab}	4.81 ± 0.08^{b}	5.01 ± 0.02^{ab}
1000	14			5.36 ± 0.02^{ac}	5.14 ± 0.01^{ac}	5.60 ± 0.02^{c}	5.69 ± 0.08^{c}
Aged soil							
200	0	5.2 ± 0.03*	5.15 ± 0.02*	5.12 ± 0.07*	5.33 ± 0.07	5.25 ± 0.07	5.26 ± 0.06
1000	0			5.3 ± 0.04*	5.19 ± 0.02	5.14 ± 0.02	5.16 ± 0.02*
200	14	5.27 ± 0.08*	5.19 ± 0.04*	5.14 ± 0.03*	5.11 ± 0.02	5.16 ± 0.02	5.12 ± 0.02
1000	14			5.45 ± 0.02*	5.28 ± 0.006	5.24 ± 0.06	5.21 ± 0.01*

Data expressed as mean ± SEM (n = 4). Different letters denote statistically significant differences ($P < 0.05$, ANOVA, Games-Howell); asterisks denote the statistically significant difference between the fresh and aged soil pH ($P < 0.05$, *t*-test).

3.3.2 Total and extractable soil copper

The exposure was monitored by measuring the total metal concentrations in the soil in both the fresh soil and aged soil experiments. In the former experiment, the control soil contained a background Cu concentration of (mean ± SEM, *n* = 4) 1.37 ± 0.2 mg Cu kg^{-1} dw and of this total Cu, 5 ± 0.6 and 55 ± 5 % were water and 0.1 M HCl-extractable respectively, indicating that at least half of the total Cu was labile. Exposure to $CuSO_4$ caused the expected increase in total soil Cu concentrations that were close to the nominal values (Figure 3.1a). The fresh soils treated with the various forms of CuO ENMs did not show the same measured total Cu concentrations. Instead, the amount of Cu measured in the soil varied depending on the type of ENM coating (Figure 3.1a). The measured total Cu concentration in the fresh soils dosed with CuO ENMs were in the following order: Core > NH_4^+ > COOH > PEG. Nonetheless, exposure was confirmed, with the total soil Cu concentrations in the ENM treatments being much higher than the unexposed control. Despite some differences in measured total soil Cu between the ENM treatments, the extractable fractions remained similar (no statistical differences), regardless of the type of coating on the ENMs (Figure 3.1a). The water-extractable fractions remained around 1-2% regardless of the ENM coating at the nominal 200 mg Cu kg^{-1} dw soil treatments (Figure 3.2). Differences emerged in the nominal 1000 mg Cu kg^{-1} dw treatments. In the nominal 1000 mg Cu kg^{-1} dw ENM treatments, the water available Cu concentrations were statistically significant different between CuO-PEG (4.2 ± 0.09 %), -COOH (2.5 ± 0.18 %), -NH_4^+ (2.2 ± 0.1 %) and -core (0.9 ± 0.1 %) treatments (mean ± SEM, *n* = 4). There were statistically significant differences between the CuO PEG and CuO-Core treatments with all other

treatments (P <0.05, Kruskal-Wallis, Dunn's test), but no significant differences between CuO-COOH and -NH_4^+ at nominal 1000 mg Cu kg^{-1} dw test concentrations (P > 0.05, Dunn's test). The 0.1 M acid extractable dominated, contributing 50 % or much more of the total Cu in the ENM treatments, with a similar pattern at both the 200 and 1000 mg Cu kg^{-1} dw exposure concentrations (Figure 3.1a); although only the CuO-PEG treated soils were significantly different from the rest.

The total concentration of Cu in soils was confirmed again prior to beginning of the aged soil experiment. The total concentration of Cu was slightly lower in all treatments but only significantly lower in the $CuSO_4$ and in the nominal 1000 mg Cu kg^{-1} dw CuO-COOH and CuO- NH_4^+ treatments, at the start of the aged soil experiment compared to a year earlier (compare Figures 3.1a and b).

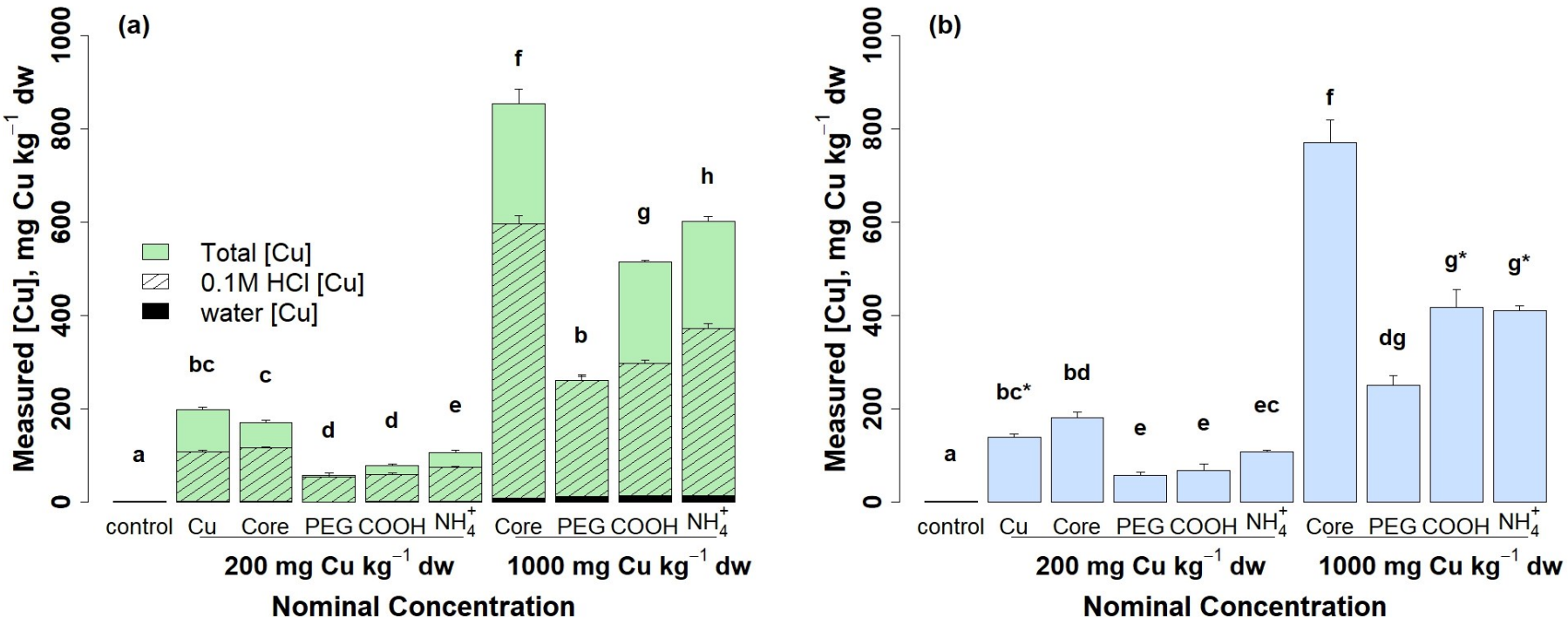

Figure 3.1 Total Cu concentration in the soil at the beginning of the experiments (a) fresh soil, (b) soils aged for one year (Cu – $CuSO_4$, Core – uncoated CuO ENMs, PEG-, COOH-, NH_4^+ - coated CuO ENMs). Data expressed as mean ± SEM (n = 12, soil samples analysed in triplicate from the fresh soil experiment; n = 8, soil samples analysed in duplicate from the aged soil experiment). In panel (a) the water or 0.1M HCl extractable Cu is presented as black or dashed bars, respectively. Treatments that do not share a letter are significantly different from the each other within each experiment (P < 0.05, ANOVA) Asterisks denote a statistically significant difference between the total Cu concentrations in the fresh compared to aged soil experiment (P < 0.05, Mann-Whitney).

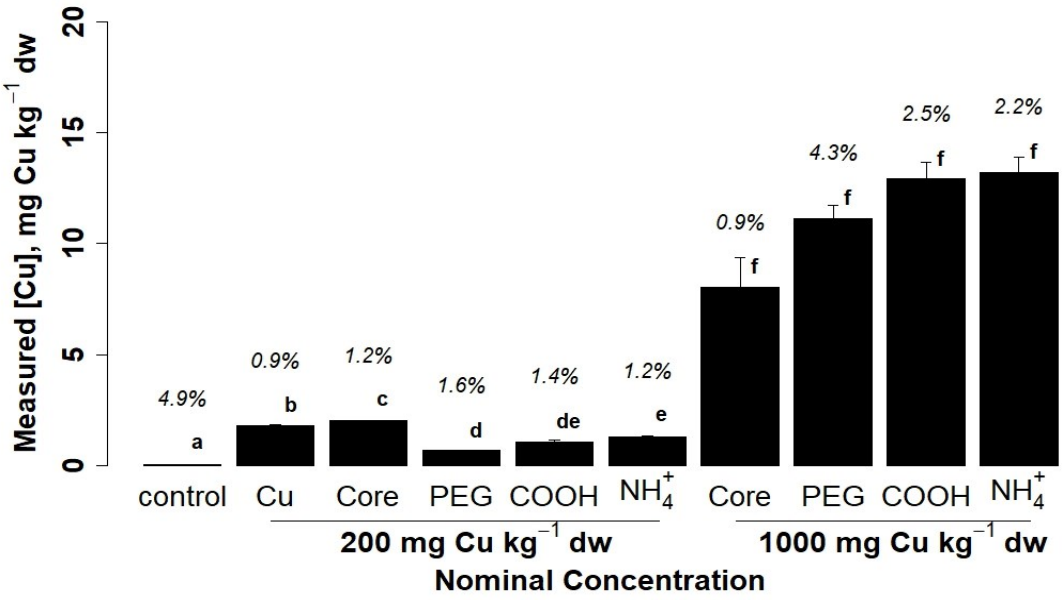

Figure 3.2 Measured water extractable concentration of Cu in the beginning of the fresh soil experiment in mg Cu kg^{-1} dw (Cu – $CuSO_4$, Core – uncoated CuO ENMs, PEG-, COOH-, NH_4^+ - coated CuO ENMs). Data presented as mean ± SEM (n = 8, soil samples analysed in duplicate from each treatment). The percent water extractable fraction of the total measured Cu in soils is presented on each treatment bar (%) (total Cu in Figure 3.1a). Statistical labels are presented for the measured water extractable Cu. Treatments that do not share a letter are statistically significantly different (P < 0.05, ANOVA, Games-Howell).

3.3.3 Metals in earthworm tissue

In addition to the soils, total Cu was also measured in whole earthworms, following depuration of the gut contents (Figure 3.3). In the fresh soil experiment, the unexposed control earthworms had a Cu concentration of 13.8 ± 2 mg Cu kg^{-1} dw at the end of the experiment (day 14). In contrast, the $CuSO_4$ and the ENM treatments all showed elevated Cu concentrations compared to the control earthworms (Figure 3.3a). There was no nano-effect on Cu accumulation with those in the $CuSO_4$ treatments being similar to the CuO core material in the fresh soil. However, there was a coating-effect within the CuO ENMs, with exposure to the CuO-PEG resulting

in less total Cu in the earthworms compared to the other treatments (Figure 3.3a and b). The total Cu concentration in the earthworms from the fresh soil experiment was correlated the measured total and water/acid extractable Cu in the soils in the beginning of the experiment (r_s = 0.8, P < 0.05, Spearman's for all, Figure3.3c). There was no statistically significant difference between the internal Cu concentrations in earthworms from day 7 to 14 in the nominal 200 mg Cu kg^{-1} dw treatments, which allows the calculation of the nBAF, since the metal BAF calculation is based on the assumption Cu has reached a steady state in the organism. Despite there being an increase in internal Cu concentrations in earthworms in the nominal 1000 mg Cu kg^{-1} dw treatments, for comparison purposes the nBAF is shown (Table 3.2). The BAF is highest in the controls as expected. The BAF in $CuSO_4$ controls is slightly lower than in the CuO-Core ENM treatments. The coated ENM treatments at the nominal 200 mg Cu kg^{-1} dw concentration have the highest nBAF which are also statistically significantly different from all other exposures. The nominal 1000 mg Cu kg^{-1} dw ENM treatments had lower nBAFs and were comparable to that of the nominal 200 mg Cu kg^{-1} dw $CuSO_4$ and CuO-core ENM treatments.

In the aged soil experiment, the control earthworms had an internal Cu concentration of 9.8 ± 0.2 mg Cu kg^{-1} dw. While an increase in Cu was seen in all of the Cu treated exposures. The same amount of Cu was taken up in the $CuSO_4$ and the CuO-Core ENM treatments. Statistically, there was no significant coating effect on the uptake of Cu in the nominal 200 mg Cu kg^{-1} dw treatments, although the uptake was lowest in the CuO-PEG treatment (Figure 3.4a). However, in the nominal 1000 mg Cu kg^{-1} dw treatments uptake of Cu was significantly higher in the CuO-COOH treatments compared to the rest of the ENM treatments. The correlation between the total body

burden and total soil Cu was similar to the fresh soil experiment (r_s = 0.8, P < 0.05, Spearman's). Compared to the fresh soil study, the uptake of Cu was more uniform between treatments as well as around two times lower in the nominal 1000 mg Cu kg^{-1} dw treatments (Figure 3.4b).

The BAF/nBAF was also calculated for the aged soil experiment. Although it is not certain if the organism had reached steady state, it is a good indicator of differences between treatments. In the aged soil experiment, the pattern of the BAF/nBAF was similar to the fresh soil experiment, the controls had the highest BAF. The BAF in the $CuSO_4$ treatment remained very similar to the fresh soil study, while nBAF in the nominal CuO-core treatment was lower than in the fresh soil study (Table 3.2). The nominal 200 mg Cu kg^{-1} dw coated ENM treatments remained the highest out of all Cu treatments, although when compared to the fresh soil, they were lower. In general, the nominal 1000 mg Cu kg^{-1} dw nBAF values were lower than the fresh soil nBAFs in the same test concentration.

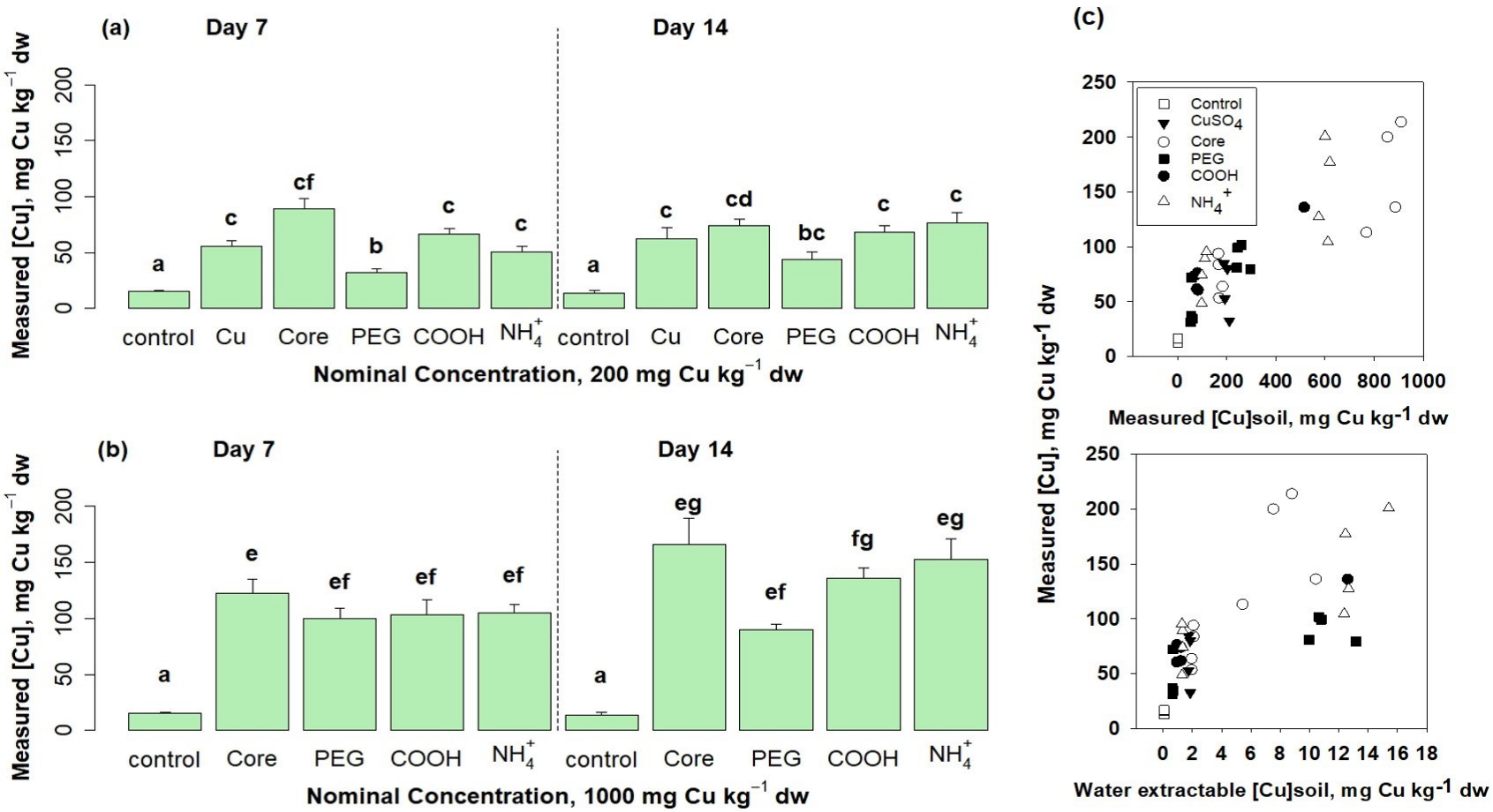

Figure 3.3 Total concentration of Cu in earthworms exposed to fresh soil containing a nominal (a) 200 or (b) 1000 mg Cu kg^{-1} dw on day 7 (left) and day 14 (right) for each treatment (Cu – $CuSO_4$, Core – uncoated CuO ENMs, PEG-, COOH-, NH_4^+ - coated CuO ENMs). Data are presented as mean ± SEM (n = 8 as number of earthworms from each treatment). Treatments that do not share a letter are statistically significantly different across panel (a) and (b) ($P < 0.05$, ANOVA).The relationship between measured concentration of Cu in earthworms and measured concentration in soil (upper panel (c)) and the water extarctable Cu in soil (lower panel (c)) is shown for reference.

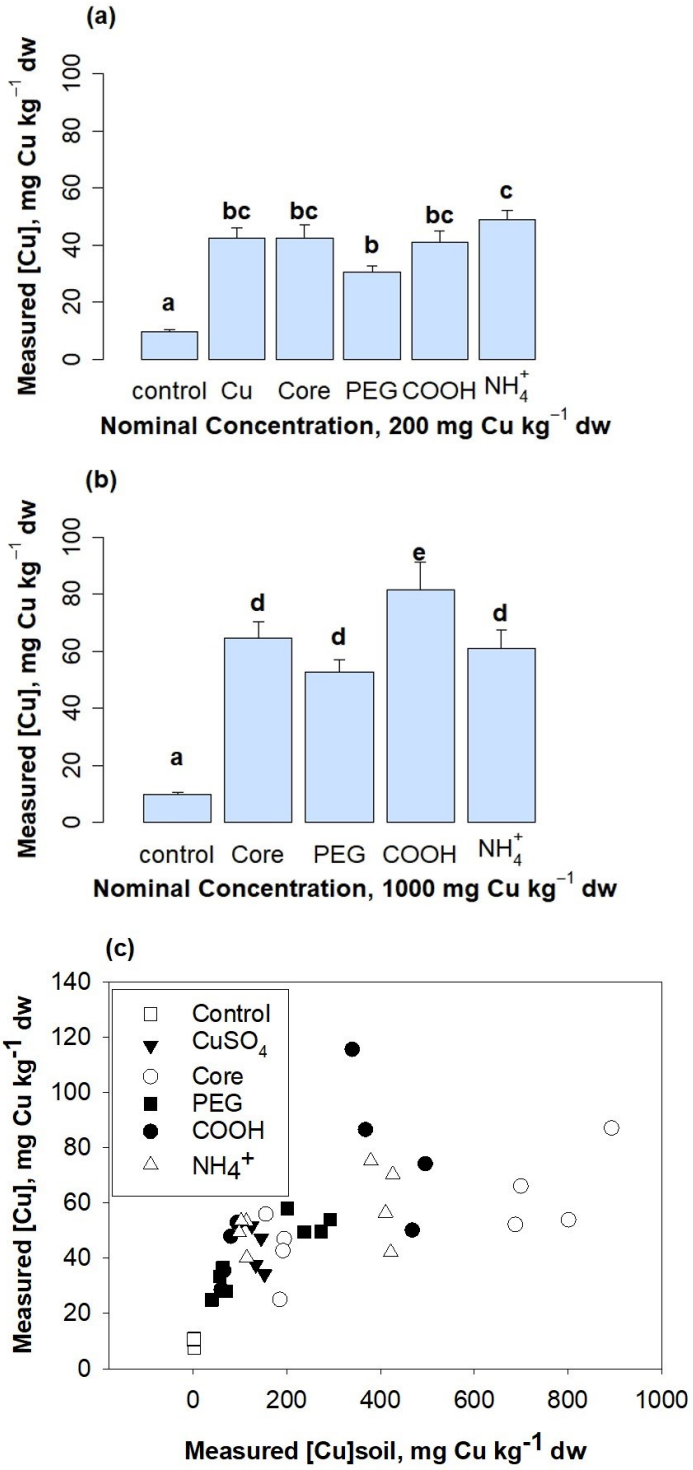

Figure 3.4 Total concentration of Cu in earthworms on day 14 (the end of the experiment) in aged soil containing a nominal (a) 200 or (b) 1000 mg Cu kg^{-1} dw. (Cu – $CuSO_4$, Core – uncoated CuO ENMs, PEG-, COOH-, NH_4^+ - coated CuO ENMs). Data are presented as mean ± SEM (*n* = 8 as no of earthworms from each treatment). Treatments that do not share a letter are significantly different from the each other within test concentration ($P < 0.05$, two-way ANOVA, Tukey HSD). The relationship between measured [Cu] in earthworms and measured total Cu concentration in soil is shown on panel (c) for reference.

Table 3.2 The nano-/bioaccumulation factors (n/BAFs)in earthworms from the the fresh and aged soil experiments.

Treatment	Nominal	Fresh Soil	Aged soil
	mg Cu kg^{-1} dw	BAF / nBAF	
control	**0**	10.4 ± 2.62[a]	5.49 ± 0.75[a]
$CuSO_4$	**200**	0.29 ± 0.06[b]	0.31 ± 0.04[b]
Core		0.35 ± 0.04[b]	0.24 ± 0.05[b]
PEG		0.74 ± 0.08[c]	0.55 ± 0.05[c]
COOH		0.76 ± 0.09[c]	0.54 ± 0.02[c]
NH_4^+		0.76 ± 0.07[c]	0.46 ± 0.04[c]
Core	**1000**	0.22 ± 0.03[b]	0.08 ± 0.01[e]
PEG		0.28 ± 0.02[b]	0.22 ± 0.0[b]
COOH		0.33*[bc]	0.21 ± 0.05[b]
NH_4^+		0.26 ± 0.03[b]	0.15 ± 0.02[be]

Data expressed as expressed as mean ± SEM (n = 4). Different letters denote statistically significant differences ($P < 0.05$, Tukey HSD/ Games-Howell).

3.3.4 Survival, growth and behaviour

In the fresh soil experiment, the control earthworms remained healthy (100 % survival) and with no statistically significant loss of biomass over time (Table 3.3). Similarly, in the $CuSO_4$ treatment, the exposure was sub-lethal with 90 % or more survival and no differences in biomass compared to controls. For the ENM treatments in fresh soil, the 200 mg Cu kg^{-1} dw nominal exposure had negligible effects on survival, with survival remaining above 95 % and similar to the $CuSO_4$ treatment. However, for the ENMs used in the higher Cu dose (1000 mg Cu kg^{-1} dw) some toxicity was observed, with the CuO-COOH and CuO-NH_4^+ treatments showing statistically significant mortality compared to the unexposed controls and other forms of the ENM by the end of the experiment (Table 3.3). Biomass also declined in all the ENM treatments compared to the controls at the 1000 mg Cu kg^{-1} dw exposure concentration at day 7, and this persisted with often further loss of biomass in the ENM-exposed groups until the end of the experiment (Table 3.3). The survival, regardless of the type of Cu presented, was also positively correlated (r_s = 0.7, $P < 0.05$, Spearman's) with the approximate individual wet weights (i.e., total biomass divided by the number of surviving animals) of the earthworms. In addition, as a general observation, earthworms in the controls and nominal 200 mg Cu kg^{-1} dw exposures were feeding normally, while in the nominal 1000 mg Cu kg^{-1} dw exposures, lack of feeding was evident. This observation also explains loss of weight.

There were also some behavioural changes in the earthworms that preceded toxicity. At the beginning of the experiment in the nominal 1000 mg Cu kg^{-1} dw CuO-COOH treatment, the earthworms were avoiding the soil, rather than burrowing. In

contrast, the earthworms in all the other treatments burrowed almost immediately. One day later, other behaviours were observed in the highest test concentrations of ENMs. In all ENM treatments, except for the CuO-core, the earthworms were observed bundled together; presumably to minimise contact with the soil. Furthermore, by day 7, even the earthworms in the higher CuO-core treatment were showing this behaviour. At the end of the experiment with fresh soils, the earthworms from the ENM treatments (except the CuO-PEG) appeared to have more soil stuck to their epidermis; perhaps indicating a change in mucus secretion or hydration state. Notably, most of the earthworms from the higher CuO treatments (core, COOH, NH_4^+ did not survive when placed on moist filter paper in a Petri dish to depurate their guts overnight, indicating that the earthworms were moribund.

The survival of earthworms in the aged soil experiment was broadly similar to those of the experiment with fresh soil (Table 3.3), with the animals from the control and $CuSO_4$ treatments surviving, as well as those from the lower concentrations of the CuO ENMs regardless of coating. In the latter for the CuO ENM, the biomass even increased. However, the higher exposure concentration of the CuO-core and CuO-NH_4^+ ENMs were toxic, decreasing survival compared to the controls. Biomass also showed a statistically significant decrease in all the higher concentrations of the ENMs, except for the CuO-PEG which also less toxic. Earthworm survival and approximate individual wet weight were positively correlated ($r_s = 0.5$, $P < 0.05$, Spearman's). In contrast to the fresh soil experiment, soil avoidance was not observed at the start of the experiment, although on day 14 earthworms in all higher CuO ENM treatments, regardless of coating, were found bundled together or curled up alone as in the fresh soil experiment.

Table 3.3 Survival and biomass of earthworms following 14 days exposure to CuO ENMs or $CuSO_4$ in fresh and aged soils.

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

Aged soil														
200	**14**	Total	5[a]	2 ± 0.1a	5[a]	1.3 ± 0.1[a]	5 ± 0.2[a]	1.4 ± 0.2[b]	5[a]	1.8 ± 0.04[ab]	5[a]	1.8 ± 0.1[ab]	5[a]	1.6 ± 0.1[ab]
		%	100	↑53 ± 8.4	100	↑0.1 ± 5.5	95 ± 5	↑29.4 ± 9.2	100	↑36.2 ± 8.5	100	↑45.8 ± 1.1	100	↑42.1 ± 6.4
1000	**14**	Total					4 ± 1[b]	0.7 ± 0.2[c]	5[a]	1.1 ± 0.1[c]	5[a]	1 ± 0.1[c]	2 ± 0.2[b]	0.2 ± 0.04[c]
		%					70 ± 10	↓26.3 ± 2.3	100	↓11.4 ± 9.3	100	↓19.4 ± 9.6	30 ± 5.8	↓37.1 ± 8.1

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

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

↑ Increase in wet weight relative to day 0

↓Decrease in wet weight relative to day 0

3.3.5 Sodium pump activity and tissue elemental composition

In the experiment with fresh soil, the control animals showed normal Na^+/K^+-ATPase activity at around 8 µmol ADP per mg^{-1} protein h^{-1} (Figure 3.5a). The earthworms exposed to $CuSO_4$ showed no change in the activity of the Na^+ pump. However, of those exposed to the 200 mg Cu kg^{-1} dw of the ENMs, only the CuO-core and CuO-PEG treatments showed a statistically significant decrease in enzyme activity compared to unexposed controls or to the $CuSO_4$ treatment. At the test concentrations of 1000 mg Cu kg^{-1} dw of the ENMs, there was an overall trend of lower sodium pump activity compared to the controls, but at the end of the experiment, this was only statistically significant for the CuO-core, CuO-PEG and CuO-NH_4^+ treatments (Figure 3.5a). Statistically significant negative correlation was found between total Cu in earthworm tissue and Na^+/K^+ ATPase activity regardless of treatment (r_s = - 0.5, $P < 0.05$, Spearman's, Figure 3.5c). However, further individual scatterplots by treatment were explored, and these revealed that exposure to the CuO-PEG ENMs did not follow a clear concentration-dependent pattern (Figure 3.5c), with the Na^+/K^+ ATPase activity being reduced regardless of the measured total Cu concentration in the earthworms; indicating the –PEG form as a potent inhibitor of the sodium pump.

The total concentrations of tissue electrolytes (Ca, Mg, K, Na) and trace elements (Mn, Fe and Zn) were measured in both the fresh and aged soil experiments (see Table 3.4). For the fresh soil experiment (Table 3.4), electrolyte concentrations showed no clear time or treatment-dependent statistically significant changes, except in the nominal 200 mg Cu kg^{-1} dw $CuSO_4$ and CuO-PEG exposures, where the concentration of K was significantly lower than the control on day 14 (Table 3.4, $P <$

0.05, ANOVA). Overall a strong positive correlation was found between Cu and Mg (r_s = 0.6, P < 0.05, Spearman's) and Cu and Zn (r_s = 0.6, P < 0.05, Spearman's) in the earthworm tissue in the fresh soil experiment.

In contrast to the fresh soil experiment, there was no statistically significant effect of exposure to any of the treatments on the activity of the sodium pump in the aged soil experiment (Figure 3.5d). However, in the most toxic treatment, the 1000 mg Cu kg^{-1} dw of CuO-NH_4^+ ENM, the Na^+/K^+-ATPase activity also the lowest measured in the aged soil experiment (3.6 ± 0.5 µmol ADP mg $protein^{-1}$ $hour^{-1}$), although not statistically significant. If the sodium pump activity was compared across the fresh and aged soil experiments by treatment, the 1000 mg Cu kg^{-1} dw CuO-core and CuO-COOH treatments were significantly higher in the latter experiment (Figure 3.5a, d). Of the electrolytes and trace elements measured in the tissues of the earthworms in the aged soil experiment, only Zn and Mn showed statistically significant treatment-dependent changes (P < 0.05, ANOVA, Table 3.4). For example, the concentration of Zn in earthworm tissue was the lowest in the nominal 1000 mg Cu kg^{-1} dw CuO-NH_4^+ exposure (54 ±4 mg Zn kg^{-1} dw). There was a strong negative correlation between measured Cu and Zn in the earthworm tissue (r_s = -0.7, P < 0.05, Spearman's). The concentration of Mn in earthworm tissue was lowest in the CuO-Core exposure (17.07 ± 2.72 mg Mn kg^{-1} dw dw). There was a strong negative correlation between and Cu and Mn (r_s = -0.5, P < 0.05, Spearman's) in the earthworm tissues from all treatments in the aged soil experiment (Table 3.4).

3.3.6 Oxidative stress markers and histological observations

Total glutathione (Figure 3.5b) and SOD activity (Figure 3.6) were also measured in the earthworms as indicators of oxidative stress. There was no clear exposure

concentration-dependent depletion of total GSH within or between treatments. However, total GSH showed statistically significant increases in the 200 mg Cu kg^{-1} dw CuO-PEG, CuO-COOH, and CuO-NH_4^+ treatments; as well as in the 1000 mg Cu kg^{-1} dw CuO-PEG treatments compared to the unexposed or $CuSO_4$ controls. The absence of glutathione depletion in the fresh soil experiment was complemented by the absence of treatment-dependent changes in SOD activity in the earthworms from different treatments, although there was a trend of increasing SOD in the highest test concentration of the coated-CuO ENM treatments (not statistically significant, $P > 0.05$, ANOVA; Figure 3.6). There was a statistically significant negative correlation between total SOD and survival at the end of the experiment ($r_s = - 0.4$, $P < 0.05$).

In the aged soil experiment, there was no evidence of glutathione depletion, and in fact, the 200 mg Cu kg^{-1}, CuO-COOH and CUO-NH_4^+ treatments showed statistically significant increases in total GSH compared to unexposed controls (Figure 3.5e). However, no induction of total GSH was observed at the higher test concentrations in the aged soil experiment. If the total glutathione response is compared to fresh and aged soils by treatment, some statistical difference is observed at the 1000 mg Cu kg^{-1} dw test concentrations of the ENMs for the CuO-core, CuO-PEG and CuO-COOH treatments; but the direction and magnitude of the changes were not consistent by material coating across the experiments.

Histology was conducted on three animals per treatment at the end of the fresh soil experiment to observe any morphological evidence of inflammation or other tissue injuries that might be indicative of oxidative stress (Figure 3.7). This end point was restricted to the control, $CuSO_4$ treatment and the 1000 mg Cu kg^{-1} dw of the various ENMs. Generally, earthworms from all treatments showed normal histology. All earthworms showed an intact cuticle. There was no indication of necrosis or reactive

hyperplasia in the epidermis. However, for all the types of CuO ENMs (except the PEG-coated), a mild hypoplasia of mucous (goblet) cells was observed in 2/2, 2/3, 3/3 animals respectively for the CuO-core, CuO-COOH, and CuO-NH_4^+ treatments compared to the controls (Figure 3.7). There was no loss of architecture of the circular and longitudinal muscles or other pathologies to these tissues. However, the presence of granular like pigment was observed in the circular muscle, the amount of pigment was not greater in the ENM treatments (Figure 3.7). There were no statistically significant differences between the thickness of the epidermis, circular or longitudinal muscle (Table 3.5). In the absence of organ pathology in the fresh soil experiment, histology was not pursued in the aged soil study.

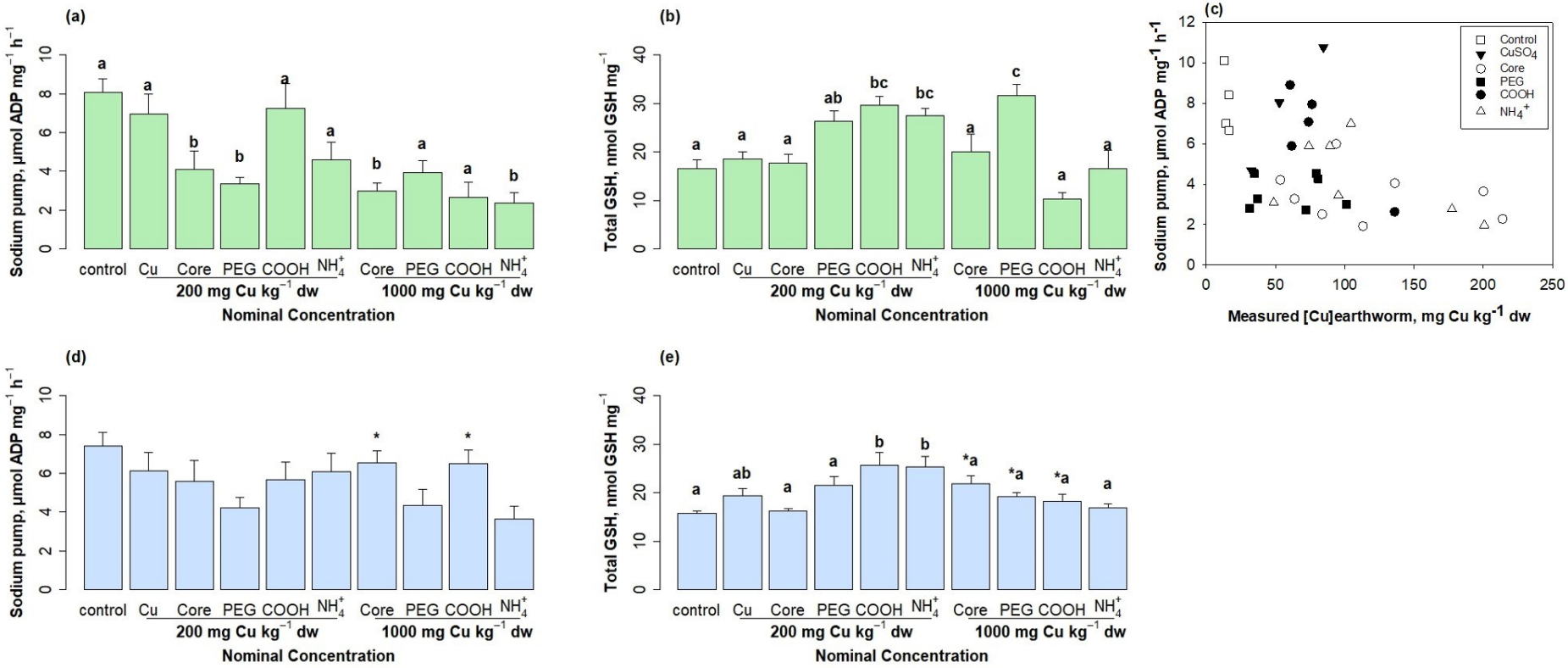

Figure 3.5 Biochemical endpoints in the fresh (panels a, b and c) and aged soils (panels d and e) at day 14, the end of the experiment. (a),(d) sodium pump (Na^+/K^+-ATPase) activity is expressed as µmol of ADP released per mg protein per hour. (d),(e)Total glutathione is expressed as nmol per mg protein. Different letters denote statistically significantly differences between treatments irrespective of test concentration ($P < 0.05$, one-way ANOVA). Asterisks denotes a statistically significantly difference from their fresh soil counterpart within treatment ($P < 0.05$, Mann-Whitney). Data are means ± SEM (n = 8, of less due to high mortality which restricted the number of earthworms for biochemistry at the high exposure concentrations). The relationship between sodium pump activity and measured Cu concentartion in earthworms is provided for reference (c).

Table 3.4 Total concentration of essential and trace elements in controls, $CuSO_4$ and CuO ENM exposed earthworms in the fresh and aged soil experiment.

Soil [Cu] Nominal, mg Cu kg^{-1} dw	Day	Electro-lytes	Measured concentration, mg kg^{-1} dw					
			Control	$CuSO_4$	Core	PEG	COOH	NH_4^+
Fresh soil								
200	**7**	**Na**	6067 ± 351	4761 ± 401	6886 ± 743	5712 ± 563	5257 ± 343	5812 ± 700
		K	10651 ± 331	8126 ± 761	10708 ± 923	9087 ± 705	9169 ± 4847	9713 ± 489
		Ca	4800 ± 373	4018 ± 380	5108 ± 424	4401 ± 487	3879 ± 321	3959 ± 435
		Mg	928 ± 35	730± 71	974 ± 79	817 ± 63	832 ± 47	906 ± 99
		Fe	395 ± 55	320 ± 88	421 ± 68	419 ± 76	328 ± 37	360 ± 51
		Mn	38 ± 6	37 ± 8	63.98 ± 7	59.± 8	60 ± 13	50 ± 10
		Zn	130 ± 8	105 ± 11	144 ± 12	112 ± 8	116 ± 7	132 ± 14
200	**14**	**Na**	5793 ± 789	4493 ± 595	5311 ± 322	4321 ± 661	6256 ± 530	7426 ± 973
		K	9592 ± 974	5509 ± 728*	6430 ± 479	4657 ± 711*	10281 ± 856	11187 ± 1450
		Ca	4233 ± 421	2733 ± 379	3058 ± 229	2873 ± 295	3940 ± 284	4229 ± 584
		Mg	832 ± 101	629 ± 84	746 ± 58	574 ± 84	876 ± 61	1087 ± 133
		Fe	406 ± 67	305 ± 54	298 ± 31	300 ± 54	392 ± 77	490 ± 53
		Mn	43 ± 6	25 ± 5	26 ± 5	31 ± 6	46 ± 7	45 ± 8
		Zn	130 ± 18	78 ± 10	97 ± 8	74 ± 10	118 ± 8	153 ± 15
1000	**7**	**Na**			5484 ± 485	5016 ± 269	4517 ± 508	4808 ± 499
		K			10161 ± 488	10193 ± 352	8955 ± 677	9462 ± 237
		Ca			4121 ± 394	4511 ± 336	3634 ± 245	4222 ± 621
		Mg			850 ± 31	861 ± 18	783 ± 35	971 ± 101
		Fe			295 ± 19	362 ± 57	279 ± 23	279 ± 16
		Mn			39 ± 7	37 ± 6	38 ± 6	42 ± 9
		Zn			133 ± 8	122 ± 3	121 ± 4	141 ± 14

1000	**14**	**Na**			7144 ± 1000	6185 ± 512	3562 ± 1248	5820 ± 1122
		K			9849 ± 1082	10408 ± 638	7856 ± 1932	10849 ± 1446
		Ca			4984 ± 597	4147 ± 375	4102 ± 421	4920 ± 610
		Mg			956 ± 46	908 ± 48	793 ± 83	1059 ± 101
		Fe			351 ± 22	325 ± 35	351 ± 144	387 ± 63
		Mn			26 ± 4	27 ± 5	26.57 ± 0	32 ± 7
		Zn			136 ± 8	121 ± 6	117 ± 24	149 ± 14
Aged Soil								
200	**14**	**Na**	5600 ± 416	5147 ± 142	4035 ± 341	4154 ± 361	5015 ± 168	5293 ± 249
		K	9520 ± 592	10587 ± 217	9362 ± 441	9018 ± 394	9473 ± 389	9591 ± 398
		Ca	3736 ± 305	4059 ± 193	3026 ± 298	3112 ± 116	3245 ± 166	3583 ± 158
		Mg	838 ± 59	938 ± 48	884 ± 52	782 ± 29	786 ± 25	814 ± 18
		Fe	419 ± 28	375 ± 47	328 ± 41	345 ± 42	434 ± 64	448 ± 83
		Mn	62 ± 11	21 ± 2	36 ± 8	31 ± 5	31 ± 5	33 ± 9
		Zn	109 ± 7	85 ± 4	86 ± 5	94 ± 5	93 ± 6	82 ± 2
1000	**14**	**Na**			4696 ± 245	4976 ± 230	6312 ± 1188	4816 ± 322
		K			8967 ± 818	9304 ± 117	10421 ± 1917	7998 ± 805
		Mg			902 ± 62	864 ± 28	1024 ± 202	942 ± 51
		Ca			3464 ± 228	3673 ± 204	4208 ± 526	3862 ± 356
		Fe			390 ± 29	317 ± 26	393 ± 36	380 ± 57
		Mn			17 ± 2 *	23 ± 3 *	25 ± 4 *	20 ± 3*
		Zn			76 ± 7 *	72 ± 6 *	71 ± 4 *	54 ± 3*

Data expressed as mean ± SEM mg element kg^{-1} dry weight (n = 8, or less due to high mortality). Asterisks denote statistically significant differences between treatments and the control ($P < 0.05$, ANOVA).

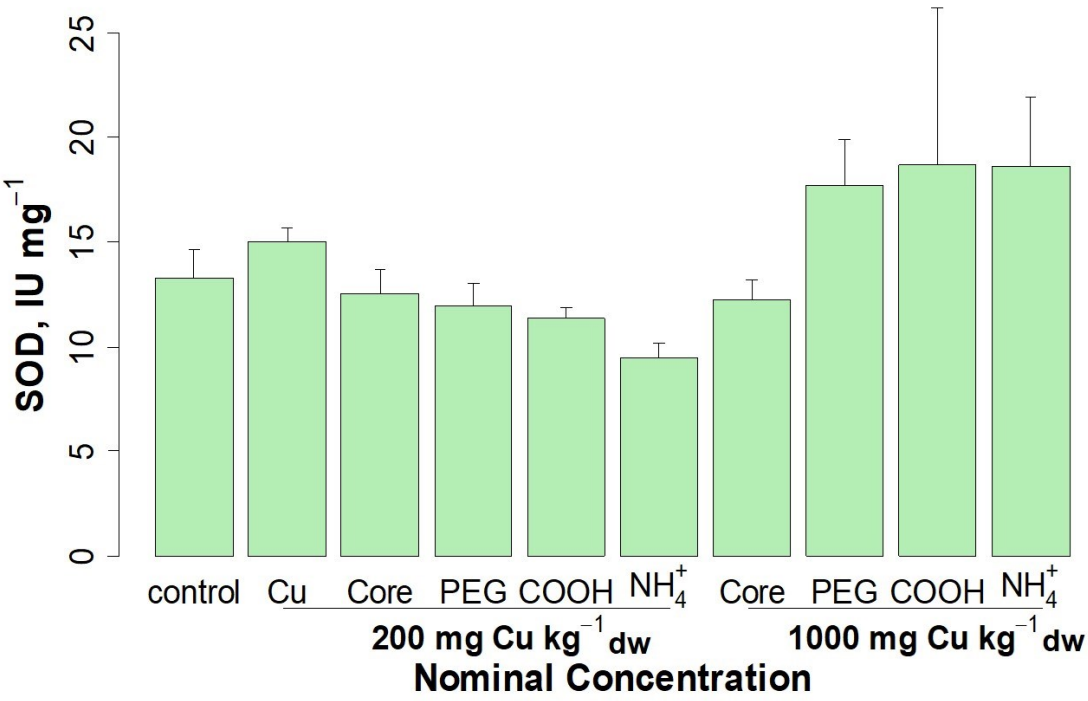

Figure 3.6 Total concentration of superoxide dismutase in earthworms in the fresh soil experiment. Data is expressed as mean ± SEM (n = 8), Total SOD unit (IU) per mg of protein in the homogenate.

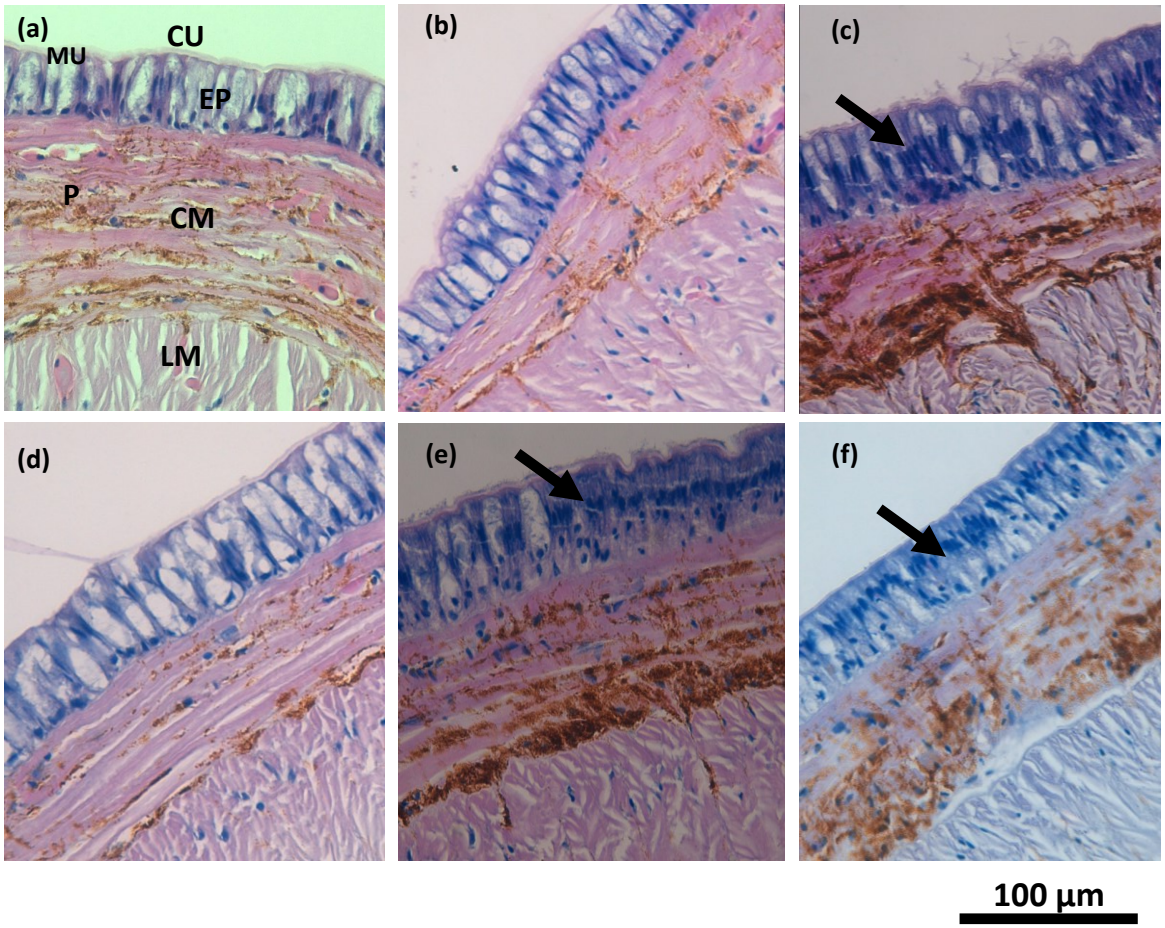

Figure 3.7 Transverse sections of segments from the anterior region of earthworms from the fresh soil experiment at day 14: (a) Control, (b) 200 mg Cu kg^{-1} dw $CuSO_4$, (c) 1000 mg Cu kg^{-1} dw CuO-Core, (d) 1000 mg Cu kg^{-1} dw CuO-PEG, (e) 1000 mg Cu kg^{-1} dw CuO-COOH and (f) 1000 mg Cu kg^{-1} dw CuO- NH_4^+ (all nominal values). CU – cuticle, EP – epidermis, CM – circular muscle, LM – longitudinal muscle, P- pigment as granular deposits, most likely porphyrins, MU – mucous cells (clear, with slight deposits, cylindrical cells). Arrows point to example areas of reduced number of mucous cells.

Table 3.6 Quantitative histology of the ectodermal layers (epidermis, the circular and longitudinal muscles) of earthworms from the fresh soil experiment.

Treatment	Nominal Concentration	Epidermis	Circular muscle	Longitudinal muscle
	mg Cu kg^{-1} dw		mm	
Control	0	0.67 ± 0.13	0.97 ± 0.32	2.70 ± 0.89
$CuSO_4$	200	0.67 ± 0.18	1.13 ± 0.44	2.40 ± 0.40
CuO-core[1]		0.30 ± 0.08	0.95 ± 0.12	2.90 ± 0.08
CuO-PEG	1000	0.67 ± 0.13	1.03 ± 0.09	3.67 ± 0.67
CuO-COOH		0.63 ± 0.13	0.77 ± 0.15	2.73 ± 0.23
CuO-NH_4^+		0.43 ± 0.07	1.17 ± 0.17	2.17 ± 0.44

Data are presented as mean ± SEM in mm (n = 3). Measured on images taken at x 4 magnification using the Leica DM device.[1] n = 2 earthworms due to high mortality.

3.4 Discussion

This study found that CuO ENMs can be toxic to earthworms, but the magnitude and types of effects are broadly similar to that of $CuSO_4$. There were also some differences in the sub-lethal effects that were dependent on the type of coatings, and although the ranking of the coating effect was not consistent across biological endpoints, the CuO-COOH material was the most hazardous overall in fresh soil. Critically, both Cu toxicity and the coating-effects, changed with soil ageing. In the latter experiment, the aged soil was generally less hazardous than fresh soil, but importantly the CuO-NH_4^+ now had the most effects on the earthworms.

3.4.1 Soil pH

Soil pH is known to be a factor in metal speciation and the ecotoxicity of soils (Forbes and Forbes, 1994), although it has not been shown to impact Cu accumulation in earthworms (review in Ardestani et al., 2014). $CuSO_4$ is known to reduce the pH of a medium, which was also seen in the results of this study. Either the addition of horse manure, earthworm activity (excretion of urea, mucus) or, indeed, the presence of the coating (COOH, NH_4^+) has induced the CuO ENM to increase in the pH over the course of the experiment. Nonetheless, the statistical analyses suggested the pH did not influence the endpoints measured.

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

Copper exposure was initially confirmed by measuring the total Cu concentrations in the soils (Figure 3.1). The background total Cu concentration in the Lufa 2.2 soil was as expected (~1.4 mg Cu kg^{-1}dw, Figure 3.1) and comparable to previous values (e.g., 1.5 mg Cu kg^{-1}dw, Bastos et al., 2015). In the fresh soil experiment, the nominal total Cu concentrations were for the $CuSO_4$ and CuO core (uncoated) material were also close to the measured values (Figure 3.1). For the coated ENMs, dosing intended on to be on a Cu mass basis of the total ENM. However, because of uncertainty of the stoichiometry and the technical challenges in measuring the mass attributed to the coating, the amount of Cu differed in the ENMs (Chapter 2.5). Thus, it was expected that the measured total Cu concentrations from the soils dosed with the coated-ENMs would be lower than the equivalent CuO core treatment, but still be a Cu exposure that was much higher than the controls (as observed, Figure 3.1). Interestingly, the thermogravimetric analysis of the batches of the material (Chapter

2, Table 2.1) indicated that the $–NH_4^+$, -COOH and –PEG coatings contributed roughly 12, 22 and 42 % of the mass of the materials, and this was broadly reflected in the measured total Cu concentrations in the soil with the CuO-NH_4^+ showing the most Cu and the CuO-PEG the least.

Regardless of the measured total Cu concentrations in the soil, the main concern for hazard assessment is the bioavailable fraction of the metal. This is normally addressed by measuring the extractable fractions of metal from the soil (Figure 3.1,3.2). In the fresh soil experiment, the water extractable fraction remained very small (~ 2.3 mg Cu kg^{-1} dw) and identical to previous reports for Cu salts (2 mg Cu kg^{-1}, Scott-Fordsmand et al., 2000). However, the dilute acid-extractable fraction dominated, accounting for 50 % or much more of the total Cu, regardless of the test concentration or type of material. For solutes, the acid-extractable fraction is intended to represent the loosely bound fraction of cations on the surface of soil grains that can be eluted by simple ion exchange (i.e., exchanging H^+ with Cu^{2+} in this case). Thus, as expected for ion competition of solutes based on molarity, charge density and mobility of the ions in water (see Handy and Eddy, 2004), more than half of the Cu added to the soil as $CuSO_4$ can be recovered in this way. However, ENMs are not solutes (see Handy et al., 2008a), and with moderate rates of Cu dissolution from the particles (Table 2.1), it is likely that the acid-extractable fraction here also represents a mixture of dissolved copper and potentially intact particles removed from (low energy) agglomerates or those loosely attached to the soil grains (e.g., by electrostatic attraction). Interferences from colloids already in the soil prevented any useful attempt of determining particle number concentrations in the extract by NTA to confirm this. Nonetheless, Cu from both $CuSO_4$ and the

different ENMs were mainly found in what is regarded as the extractable fraction of the soil.

Copper exposure was mainly confirmed by measuring the total Cu concentrations in the earthworms (Figure 3.3). The background Cu concentrations in earthworms from the control soils were low (~8 - 10 mg Cu kg^{-1} dw, Figure 3.3) and similar to previous values for this species (e.g., 8 mg Cu kg^{-1}dw, Streit, 1984). Following the notion of the dissolved metal paradigm, in the fresh soil experiment (Figure 3.3), Cu from the $CuSO_4$ treatment was also accumulated in the tissues within 7 days and persisted to the end of the experiment. Notably, this was no different from the CuO core material at the nominal 200 mg Cu kg^{-1} dw exposure in fresh soil; suggesting the bioavailability of Cu from $CuSO_4$ and the uncoated CuO are similar in the conditions here (Figure 3.3).

A key concern is whether the surface coating of ENMs imparts any additional bioaccumulation risk compared to uncoated ENMs. All of the ENM treatments in the fresh soil experiment, regardless of coating or not, showed concentration-dependent increases in the earthworms that were generally consistent with the notion of dose-response (Figure 3.3). However, there was a coating-effect on tissue Cu concentrations in fresh soil. Despite the presence of lower total Cu in the soil with the coated ENMs compared to the CuO core (Figure 3.1), there was generally no difference between coatings in terms of total Cu concentration in the earthworms (Figure 3.3). Thus in fresh soil, proportionally more total Cu is transferred from the soil to the earthworms, depending on the type of ENM coating. The nano-BAF values clearly demonstrate that the coated forms of the ENM are more bioavailable than the CuO-core (Table 3.2).

In the aged soil study, Cu accumulation in newly exposed earthworms was assessed after ageing the soils for one year. The aged soil contained the remains of any Cu from the original dosing the previous year, and the pattern of total Cu concentrations were consistent with the original measurements in fresh soil (compare Figure 3.1b with Figure 3.1a). Some of the total Cu concentration in the soil at the start of the aged soil experiment were a little lower (statistically different) to the previous year. However, this is explained by the total metal removed from the soil by the earthworms in the first experiment, plus that lost to plants spontaneously growing in the soil during the year (see Chapter 6 for details). Nonetheless, exposure of new earthworms to the one-year aged soils for 14 days did result in elevated total Cu concentrations in the earthworms (Figure 3.4). The measured total metal concentrations in the earthworms were generally less than the equivalent earthworms in the fresh soil experiment, partly for the reason above, but overall the pattern of accumulation by material-type was generally the same (compare Figures 3.3 and 3.4). However, when the ratio of the total Cu measured in the aged soil to that in the earthworms is calculated (or the nBAF, Table 3.2), some key soil ageing effects are revealed. The apparent nano bioaccumulation factors are less than the fresh soil experiment, but the ranking has also changed with the CuO core being less available than the $CuSO_4$. Moreover, the copper from CuO–PEG and –COOH coated ENMs is more available than the $–NH_4^+$ form. These changes in ranking are also reflected in the sub-lethal biological responses (see below). An interaction between ENM coating and soil ageing has not been previously reported. However, the finding on $CuSO_4$ are consistent with previous results of lower Cu accumulation in earthworms from aged soils (Lock and Janssen, 2003).

3.4.3 Survival, growth and behaviour

Earthworms were from a healthy population as confirmed by the survival and normal behaviour of the control animals in both fresh and aged soil experiments. The nominal concentration of 200 mg Cu kg^{-1} dw soil was intended as a sub-lethal exposure and this was the case with 92 % survival in the $CuSO_4$ treatment in the fresh soil experiment, in keeping with previous findings on earthworms at the same concentration (95 % survival after 8 weeks, Spurgeon et al., 1994). The survival was also good for the earthworms exposed to 200 mg Cu kg^{-1} dw soil as ENMs (95 % or more, Table 2). However, a coating-effect on survival was revealed at the higher nominal concentration of 1000 mg Cu kg^{-1} dw soil, with the survival ranked in the following order by material in the fresh soil experiment: CuO-PEG < CuO-core < CuO-NH_4^+ < CuO-COOH (Table 3.2). The decreases in survival were also reflected in reduced biomass (declining growth) of the earthworms (Table 3.2). Some of the mortality and slower growth could be attributed to reduced feeding since the earthworms avoided burrowing at the high exposure concentrations. There was also evidence of specific trace element deficiencies (see below). Avoidance behaviour has been seen in exposures to Ag ENMs at 6.92 mg Ag kg^{-1} dw (10 nm PVP-coated, Shoults-Wilson et al. 2011) and TiO_2 ENMs at 1000 - 5000 mg TiO_2 kg^{-1} dw (5 – 300 nm anatase, McShane et al., 2012). Reduction in biomass have also been observed (~ 27 % weight loss at 1000 mg Ag ENM kg^{-1}, 30 – 50 nm, Heckmann et al., 2011).

In the aged soil experiment, the animals all survived at the nominal 200 mg Cu kg^{-1}, similar to the fresh soil study. However, unlike the situation in fresh soil where animals decreased in weight, the animals gained biomass in the aged soil (Table 3.2). This may be partly explained by the presence of different food in the soil, as the

aged soil experiment had plant matter (roots) that the earthworms could feed on in addition to the added horse manure at the start of the aged soil experiment. However, any benefit was lost at the higher 1000 mg Cu kg^{-1} dw nominal concentration in aged soil, as in the fresh soil experiment – but with one important difference. In the aged soil, the toxicity ranking by material was: CuO-PEG = CuO-COOH < CuO core < CuO-NH_4^+. Thus, the ammonium coating was now the most hazardous in aged soils, even though the total Cu accumulation in the earthworms was not the highest (Figure 3.4). Why the ranking has changed in the aged soil is unclear.

3.4.4 Effects on ionic regulation

Earthworms actively osmoregulate to maintain their body fluids in a hyperosmotic state relative to the surrounding media and normally produce hypo-osmotic urine that is less concentrated than the body fluids (Prosser and Brown, 1965; Dietz and Alvarado, 1970). However, despite this knowledge of the osmoregulatory strategy of earthworms, the sodium pump activity and electrolyte composition of earthworms is not often measured in studies on metal toxicity. The crude homogenates of the control earthworms had an Na^+/K^+-ATPase activity of 6 – 8 µmol ADP mg^{-1} protein h^{-1} (Figure 3.5) and this consistent with at least one other report for *E. fetida* (4 – 8 µmol Pi mg h^{-1} in Luo et al., 2010). The tissue K^+ concentrations (8000 – 10 000 mg kg^{-1} dw)are also comparable with previous reports (~ 8000 mg kg^{-1}, Janssen et al., 1998).

Dissolved Cu is a well-known inhibitor of the ubiquitous Na^+/K^+-ATPase (Li et al., 1996), and while there was a trend of decreasing Na^+ pump activity, the $CuSO_4$

concentration used here were not sufficient to cause statistically significant inhibition of the enzyme (Figure 3.5a and c). Nonetheless, some variability of tissue K^+ concentrations were noted in the $CuSO_4$ treatment compared to controls (Table 3.4) suggesting that the earthworms were on the threshold of osmotic disturbance in the fresh soil experiment. At the same nominal Cu concentration, the CuO-core or CuO-PEG inhibited the Na^+ pump in the fresh soil experiment, and at the highest exposure concentration all the ENMs caused inhibition of the Na^+/K^+-ATPase (Figure 3.5). The CuO-core and CuO-PEG materials were the most potent, but this potency is not explained by higher Cu body burden in those treatments (not observed, Figure 3.3a) or oxidative damage to the Na^+ pump (no depletion of GSH, Figure 3.5). Notably, this osmoregulatory hazard was not observed in the aged soil experiment with no inhibition of the Na^+ pump (Figure 3.5) or electrolyte losses (Table 3.4), probably because the Cu present was less bioavailable (less Cu in the tissues, Figure 3.4). The mechanism of coating-dependent inhibition of the Na^+ pump requires further investigation, but the CuO ENMs in fresh soil do present an osmoregulatory hazard that is generally greater than the metal salt.

Cu is also known to have some specific interactions with other trace elements, especially Fe and Zn due to shared uptake and/or excretion pathways (see Bury and Handy, 2010). No interferences with Fe were observed in either the fresh or aged soil experiments, but there were some losses of manganese and zinc. In the aged soil study, significantly lower tissue manganese was associated with $CuSO_4$ exposure and the nominal 1000 mg Cu kg^{-1} dw ENM exposures only (more than 50 % depletion of Mn, Table 3.4). This is not a nutritional defect attributed to the indirect effects of Cu on Mn via Fe status (see Andersen et al., 1996), since Fe concentrations did not change (Tables 3.4, 3.5). Taken together, these trace element

depletions could partly explain the retarded growth of the earthworms. For example, Zn concentrations in earthworms are regulated to around 80 - 120 mg kg^{-1} dw and are nutritionally required (Van Gestel et al., 2010). Thus the values reported here (~40 mg Zn kg^{-1}dw, Tables 3.4-3.5), could represent a Zn deficiency. However, any solute transporter explanation of Cu out competing Zn would require the CuO ENMs to dissolve inside the earthworms. The dissolution of micro molar amounts of Cu (Table 3.1), if it occurred inside the earthworms, would enable some stimulation of these high affinity transporters (Eide, 2006). These phenomena require further research to understand the mechanism behind such changes in tissue ion concentrations.

3.4.5 Absence of oxidative stress

The sub-lethal effects observed in the present study on growth and osmotic regulation are not explained by oxidative stress because no inflammation pathology was evident in the dermis or muscularis of the earthworms (Figure 3.7, Table 3.6) and no glutathione depletion was observed (Figure 3.5) or significant changes to SOD activity (Figure 3.6). The background total GSH in the earthworms of 16.5 nmol mg^{-1} protein (which equates to ~ 1.2 µg g^{-1} fresh weight) (Figure 3.5) were similar to previous reports of around 1.3 µg g^{-1} fresh weight (Ribera et al., 2011). However, some induction of total GSH was observed, but only in the coated forms of the ENMs (Figure 3.5). Increases in total glutathione has been observed previous with *E. fetida* in response to Ag ENMs (49 ± 8 nm, Gomes et al., 2015). This might be interpreted as a premature defence to prevent oxidative stress, or an aspect of metal resistance since glutathione is also an intracellular Cu carrier. However, why the response occurs only with the coated materials and not the CuO core or the $CuSO_4$ is unclear.

3.4.6 Histological observations

The epidermal mucus layer creates an interface for gas exchange and serves as a first-line protection from pathogens and contaminants (Wang et al., 2011) and has also been found as a barrier for rapid entry of metal ions (Fleming and Richards, 1982). There was some indication of a slight reduction in the mucous cells and their size in the earthworms from core-, COOH- and CuO- NH_4^+ ENM treatments. This could explain why the soil was adhering to the earthworms' epidermis, since they were potentially producing less mucus.

3.4.7 Conclusions

In the present study, the effects of CuO ENMs were broadly of the same magnitude as $CuSO_4$, depending on the endpoint measured and the exposure duration. At environmentally-relevant total concentrations of Cu in a contaminated area, modes of action were also similar; with the ENMs causing ionregulatory disturbances and Na^+ pump inhibition that are well-known for Cu. The CuO ENMs could cause lethal toxicity, but only at a high concentration of 1000 mg Cu kg^{-1} dw soil, where the effects of surface coatings on the materials was also especially revealed. The hazard ranking of the coatings was not readily explained by a bioavailable fraction of metal according to the dissolved metal paradigm in soils. The ranking also changed with ageing of the soil, and the CuO-NH_4^+ ENMs became more toxic one year on. Thus, the surface coating and soil ageing should be considered in any risk assessment.

Chapter 4. The uptake and elimination of uncoated and coated CuO ENMs in earthworms compared to $CuSO_4$

4.1 Introduction

The appropriate risk assessment of ENMs requires, not only the acute and sub-lethal toxicity data, but also an understanding of whether they have the potential to bioaccumulate; which in turn can lead to biomagnification and trophic transfer. Earthworms are known to have a permeable cuticle and an effective osmoregulation mechanism to maintain homeostasis (Streit, 1984). Essential metals are known to be regulated by earthworms and non-essential metals stored as insoluble forms (non-toxic / less toxic) and/or eventually excreted (review by Ardestani et al., 2014). Numerous studies have been carried out to assess the uptake and excretion of metals in earthworms (detailed review by Nahmani et al., 2007). Overall, dissolved Cu is well regulated by earthworms and thus it is not regarded as bioaccumulative, since it's BAF in earthworms is around 0.3 (e.g. Svendsen and Weeks, 1997; Spurgeon and Hopkin, 1999).

Studies on ENMs that have been focussing on uptake kinetics (Ag ENMs, Diez-Ortis et al., 2015; ZnO ENMs, Laycock et al., 2015) have found the main uptake route for these ENMs to be via ingestion rather than dermal uptake; the opposite to what is known of dissolved metal accumulation (Vijver et al., 2003). This raises concerns over their accumulation. If the routes of uptake are different, so might be the bioaccumulation potential compared to their dissolved metal counterpart. Uncoated Cu ENMs had a low bioaccumulation potential in earthworms (10 – 20 nm, Unrine et al., 2010). However, some ENMs have also been found to have a higher bioaccumulation potential. For example, cobalt (Co) ENMs were found to be excreted only after 4 months (DT_{50} = 4 months), although Co dissolution from the ENMs was rapid, indicating potential dissolved Co accumulation (4 nm, Coutris et al., 2012).

Surface modifications may affect the accumulation of ENMs, as seen in studies with the crustacean *Daphnia magna* exposed to similarly coated ENMs as used in this study, PEG (neutral polyethylene glycol, 10-20 nm), COOH (negatively charged carboxylate, 10-20 nm) and NH_2 (positively charged, amine, 10-20 nm) CdTe QDs. The authors found the COOH coated ENMs were most accumulated compared to the rest of the materials (Feswick et al., 2013). However, this difference in accumulation may be coating / ENM specific, or indeed, species specific, since differently coated Ag ENMs (PVP and OA) were not found to be differently bioaccumulated in earthworms (Shoults-Wilson et al., 2011). Overall, there have been many studies assessing the total metal concentration in earthworms, following the exposure to metal based ENMs, although only a handful have studied the elimination of ENMs. Recently, Carbone et al. (2016) found cerium and tin ENMs (CeO_2 and SnO_2) not to be bioaccumulative, as shown by rapid excretion of the two, when earthworms were placed into clean soil.

The purpose of the current study was to provide preliminary insight to uptake and excretion of uncoated and coated CuO ENMs compared to $CuSO_4$ in a standard natural sandy loam soil (Lufa 2.2), and thus assess if the ENMs pose a risk as having the potential to bioaccumulate. The study design incorporated a range of coatings on a common CuO core ENM to represent anionic, carboxylate (COOH), cationic, ammonium (NH_4^+) and neutral ligands, polyethylene glycol (PEG) on the surface of the particles. As an addition to Cu concentration in the earthworms, tissue essential and trace metals were measured alongside Cu to assess any ion disturbances that may have been caused by excess Cu or CuO ENMs in earthworm tissues.

4.2 Methodology

4.2.1 Test soil, organisms, conditions and engineered nanomaterials

Test soil composition, animals and conditions were exactly as previously described (see Chapter 2 details). Earthworms were placed in Lufa 2.2 soil, with feed, a week prior to the experiment to acclimatise to the conditions. Experiments were conducted at 20 ± 2^{o}C and with a 12:12 h light:dark cycle under 400-800 lux lighting. During the experiment the earthworms were not fed to avoid the potential effect of the presence of feed (increased organic matter) on the bioavailability of the test substances added to the soil. The ENMs used are detailed in Chapter 2.2.

4.2.2 Experimental design and dosing of the ENMs into the soil

The approach broadly followed a shortened version of the OECD TG no. 317 for bioaccumulation in terrestrial oligochaetes (OECD, 2010), using fewer earthworms that were exposed in a batch rather than individually and a 14-day test duration. This decision was based on the finding of previous experiments with the CuO materials that showed 14 days was sufficient to achieve a reliable metal accumulation measurement. The experiment included a soil control (no added Cu), a metal salt control (Cu as $CuSO_4$), and four variations of CuO ENMs, a CuO-core material and CuO ENMs with different coatings: COOH, PEG and NH_4^+. After considering the results of previous experiments, one sub-lethal concentration of each of the CuO ENMs was chosen to explore uptake and excretion kinetics over time (400 mg kg^{-1} dw) compared to a sub-lethal concentration of the Cu as $CuSO_4$ (200 mg kg^{-1} dw). The latter was used, since the 400 mg Cu kg^{-1} dw as $CuSO_4$ would have resulted in earthworm mortality (very low pH). The nominal concentration of 400 mg Cu kg^{-1} dw

was chosen for ENMs since it was a known sublethal concentration and represents an intermediary between the two concentrations used in the previous study (200 and 1000 mg Cu kg^{-1} dw in Chapter 3). Adult *Eisenia fetida*, with a mean weight of 1.95 ± 0.06 g for a sub-sample of 8 earthworms (n = 19 treatments, mean ± SEM, individual weight of worms ~ 0.25 g) were exposed in 3 replicates (*n* = 24 earthworms per treatment).

Dosing of the test soils with ENMs was achieved using a dry mixing method as previously described Chapter 2.2. Since three replicates were used, the dosing of the soil was achieved by first mixing the mass of ENM powder required to dose all 3 replicates within each treatment into an initial sample of 50 g of the test soil. This was thoroughly mixed by hand for 10 minutes. The 50 g sub-sample of soil containing the ENM was then added to the remaining amount of soil (850 g) and mixed by hand to make sure the ENM powder was evenly distributed. The soil was divided into the triplicate containers (300 g soil per container) and were left to equilibrate overnight (at least 24 h) before the earthworms were introduced.

In order to construct a bioaccumulation curve over time, earthworms were sampled at day 0, 1, 4, 7 and 14 of exposure for metal analysis. At each time point, one worm was removed from each replicate (n = 3 per treatment), rinsed in water, and placed onto a moist filter paper in a Petri dish for 24 h in the dark at 20 ºC to allow time for the depuration of the soil in the gut of the animal. The filter paper was changed after 12 h to remove excreted soil. After 24 h to void their gut contents, each worm was individually frozen and stored at -20ºC until further analysis. To determine excretion rates, exposed worms also needed to be transferred back to clean soil. This was done with half of the worms at day 7, while others continued with exposure for a

further week. At 7 days, worms (n = 3 per treatment replicate) were taken from the exposure soils, washed, and placed into clean Lufa 2.2 soil for the elimination part of the experiment to start. One worm was taken from each replicate of clean soil at day 1, 4, and 7 of the elimination phase, rinsed and then immediately depurated of gut contents as above, and stored frozen until required for metal analysis. Further to the earthworm sampling, notes on earthworm survival and appearance were made throughout the experiment. Soil samples were also taken at the start of the experiment (day 0) and soil pH was measured using a glass combination probe (Corning instruments) on days 0, 7 and 14 in a 1:1 soil:water slurry made from the soils.

4.2.3 Total and extractable metal analysis in samples

The total copper concentration was measured in soil samples taken at the beginning of the experiment (0 days), to measure total and extractable copper concentrations. Total copper concentration, along with a suite of other elements, were measured in earthworms (n = 3) that were sampled at each stated time-point from each treatment. Details of all metal analyses are described in Chapter 2.

4.2.4 Statistical analyses and data presentation

Statistical analyses were performed following Chapter 2.8. The uptake and elimination data points were initially plotted against time. The resulting data indicated the concentrations in earthworms plateaued after 4 days of uptake and after 1 day of depuration. Therefore, assuming equilibrium, non-linear regression analyses of Cu

uptake kinetics were performed with a hyperbolic curve fit (single rectangular hyperbola, two parameter y = a * x /(b + x); SigmaPlot version 14.0; Systat Inc., Chicago, IL, USA) in order to fit the parameters of the Michaelis–Menten equation: C = C_{max} * t / K_m + t; where C = concentration of metal in a given time (mg Cu kg^{-1} dw) , C_{max} = the maximum or saturation concentration (mg Cu kg^{-1} dw), t = time in days and Km = the time taken to reach half the value of C_{max}. The non-linear regression analyses of Cu elimination kinetics were performed with a hyperbolic decay (two parameter, y = (a * b) / (b + x); SigmaPlot version 14.0, Systat Inc., Chicago, IL, USA). The average uptake and elimination rate (referred to as A k1 and k2) were derived using the software derived values for Km (or b). The maximum uptake and elimination rate (referred to as max k_1 and k_2) were derived manually from the steepest part of the uptake and elimination curves (k1 or k2 = y / x), respectively. In order to derive a bioaccumulation factor based on the derived rates, k1 was divided by k2 resulting in a kinetic BAF or apparent nano-BAF. Another measure was calculated based on the total copper concentration in the earthworms, divided by the total concentration of copper soils ($[Cu]_{earthworm}/[Cu]_{soil}$), and in text it is referred as nBAF* to distinguish it from the kinetic nBAF. The statistical significance level (α) for all tests was set at 0.05. In all figures “Cu” represents the $CuSO_4$ treatments and ‘core’ as the uncoated and ‘PEG’, ‘COOH’ and ‘NH_4^+’ to refer the coated CuO ENMs.

4.3 Results

4.3.1 Soil pH

Soil pH varied between treatments at the beginning of the experiment and after 7 days in the uptake experiment (Table 4.1). There was no overall difference in pH between the different time-points 0, 7 and 0, 14 and 7, 14 days ($P > 0.05$, Student's *t*-test) in each treatment, although the pH did differ between treatments (Table 4.1). Soil pH did not differ between excretion experiment soils and was 5.3 ± 0.01 (mean ± SEM, pooled data of n = 16). Soil pH was not found as a co-factor in the accumulation of Cu in earthworms ($P > 0.05$, ANCOVA).

Table 4.1 Soil pH in in the uptake soils at T (day) = 0, 7, 14 (nominal 400 mg Cu kg^{-1} as CuO ENMs or 200 mg Cu kg^{-1} dw as $CuSO_4$)

Time-point	**Treatment**					
Days	**Control**	**$CuSO_4$**	**Core**	**PEG**	**COOH**	**NH_4^+**
0	5.25 ± 0.06^a	4.82 ± 0.02^c	5.27 ± 0.01^a	5.09 ± 0.01^b	5.07 ± 0.01^b	5.16 ± 0.02^{ab}
7	5.11 ± 0.03^a	4.81 ± 0.03^b	5.34 ± 0.01^c	5.15 ± 0.01^a	5.11 ± 0.01^a	5.21 ± 0.01^a
14	5.23 ± 0.04^a	4.86 ± 0.04^b	5.33 ± 0.01^a	5.19 ± 0.01^a	5.14 ± 0^a	5.25 ± 0.01^a

Data expressed as mean ± SEM (n = 3). Different letters denote statistically significant differences ($P < 0.05$, ANOVA, Tukey HSD).

4.3.2 Total and extractable copper in soils

The exposure was confirmed by measuring the total metal concentrations in the soil. The control soil contained a background Cu concentration of (mean ± SEM, *n* = 8) 2.9 ± 0.1 mg Cu kg^{-1} dw, and of this total Cu 13.5 ± 0.8 and 47.1 ± 2.1 % was water and 0.1 M HCl-extractable respectively, indicating that at least half of the total Cu was labile. Exposure to $CuSO_4$ resulted in an expected increase in total soil Cu concentrations that were close to the nominal values (Figure 4.1, more details in Table 4.4). The soils treated with the various forms of CuO ENMs did not show the same measured total Cu concentrations. Instead, the amount of Cu measured in the soil varied depending on the type of ENM coating, which was due to the differences in the mass of each coating (Figure 4.1, discussed in detail in Chapter 3.3.2). There were differences between the amount of water and acid extractable Cu (mg Cu kg^{-1} dw), but this was related to the total Cu in the soil (Figure 4.1, Table 4.2). The percent water available and acid extractable fractions were similar between the different ENM exposures. However, some differences remained, mainly CuO-COOH ENMs being most extractable in the water compared to the rest of the treatments (Table 4.4, $P < 0.05$, ANOVA, Tukey HSD). There was a strong positive correlation between total copper in soil and water extractable Cu in soil as well as between total copper in soil and 0.1 M HCl extractable Cu ($r_s = 0.8$, $P < 0.05$, Spearman's). There was no significant correlation between the total Cu in soil and total percent water extractable Cu and between total Cu in soil and 0.1 M HCl extractable Cu.

The total copper concentration in the excretion phase was not measured since it came from the same batch as the control soils, thus it was assumed the concentration of Cu is the same as in the control soils.

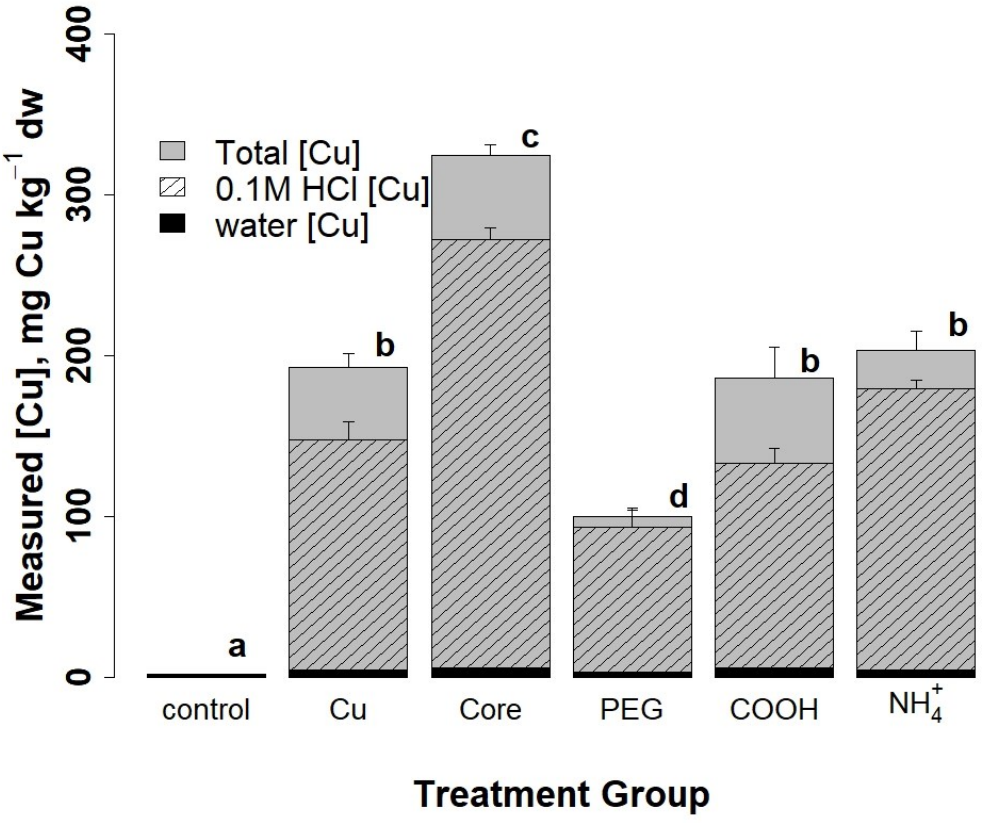

Figure 4.1 Total, acid (0.1 M HCl) and water extractable [Cu] in the experimental soil at a nominal concentration of 200 mg Cu kg^{-1} dw for Cu as $CuSO_4$, and 400 mg Cu kg^{-1} dw for coated CuO ENM variants. Data presented as mean ± SEM (n = 6, duplicate analysis of three treatment replicates). Different letters denote statistically significant differences between the total (and including acid extractable) [Cu] in the soil ($P < 0.05$, ANOVA, Tukey HSD).

4.3.3 Uptake and excretion of copper in earthworms

To assess the uptake and excretion, the total copper concentration in earthworms was measured at the time-points identified. The total copper concentration in earthworms as mg Cu kg^{-1} dw are shown (Figure 4.2) with the relevant uptake and excretion curves fitted (all curves had a $R^2 > 0.9$). The unexposed control earthworms, as expected, showed background variation over time with a mean tissue [Cu] at 7.55 ± 2 mg Cu kg^{-1} dw (mean ± SEM, n = 21). Earthworms exposed to

$CuSO_4$ showed a clear time-dependent increase in tissue Cu concentration following the expected rectangular hyperbola of the dissolved metal salt. The earthworms removed from the $CuSO_4$ contaminated soil at day 7 showed rapid clearance of the body burden to achieve close to control tissue concentrations by day 14 of the experiment (day 7 of the clearance phase). Exposure to the CuO ENMs showed very similar hyperbolic uptake and excretion curves to the metal salt. The accumulation of Cu was fastest in the CuO-COOH exposures, and slowest in the CuO-PEG exposures. In the uptake phase, a two-way ANOVA showed that for all ENMs, regardless of coating, the whole-body burden of Cu on days 7 and 14 were similar ($P > 0.05$), indicating that the uptake phase was beginning to saturate between days 7-14, in keeping with previous findings (Chapter 3). The earthworms that were transferred to clean soil on day 7 also showed rapid excretion of the total Cu body burden after being in the soil for 1 day (and an additional 24 h on the filter paper). The body burden returned to initial levels in all cases, except CuO-core exposures, where earthworms sustained internal concentrations slightly higher, around 20 mg Cu kg^{-1}, than the baseline. Furthermore, the elimination of Cu was much slower in the CuO-PEG exposures when compared to the rest of the treatments. A two-way ANOVA showed that there was no difference between internal Cu concentration between days 4 and 7 of the clearance phase (day 11 and 14 on Figures 4.2 and 4.3). Further statistical labels for each time-point and treatment specific differences are presented in Table 4.2. The specific equations and goodness of fit (r^2) for each curve are presented in Table 4.3.

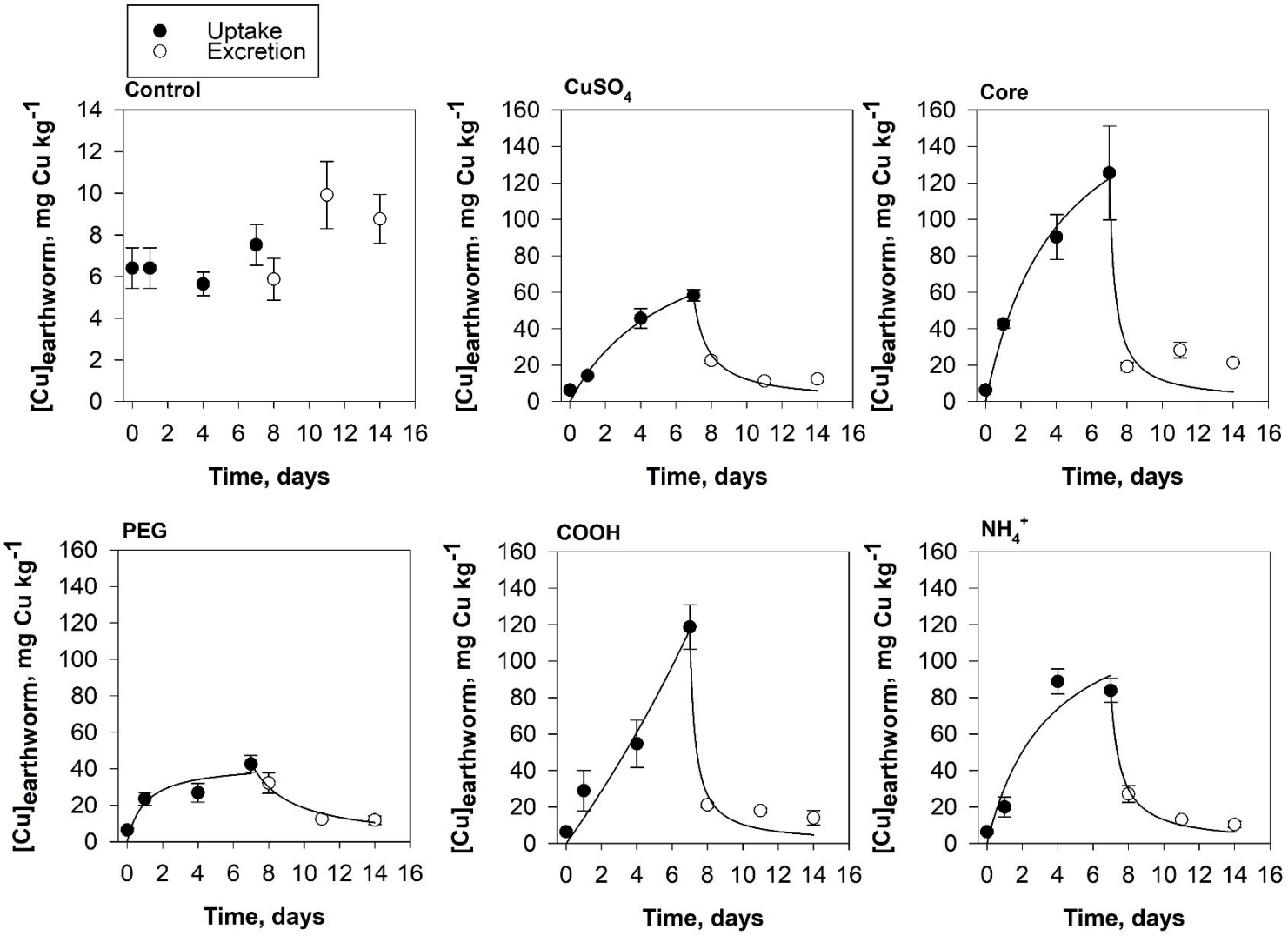

Figure 4.2 Total [Cu] earthworm as mg Cu kg^{-1}, dry weight with uptake (7 days) and excretion (7 days) curves for different CuO ENM variants and $CuSO_4$. Concentrations in earthworms from test soil controls are shown at different time-points. Data presented as mean ± SEM (n = 3).

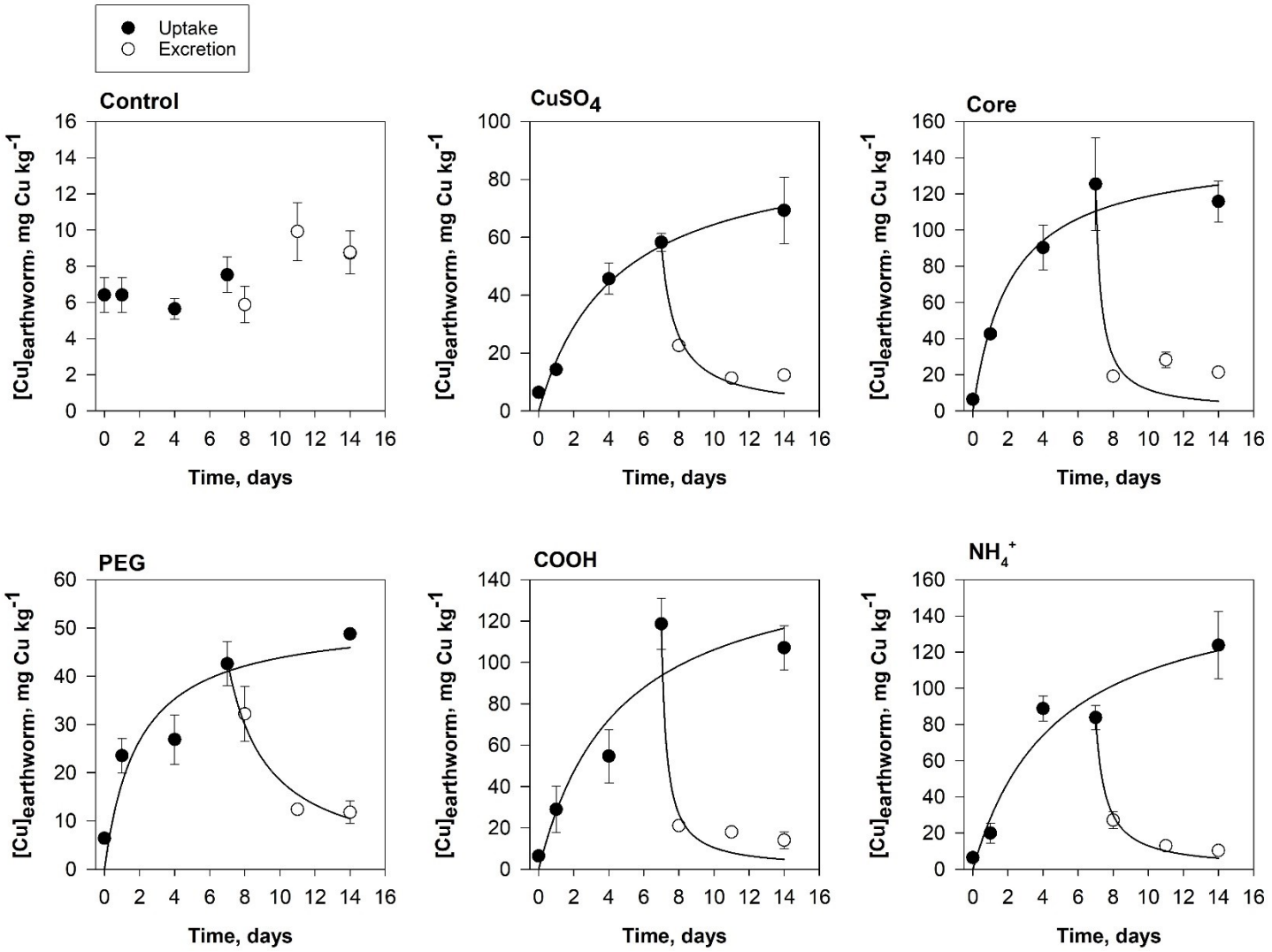

Figure 4.3 Total [Cu] earthworm as mg Cu kg^{-1}, dry weight with uptake (14 days) and excretion (7 days) curves for different CuO ENM variants and CuSO4. Concentrations in earthworms from test soil controls are shown at different time-points. Data presented as mean ± SEM (n = 3).

Table 4.2 Statistical labels for total concentration of Cu in earthworm tissue to complement Figures 4.2 and 4.3.

Time, days	Phase	Treatment										
		Control	$CuSO_4$ Time	$CuSO_4$ Treatment	Core Time	Core Treatment	PEG Time	PEG Treatment	COOH Time	COOH Treatment	NH_4^+ Time	NH_4^+ Treatment
1	**Uptake**	a	A	B	AB	b	AB	ab	A	ab	A	ab
4		a	BC	b	AB	c	ABC	ab	A	bc	B	c
7		a	C	b	AC	c	DC	b	B	c	B	bc
14		a	C	bc	C	db	D	c	B	db	B	d
1 (8)	**Excretion**	a	A	b	C	ab	DCB	b	A	b	A	b
4 (11)		a	A	ab	B	b	B	a	A	b	A	a
7 (14)		a	A	ab	B	b	B	ab	A	ab	A	a

Time - uppercase letters denote differences between total Cu at different time-points within a treatment. Treatment - lowercase letters denote statistically significant differences between treatments at a specific time-point ($P < 0.05$, Two-way-ANOVA, Tukey HSD). The number in brackets in the column titled Time, denotes the day on Figure 4.2 and 4.3.

Table 4.3 The equations to support Figure 4.2 and 4.3 and respective r^2 values.

Treatment	Duration, days	Uptake	r^2	Elimination	r^2
$CuSO_4$	7	y=108.03*x/(5.8+x)	0.97	y=(-7.6*-6.1)/(-6.1+x)	0.96
Core	7	y=193.82*x/(4.08+x)	0.98	y=(-5.89*-6.68)/(-6.68+x)	0.90
PEG	7	y=42.72*x/(1.01+x)	0.80	y=(-20.33*-4.76)/(-4.76+x)	0.97
COOH	7	y=-511.62*x/(-37.57+x)	0.95	y=(-5.14*-6.7)/(-6.7+x)	0.97
NH_4^+	7	y=138.36*x/(3.5+x)	0.91	y=(-7.14*-6.4)/(-6.4+x)	0.99
$CuSO_4$	14	y=92.53*x/(4.3+x)	0.96	NA	
Core	14	y=143.76*x/(2.11+x)	0.96	NA	
PEG	14	y=52.22*x/(1.9+x)	0.97	NA	
COOH	14	y=154.31*x/(4.53+x)	0.97	NA	
NH_4^+	14	y=161.73*x/(4.7+x)	0.99	NA	

Equations explained in section 4.2.4.

The free metal ion of the total metal concentration is regarded as the bioavailable fraction in metal toxicology, and thus considered as a cause for concern. Further to Figure 4.1, Table 4.4 presents the proportion of the total soil Cu concentration that was water or acid extractable. The former was consistent and low, with the majority of the metal in the soil being labile, as demonstrated by acid extraction. The method did not allow the determination of intact particles in the extracts, but does show a likely substantially bioavailable fraction. This is consistent with the rapid uptake of total Cu in the worms over 7 and 14 days (Figure 4.2 and 4.3).

A key concern for Environmental Risk Assessment is whether or not a substance is demonstrated to be bioaccumulative in a long-term study, or shows bioaccumulation potential for the kinetic results of shorter experiments. For the latter, the average (A) and maximum (max) rates of apparent total metal uptake (i.e., k_1) and excretion (k_2)

are shown (Table 4.4). If k_1 exceeds k_2 then the substance is considered bioaccumulative (i.e., the apparent nano-bioaccumulation factor, or nBAF is >1). The average rate represents the mean rate up to the 50% saturation point of the curves, and is often a default value from curve fitting software, giving a slightly conservative estimate of any nBAF. However, for the most accurate value, it is best to use the maximum rate of uptake and excretion, calculated manually from the initial step part of each curve. The maximum nBAF values calculated are all < 1, indicating the materials are not predicted to be bioaccumulative. However, the CuO-PEG has a value of 0.8, which is not far from the threshold for concern. This latter value is not an artefact of the uncertainty over the stoichiometry of the coating to metal content of the particles as the values are based on measured total metal concentrations. It is also reflected in the slower excretion curve for the CuO-PEG material (Figure 4.2 and 4.3).

For comparison purposes the nBAF* was also calculated as the ratio of the total copper concentration in earthworm (day 7) and total copper concentration at the beginning of the experiment (Table 4). For the control soils, it is expected that the apparent bioaccumulation factor is high, because the Lufa 2.2 soil is very low in copper, and earthworms have a higher background Cu concentration. The $CuSO_4$ exposures showed higher BAF* than kinetic BAF. There were bigger differences however when ENMs were concerned. The nBAF* was higher than the kinetic nBAF in the CuO-core, -COOH and NH_4^+ exposures, but lower in the CuO-PEG exposures (Table 4.4).

Table 4.4 Total, water and 0.1 M acid extractable [Cu] in experimental soils, percent water/ acid extractable [Cu] of the measured total [Cu]. Maximum (Max) and average (A) uptake (k_1) and excretion (k_2) rate for the seven-day uptake and excretion phases.

Treat-ment	**Nomi-nal [Cu]**	**Water extractable [Cu]**	**Water extract-able [Cu] % of measured**	**0.1M HCl extract-able [Cu]**	**0.1M HCl extract- able [Cu] % of measured**	**Measured [Cu]**	**A k_1**	**A k_2**	**Max k_1**	**Max k_2**	**nBAF**	**nBAF ***
		mg Cu kg^{-1} dw	***%***	***mg Cu kg^{-1}dw***	***%***	***mg Cu kg^{-1} dw***	***mg Cu kg^{-1}day^{-1}dw***					
Control	NA	0.4 ± 0.01^{a}	13.5 ± 0.8^{a}	1.4 ± 0.1^{a}	47.1 ± 2.1^{a}	2.9 ± 0.1^{a}	NA	NA	NA	NA	NA	3.8[1]
$CuSO_4$	200	2.6 ± 0.1^{b}	1.3 ± 0.08^{b}	145.9 ± 12.2^{b}	74.1 ± 5.8^{b}	197.1 ± 8.5^{b}	9.3	35.3	16.	79.9	0.2[1]	0.3[1]
Core	400	6.3 ± 0.3^{c}	1.9 ± 0.1^{c}	271.8 ± 6.7^{c}	82.9 ± 2.5^{b}	328.5 ± 6.1^{c}	23.9	194.1	43.8	148.4	0.3	0.4
PEG	400	2.7 ± 0.2^{d}	2.8 ± 0.1^{d}	83.4 ± 8.3^{d}	85.5 ± 7.8^{b}	97.3 ± 2.6^{d}	21.1	9.7	29.	36	0.8	0.4
COOH	400	5.9 ± 0.5^{c}	3.6 ± 0.5^{d}	132.3 ± 10.2^{b}	80.6 ± 12.5^{b}	185.7 ± 21.9^{b}	15.4	199.1	17.3	103.3	0.2	0.7
NH_4^+	400	4.5 ± 0.3^{c}	2.2 ± 0.2^{d}	175.4 ± 4.0^{b}	69.2 ± 16.6^{b}	206.1 ± 12.4^{b}	19.8	86	31.8	145.4	0.2	0.5

[1] BAF – bioaccumulation factor rather than nano-BAF. nBAF – is based on the Max k_1 and Max k_2. nBAF* is based on the ratio of total Cu concentration in earthworms on day 7 to the total copper concentration in soils. All total or extractable [Cu] data presented as mean ± SEM (n = 6, duplicate of 3 treatment replicates). Different letters denote groups that are statistically significantly different ($P < 0.05$, ANOVA, Tukey HSD). All results based on dry weight.

4.3.4 Survival and appearance of earthworms

It was expected that the earthworms would survive the duration of the experiment, which was the case in the controls, $CuSO_4$ and coated ENM exposures (100 % survival accompanied by normal appearance and response to stimuli). In the CuO-core exposure, however, there was one mortality on day 14, thus leaving only two earthworms for analysis. The two surviving earthworms were lethargic and slower to respond when compared to the controls and other exposures on day 14.

4.3.5 Essential and trace metals in earthworm tissue at different time-points

Different essential (Ca, Mg, K, Na) and trace (Mn, Fe and Zn) metal concentrations were analysed alongside Cu throughout the uptake and excretion phase, to assess any ion-imbalances and relate this to either exposure to Cu or CuO ENMs. When all data was analysed together (uptake and excretion phase at each time-point) there were no clear time or treatment-dependent statistically significant changes ($P > 0.05$, two-way ANOVA). This may be an overestimation as individual scatterplots revealed some differences in the data. After investigating each individual element within each exposure, and each time-point for treatment specific differences, statistical differences were evident. The results are shown with appropriate statistical labels in Table 4.5, where the capital letters denote statistically significant differences at different time-points for each element within one exposure, and non-capital letters denote statistically significant differences between treatments at each time-point ($P < 0.05$, ANOVA, Tukey HSD).

The control earthworms showed expected tissue concentrations of most of the elements, compared to other studies, throughout the experiment (Chapter 3 and 5). However, some differences in tissue Mn and Fe were evident, mostly towards the end of the exposure. Earthworms exposed to $CuSO_4$ did not show any time-specific effects, while earthworms exposed to CuO-core significantly increased in Fe concentration when they were placed in the clean Lufa 2.2 soil, and continued to do so until the end of the elimination phase of the experiment. Out of all the coated CuO ENMs, CuO-PEG was the only exposure to show some differences in the data. CuO-PEG exposed earthworms showed most variation and significant differences in essential and trace metal concentrations throughout the exposures, compared to the rest of the treatments (Table 4.5).

Treatment effects were present only on day 14 in the uptake phase, and on day 1 and 4 of the elimination phase. Manganese was found to be lower in all Cu or CuO ENM exposures, and lowest in the $CuSO_4$ and CuO-core exposures when compared to the control. However, Mn concentration was low at certain points throughout the experiments in the control animals as well (e.g. uptake phase day 7, elimination phase day 4), although it never dropped below 11 mg Mn kg^{-1} dw dw, as in the $CuSO_4$ and CuO-core exposures. On day 4 of the excretion phase, Fe concentration was different between earthworm tissues, with the controls having the lowest concentration, and CuO-PEG exposed earthworms the highest. Furthermore, on day one of the elimination phase, Zn concentration was highest in CuO- PEG exposed earthworms when compared to the rest of the exposures.

Table 4.5 The measured concentration of essential and trace metals in earthworms at different time points throughout the uptake and excretion experiment.

Element		**Phase**	**Treatment**					
mg kg^{-1} dw	Time		Control	$CuSO_4$	Core	PEG	COOH	NH_4^+
Na	1	Uptake	5094 ± 1929^{a}	3914 ± 313^{ab}	4458 ± 684^{b}	3964 ± 582^{b}	4395 ± 284^{ab}	4226 ± 73^{ab}
	4		3584 ± 155	3860 ± 326	4922 ± 512	4723 ± 117	4264 ± 544	3966 ± 211
	7		3652 ± 404	4687 ± 495	4434 ± 305	5737 ± 914	4308 ± 459	5248 ± 1530
	14		6984 ± 794	5086 ± 809	3575 ± 213	4344 ± 196	5876 ± 313	4545 ± 319
	1	Excretion	4655 ± 120	4672 ± 210	4734 ± 483	6125 ± 274	4999 ± 77	4828 ± 678
	4		5803 ± 128	5705 ± 491	7162 ± 1327	4992 ± 525	6325 ± 118	4857 ± 588
	7		6401 ± 369	6035 ± 1118	6134 ± 1376	5617 ± 305	5320 ± 1385	3903 ± 225
K	1	Uptake	9687 ± 4514	8640 ± 262	7933 ± 338	8119 ± 1134^{A}	9984 ± 444	10423 ± 636
	4		7449 ± 279	8311 ± 746	10172 ± 1092	8100 ± 301^{A}	9721 ± 342	9170 ± 1073
	7		7940 ± 752	7786 ± 786	8383 ± 958	11686 ± 930^{B}	9414 ± 771	10062 ± 2554
	14		11018 ± 1698	7988 ± 532	8119 ± 102	8904 ± 313^{AB}	8760 ± 132	8329 ± 408
	1	Excretion	8328 ± 248	7947 ± 207	8425 ± 889	11470 ± 930^{AB}	9379 ± 471	8639 ± 1473
	4		9856 ± 435	9044 ± 1177	12041 ± 1761	8355 ± 516^{AB}	9703 ± 566	8262 ± 735
	7		10130 ± 1575	9525 ± 1199	11507 ± 2424	9886 ± 120^{AB}	8300 ± 2492	6789 ± 540
Ca	1	Uptake	3579 ± 1589	3445 ± 286	2925 ± 377	2966 ± 318^{A}	3645 ± 210	3319 ± 33

	4		3226 ± 55	3986 ± 103	4607 ± 203	3758 ± 89 ^{AB}	3929 ± 369	3824 ± 465
	7		3573 ± 141	4756 ± 55	4014 ± 461	4939 ± 654^{B}	4327 ± 372	4421 ± 1281
	14		4767 ± 424	3962 ± 387	3031 ± 211	3682 ± 186^{AB}	3777 ± 312	4100 ± 352
	1	Excretion	3810 ± 430	3724 ± 109	4044 ± 423	5184 ± 521^{B}	3770 ± 130	4256 ± 613
	4		4429 ± 204	4236 ± 564	5111 ± 823	3804 ± 207^{AB}	4330 ± 128	3750 ± 99
	7		4737 ± 555	4153 ± 233	5133 ± 461	4368 ± 143^{AB}	4235 ± 1094	2811 ± 299
Mg	1	Uptake	868 ± 361	808 ± 37	886 ± 88	682 ± 48^{A}	857 ± 16	906 ± 69
	4		640 ± 25	715 ± 36	891 ± 60	742 ± 15^{AB}	808 ± 32	829 ± 111
	7		679 ± 72	738 ± 47	735 ± 75	967 ± 49^{B}	842 ± 37	854 ± 207
	14		1052 ± 146	693 ± 90	734 ± 17	733 ± 22^{AB}	700 ± 12	671 ± 21
	1	Excretion	721 ± 58	746 ± 35	765 ± 68	971 ± 63^{B}	811 ± 3	835 ± 72
	4		799 ± 16	814 ± 100	1062 ± 168	766 ± 57^{AB}	814 ± 29	706 ± 61
	7		851 ± 93	851 ± 72	1095 ± 191	840 ± 28^{AB}	755 ± 200	609 ± 32
Fe	1	Uptake	774 ± 168^{A}	423 ± 81	351 ± 5^{A}	525 ± 193	363 ± 14	440 ± 111
	4		342 ± 59^{ABC}	664 ± 22	517 ± 111^{AB}	958 ± 213	357 ± 37	391 ± 66
	7		262 ± 29^{C}	671 ± 143	592 ± 123^{AB}	618 ± 127	636 ± 168	657 ± 196
	14		723 ± 65^{aAB}	384 ± 30^{b}	322 ± 23^{bA}	385 ± 61^{b}	415 ± 40^{b}	355 ± 19^{b}
	1	Excretion	503 ± 107^{ABC}	502 ± 173	808 ± 183^{AB}	601 ± 191	848 ± 213	868 ± 99
	4		311 ± 44^{aBC}	716 ± 61^{ab}	520 ± 99^{abAB}	942 ± 261^{b}	457 ± 63^{ab}	538 ± 100^{ab}

	7		770 ± 59^{AB}	693 ± 123	978 ± 152^{B}	505 ± 30	664 ± 142	514 ± 246
Mn	1	Uptake	54 ± 4^{A}	17 ± 2	28 ± 11	25 ± 7^{A}	29 ± 15	21 ± 9
	4		28 ± 1^{AB}	25 ± 1	21 ± 7	47 ± 15^{A}	16 ± 1	22 ± 4
	7		18 ± 0^{B}	22 ± 6	28 ± 7	20 ± 3^{A}	28 ± 9	33 ± 6
	14		33 ± 6^{aAB}	10 ± 0^{b}	9 ± 0^{b}	14 ± 4^{abB}	12 ± 1^{ab}	16 ± 4^{ab}
	1	Excretion	24 ± 5^{B}	21 ± 5	41 ± 7	27 ± 5^{A}	33 ± 6	43 ± 3
	4		19 ± 1^{B}	25 ± 1	33 ± 9	44 ± 13^{A}	19 ± 2	19 ± 4
	7		29 ± 12^{AB}	26 ± 9	42 ± 6	23 ± 2^{AB}	32 ± 7	19 ± 10
Zn	1	Uptake	112 ± 50	97 ± 8	123 ± 9	87 ± 12^{A}	110 ± 7	112 ± 10
	4		84 ± 2	93 ± 10	120 ± 7	99 ± 5^{ABC}	114 ± 4	111 ± 14
	7		95 ± 11	104 ± 8	105 ± 7	132 ± 7^{BC}	113 ± 9	114 ± 29
	14		107 ± 14	91 ± 10	103 ± 0	90 ± 3^{A}	99 ± 8	92 ± 4
	1	Excretion	95 ± 4^{a}	82 ± 5^{a}	105 ± 9^{ab}	136 ± 7^{bC}	104 ± 6^{ab}	108 ± 9^{ab}
	4		97 ± 2	86 ± 9	138 ± 24	97 ± 10^{AB}	104 ± 1	84 ± 4
	7		95 ± 8	100 ± 13	131 ± 26	96 ± 2^{AB}	95 ± 28	67 ± 5

Data expressed as mean ± SEM (n = 3) and is rounded to nearest mg. Time differences are denoted with upper case letters. The groups that share upper case letters are not different ($P > 0.05$, Two-way ANOVA, Tukey HSD). Different lower-case letters denote statistically significant treatments at the specific time-point ($P < 0.05$, ANOVA, Tukey HSD).

4.4 Discussion

Taken together, the results provide new insights to CuO ENM bioaccumulation potential that is in line with the traditional metal uptake and elimination kinetics. Cu ENMs were not found to persist in the organism, thus these results fit the essential trace metal paradigm, where the animals are able to regulate the tissue metal concentrations for internal homeostasis.

4.4.1 Soil pH

Soil pH is known to impact the mobility of metals in soil, as well as their bioavailability to organisms. Although accumulation of copper has not been found to be affected by pH (review by Ardestani et al., 2014), there is currently no conclusive evidence on whether the pH can impact the bioavailability of CuO ENMs in soil. In this study, there was no evidence that soil pH was a factor in the Cu / CuO ENM accumulation. Studies with other ENMs, e.g., ZnO ENMs, have found that lower pH can induce the dissolution of Zn and therefore increase accumulation (Heggelund et al., 2014).

4.4.2 Total and extractable Cu in soils

Exposure to copper was confirmed and extractable fractions were assessed to aid the interpretation of results (Figure 4.1). Total copper in controls was higher than previous studies have shown (e.g. Chapter 3), although it is close to other studies published (~ 4 mg Cu kg^{-1}dw, Ardestani and van Gestel, 2013) and may be explained by the different batches of soil used in the experiment. Overall, results in

Chapter 3 and this study are in the range published values of up to 17 mg Cu kg^{-1} dw (ECI, 2008). The water and acid extractable Cu was less than 14 and 50 % respectively in the control soils, similar to previous findings (Chapter 3). However, overall the water extractable fraction is higher than expected of natural soils (< 1 %, Svendsen and Weeks, 1997) and previous reports with Lufa 2.2 (< 1 % Ardestani and Gestel, 2013). This may be due to differences in the extraction methods, as in this study the soil is also centrifuged after mixing with water. Copper concentration in the $CuSO_4$ treated soil was close to nominal, and the extractable fractions were similar to recent published studies, although around 0.3 % higher (~ 1 % in Scott-Fordsmand et al., 2000 and 0.9 % in Chapter 3). CuO-core ENMs were also close to nominal as expected, and the coated CuO ENMs varied in Cu concentration, which was anticipated due to the differences in the mass of the coating (discussed in Chapter 3). However, as before, the exposures had significantly higher Cu than the control soils. Despite the differences in the total Cu concentration in soils, the percent water and dilute acid extractable fraction was similar between ENM treatments. This finding supports earlier results (Chapter 3), suggesting the coating determines the water extractability of either Cu or intact CuO ENMs (as it was not possible to measure the particles in the extracts, it is assumed a mixture of dissolved and particulate Cu was likely present) (Figure 4.1, Table 4.3). Although, overall the extractability of Cu or CuO ENMs was similar across ENM treatments, it was highest in the CuO-COOH exposures. Furthermore, the lack of correlation between the total Cu in soils and the percent water extractable Cu and between the total and the percent acid extractable Cu, supports the notion that the coating influences the extractability of the ENMs, or in fact the dissolution of Cu.

4.4.3 Uptake and excretion of copper in earthworms

The total copper accumulation in earthworms was measured to assess the uptake and elimination of Cu or CuO ENMs. The control earthworms were monitored throughout the experiment and they sustained an expected background Cu concentration of ~ 7.5 mg Cu kg^{-1} dw (see Chapter 3 and ~ 8 mg Cu kg^{-1} dw in Streit, 1984). The culture medium, as well as the Lufa 2.2 soil, are low in Cu, almost classed as Cu deficient, thus the bioaccumulation factor (BAF*) based on the total metal concentration in the control earthworms was high (Table 4.4). This is common and has been noted by other researchers (e.g., Svendsen and Weeks, 1997). The uptake and elimination of Cu in naturally contaminated field soils showed almost identical curves to the results of $CuSO_4$ exposure presented in this study. They were characterised by a rapid uptake, which plateaued within a few days, and almost immediate clearance of Cu when earthworms were placed into clean soils (Spurgeon and Hopkin, 1999). Furthermore, the calculated BAF, as well as the BAF* based on total copper in soils, were close to or identical to those published before (0.2 and 0.3 in this study, compared to 0.3 in Chapter 3, and 0.3 in Svendsen and Weeks, 1997).

Compared to the $CuSO_4$ earthworms exposed to CuO-core showed higher BAF and BAF* values (Table 4.4), despite the total Cu concentration in the soil being higher, which should result in lower BAF values in the case of metals (Adams and Chapman, 2007). Similar findings have been reported by Heggelund et al. (2014) where exposure to ZnO ENMs resulted in higher nBAF values than dissolved Zn. This could potentially be due to the differential accumulation of the ENMs via skin and gut that allows the higher uptake (Laycock et al., 2016; Diez-Ortiz et al., 2015). There was an evident coating effect on the accumulation of copper in the

earthworms (Figures 4.2 and 4.3), which may be related to the water extractable fraction (discussed above), since the highest accumulation of Cu in earthworms was seen in CuO-COOH exposures, which also had the highest amount of water extractable Cu / CuO particles. Furthermore, there was a non-significant trend of higher Cu on day 14 in the CuO-NH_4^+ exposures. This trend was also seen in Chapter 3, and may indicate a coating effect on continued accumulation (in relation to slow release of Cu), which was not evident in any of the other exposures. Despite the differences between the accumulation and excretion rates of the ENMs, the nBAF values for all were < 1. However, in the CuO-PEG exposures, the nBAF was 0.8, which is close to a threshold for concern. Though this may have been due to the overall lower amount of copper in the soil (compared to the other ENM and $CuSO_4$ exposures) and the earthworms being in a copper deficient culture soil thus holding on to additional Cu, or whether it is a ENM specific phenomena.

Many non-kinetic studies use either the bioconcentration factor (BCF) or BAF based on measured total metal concentration in the earthworms and soils, at a fixed time-point, to estimate the bioaccumulation potential. In this study, the results of the non-kinetic nBAF (nBAF*) further support the differences in the accumulation between the different materials, irrespective of the total measured concentrations in the soil. The two calculated factors are only comparable in the $CuSO_4$ and CuO core exposures, but in the coated ENM exposures the differences are much bigger, and different for each coating, with the COOH material having the highest nBAF* followed by NH_4^+ and PEG (Table 4.4). These results demonstrate the inconsistent variability that arises from different surface coatings using the two sets of data, which is not to do with the mass of the coating since all values are based on measured Cu concentrations.

4.4.4 Additional endpoints: survival and differences in essential and trace metal concentrations in earthworms

The control earthworms and those exposed to $CuSO_4$ remained healthy and survived throughout the experiment as expected. In the ENM exposures, only the CuO-Core ENMs resulted in one mortality, which is likely due to high internal Cu concentrations (well exceeding the critical body residues (CBRs) of 60 mg Cu kg^{-1} dw in *L. rubellus* reported in Ma, 2005). However, earthworms exposed to CuO-COOH and NH_4^+ EMNs reached similar body burdens, albeit with no mortalities (or abnormal behaviour, e.g., lethargy). This could indicate the earthworms were under more stress, due to the higher external Cu, and placing most of their energy on actively regulating internal Cu. Moreover, it may be due the accumulation of CuO ENMs, which are not as toxic as dissolved Cu, since the internal Cu concentration of the earthworms are around double the CBR reported in Ma (2005).

The ionic balance in organisms can be influenced by excess of heavy metals, however it is not certain if metal based ENMs can cause similar effects. The control earthworms sustained expected tissue concentrations of the electrolytes and trace elements (Table 5, compare to Chapters 3 and 5), although some changes to Mn and Fe were evident on day 7 of the uptake phase, which may indicate natural variation or lack of sufficient nutrition. The latter explanations seem possible because both of these elements are actively regulated (Ireland, 1978, Procházková et al., 2014) as well as nutritionally important. The effects on Mn depletion were seen in only $CuSO_4$ and CuO-core exposures on day 14 (Table 4.5), which may indicate a similar mechanism in the two exposures of dissolved Cu competing with Mn. Copper is known to interfere with Fe metabolism (Linder, 1991), and the effects seen on Fe

concentration (Table 4.5) on day 14 may be due to internalised Cu. The earthworms exposed to CuO-PEG ENMs showed the most differences to the varying tissue metal concentrations throughout the experiment, which could have been caused by the accumulation of the intact ENMs, or could have been an effect of the coating. The PEG coating, if taken up, may have interfered with the ion regulation, since tissue Cu concentration was below any of the other exposures (although the ambient concentration of Cu was also lower than any of the other exposures).

4.5 Conclusions

The European Copper Institute's voluntary risk assessment on copper (ECI, 2008) concluded that Cu is not bioaccumulative or likely to be biomagnified in the food chain. The maximum nBAF values calculated for all ENMs were < 1, indicating the specific CuO ENMs used in this study are not bioaccumulative in earthworms. However, the CuO-PEG ENMs had the highest nBAF value of 0.8, which was reflected in the slower clearance of Cu in these exposures, which may have been due to the overall lower Cu concentration in the soil, or, in fact an effect of the coating. Trace metal disturbances were observed in earthworms that could be related to malnutrition coupled with Cu / CuO ENM exposure. In summary, the results fit the dissolved Cu (essential trace metal) kinetics where the animals are able to regulate the internal concentrations to maintain homeostasis. Studies into the effects of coating, and potentially measuring the accumulation of the coating in earthworms, warrants further investigation, in order to reliably assess the environmental risk of differently coated ENMs.

Chapter 5. The biological effects of CdTe QDs on earthworms

5.1 Introduction

Quantum technology is a growing market with substantial forecasts for more efficient and sustainable future technologies. Quantum dots (QDs) with their unique electronic and optical properties were first synthesised in the early 1980s and in 1988, Reed et al. (1988) first used the phrase “quantum dot” to describe these semiconductor nanocrystals. Consequently, they have many potential applications including bio-imaging, drug delivery, cell labelling, a variety of visual light-emitting diode (LED) technologies, quantum information processing and more (Hardman, 2009). Cadmium-telluride quantum dots are efficient semiconductors; thus, one of their most common use is in photovoltaics (in solar panels) that offer alternative energy solutions (Sinha et al., 2012). Additionally, CdTe QDs are also used in biolabeling (Thuy et al., 2011). Quantum dots are synthesised using various methods, mostly physio-chemically (Dabbousi et al., 1997), although biological ways have been also discovered which could potentially increase the biocompatibility to enhance drug delivery and other medical applications. Bacteria (Kominkova et al., 2014) and yeasts (Cui et al., 2009) have been found to biosynthesise quantum dots when exposed to a combination of Cd and Te, or Cd and Se, metal salts. More recently, earthworms and their natural protective mechanism, like the ability to reduce metals into inorganic forms, have been used to synthesise CdTe dots (Stürzenbaum et al., 2013; Kominkova et al., 2014). Little is known about their environmental fate and toxicity in soil, that is ultimately, one of the last sinks for many man-made contaminants.

One of the biggest concerns of cadmium-based quantum dots is the use of a known toxic metal that may result in the release of dissolved Cd from the particles. Thus far, studies have shown CdTe QD specific effects, e.g., related to their potential to

generate reactive oxygen species (Lopes Rocha et al., 2017). But also toxicity related to Cd dissolution has been shown in various organisms (comprehensive review in Lopes Rocha et al., 2017). There are soil guideline values set in the UK for total Cd depending on land use, 1.8, 10 and 230 mg Cd kg^{-1}, for allotment, residential and commercial land, respectively (EA, 2009). A natural background concentration of around 0.49 mg Cd kg^{-1} dw can be found in the UK soils (Spurgeon et al., 2008). Anthropogenic sources of Cd include mining and refining, the burning of fossil fuel, waste from the electronics industry and some fertilisers contain Cd as a trace metal (Alloway, 1995). It is a concern that the production and use of CdTe QDs will add to the contamination (via waste disposal). Once in the environment CdTe QDs may slowly degrade and release hazardous dissolved metals (Navarro et al., 2008), or even create new hotspot for metal contamination. And since CdTe QDs and other QDs are increasingly used in technology the global problem of e-waste burning in developing areas may lead to a further pollution from CdTe QDs (Luo et all., 2011).

Cadmium is a non-essential metal for eukaryotes, and is known to bioaccumulate in earthworms (Hopkin, 1989). Dissolved cadmium acute toxicity is well known in earthworms; 4 week $^{mortality}LC_{50}$ = 588 mg Cd kg^{-1} dw in artificial soil (van Gestel et al., 1991). As well as the reproductive toxicity with the 56 day $^{reproduction}EC_{50}$ = 46.3 mg Cd kg^{-1} dw in artificial soil, Spurgeon et al., (1994). The fate and effects of Cd in earthworms has been extensively studied (Spurgeon et al., 2004; Stürzenbaum et al., 2004 and references therein). Cd increases the production of metallothionein (MT) in earthworms and a cadmium specific-metallothionein (worm metallothionein-2 or wMT2) has been identified by Stürzenbaum et al. (2001). This specific cysteine rich protein sequesters Cd in the chloragogenous tissue, from where it's eventually

released into either the earthworm's typhlosole or into the coelom and slowly excreted (Stürzenbaum et al., 2004). Most importantly, when bound to wMT2, Cd is not toxicologically active in the earthworm, allowing it to survive very high total tissue Cd concentrations (Ardestani et al., 2014). Although at high tissue Cd concentrations, often reproduction and growth are sacrificed (Spurgeon et al., 1994; 2004).

In contrast to Cd, the ecotoxicity of tellurium is poorly understood and there are no know guideline values for tellurium in soils. Tellurium is never likely to be present in the environment as Te^0, but rather as tellurite (Te(IV)) or tellurate (Te (VI)). Tellurium is a very rare element, with background concentrations in soil being in the μg kg^{-1} dw range (Belzile et al., 2015). Due to its biogeochemistry, it is a difficult metal to measure in complex matrices, thus only recently measurements of Te in soil and sediments are emerging (Belzile et al., 2015). Tellurium is mostly obtained as a by-product of copper mining and refining. While before the era of quantum dot technology, it was mostly used in copper alloys, stainless steel and as a colorant in glass and ceramics (Belzile et al., 2015). Tellurium has no known biological functions in eukaryotic cells (Ba et al., 2010) and not much is known about its toxicity to soil organisms. The soluble oxyanions of tellurium, tellurite and tellurate, however, have been found to be toxic to organisms at low concentrations (< 1 mg l^{-1} Chasteen et al., 2009 and references therein). So far, the only known biological function of tellurium has been found in fungi, that can use tellurite instead of sulphur for production of amino acids when sulphur is limited or absent (Ramadan et al., 1989).

Since there is a lack of knowledge on the acute and chronic (reproductive) effects of CdTe QDs in earthworms, this experiment aimed to provide a baseline dataset for

soil hazard assessment. The study design included a control soil, CdTe-bulk QDs, which are in the micron size range, and three differently capped CdTe QD ENMs to represent anionic, carboxylate (COOH), cationic, ammonium (NH_4^+) and neutral organic ligands, polyethylene glycol (PEG) on the surface of the quantum dots. In addition to establishing a baseline dataset, the potentially toxic component(s) of the composite of the QDs were identified from accumulation of Cd and Te in earthworm tissues. Further biological effects were assessed to ascertain ion-regulatory toxicity or induction of oxidative stress, and compared against known dissolved Cd toxicity. Having established the response in first experiment, a second experiment including the same endpoints, was carried out with newly exposed earthworms after a six-month period of ageing the soils.

5.2 Methodology

Two experiments were carried out using a low (50), medium (500) and high (2000) nominal CdTe QD ENM concentration in mg CdTe kg^{-1} dw in quadruplicate test design with a test soil and CdTe micron size control. The first experiment was conducted with a freshly spiked soil, and the second using the same soil after six months of ageing, in the text referred to as fresh and aged soil experiments, respectively.

5.2.1 Test soil, organisms, conditions and engineered nanomaterials

Adult *E. fetida* were used from an internal synchronous laboratory breeding culture held at University of Plymouth in the fresh and aged soil experiments (details in

Chapter 2.4). A standard sandy loam Lufa 2.2 soil (LUFA Speyer, Germany) was used in experiments, details of which are presented in Chapter 2.5. The soil used in the aged soil experiment, was the same used in the initial fresh soil experiment. It was left for 6 months after the first experiment in the same containers (pierced lids) to provide airflow. One day prior to the experiment plant material was quantified and above ground material was removed (details in Chapter 6). Soil moisture content was adjusted to 50 - 60 % and soil pH was measured (detailed methods in Chapter 2.2).

The ENMs used in the experiments were provided by PlasmaChem and were supplied with characterisation information carried out by the supplier or partner institutions of the research project (Table 5.1). The details of the coatings and the synthesis are commercially sensitive information, therefore detailed descriptions were not provided. The different coatings of polyethylene glycol, carboxylate and ammonium are referred to as PEG, COOH and NH_4^+ in text, respectively. Appropriate micron sized CdTe QD powder (size described as < 250 µm**)** was used as a control, purchased from Sigma (CAS 1306-25-8), and referred to as the CdTe-bulk hereafter. The QD powders supplied were dark red (NH_4^+ coated) or bright red (PEG, COOH coated) in colour and no impurities were identified by the supplier. The CdTe-bulk powder was black in colour and no impurities were noted by Sigma. All other chemicals used were analytical grade and purchased from Sigma unless stated otherwise.

Table 5.1. Manufacturer's information on the CdTe QD ENMs used in the experiments.

ENM variant	[1]Manufacturer's Information	[2]Estimated primary particle size (nm)	[3]NTA, hydrodynamic diameter (nm)	[4]TGA, degree of functionalisation (% weight loss)	[5]Maximum rate of dissolution in Milli Q water (μg h^{-1})	
					Cd	Te
CdTe-Polyethylene Glycol	Lot No.YF140402, 99% purity, size 3-5 nm.	< 4	156 ± 72	50.4 ± 8.3	0.017	0.008
CdTe- Carboxylate	Lot No.YF140402, 99% purity, size 3-5 nm.	< 4	84 ± 58	23.4 ± 4.2	0.036	0.031
CdTe- Ammonium	Lot No. YF140402, 99% purity, size 3-5 nm.	< 4	75 ± 50	8.8 ± 0.5	0.146	3.134

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

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

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

[4]TGA – thermogravimetric analysis. 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.

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

5.2.2 Total and extractable metal analysis in samples

Total metal analysis in soils was carried out as described in Chapter 2.5 in the beginning of both, fresh and aged soil, experiments. To determine the accessible fractions of Cd and Te in soils water and 0.1 M HCl extractable fractions were determined in duplicate in the nominal 2000 mg CdTe kg^{-1} dw exposures (details in Chapter 2.6). Total Cd, Te and a suite of essential and trace metals were analysed in depurated earthworms following the method in Chapter 2.7, from both, fresh and aged soil, experiments at the end of 28 days.

5.2.3 Experimental design

5.2.3.1 Fresh soil experiment

The experiment was conducted using the standard guideline for the earthworm reproduction test per OECD TG 222 (OECD, 2004), but with a reduced number of earthworms and additional sub-lethal endpoints. The study design included a control soil, CdTe bulk powder and 3 variations of coated CdTe QDs: PEG, COOH, NH_4^+. Each test vessel contained 250 g (dry weight) of soil. Three concentrations were chosen for the CdTe QDs at 50, 500 and 2000 mg CdTe kg^{-1} dw based on known Cd toxicity to earthworms (van Gestel et al., 1991; Spurgeon et al., 1994) which were aimed to produce a dose-response. Earthworms (n = 5 earthworms/box of soil) were exposed in 4 replicates (n = 20 earthworms per treatment). Total body burden of Cd, Te and other cations and biochemical endpoints were measured at the end of the experiment (n = 8 earthworms for each analysis per treatment). The test materials were added to the soil as dry powders following wetting of the soil, as described in Chapter 2.2. Soil pH was measured at the beginning and end of the experiment;

moisture loss from the soils was replenished every 2 weeks throughout the experiment.

Adult *E. fetida* (*n* = 5 earthworms) with a mean weight of 1.86 ± 0.02 g for 5 earthworms (mean ± SEM, *n* = 52, individual earthworm weight ~ 0.36 g) were exposed at 20 ± 1 ^{o}C at 12:12 light:dark cycle. The earthworms were fed dried horse manure (1 g/earthworm) wetted to 70 % of its water holding capacity (WHC) with Milli-Q water. Life history traits such as survival and weight of the earthworms were recorded at the beginning of the experiment and on days 14 and 28 (half-way and at the end of the experiment, respectively). Behavioural changes such as avoidance of burrowing into the soil, were recorded following visual assessment in all treatments in the beginning of the fresh soil experiment. Further endpoints such as total concentration of Cd, Te and other elements in earthworm tissue and total glutathione and Na^{+}/K^{+}-ATPase activity, were measured on day 28. In addition, these specific endpoints were chosen to aid comparison between CuO ENM (Chapter 3) and CdTe QD effects. After 28 days, when all the adults were removed from soils, additional feed was added (5 g, dw, wetted to 70 % WHC with Milli-Q water) and soils were left for a further 28 days to allow juveniles to hatch from cocoons. The juveniles were counted following the OECD TG 222 (2010). Briefly, each test vessel was placed in a water bath (60ºC) and the juveniles that emerged to the surface were collected and counted. After counting, soils were manually checked to ensure no juveniles remained in the soil. Collected juveniles from each treatment were washed in deionised water, blotted dry and weighed, to allow the assessment of the treatment effect on juvenile growth.

Aged soil experiment

The same study design and methods were used in the aged soil experiment as described in the fresh soil experiment. Five earthworms with a mean weight of 1.89 ± 0.03 g (mean ± SEM, *n* = 52, individual weight ~0.38 g) were used in the experiment. On days 0, 14 and 28 endpoints such as survival, biomass and appearance were recorded. Reproduction was assessed after 56 days of exposure (described above). In addition, total concentration of Cd, Te and other cations in earthworm tissues were measured on day 28. Total glutathione and Na^+/K^+-ATPase were assessed at the end of the exposure on day 28. Juvenile weight was not assessed in the aged soil study due to the difficulty of obtaining the alive and intact juveniles from the soil. This was due to the presence of plant roots several individuals were stuck in the soil for too long and, unfortunately, did not survive.

5.2.4 Biochemical analyses

All analyses were carried out as described in Chapter 2.7.

Due to a technical problem with the -80°C freezers, the samples from the aged CdTe QD experiment were accidentally defrosted twice. This resulted in abnormal and unexpected values for the biochemical endpoints. To understand the effect of this repeated defrosting of samples, a precautionary small-scale trial was carried out with unexposed earthworms from the internal earthworm culture following the methods described in Chapter 2.7.

5.2.4.1 Defrosting effect on biochemical endpoints

Twelve adult fully clitellated earthworms were taken from the internal culture, washed in deionised water, blotted dry, snap frozen in liquid nitrogen and stored at – 80°C until analysis.

Earthworms were divided into 3 groups (A, B, C), each with *n* = 4 earthworms. The group details were as follows:

A) Freeze – defrost at 4ºC – homogenise – analyse.

B) Freeze – defrost at 4ºC – freeze (1 day) – defrost at 4ºC – homogenise - analyse.

C) Freeze – defrost at 4ºC – freeze (1 day) – defrost at 4ºC – freeze (1 day) – defrost at 4ºC – homogenise – analyse.

Samples (groups B and C) that were subject to defrosting and re-freezing were defrosted for 3 h each time in the fridge at 4ºC, and re-frozen at the – 80ºC freezers. The results shown in Table 5.2 indicate that defrosting samples three times prior to analysis influences the total glutathione content, Na^+/K^+-ATPase activity but not the total protein concentration.

Table 5.2. Total protein content, total glutathione and Na^+/K^+-ATPase (sodium pump) activity in earthworms.

Sample group	**Total protein in homogenate**	**Total Glutathione**	**Na^+/K^+-ATPase activity**
	mg ml^{-1}	nmol GSH mg^{-1} protein	µmol ADP mg^{-1} protein h^{-1}
A	9.5 ± 1.2	7.9 ± 1.2[a]	8.0 ± 1.2[a]
B	6.1 ± 0.3	5.9 ± 1.1[a]	9.2 ± 0.7[a]
C	8.5 ± 1.6	2.3 ± 0.3[b]	1.1 ± 0.4[b]

(A) defrosted once, (B) defrosted twice, (C) defrosted three times. Results presented as mean ± SEM (*n* = 4). Different letters denote statistically significant groups ($P < 0.05$, ANOVA, Tukey HSD).

Based on the small-scale trial it is possible that defrosting samples and freezing them two times (defrosting three times in total) has a detrimental effect on the total glutathione content and Na^+/K^+-ATPase enzyme activity. Repeated freezing and thawing has been shown to cause considerable loss of glutathione reductase activity (Owens and Belcher, 1965).

5.2.5 Statistical analyses and data presentation

All statistical analyses were performed as described in Chapter 2.8. The reproduction results were normalised to the control values and the 50 % effect concentration (EC_{50}) values and its 95 % confidence intervals were estimated in

SigmaPlot 13.0 by carrying out a logistic nonlinear regression analysis (sigmoid) on raw data using the log-transformed nominal concentrations. To estimate the nominal effect value, the log-transformed value was reversed. The ratio of the total concentration of a metal in earthworms to the concentration of total metal as a representation of either metal from the QDs or metal incorporated in the QDs in the soil, or the bioaccumulation factor (BAF), was calculated in Excel ($[Cd]_{soil}$ or $[Te]_{soil}$ / $[Cd]_{earthworm}$ or $[Te]_{earthworm}$). As discussed in (Chapter 4), the term nano-BAF is used, since it cannot be determined if the dissolved metal or intact particle is accumulated. Nonetheless, the apparent nBAF offers an insight to differences in accumulation, although it is yet uncertain whether the principles of BAF apply to engineered nanomaterials. In all figures 'bulk' to represent the micron size CdTe QDs and the uncoated and 'PEG', 'COOH' and 'NH_4^+' to refer the coated CdTe QDs.

5.3 Results

5.3.1 Soil pH

5.3.1.1 Fresh and aged soil experiment

In the beginning of the fresh soil experiment, soil pH differed between QD treatments and the control soils, while by the end of the exposure (day 28) there were no differences between the control soils and the soils treated with QDs (Table 5.3). But differences between the different QD exposures remained. There were no statistically significant differences between the control soils and the treatments in the beginning and the end of the aged soil experiment. However, there were some statistically significant differences between QD exposures (Table 5.3). The aged soil pH, both at the beginning and the end of the experiment, did not statistically

significantly differ from the pH in the fresh soil experiment ($P > 0.05$, Student's *t*-test). Due to the differences, soil pH was included as a cofactor in all endpoint analysis, but no significant interactions were found ($P > 0.05$, ANCOVA).

Table 5.3. Soil pH in the beginning and end of the fresh and aged CdTe QD experiment.

Nominal [CdTe QD]$_{soil}$, mg kg^{-1}	**Time Days**	**Control**	**CdTe-bulk**	**CdTe-PEG** pH	**CdTe-COOH**	**CdTe- NH_4^+**
			Fresh soil			
50	0	5.59 ± 0.04^{ab}	5.61 ± 0.05^{ab}	5.52 ± 0.01^{bc}	5.53 ± 0.02^{abc}	5.38 ± 0.05^{cfg}
500	0		5.28 ± 0.06^{fg}	5.89 ± 0.03^{d}	5.36 ± 0.02^{cfg}	5.71 ± 0.08^{ae}
2000	0		5.87 ± 0.09^{de}	5.52 ± 0.04^{bc}	5.21 ± 0.04^{g}	5.44 ± 0.05^{bcf}
50	28	5.52 ± 0.01^{ab}	5.39 ± 0.02^{b}	5.78 ± 0.1^{ac}	5.57 ± 0.02^{a}	5.51 ± 0.01^{ab}
500	28		5.39 ± 0.01^{b}	5.51 ± 0.02^{ab}	5.60 ± 0.01^{ab}	5.48 ± 0.02^{ab}
2000	28		5.70 ± 0.01^{a}	5.76 ± 0.01^{a}	5.70 ± 0.01^{a}	5.78 ± 0.02^{a}
			Aged soil			
50	0	5.50 ± 0.03^{abc}	5.38 ± 0.01^{c}	5.61 ± 0.14^{abc}	5.60 ± 0.03^{abc}	5.44 ± 0.06^{bc}
500	0		5.42 ± 0.04^{bc}	5.71 ± 0.13^{a}	5.63 ± 0.03^{ab}	5.70 ± 0.1^{a}
2000	0		5.64 ± 0.05^{ab}	5.70 ± 0.01^{a}	5.59 ± 0.05^{abc}	5.49 ± 0.02^{abc}
50	28	5.53 ± 0.06^{ab}	5.38 ± 0.04^{a}	5.60 ± 0.22^{ab}	5.61 ± 0.03^{ab}	5.41 ± 0.05^{ac}
500	28		5.48 ± 0.04^{ab}	5.55 ± 0.13^{ab}	5.58 ± 0.06^{ab}	5.56 ± 0.03^{ab}
2000	28		5.56 ± 0.04^{ab}	5.69 ± 0.04^{b}	5.65 ± 0.03^{bc}	5.57 ± 0.04^{ab}

Data presented as mean ± SEM (n = 4). Treatments that do not share a letter are statistically significantly different ($P < 0.05$, ANOVA, Tukey HSD or Kruskal-Wallis, Dunn's test).

5.3.2 Total and extractable Cd and Te concentrations in soil

To enable the interpretation of the results, the total concentration of Cd and Te was measured at the beginning of both the fresh and aged soil experiments. In the former experiment, the control soil contained a background Cd concentration of 0.61 ± 0.05 mg Cd kg^{-1} dw (mean ± SEM, n = 8) of this total Cd, 9.2 ± 1.4 and 10 ± 1.5 % was water and 0.1 M HCl-extractable respectively, indicating that less than 10 % of the Cd was accessible. Te was not detected in the control soil, as the results were below the limit of detection, < 0.1 µg Te kg^{-1} dw (Chapter 2.5). The nominal values were based on the total mass of CdTe QDs added, thus the total Cd and Te concentrations were not expected to be close to the nominal values of the QDs. Exposure to CdTe bulk and differently coated CdTe QD ENMs caused the expected increase in total soil Cd concentrations that did not differ significantly between the type of coated material at each nominal test concentration (Figure 5.1a, $P > 0.05$, ANOVA). In contrast, total tellurium concentration varied significantly between treatments in each nominal test concentration (Figure 5.1b). The concentration of Te in soil ranged from 7.9 ± 1.6 – 26 ± 6 mg Te kg^{-1} dw in the low (50 mg CdTe kg^{-1} dw) exposures, with PEG- and COOH-coated materials having the lowest concentration of Te. The same trend followed in the intermediate (500 mg CdTe kg^{-1} dw) exposures, where the concentration ranged from 26.3 ± 1.2 – 175.6 ± 22.8 mg Te kg^{-1} dw and the top (2000 mg CdTe kg^{-1} dw)exposures with 95.3 ± 3 – 602.5 ± 5 mg Te kg^{-1} dw. The amount of Te in the exposures followed the order of: CdTe-bulk > NH_4^+ > PEG ≥ COOH. In the CdTe-bulk exposures, less than 1 % of cadmium and tellurium were extractable. The percent water available Cd fraction of the total cadmium in soil was: 0.02 ± 0.01, 1.32 ± 0.13, 1.78 ± 0.12, 2.42 ± 0.28 % in the CdTe-bulk, -PEG, -COOH, -NH_4^+ QD treated soils, respectively. And for Te, 0.001 ±

0.001, 0.51 ± 0.08, 0.36 ± 0.72, 0.39 ± 0.05 % in the CdTe-bulk, -PEG, -COOH, -NH_4^+ QD treated soils, respectively. The percent 0.1 M HCl available Cd of the total was 1.19 ± 0.22, 33.4 ± 13.7, 52.6 ± 10.5, 102. ± 9.8 % in the CdTe-bulk, -PEG, -COOH, -NH_4^+ QD treatments, respectively; and for Te, 0.74 ± 0.17, 1.29 ± 0.49, 8.39 ± 1.19, 23.4 ± 2.54 % in the CdTe-bulk, -PEG, -COOH, -NH_4^+ QD treatments, respectively. The water available fraction of Cd and Te was the highest from CdTe-NH_4^+ QD exposures (Figure 5.2a, b).

The total concentration of Cd and Te in the soil were measured again prior to beginning of the aged soil experiment. In general, the concentration of Cd and Te in the aged soil followed a very similar pattern to the fresh soil experiment, but with a trend of slightly lower values than in the fresh soil experiment. However, statistical analysis showed that most of the treatments were not statistically significant. In the CdTe-bulk exposures the difference between Cd in the fresh and aged soil was not significant ($P > 0.05$, *t*-test, paired, Figure 5.1c), and borderline in the nominal test concentrations 500 and 2000 mg CdTe kg^{-1} dw (P = 0.06, *t*-test, paired). When further comparing each CdTe QD ENM treatment individually to the fresh soil equivalent, the concentration of Cd was significantly lower only in the CdTe-PEG exposures, in the nominal 50 mg CdTe kg^{-1} dw CdTe-NH_4^+ exposure and in the nominal 2000 mg Cd kg^{-1} dw CdTe-COOH exposure ($P < 0.05$, paired *t*-test, Figure 5.1c). Te was significantly lower in the nominal 2000 mg CdTe kg^{-1} dw CdTe-PEG and COOH and 50 mg Cd CdTe-NH_4^+ exposures ($P < 0.05$, paired *t*-test, Figure 5.1d).

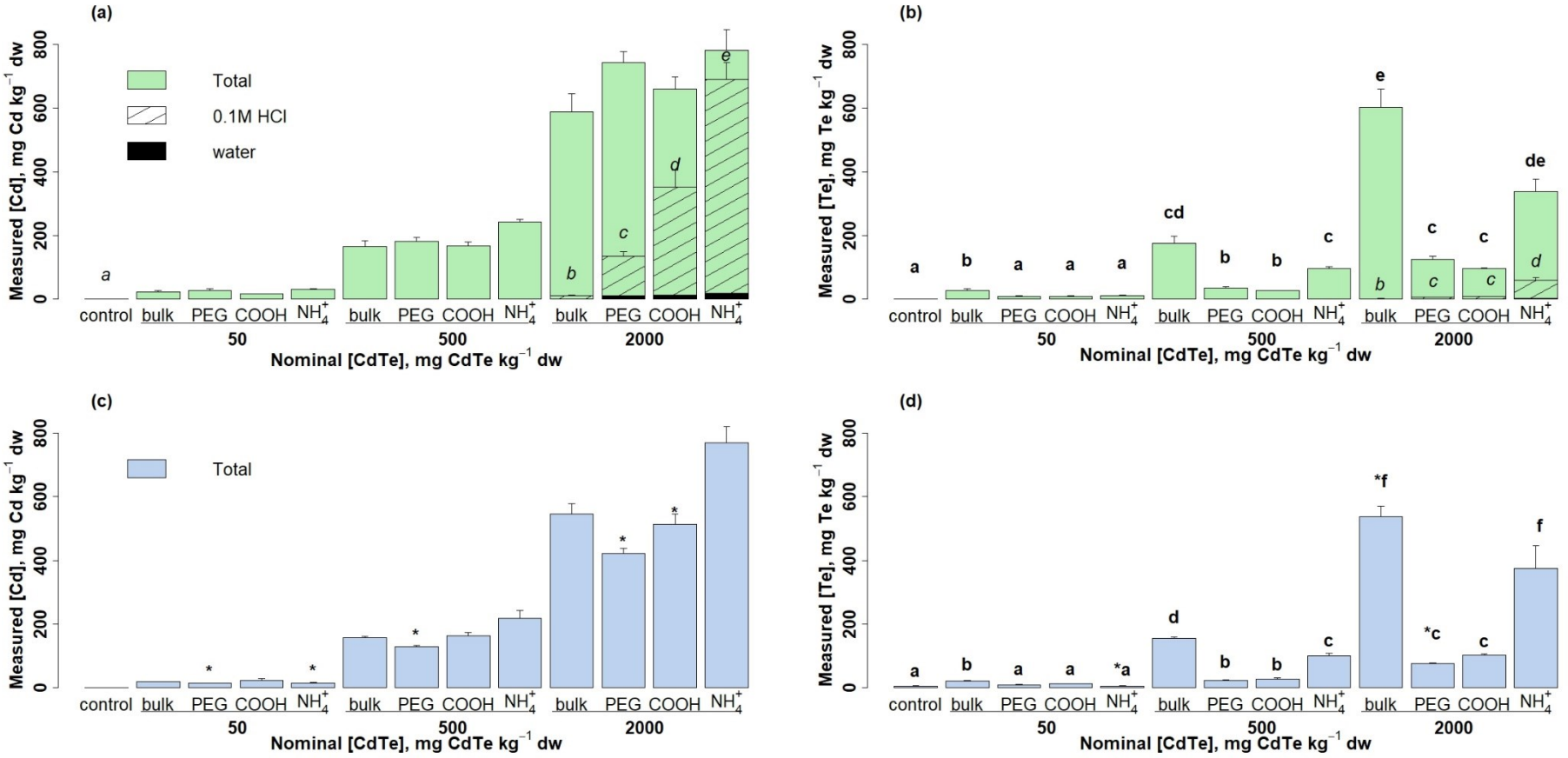

Figure 5.1 (a),(c) Total Cd concentration (mg Cd kg^{-1} dw) (b),(d) total Te concentration (mg Te kg^{-1} dw) in soils at the beginning of the fresh (top bars) and aged (bottom bars) soil experiment in mg kg^{-1} dw . Data expressed as mean ± SEM (n = 8). Water and 0.1M HCl available fraction is shown only in the nominal 2000 mg CdTe kg^{-1} dw treatment (See Figure 5.2 for water-available fraction). Different letters show statistically significant differences between treatments. Italicised letters denote differences between acid extractable Cd or Te (P < 0.05, ANOVA). Asterisks denote differences between fresh and aged soil (P < 0.05, t-test, paired).

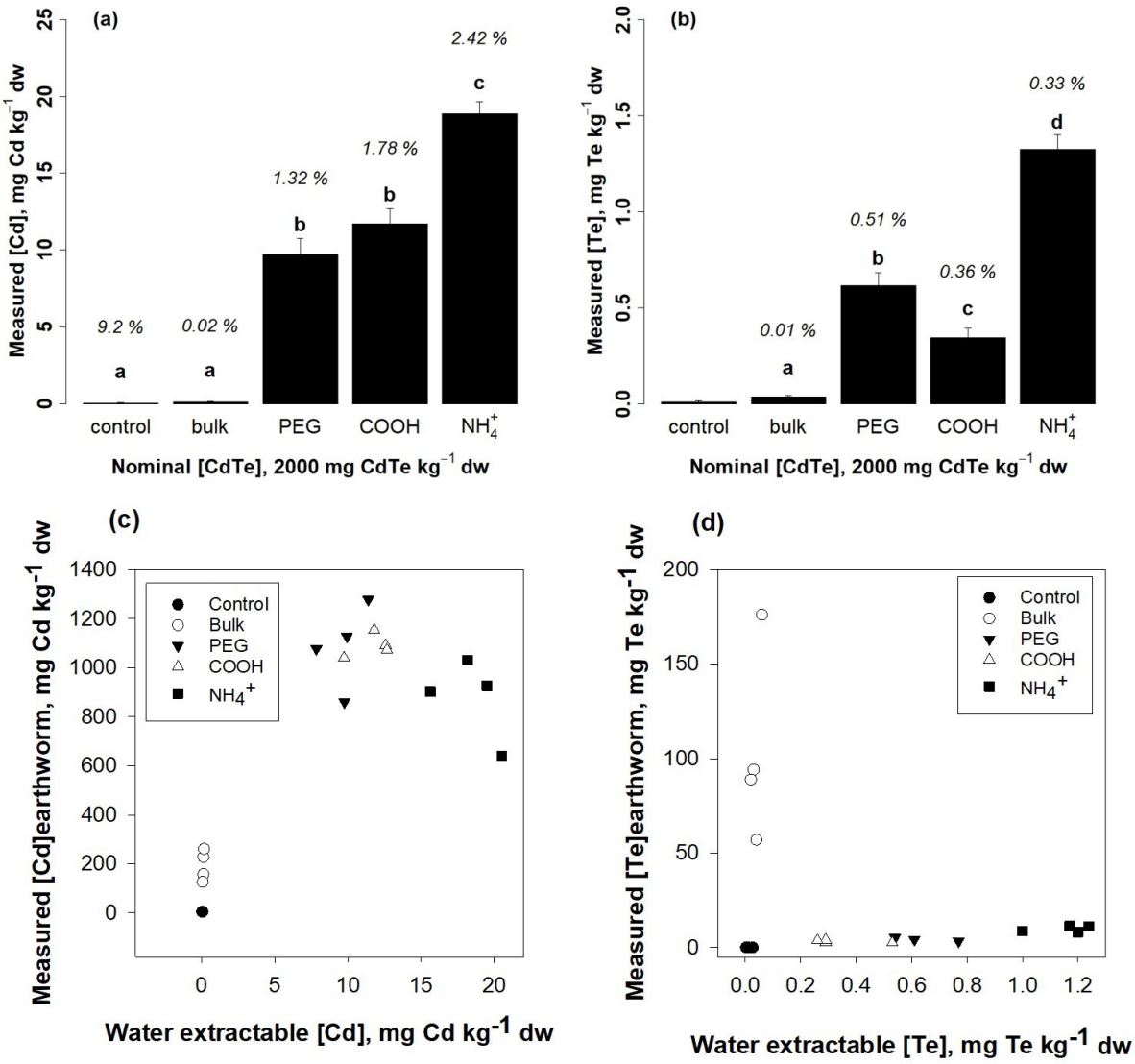

Figure 5.2 (a) Water extractable Cd and (b) Te concentration in the highest nominal CdTe QD treatments (see Figure 5.1 for total [Cd] and [Te]). Data presented as mean ± SEM (n = 8). Different letters denote statistically significant differences between treatments ($P < 0.05$, ANOVA, Tukey HSD). The percentage of the water extractable Cd or Te of the total Cd or Te is noted on each bar for reference. (c) Relationship between the concentration of water extractable Cd and earthworm tissue Cd mg Cd kg^{-1} dw; (d) relationship between the concentration of water extractable Te and earthworm tissue Te, mg Te kg^{-1} dw in the nominal [CdTe] mg CdTe kg^{-1} dw exposures.

5.3.3 Total Cd and Te in earthworm tissue

The total concentration of Cd and Te was also measured in earthworm tissues. The control earthworms in both fresh and aged experiments had some background Cd in the tissues (4.23 + 0.34 mg Cd kg^{-1}, mean ± SEM, n = 8), while no tellurium was detected (all readings below the LOD of ICP-MS, Chapter 2.5). A concentration dependent increase in the total body burden of Cd was seen in all CdTe-bulk and all CdTe QD ENM exposures (Figure 5.3a). The accumulation of Cd was significantly lower in the CdTe-bulk when compared to the ENM exposures, despite similar amounts of total Cd in the soil. There was no significant difference between the total concentrations of Cd in earthworm tissues between the different CdTe ENM variants in each nominal test concentration (Figure 5.3a, $P > 0.05$, ANOVA). More Cd was accumulated by earthworms exposed to the CdTe QD ENMs than the CdTe-bulk. Total concentration of Te in earthworm tissues was increased in a concentration dependent manner in the CdTe-bulk exposures, but it was uniformly low across the different CdTe ENM types and test concentrations (Figure 5.3b). The accumulation of Cd and Te were strongly correlated in each treatment group (r_s= 0.9, 0.8, 0.8 and 0.9 for CdTe-bulk, -PEG, -COOH and -NH_4^+ exposures, $P < 0.05$, Spearman). The total Cd or Te in the soil was significantly correlated to the total Cd or Te in earthworms when analysing all the data together (r_s = 0.8 and 0.7 for Cd and Te, $P <$ 0.05, Spearman). Note, comparable results were obtained in individual per treatment analysis. Individual scatterplots revealed the water and acid extractable fractions were not related to the accumulation of Cd and Te in the CdTe-bulk earthworm tissues (Figure 5.2c,d). Furthermore, the water and acid extractable Cd were not related to Cd accumulation in CdTe QD ENM exposures. However, the water and

acid extractable Te was related to accumulation of Te in earthworm tissues (compare Figures 5.1a, b and 5.3a, b, Figure 5.2b).

To further assess the likelihood of accumulation of intact CdTe QDs in earthworms the concentration ratio of Cd to Te was calculated in the fresh and aged soil studies. This was compared against the ratio of Cd to Te in soils and extractable fractions (latter only in the fresh soil study). The ratios were similar across test concentrations thus only results for the 2000 mg CdTe kg^{-1} dw test concentration are shown (Table 5.4).

The concentration of Cd and Te in the earthworms was only measured at the end of the experiment thus it was not possible to ascertain if the Cd and Te concentration in the earthworm tissues had reached a steady state. Despite this limitation, for comparison purposes a nBAF was calculated for each test concentration (Table 5.5). The background concentration of Cd in the control soils resulted in a high nBAF in the earthworms not exposed to added Cd. The ENM treated earthworms had higher nBAF values for Cd than the bulk CdTe QD treated earthworms, while the nBAF values for Te remained similar between the different QD types (Table 5.5).

In the aged soil, the accumulation of Cd and Te was overall higher than in the fresh soil experiment. In the CdTe-bulk exposures the uptake was significantly higher than in the fresh soil study, and the concentrations were closer to the CdTe QD ENM exposures (Figure 5.3c). In the nominal 50 mg CdTe kg^{-1} dw test concentration there were no significant differences in the concentration of Cd between the different CdTe QD types. In the nominal 500 or 2000 mg CdTe kg^{-1} dw test concentrations there were no statistically significant differences between the concentrations of Cd, but there was a non-significant trend of higher tissue Cd concentration in earthworms

exposed to the differently coated CdTe QD ENMs when compared to the CdTe-bulk (Figure 5.3c). In the aged soil, the concentration of Te followed a similar pattern of accumulation to the fresh soil experiment (compare Figure 5.3b to 5.3d). The highest amount of Te was found in earthworms exposed to CdTe-bulk QDs in all test concentrations (Figure 5.3d). In the nominal 50 mg CdTe kg^{-1} dw test concentration, there were no statistically significant differences between the different CdTe QD variants. The differences in accumulation emerged in the 500 mg CdTe kg^{-1} dw test concentration. The accumulation of Te was similar in the CdTe-PEG and -COOH QD exposures, while it was the highest in the CdTe-NH_4^+ QD exposure at 500 mg CdTe kg^{-1} dw test concentration. In the nominal 2000 mg CdTe kg^{-1} dw test concentration the accumulation of Te was not statistically significantly different between the different CdTe QD ENM exposures, although there was a non-significant trend of higher Te in the CdTe-NH_4^+ groups. Similarly, to the fresh soil experiment the nBAF was calculated for comparison purposes. The values were higher than in the fresh soil study in line with the higher accumulation of Cd and Te (Table 5.5).

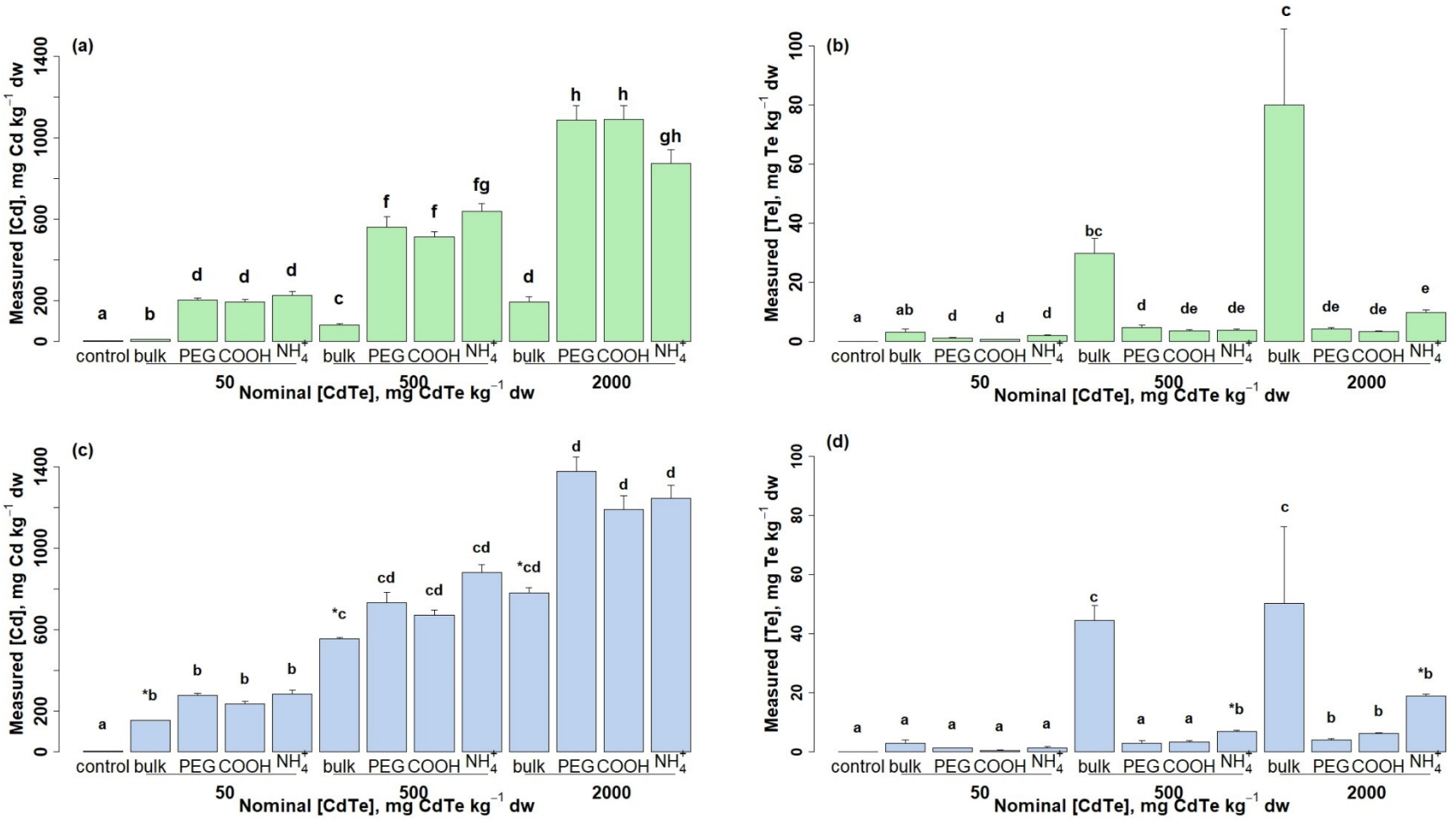

Figure 5.3 (a),(b) Total concentration of Cd as mg Cd kg^{-1} dw and (b), (d) Te in earthworms as mg Te kg^{-1} dw on day 28 in controls and CdTe QD treatment groups from the fresh (top) and aged (bottom) soil experiments. Different letters denote the statistically significant differences (P < 0.05, ANOVA, Tukey HSD). Asterisks denote statistically significant differences between treatments in the fresh and aged exposures (P < 0.05, t-test, unpaired).

Table 5.4. Total concentration ratio of Cd to Te in soil, different extracts and earthworms in the fresh and aged soils.

Treatment	Nominal	The concentration ratio of Cd to Te			
	mg CdTe kg^{-1} dw	Fresh soil		Aged soil	
		Soil	Earthworms	Soil	Earthworms
control	**0**	NA	NA	NA	NA
bulk	**Total**	0.98 ± 0.01	1.7 ± 0.15	1.02 ± 0.01	18.28 ± 11.43
PEG		6 ± 0.2	257.9 ± 14.53	5.6±0.2	314.9 ± 208.58
COOH		6.9 ± 0.2	356.4 ± 35.5	4.98 ± 0.26	280 ± 241
NH_4^+		2.2 ± 0.07	91.6 ± 8.84	2.24 ± 0.37	85 ± 47.11
bulk	**Water extractable**	3.95 ± 0.5			
PEG		16.25 ± 2			
COOH		36.69 ± 5.3			
NH_4^+		20.7 ± 6			
bulk	**0.1 HCl extractable**	12.57 ± 4.9			
PEG		24.44 ± 1.12			
COOH		41.92 ± 3.41			
NH_4^+		10.42 ± 0.9			

Data presented as mean ± SEM (n = 4). Note, water and acid extractions were only carried out with fresh soil samples.

Table 5.5. Total earthworm [Cd] or [Te] to total soil [Cd] or [Te] ratio, or the nano bioaccumulation factor (nBAF).

Treatment	Nominal	Fresh Soil, nBAF		Aged Soil, nBAF	
	mg CdTe kg^{-1}dw	Cd	Te	Cd	Te
control	**0**	6.52 ± 0.85^{a}	0^{a}	5.01 ± 0.56^{a}	0^{a}
bulk	**50**	0.45 ± 0.1^{b}	0.12 ± 0.04^{cd}	9.27 ± 1.09^{abc}	0.10 ± 0.03^{b}
PEG		8.05 ± 1.35^{a}	0.16 ± 0.03^{d}	15.97 ± 1.75^{cd}	0.15 ± 0.04^{b}
COOH		11.77 ± 1.24^{a}	0.08 ± 0.03^{bcd}	10.34 ± 1.88^{bc}	0.05 ± 0.03^{ab}
NH_4^+		7.56 ± 0.4^{a}	0.18 ± 0.03^{d}	19.35 ± 2.33^{d}	0.28 ± 0.06^{ab}
bulk	**500**	0.49 ± 0.03^{e}	0.17 ± 0.03^{d}	3.56 ± 0.35^{aef}	0.29 ± 0.15^{ab}
PEG		3.01 ± 0.34^{b}	0.13 ± 0.01^{d}	5.50 ± 0.23^{ab}	0.07 ± 0.02^{b}
COOH		3.13 ± 0.32^{b}	0.14 ± 0.01^{d}	4.70 ± 0.33^{a}	0.10 ± 0.03^{b}
NH_4^+		2.62 ± 0.1^{bc}	0.04^{bc}	4.39 ± 0.56^{ae}	0.07 ± 0.01^{b}
bulk	**2000**	0.33 ± 0.06^{e}	0.13 ± 0.04^{cd}	1.47 ± 0.15^{g}	0.11 ± 0.03^{b}
PEG		1.46 ± 0.12^{cd}	0.04^{b}	2.55 ± 0.21^{efg}	0.09 ± 0.03^{b}
COOH		1.66 ± 0.07^{bcd}	0.03^{b}	2.43 ± 0.37^{fg}	0.07 ± 0.01^{b}
NH_4^+		1.16 ± 0.17^{d}	0.03^{b}	1.49 ± 0.14^{g}	0.06 ± 0.02^{b}

Data expressed as mean ± SEM (n = 4). Different letters denote statistically significant differences (P < 0.05, ANOVA, Tukey HSD/ Games-Howell).

5.3.4 Survival, biomass and appearance of earthworms

The control animals were healthy and increased in weight (biomass). In the fresh soil experiment, there were no significant changes in the survival in any of the treatments throughout the experiment, except some minor mortalities < 10 %. The biomass of earthworms did not decrease in the CdTe-bulk exposures, but rather, increased by around 20 % (Table 5.6). Furthermore, the biomass was similarly increased in the nominal 50 mg CdTe kg^{-1} dw coated CdTe QD ENM exposures. In the nominal 500 mg CdTe kg^{-1} dw test concentration the biomass was increased in the PEG- and COOH-coated exposures while it was decreased by around 15 % in the NH_4^+ exposures. In the nominal 2000 mg CdTe kg^{-1} dw CdTe QD ENM exposures, the biomass was significantly reduced by almost half when compared to the biomass in the beginning of the experiment (Table 5.6).

There were no behavioural or appearance changes in earthworms exposed to CdTe-bulk and the nominal 50 and 500 mg CdTe kg^{-1} dw CdTe QDs ENMs. However, earthworms from all the CdTe QD ENM exposures at the nominal 2000 mg CdTe kg^{-1} dw test concentration were sluggish and slow to respond and were not actively feeding (evidence of left-over manure), although they were still moving in the soil. Furthermore, morphological changes were evident in earthworms from the 2000 mg CdTe kg^{-1} dw CdTe QD ENM exposures. The earthworms were shorter in length and showed evidence of shedding of the posterior segments (the last segments were clearly lighter coloured with signs of regeneration). A strong negative correlation was found between tissue Cd or Te and biomass when analysing all the data from all treatments together (r_s = 0.6 and 0.5 for Cd and Te respectively, $P < 0.05$, Spearman).

In the aged soil study, the control earthworms were healthy in appearance, however, a non-significant trend of biomass loss was noted. The earthworms survived in all the CdTe-bulk exposures with only minor mortalities < 10 % at the nominal 2000 mg CdTe kg^{-1} dw exposures. In the CdTe-bulk exposures earthworms significantly lost biomass only at the nominal 2000 mg CdTe kg^{-1} dw test concentration (Table 5.6). In the different ENM exposures, the CdTe-NH_4^+ QDs were toxic to earthworms at the 2000 mg CdTe kg^{-1} dw test concentration where survival was reduced to 60 ± 25 % (mean ± SEM, n = 4, Table 5.6). In addition, earthworms from this treatment showed statistically significant weight loss. In earthworms from other CdTe QD ENM treatments, survival was not significantly affected, however, biomass was reduced in the nominal 500 and 2000 mg CdTe kg^{-1} dw test concentrations (Table 5.6).

There were no obvious behavioural or appearance changes in the CdTe-bulk exposures or the nominal 50 mg CdTe kg^{-1} dw CdTe QD ENM exposures. Some earthworms appeared lethargic and non-responsive in the 500 and 2000 mg CdTe kg^{-1} dw CdTe QD ENM exposures and similarly to the fresh soil study, there was evidence of shedding of the posterior segments and lack of feeding (presence of surplus feed).

Table 5.6 Survival and biomass of earthworms following 14 and 28 days exposure to CdTe QDs in fresh and aged soils (only 28 days for aged soil experiment).

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

Survival of earthworms shown as the total number of earthworms (total) and percent survival (%) and biomass of earthworms shown as the total biomass of survived earthworms (total) (wet weight, mg) and percent weight increase/decrease relative to day 0 (%). Data are shown for

each treatment for days 14 and 28 in fresh soil and day 28 in aged soil experiments. Data are presented as mean ± SEM (n = 4). Different letters denote statistically significant difference between the treatments on the day ($P < 0.05$ repeated measures ANOVA for biomass data or Kruskal-Wallis, Dunn's test for survival data).

Day 0 mean wet weight was 1.86 ± 0.02 g (mean ± SEM, for a subsample of 5 of the initial earthworms, n = 52 treatments) in fresh soil experiment. Day 0 mean wet weight of 1.89 ± 0.03 g of per exposure replicate (mean ± SEM, for a subsample of 5 of the initial earthworms, *n* = 52 treatments).

↑ Weight increase relative to day 0; ↓Weight decrease relative to day 0.

5.3.5 Reproduction

Reproduction is one of the most sensitive endpoints in earthworm studies. In the experiment with fresh and aged soil, the control earthworms produced a healthy number of juveniles per each earthworm with a coefficient of variation (CV %) in the controls < 30 % (Table 5.7; OECD, 2004). Reproduction was not significantly reduced in any of the CdTe-bulk test concentrations or in the nominal 50 mg CdTe kg^{-1} dw CdTe QD ENM exposures. While it was significantly reduced in the CdTe QD ENM treatments at the nominal 500 mg CdTe kg^{-1} dw test concentration and completely inhibited at 2000 mg CdTe kg^{-1} dw test concentration in the CdTe QD ENM exposures. The 50 % effect concentration (EC_{50}) for reproductive success for the different materials is presented in Table 5.7. The estimated EC_{50} values were calculated for the nominal CdTe QD concentrations (curves presented in Figure 5.4). The EC_{50} values decrease in the following order: CdTe-COOH < -NH_4^+ < -PEG < -bulk QDs. In the latter treatment, there was no complete inhibition of reproduction, thus a value was not estimated. The fresh wet weight of the juveniles was measured, once they were collected washed and dried, from each treatment (Table 5.8) and it differed between exposures. The differences followed the same order as the total number of juveniles produced, although, the CdTe-bulk exposed juveniles were significantly smaller in size than the control earthworms and other CdTe-bulk test concentrations.

In the aged soil experiment, the controls produced more juveniles than in the fresh soil experiment. In the CdTe QD exposures there were more pronounced effects on reproductive success. Reproduction was reduced in a concentration dependent manner in the CdTe-bulk exposures with a complete inhibition of reproduction at the

nominal 2000 mg CdTe kg^{-1} dw test concentration. The number of juveniles produced was significantly reduced or completely inhibited in all the different CdTe QD ENM exposures at 500 and 2000 mg CdTe kg^{-1} dw test concentrations (Table 5.7). The EC_{50} values for reproductive success decreased in the following order CdTe-NH_4^+ < -COOH < -PEG < -bulk, however, the reliability of this order is low, due to three data-points and high effect values (Table 5.7, Figure 5.4).

Table 5.7 Total number of juveniles produced and the EC_{50} values in each treatment in the fresh and aged soil experiments.

Nominal $[CdTe]_{soil}$	**Control**	**CdTe-Bulk**	**CdTe-PEG**	**CdTe-COOH**	**CdTe-NH_4^+**
mg CdTe kg^{-1} dw	**Total number of juveniles / EC_{50} (95 % confidence intervals)**				
			Fresh Soil		
50	25 ± 5[a]	22 ± 6[a]	18 ± 1[a]	18.25 ± 4[a]	20 ± 3[a]
500		18 ± 2[a]	3 ± 2[b]	3 ± 1[b]	1 ± 0[b]
2000		19 ± 3[a]	0[b]	0[b]	0[b]
EC_{50}		NA	108 (72-165)	65 (30-142)	96 (20-610)
			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)

Data presented as mean ± SEM (n = 4). Different letters denote statistically significant differences between treatments ($P < 0.05$, ANOVA, Tukey HSD). EC_{50} values for 50 % reduction in reproduction after 4 weeks of exposure in fresh and aged soils with CdTe QDs are presented with 95 % confidence intervals in the brackets. NA – not possible to calculate EC_{50} due to no effect; not possible to calculate confidence intervals due to high effect

Table 5.8 Total fresh (wet) weight of the earthworm juveniles (mg) in the fresh soil experiment.

Nominal $[CdTe]_{soil}$		**Control**	**CdTe-Bulk**	**CdTe-PEG**	**CdTe-COOH**	**CdTe-NH_4^+**
mg CdTe kg^{-1} dw		**Fresh (wet) weight, mg**				
50	Total	370 ± 58[a]	222 ± 90[a]	267 ± 57.6[a]	220 ± 80[a]	307 ± 84.3[a]
	Individual	13.3 ± 2.1[a]	10.6 ± 1.3[a]	15.15 ± 1.2[a]	12.64 ± 2.26[a]	17.8 ± 5.48[a]
500	Total		250 ± 61[a]	33.6 ± 26.2[c]	5.01 ± 3.35[c]	9 ± 3[c]
	Individual		14.6 ± 2.4[a]	15.1 ± 8.2[ab]	3.24 ± 1.28[b]	6[b]
2000	Total		142 ± 24[b]	ND	ND	ND
	Individual		8.41 ± 1.9[b]			

Data is presented as total weight of all the juveniles (Total) and estimated individual wet weight (Individual). Data presented as mean ± SEM, n = 4). Different letters denote statistically significant differences between treatments ($P < 0.05$, ANOVA, Tukey HSD).

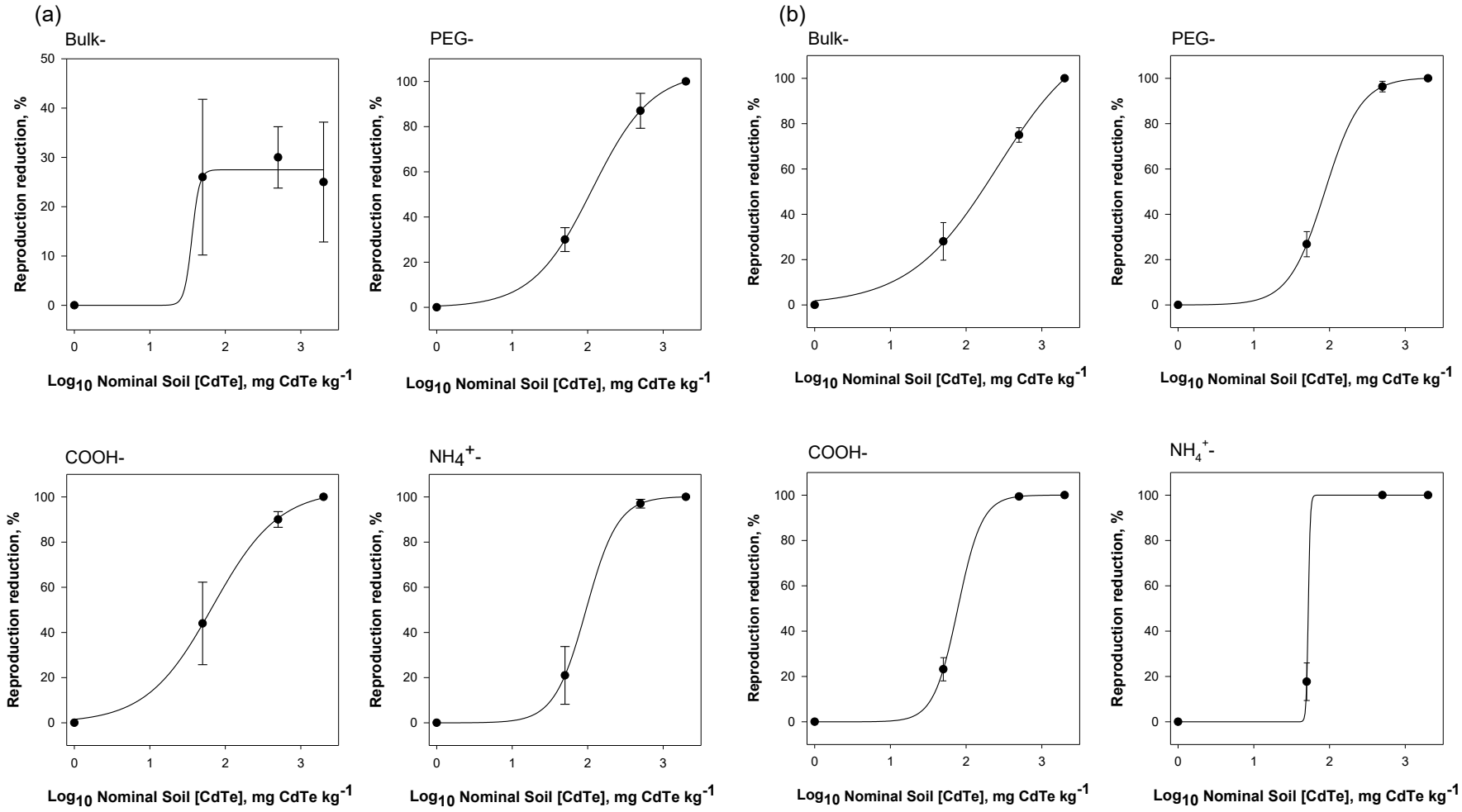

Figure 5.4 The reproduction curves in the (a) fresh and (b) aged soils. The Log_{10} transformed concentrations 1.7, 2.6 and 3.3 equate to nominal concentrations 50, 500 and 2000 mg CdTe QD kg^{-1}dw, respectively. The sigmoid curves were fitted to raw data ($r^2 > 0.8$ for curve fits) while the mean ± SEM (n = 4) is shown. Statistical differences can be seen in the raw data Table 5.7.

5.3.6 Sodium pump activity and tissue elemental composition

In the experiment with the fresh soil the control animals showed a normal Na^+/K^+-ATPase (sodium pump) activity as seen in previous work (Figure 3.5a, Chapter 3). Due to the repeated freezing and thawing of the aged soil experiment samples, the results were considered unreliable due to loss of enzyme activity (discussed above, section 5.2.4.1) and thus not shown. In the fresh soil experiment, there was an overall trend of lower sodium pump activity in all test concentrations of the CdTe-bulk exposures, with significantly reduced activities in the nominal 500 and 2000 mg CdTe kg^{-1} dw test concentration (Figure 5.5a). The non-significant lower trend of sodium pump activity was also evident in the CdTe-PEG exposures, although, the reduction was only significant in the nominal 500 mg CdTe kg^{-1} dw test concentration. In the CdTe-NH_4^+ exposures there was an overall lower trend of sodium pump activity, but the activity was significantly reduced only in the nominal 2000 mg CdTe kg^{-1} dw test concentration. In the CdTe-COOH exposures the sodium pump activity did not show an obvious trend different to the control earthworms (Figure 5.5a). There was a weak correlation between the sodium pump activity and the concentration of Te in earthworm tissues when analysing all the data together (r_s = -0.3, $P < 0.05$, Spearman). There was no clear effect on the concentration of Na^+ in earthworm tissues, however, the concentration of K^+ was significantly lower in the nominal 500 and 2000 mg CdTe kg^{-1} dw CdTe-NH_4^+ exposures. Correlation analysis also revealed that lower tissue K^+ concentration was related to water and acid extractable Cd and Te in soil (r_s = -0.6 for both, $P < 0.05$, Spearman) in soil rather than tissue concentration of either of the metals. In the aged soil study, there was no depletion or significant increase in the concentration of Na^+ and K^+ between the test concentrations and the control (Table 5.9).

In the fresh and aged soil study, other essential and trace metals that are regulated were measured, and remained in the expected range for a healthy earthworm population (Table 5.9). The earthworms in the fresh soil study showed significant treatment dependent changes (Table 5.9). There was no effect in the CdTe-bulk exposures at different test concentrations. Differences in the metal concentrations were evident in the differently coated CdTe QD ENM exposures at 500 and 2000 mg CdTe kg^{-1} dw test concentrations. The concentration of essential metals Ca, Mg and Fe were significantly reduced in the nominal 500 and 2000 mg CdTe kg^{-1} dw CdTe-NH_4^+ exposures. There was a significant negative correlation between the tissue concentration of Cd and the influenced metals (r_s = -0.3, -0.4 and -0.3 for Ca, Mg and Fe respectively, $P < 0.05$, Spearman). Earthworm tissue copper concentrations were significantly reduced in nominal 500 mg CdTe kg^{-1} dw CdTe-COOH and NH_4^+ and nominal 2000 mg CdTe kg^{-1} dw CdTe-COOH exposures. Manganese (Mn) was significantly depleted in earthworm tissues from the nominal 500 mg CdTe kg^{-1} dw CdTe-NH_4^+ and nominal 2000 mg CdTe kg^{-1} dw CdTe-PEG, COOH and NH_4^+ exposures. A significant strong negative correlation was found between the tissue Cd and Cu or Mn (r_s = -0.6 for both, $P < 0.05$, Spearman).

In the experiment with the aged soil, there were no significant effects on the essential metals, however trace metals, Cu and Mn, were affected. The concentration of Cu was significantly reduced in the nominal 50 and 500 mg CdTe kg^{-1} dw CdTe-COOH and NH_4^+ and in all the 2000 mg CdTe kg^{-1} dw exposures. The concentrations of Mn were significantly reduced only in the nominal 2000 mg CdTe kg^{-1} dw CdTe-COOH and NH_4^+ exposures. There was a significantly strong negative correlation between the tissue concentration of Cd and Cu or Mn (r_s = -0.5 for both, $P < 0.05$, Spearman).

5.3.7 Total glutathione as an oxidative stress marker

Total glutathione (GSH) concentrations were measured as oxidative stress markers in earthworms. Earthworms from the fresh soil experiment sustained a normal total glutathione concentration (compare concentrations in Chapter 3 and this chapter). In the CdTe-bulk exposures there was no significant effect on the total GSH concentration. Also, there was no significant effect in the differently-coated CdTe QD exposures when compared to the controls ($P > 0.05$, ANOVA, Tukey HSD). There was a non-significant trend of lower total glutathione content in earthworms exposed to the nominal 50 mg CdTe kg^{-1} dw CdTe-COOH and NH_4^+ QDs. Furthermore, there was a non-significant increase in the total glutathione content in earthworms exposed to the nominal 2000 mg CdTe kg^{-1} dw CdTe-COOH QDs. Total glutathione was significantly correlated to earthworm tissue Cd concentration ($r_s = 0.4$, $P < 0.05$, Spearman). The results of the aged soil study were not considered reliable thus not presented.

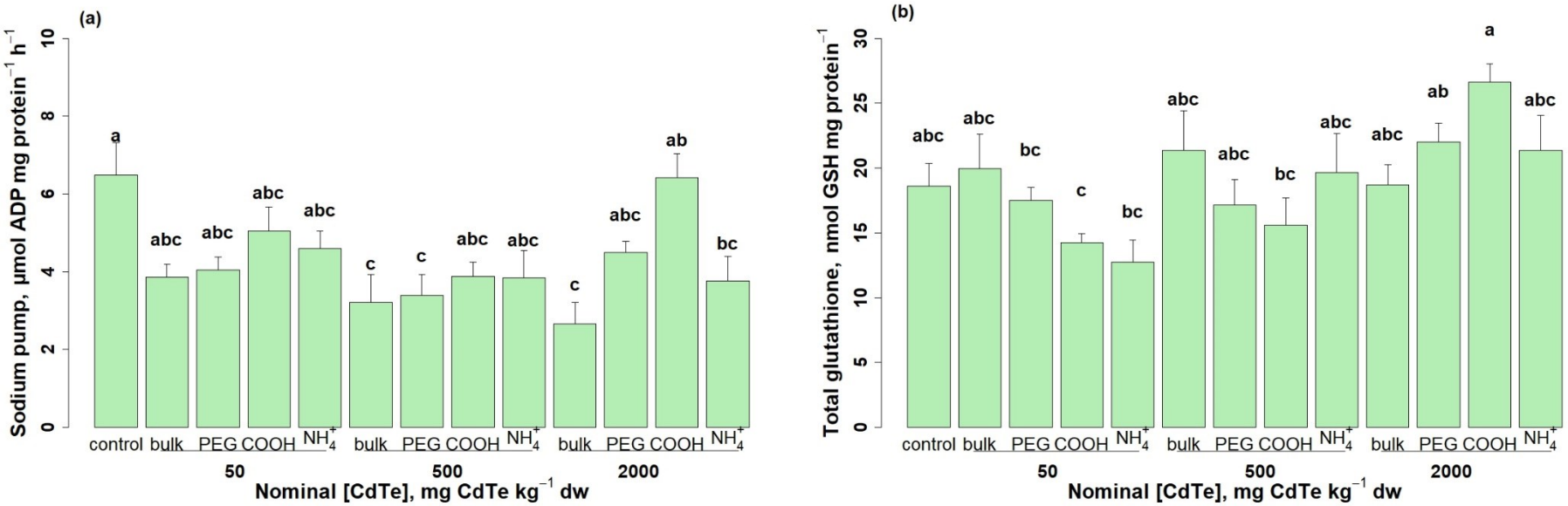

Figure 5.5 (a) Na^+/K^+-ATPase activity (sodium pump) expressed as ADP µmol released per mg protein per hour in earthworms in the fresh soil experiment. (b) Total glutathione nmol per mg protein in the earthworms in the fresh soil experiment. Data is presented as mean ± SEM (n = 8). Different letters denote statistically significant differences between treatments irrespective of test concentrations ($P < 0.05$, ANOVA, Tukey HSD).

Table 5.9 Total concentration of electrolytes in CdTe QD or CdTe-bulk QD exposed earthworms in the fresh and aged soil experiments.

Soil [CdTe]	Time		Measured concentration, mg kg^{-1}dw				
Nominal, mg Cu kg^{-1} dw	Day	Electrolytes	Control	Bulk	PEG	COOH	NH_4^+
				Fresh Soil			
50	28	Na	5001 ± 289^{bcd}	5077 ± 264^{abcd}	5130 256^{abcd}	4932 ± 218^{cd}	4334 ± 314^{cd}
		K	8166 ± 386^{ab}	8060 ± 491^{abc}	8434 ± 482^{abcd}	7815 ± 350^{ab}	7115 554^{abcd}
		Ca	4020 ± 327^{a}	3839 ± 220^{a}	4266 ± 351^{a}	4215 ± 150^{a}	3689 ± 287^{ab}
		Mg	751 ± 35^{ab}	737 ± 30^{ab}	741 ± 38^{ab}	718 ± 33^{ab}	672 ± 41^{abc}
		Fe	554 ± 85^{a}	624 ± 80^{a}	329 ± 47^{ab}	468 ± 85^{ab}	662 ± 113^{a}
		Mn	43 ± 6^{ab}	49 ± 7^{ab}	27 ± 5^{bc}	34 ± 4^{abc}	52 ± 5^{ab}
		Zn	98 ± 5^{bcd}	98 ± 4^{bcd}	104 ± 5^{bcd}	102 ± 6^{bcd}	95 ± 6^{bcd}
		Cu	10 ± 0^{ab}	11 ± 0^{a}	8 ± 0^{abc}	8 ± 0^{bc}	8 ± 0^{bc}
500	28	Na		5628 ± 342^{abcd}	5211 ± 362^{abcd}	4129 ± 207^{d}	3503 ± 432^{cd}
		K		8296 ± 419^{ab}	8495 ± 451^{ab}	6632 ± 259^{bcd}	5990 ± 494^{cd}
		Ca		4262 ± 244^{a}	4542 ± 267^{a}	3753 ± 308^{ab}	3328 ± 325a^{b}
		Mg		789 ± 42^{a}	754 ± 38^{ab}	593 ± 34^{bcd}	485 ± 32^{d}
		Fe		592 ± 68^{a}	652 ± 120^{a}	485 ± 73^{ab}	246 ± 24^{b}
		Mn		56 ± 6^{a}	45 ± 6^{ab}	37 ± 5^{abc}	13 ± 3^{c}

		Zn		111 ± 5ab^{c}	108 ± 3^{bc}	83 ± 3^{d}	80 ± 5^{cd}
		Cu		11 ± 0^{ab}	9 ± 0a^{bc}	5 ± 0^{c}	6 ± 0^{c}
2000	**28**	**Na**		5416 ± 207^{abc}	7533 ± 479^{a}	6640 ± 335^{ab}	4630 ± 691^{abcd}
		K		8648 ± 193^{ab}	8909 ± 547^{a}	8757 ± 196^{ab}	5806 ± 715^{d}
		Ca		4276 ± 168^{a}	4602 ± 384^{a}	4026 ± 264^{a}	2388 ± 349^{b}
		Mg		806 ± 20^{a}	762 ± 36^{ab}	747 ± 32^{ab}	529 ± 56^{cd}
		Fe		572 ± 97^{a}	653 ± 123^{a}	586 ± 60^{a}	374 ± 63^{ab}
		Mn		44 ± 5^{ab}	14 ± 2^{c}	15 ± 3^{c}	14 ± 4^{c}
		Zn		108 ± 2^{b}	130 ± 5^{ab}	135 ± 3^{a}	91 ± 11^{abcd}
		Cu		10 ± 0^{ab}	8 ± 0^{abc}	6 ± 0^{c}	9 ± 1^{abc}
				Aged soil			
50	**28**	**Na**	6176 ± 435	7281 ± 297	7344 ± 413	6227 ± 776	5569 ± 556
		K	8646 ± 527^{ab}	10937 ± 719^{a}	9445 ± 185^{ab}	9889 ± 692^{ab}	8523 ± 791^{ab}
		Ca	3413 ± 203^{abc}	4751 ± 244^{ab}	5105 ± 447^{a}	4238 ± 203^{bcd}	3801 ± 455^{bcd}
		Mg	915 ± 61^{ab}	1153 65^{a}	1141 44^{a}	961 49^{ab}	887 89^{ab}
		Fe	432 ± 54	440 ± 78	672 ± 76	565 ± 104	463 ± 78
		Mn	54 ± 8^{ab}	65 ± 8^{a}	68 ± 9^{a}	59 ± 8^{ab}	50 ± 4^{ab}
		Zn	114 ± 7	144 ± 5	143 ± 3	124 ± 10	124 ± 13
		Cu	10 ± 1^{a}	9 ± 0^{a}	10 ± 0^{a}	4 ± 0b^{cd}	4 ± 0^{bcd}

500	**28**	**Na**	6463 ± 283	8127 ± 531	5880 ± 371	6290 ± 639
		K	8788 ± 379^{ab}	9817 ± 316^{ab}	9639 ± 600^{ab}	10593 ± 1113^{ab}
		Ca	4436 ± 218^{abc}	4563 ± 460^{ab}	4197 ± 337^{abc}	3999 ± 443^{abc}
		Mg	969 ± 31^{ab}	1028 ± 50^{ab}	921 ± 56^{ab}	985 ± 107^{ab}
		Fe	526 ± 92	359 ± 36	540 ± 67	579 ± 85
		Mn	55 ± 7^{ab}	43 ± 5^{ab}	53 ± 10^{ab}	45 ± 11^{ab}
		Zn	126 ± 4	129 ± 5	139 ± 10	144 ± 13
		Cu	7 ± 0^{ab}	6 ± 0^{abc}	3 ± 0^{cd}	4 ± 0^{bcd}
2000	**28**	**Na**	6053 ± 668	7591 ± 645	6694 ± 747	6969 ± 470
		K	7570 ± 705^{b}	8107 ± 646^{ab}	9381 ± 713^{ab}	9944 ± 457^{ab}
		Ca	3249 ± 217^{bc}	3378 ± 551^{abc}	3130 ± 316^{bc}	2632 ± 263^{c}
		Mg	741 ± 66^{b}	836 ± 69^{ab}	844 ± 60^{b}	845 ± 37^{b}
		Fe	437 ± 34	558 ± 64	533 ± 64	578 ± 42
		Mn	24 ± 5^{b}	28 ± 8^{ab}	19 ± 4^{c}	18 ± 3^{c}
		Zn	109 ± 7	135 ± 9	137 ± 6	141 ± 3
		Cu	4 ± 0^{bc}	5 ± 1^{abcd}	2 ± 0^{d}	3 ± 0^{bcd}

Data expressed as mean ± SEM (n = 8) mg element kg^{-1} dw and is rounded to the nearest mg. Different letters denote statistically significant differences for each element ($P < 0.05$, ANOVA, Tukey HSD/ Games-Howell).

5.4 Discussion

This is the first study to investigate the effects of differently coated CdTe QD ENMs on earthworms, compared against a micron size CdTe-bulk material. Furthermore, these effects were assessed after 6 months of ageing the soils. In the fresh soil experiment, none of the CdTe QDs (ENMs and bulk) were toxic, but exposure to CdTe QD ENMs caused significant reduction in juvenile production and disturbances to essential metal concentrations in earthworm tissues. There was evidence of a coating specific effect, potentially related to release of dissolved Cd and Te, or their oxidised states. In the aged soil experiment, the CdTe-NH_4^+ QDs were toxic. Both, CdTe-bulk and CdTe QD ENMs induced more pronounced effects on juvenile production in the aged soil experiment, but still, the CdTe QD ENMs remained more toxic than CdTe-bulk QDs.

5.4.1 Soil pH

Soil pH is known to impact nanomaterial availability and mobility in soil (Heggelund et al., 2014). The results of this study (Table 5.3) suggest the addition of the either CdTe-bulk QDs or CdTe QD ENMs does not change soil pH.

5.4.2 Total and extractable Cd and Te fractions in soil in relation to Cd and Te accumulation in earthworms

Although it was not possible to measure the particle number concentration of CdTe QD particles in the soil, the exposures were confirmed by measuring the total concentration of Cd and Te. The background concentration of Cd in Lufa 2.2 soils

was consistent with previous reports, albeit, slightly higher (0.6 ± 0.2 mg Cd kg^{-1} dw this study, 0.38 mg Cd kg^{-1} dw in Keshavarz Jamshidian et al., 2017). Less than 10 % of the total Cd was water and acid extractable (Figure 5.2), indicating that the background Cd in control soil was not accessible. As expected, no Te was detected in the control soils since it is a rare metalloid with very low background concentrations in unpolluted areas (values < 5 µg kg^{-1} dw as reported in the review by Belzile et al., 2015). The background concentration of Cd in earthworms was also in agreement with previous studies using the same soils (4.23 ± 0.34 mg Cd kg^{-1} dw this study; 5.6 mg Cd kg^{-1}, Gonzalez et al., 2013) while Te concentration remained below the detection limit.

Soils treated with CdTe QDs had elevated Cd and Te concentrations. The concentration ratio of Cd and Te based on the measured values was approximately 1 in the CdTe-bulk treated soils. This indicates equal amounts of Cd and Te in the quantum dots(Table 5.4). There was a lot more Cd in the QD ENMsthan Te. The variation in the amounts is likely due to the differences in the coating stoichiometry, i.e. the different coatings are of different mass and size (Table 5.1). This was very similar in the CuO ENMs that had the same coatings PEG, COOH and NH_4^+ (discussed in Chapter 3).

The water and acid extractions of the soils revealed less than 1 % of extractable Cd and Te in the CdTe-bulk exposures, suggesting the very low accessibility of dissolved Cd and Te and / or intact CdTe QDs of a size < 0.22 µm. Despite the low accessibility of Cd and Te in the soils, as well as known low dissolution (Vassallo, 2017, personal communication), earthworms accumulated Cd and Te in all test concentrations in the CdTe-bulk exposures. The concentration ratio of Cd and Te

was around 1.7 which indicates some accumulation of intact CdTe-bulk quantum dots as it was closer to the total Cd and Te ratio in the soil (Table 5.4). Although this was not confirmed by any further tissue analysis in this case, some accumulation and retention of intact CdTe QD ENMs has been seen in the alimentary system of the nematode *C. elegans* (5-6 nm, Qu et al., 2011) and in insects (moths, leafrollers) fed differently capped CdSe QD ENMs as larvae (6.5 nm, Al-Salim et al., 2011).

There was an evident coating effect on the extractability of Cd and Te in the CdTe QD ENM exposures (i.e., more Cd was extractable from NH_4^+ coated QDs Figure 5.1a, b and 5.2a, b), though this coating effect was not reflected in accumulation of Cd and Te in earthworms (Figure 5.3a, b). In addition, the concentration ratio of Cd to Te in earthworms was clearly higher than in soil (Table 5.4) supporting the notion of very high Cd accumulation in earthworms (Hopkin, 1989). This finding further indicates that CdTe QD ENMs likely degraded in soil (also suggested in Navarro et al., 2013) and released dissolved Cd which was accumulated by earthworms. Similar findings have been found in earthworms exposed to CdSe QDs (QDs 9-20 nm, Stewart et al., 2013), where the Cd to Se ratio was higher in earthworms than in the freshly prepared particles added to the soil. Similar extent of cadmium accumulation (Figure 5.3a) has been seen in earthworms exposed to dissolved Cd (e.g ~1000 mg Cd kg^{-1} dw in Spurgeon et al., 2005).

Thus far, no studies have measured Te accumulation in earthworms. Clearly, Te was not accumulated as strongly as Cd (Figure 5.3a *vs* 5.3b) and there was also no clear dose-dependent increase. This may suggest that either earthworms are effectively excreting Te, or potentially Te was not as accessible to earthworms as Cd was. In contrast, it has been shown that in the presence of dissolved Cd, Te is also accumulated in the earthworm tissue (Stürzenbaum et al., 2013) to form CdTe QDs.

Although it is likely that the Cd or Te concentration had not reached steady state in the earthworms, the nano-BAF was worked out to aid the interpretation of the results. The trigger value for bioaccumulation potential in environmental risk assessment is 1, when BAF > 1, the metal is considered bioaccumulative, however, if the value is < 1, it is not. Based on the results, neither Cd nor Te from the CdTe-bulk QDs is bioaccumulative. In addition, the nBAF values for Cd and Te from the bulk materials did not follow the traditional pattern of decreasing BAF with increasing soil metal concentration (Spurgeon and Hopkin, 1996). This observation may further suggest the accumulation of intact particles. In line with studies with dissolved Cd accumulation in earthworms, the results show a similar decrease in the bioaccumulation with increasing Cd concentration in soil in the CdTe QD ENM exposures (Cd BAF ~ 2.6 in Reinecke et al., 1999). These results support that Cd from CdTe QDs is bioaccumulative in earthworms, which may be due to dissolved Cd. In contrast, Te was not bioaccumulative (Table 5.3). It is possible Te was in a much less available form.

In the aged soil study, the total concentration of Cd and Te in soil was lower, which is likely due to uptake by earthworms in the fresh soil study, potentially plants that were growing in the exposure containers, where relevant (see Chapter 6 for details), and potentially adhesion to the exposure containers. The reduction in Cd in the soils was much more pronounced than the reduction in Te, which is reflected also in lower concentration ratio of Cd and Te in soils (Table 5.4).

The accumulation of Cd and Te by earthworms was overall higher in the aged soil experiment. In the increased Cd to Te ratio in the CdTe-bulk exposures indicates that over time The CdTe-bulk materials degrade and potentially release more Cd and thus increasing its bioavailability. While it is likely Te remained in the more

inaccessible state (Te accumulation was reduced, albeit non-significantly). In the CdTe QD ENM exposures, accumulation of Cd was similar to the fresh soil study, although slightly higher (Figure 5.3c). While a coating effect was evident in Te accumulation, which might suggest CdTe-NH_4^+ QD degradation over time that led to higher Te bioavailability to earthworms.

The higher nBAF values (Table 5.3) highlights continued Cd accumulation in earthworms, even after a period of ageing the soil, suggesting that Cd from both, CdTe QD ENMs and CdTe-bulk QDs is becoming more bioavailable (although, it is likely it will not be in a bioavailable form in the earthworms, as discussed earlier).

5.4.3 Survival, weight and appearance

Control earthworms survived in both experiments. They did not significantly decrease in weight or show any abnormalities in appearance indicating a healthy population of earthworms were used in the fresh and aged soil experiments. CdTe-bulk or coated CdTe QD ENMs were not acutely toxic. This was expected as earthworms are known to tolerate very high concentrations of dissolved Cd (28 day mortality LC_{50} = 588 (525 – 958) mg Cd kg^{-1} dw, van Gestel et al., 1991), since has been shown to be bound by metallothionein (wMT-2) which reduces its bioreactivity and thus toxicity in the organism (Stürzenbaum et al., 2001). Furthermore, the extractable Cd from the soil was far less than the expected lethal concentration in most of the QD exposures, except in the nominal 2000 mg CdTe kg^{-1} dw CdTe-NH_4^+ QD exposures.

Critical body residues (CBR) have been developed as bioindicators and can be used to determine the threshold after which adverse toxic effects occur in an organism (van Wensem et al., 1994; Ma, 2005). In earthworms, a CBR of around 642 mg Cd kg^{-1} (dw) would result in 50 % mortality (Conder et al., 2000). This was generally exceeded in earthworms exposed to the highest concentrations of CdTe QDs (Figure 5.3), while all earthworms survived. The earthworms from those treatments significantly lost biomass which was supported by the lack of feeding coupled with potential shedding of the posterior segments. The latter has been interpreted as a survival and an excretion mechanism in earthworms (Sims and Gerald, 1987). It is possible the earthworms in the CdTe QD exposures were on the borderline of survival, which was due malnutrition and high tissue Cd and Te concentration (supported by the negative correlation between the metals and biomass). There are currently no studies on CdTe QD toxicity in earthworms, while studies on other organisms have found the toxicity is related to the release of the metallic/metalloid components, although some QD specific effects have been seen (reviewed in Lopes Rocha et al., 2017).

In the aged soil experiment, like in the fresh soil study, survival was not affected in the CdTe-bulk exposures. However, the aged CdTe-bulk nominal 2000 mg CdTe kg^{-1} dw test concentration induced a reduction in biomass which indicates higher toxicity after a period of 6 months and is related to higher tissue Cd concentrations (discussed above). Out of the CdTe QD ENMs tested, a coating effect on survival was apparent, since, the NH_4^+ coated CdTe QDs were acutely toxic. The effects on appearance and feeding were also evident at lower concentrations, indicating the aged CdTe QDs had a greater effect on the earthworms' health. The shedding of the

posterior segments was likely an excretion mechanism due to increased inorganic waste in the earthworm tissues as Cd-wMT2 complexes (Stürzenbaum et al., 2001).

5.4.4 Reproduction

The control earthworms produced the expected number of juveniles in both, fresh and aged soil experiments, meeting the validity criteria of the OECD TG 222 (3 juveniles per earthworm, OECD, 2004). In the fresh soil experiment, the number of juveniles produced was not affected in the CdTe-bulk exposures. A coating specific effect was evident on reproduction (Table 5.7). If the total concentration of Cd in the exposure soils is considered, the results are comparable to the 56 day $^{reproduction}EC_{50}$ = 46.3 mg Cd kg^{-1} dw reported in Spurgeon et al. (1994), which suggests the reproductive toxicity of the CdTe QDs in the fresh soil study is potentially related to dissolved Cd accumulation. As discussed in the previous section the earthworms were clearly under stress shown by reduction in biomass and lack of feeding, thus they were not investing energy in reproducing but rather surviving. The differences in juvenile biomass followed the same pattern as overall effects on reproduction (Table 5.7 *vs* Table 5.8). Although in the CdTe-bulk exposures the number of juveniles was similar to the controls, the weight of the juveniles was significantly lower. This may be related to the growth rate of the juveniles or the fact that they emerged from the cocoons later, nonetheless, an effect is evident.

In the aged soil experiment, the control earthworms produced more juveniles than in the fresh soil study highlighting the variations in a population of earthworms. Also, this may have been due to the likely higher organic matter (plant roots) in the soil that encouraged earthworms to reproduce more offspring (Lahive et al., 2017). The

CdTe-bulk QDs were more toxic in the aged soil study compared to the fresh, potentially due to higher Cd availability (Figure 5.3c). Furthermore, the reproductive toxicity of CdTe QD ENMs also increased. The further reduction in reproduction may be explained by the higher tissue Cd (and Te) concentrations coupled with the acute effects on earthworm biomass.

5.4.5 Effects on ion regulation

Cadmium is known to cause ion regulatory disturbances in organisms (Stürzenbaum et al., 2004), therefore, the sodium pump activity was assessed. In the fresh soil study, earthworms in the controls sustained an expected sodium pump activity, as well as an overall concentration of Na^+ and K^+, expected in the earthworms (see Chapter 3). The sodium pump activity was overall lowest in the CdTe-bulk exposures (Figure 5.5a), this is a new finding in earthworms, and no studies exist in the published literature assessing the CdTe QD effects on such a vital ion-regulatory mechanism. There were no clear trends in CdTe QD exposures, while the CdTe-PEG and -NH_4^+ QDs were most potent in inhibiting the sodium pump activity.

An increase in tissue Te concentration was found to be related to the lower sodium pump activity, although the relationship was not strong, this may offer some insight into the mechanisms of Te toxicity. In general, not much is known about Te or the effects of its compounds on the ion regulation in organisms. But (organo)chalcogens, such as butyl(styryl)telluride, have been found to inhibit the sodium pump activity in human blood cells *in vitro* in a concentration dependent matter (Santos et al., 2009). Tellurite has also been found to inhibit saponin-sensitive ATPases in human erythrocytes (Mircevova and Kodicek, 1991). Tellurite is usually a precursor in CdTe

synthesis, thus, it may be that degradation of the CdTe QDs leaded to release of tellurium which resulted in the formation of tellurite in the soils / earthworm tissue. In the nominal 500 and 2000 mg CdTe kg^{-1} dw CdTe NH_4^+ exposures tissue K^+ concentrations were significantly reduced. This was unrelated to high tissue total Cd concentration, and thus might be explained by Te inhibition of the sodium pump (therefore resulting in limited K^+ movement into the cells). Furthermore, the CdTe NH_4^+ have shown the most potential to release Cd and Te from the QDs (Table 5.1 supplier's information on dissolution). This observation might further support the interaction of Te with ion regulation. Another hypothesis may be that an overall damage to the earthworm cells was caused in the CdTe-NH_4^+ exposures (500 and 2000 mg CdTe kg^{-1} dw), resulting in a passive non-specific electrolyte leak since other essential elements such as Ca and Mg concentrations were significantly reduced compared to the controls and other CdTe QD exposures (Table 5.4).

The two essential trace metals for earthworm health, copper and manganese, were also measured, and control earthworms showed the expected tissue Cu (see Chapter 3-4 for references) and Mn concentrations (43-54 mg Mn kg^{-1} dw this study; ~ 50 mg Mn kg^{-1} dw in *L. rubellus*, Oste et al., 2001). Manganese concentration was reduced by almost 3 times in the CdTe QD ENM exposures. Copper depletion was related to higher tissue Cd, although it was only significantly depleted in the CdTe-COOH exposures (500 and 2000 mg CdTe kg^{-1} dw) and CdTe-NH_4^+ exposure (500 mg CdTe kg^{-1} dw). Cu is a regulated metal in earthworms (Chapter 3-4 discussions) and as such any Cd^{2+} that may have not been bound to the metallothionein isoform (wMT2) may be competing with Cu in earthworm tissue. Manganese depletion was the most prominent in the CdTe QD ENM exposures, where the tissue Cd exceeded 600 mg Cd kg^{-1}, this might potentially due to the excess Cd in earthworm tissues

competing with Mn (similar observation in plant cells, Topperwien et al., 2007; Robinson et al., 2006).

In the aged soil study, the sodium pump activity results were not considered reliable due to the repeated freezing and thawing of the samples. The concentration of essential metals in earthworm tissues was not influenced in the aged soil study, despite the higher tissue concentrations of Cd and Te. However, trace metals such as Cu and Mn were influenced similarly to the fresh soil study with the same coated QDs, COOH and NH_4^+, being most potent (Table 5.5). The reasons for the trace metal depletion are likely due to competition with increased tissue concentration of Cd^{2+} (discussed above).

5.4.6 Absence of oxidative stress

The control earthworms in the fresh soil study had a normal total glutathione content at the end of the 28-day exposure (see Chapter 3, Fig 3.4). There were no clear exposure dependent changes to the total glutathione pool in the exposed earthworms. Cadmium sulphide (CdS) QDs have been shown to elevate total GSH concentrations in fish (Sanders et al., 2008) and other oxidative stress markers have been either increased or decreased in mussels (Lopes Rocha et al., 2015). The lack of more clear changes to the total glutathione pool may have been that either, there was no significant oxidative stress caused in the earthworms, or, that different antioxidants were utilised. Saez et al., (2015) exposed *E. fetida* and *H. diversicolor* to CdSe/ZnS QDs (5 nm, or aggregated size at 100 nm) by injection to the coelom. Following this the coelomocytes were harvested and authors found no significant

effect on SOD or TBARS in the *E. fetida* cells, while SOD activity was increased in *H. diversicolor* cells (Saez et al., 2015).

5.4.7 Conclusions

Taken together, the results compared to the known toxicity of Cd in earthworms indicate the CdTe QD ENMs are of similar acute and reproductive toxicity in earthworms. In contrast, the CdTe QD ENMs were more toxic than the CdTe-bulk counterpart, which was related to higher Cd accumulation in the former exposures. There was a coating effect, where the most toxic QD type was COOH- which was closely followed by NH_4^+ coated CdTe QDs (reproductive toxicity). It is likely that over time the QDs degraded further which led to higher Cd accumulation in tissues which resulted in lower biomass, reduced survival in the CdTe-NH_4^+ exposures and higher reproductive toxicity. Although no significant toxic effects were observed in the likely environmental concentrations of QDs, which at present are not known, but are likely in the low μg kg^{-1} dw range, like other ENMs have been modelled. Nonetheless, the study raises three important concerns, first, the CdTe QD ENMs are more toxic than the CdTe-bulk QDs; second, the effects are different between the differently capped CdTe QDs; third, the toxicity increases after a period of ageing which also renders, the so far known inert, CdTe-bulk materials more toxic. In addition to this, the mechanisms of toxicity are likely due to dissolved Cd, while the Te specific effects of sodium pump activity require further investigation.

Chapter 6. Plant growth in soils contaminated with coated and uncoated CuO ENMs or CdTe QD ENMs

6.1 Introduction

Plants require trace metals for normal functioning, however, excess of such can cause damage. The effects of metals on plants have been thoroughly studied (Kupper and Andersen, 2016). Copper is one of the essential trace elements in plants, playing a crucial role in respiration, photosynthesis and oxygen production (Linder, 1991). The primary route of uptake in vascular plants is via roots, where excess Cu is also stored (Linder, 1991). Excess copper has been found to inhibit enzymes, cause oxidative stress and elemental leak in grown vascular plants (Fernandes and Henriques, 1991). Cadmium is not an essential element in plants, however, due to the natural background of Cd in soils, as well as continued input from various anthropogenic sources, some vascular plants do store it. When in excess, cadmium has been found to cause the stunting of growth and chlorosis. It also causes interference with water retention, causing membrane damage and the generation of free radicals in the tissues, which lead to oxidative stress (Prasad, 1995). The effects of tellurium / telluride and its compounds has been studied less in plants. It is likely that plants accumulate and detoxify Te via selenium pathways, but no clear toxic effects have been reported in plants (Ba et al., 2010).

Non-vascular plants, such as bryophytes, have also been subject to research in metal contamination. In general, bryophytes have been used as bio-monitors to estimate the airborne metal contamination since they acquire nutrients and water from air (Brown and Wells, 1990). In addition, acrocarpous mosses can acquire metals from soil solution via rising capillary water (Tyler, 1990). In some cases, heavy metals are accumulated by mosses, and negative growth effects have been observed. Research has shown Cu exposure interferes with bryophyte photosynthesis (Brown and Wells, 1990), and Cd has been found to retard the

growth (Lepp and Roberts, 1977). The information on nanomaterial toxicity on mosses, or bryophytes in general, is limited. However, air pollution with ENMs is a very real problem, especially from fuel additives and combustion processes (Gantt et al., 2015). A recent study looking at the effects of Fe ENMs on the spreading earthmoss, *Physcomitrella patens,* (which is commonly used as a model organism for plant development and physiology) did not find a significant increase in reactive oxygen species or ATP concentrations, after the apparent internalisation of the particles (Canivet et al., 2014 and 2015). However, an increase in glutathione content was found, which may indicate a protective induction of this chelator to preserve tissue functions (Canivet et al., 2015).

Reports on the effects of ENMs on vascular plants are increasingly available since ENMs are being utilised in agriculture (Navarro et al 2008; Servin and White, 2016). The results so far indicate that ENMs can be taken up by plants (Ce_2O in kidney beans, Majumdar et al., 2014; Cu ENMs, 20 – 30 nm, in lettuce, Trujilo-Reyes et al., 2015). Copper ENMs have also been found to inhibit plant growth, and this effect may not be related to Cu dissolving from the ENMs (exposed on agar, Lee et al., 2008). In addition, Cu ENMs have been found to cause ionic disturbances, such as Mn reduction in lettuce exposed to Cu ENMs (Trujillo-Reyes et al., 2015). CdTe QDs have been found to reduce root growth as well as seedling development and lead to cell death (Chen et al., 2014) when exposed hydroponically, but whether these effects were due to dissolution is unclear.

The present work aimed to maximise the scientific value of the standard OECD (TGs 207 and 222) earthworm studies (Chapter 3 and 5) by keeping the exposure soils and allowing plants to grow without any disturbance and with no added moisture.

This meant any further moisture would have been from the surrounding air, from the plants transpiring which the resulted in condensation on the containers. The additional endpoint included assessing plant cover in each exposure in the CuO ENM and CdTe QD exposures. In addition, in the CuO ENM exposures, the total Cu concentration was measured along with essential elements (K, Na, Ca, Mg, Fe, Mn, Zn), to assess Cu accumulation and any signs of ionic disturbance in the plants. The analyses aimed to provide an insight into whether seeds would germinate and develop into plants in ENM contaminated soils. It was anticipated that it was not a controlled experiment with known additions of plant species, but instead an observational experiment to determine what incidental plant life germinated in the soils. Nonetheless, it can potentially provide insights into the effects on plants in the presence of ENMs, or the equivalent metal salts or bulk material controls, as well as contrasting the essential metal Cu with ENMs made of non-essential metals, such as Cd and Te.

6.2 Methodology

The approach used here was not based on any published guidelines for plant toxicity tests, since it was a follow-on from the earthworm tests conducted with CuO ENMs (Chapter 3) and CdTe QDs (Chapter 5).

6.2.1 CuO ENM experiment

At the end of the CuO ENM experiment (Chapter 3) the test containers were left for a further 23 weeks, in total making the exposure 25 weeks, to assess the plant growth in the exposed soils. At the end of the 25 weeks all containers were photographed,

the only vascular plants were identified to at least family level using species identification keys, and their percent cover of the container estimated. Bryophytes were not identified, but the % cover was estimated for the whole group. In addition, all vascular plants were harvested (above ground parts only) for biomass determination. Briefly, the cuttings were washed in deionised water, weighed and oven dried for 24 h (50 ^{o}C) to a constant weight. The dry weight of the plants was recorded, following which they were ground using a pestle and mortar. The ground samples were acid digested with 10 ml of 70 % HNO_3 at 80°C for 4 hours, cooled and diluted with 40 ml of Milli-Q water (method based on Campbell and Plant, 1998). Plants of small mass were grouped by species type and the pooled samples were measured together. The samples were analysed for total copper concentration by ICP-OES, under the operating conditions described in Chapter 2.5. The ICP-OES analysis included matrix matched standards, a CRM (Table 2.3) and procedural blanks.

6.2.2 CdTe QD experiment

At the end of the CdTe QD ENM experiment (Chapter 5) the test containers were left for a further 23 weeks, in total making the exposure 25 weeks to assess the plant growth in the exposed soils. At the end of the 25 weeks all containers were photographed, plants were identified and their percent cover of the container estimated.

6.2.3 Statistical analyses and data presentation

All analyses were carried out as described in Chapter 2.6. Where sample size was very small and limited, no statistical analyses were carried out (metal concentration in certain plant species). In all figures "Cu" represents the $CuSO_4$ treatments, 'bulk' to represent the micron size CuO particles, 'core' as the uncoated and 'PEG', 'COOH' and 'NH_4^+' to refer the coated CuO ENMs.

6.3 Results: CuO ENM study

The cover of vascular plants and mosses were estimated as well as imaged (Figures 6.1 and 6.2-7). The controls displayed an abundance of plants, with near 100 % cover of the soil, of which ~ 60 % was moss cover (Figure 6.1 and 6.2a). The $CuSO_4$ exposed soils displayed considerably less vascular plant and moss cover, however, some species that were not present in the controls were found in the $CuSO_4$ soils, e.g., chamomile. Overall, plant cover was statistically significantly lower in the $CuSO_4$ exposures when compared to the control and CuO ENM exposures at the nominal 200 mg Cu kg^{-1} dw exposures (Figure 6.1). The CuO-core nominal 200 mg Cu kg^{-1} dw exposures did not significantly differ from controls, and had higher abundance of plants than in the $CuSO_4$ exposure (Figures 6.1 and 6.2). The coated CuO ENM exposures at the nominal 200 mg Cu kg^{-1} dw did not differ from the controls or the CuO-core, while they had higher cover of plants than the $CuSO_4$. The variety of species was highest in the CuO-PEG exposures at the nominal 200 mg Cu kg^{-1} dw (Table 6.1, Figure 6.3b). None of the nominal 1000 mg Cu kg^{-1} dw coated ENM exposures had any appreciable vascular plant cover, expect the CuO-PEG. Similarly, to CuO-core at the nominal 1000 mg Cu kg^{-1}, CuO-COOH exposures had

a few very small plants that exhibited discolouration, with reddening of the leaves (Figure 6.4c and Figure 6.6a, b). Moss was found in all exposures, although, it was significantly lower in the CuO-NH_4^+ exposures at nominal 1000 mg Cu kg^{-1} dw when compared to the controls.

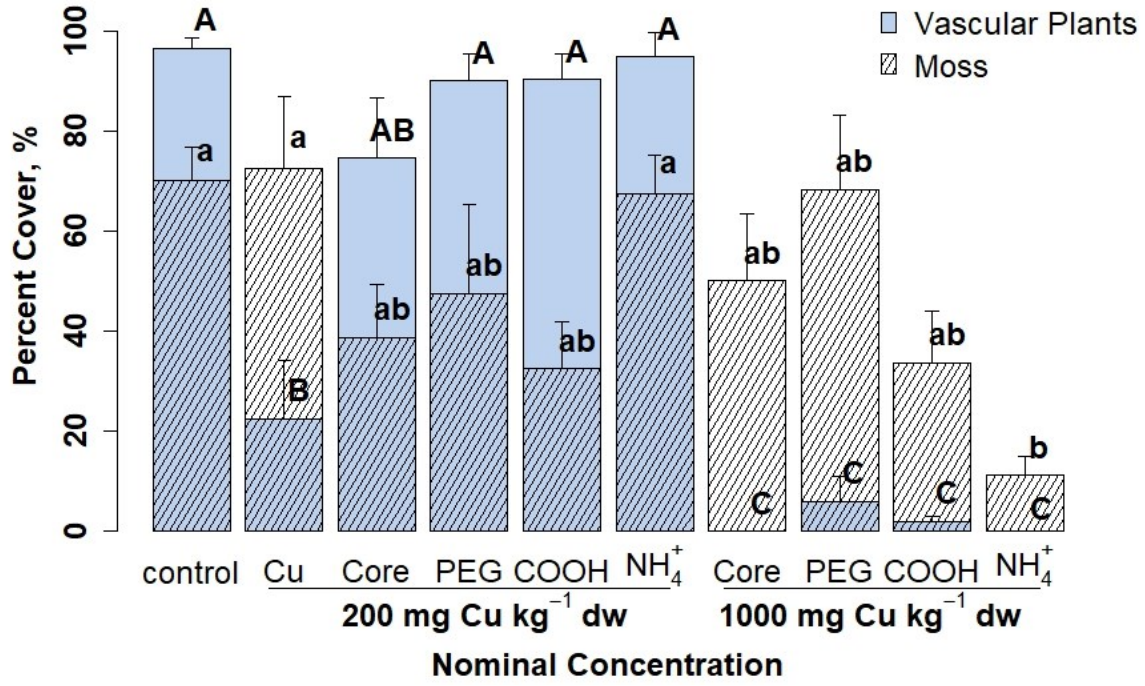

Figure 6.1 The total cover of vascular plants (solid bars) and moss (dashed bars) on the soil from exposure to CuSO4 (as Cu) at nominal 200 mg Cu kg^{-1} dw and differently coated CuO ENMs at nominal 200 and 1000 mg Cu kg^{-1} dw. Data shown as the percent cover of 4 replicate boxes of soil (mean ± SEM, n = 4). Different letters denote statistically significantly different treatments ($P < 0.05$, ANOVA). Capital letters represent differences between vascular plant cover and non-capital letters represent differences between moss cover.

The species were identified (Table 6.1) in all treatment replicates. The most abundant species across all treatments, in terms of both cover and mass, was *Hypericum humifusum* (trailing St John's wort), with *Glechoma hederacea* (ground ivy) being the second most abundant. All grass species were grouped and referred to as "grasses". Other species included chamomile, *Chamamelum nobile*, (in $CuSO_4$ treatment), broad-leaved plantain, *Plantago major*, two unidentifiable plants (all found in CuO-PEG nominal 200 mg Cu kg^{-1} dw exposure), and common mouse-ear *Cerastium fontanum*, (in CuO-NH_4^+ treatment). The wet and dry weight of plants is reflected by their percent cover in each treatment (Figure 6.1 and Table 6.1). In the controls, $CuSO_4$ and CuO-PEG exposures, the percent of dry tissue mass of the total mass (wet weight) was around 20 %. It was considerably less in the CuO-COOH, followed by CuO-NH_4^+ and -core exposures (Table 6.1).

Table 6.1 Summary of the total wet mass of each species of plants in each treatment measured above soil only (excluding roots).

Nominal $[Cu]_{soil}$		**Total plant wet mass (above soil), g**					
mg Cu kg^{-1} dw	**Plant species**	**Control**	**$CuSO_4$**	**Core**	**PEG**	**COOH**	**NH4+**
200	***Hypericum humifusum,***						
	wet weight	11.25 ± 0.65[a]	0.57 ± 0.07[b]	5.17 ± 0.22[c]	11.2 ± 0.8[a]	16.22 ± 1.45[d]	6.43 ± 0.3[c]
	dry weight	2.24 ± 0.11[a]	0.15 ± 0.02[b]	0.80 ± 0.04[c]	2.09 ± 0.16[a]	2.26 ± 0.21[a]	0.93 ± 0.04[c]
	percent dry weight of wet weight	19.90 %	26.80 %	15.55 %	18.60 %	13.90 %	14.42 %
		n = 4	n = 3	n = 4	n = 4	n = 4	n = 4
	Glechoma hederacea	4.86 ± 2.08	0.42 ± 0.05	5.44 ± 0.46	9.59 ± 0.69	6.88 ± 1.55	0.07
		n = 2	n = 2	n = 3	n = 3	n = 3	n = 1
	Stellaria media	1.3	NA	2.45 ± 1.22	NA	NA	1.13
		n = 1		n = 2			n = 1
	Grass	0.64 ± 0.1	1.27 ± 0.46	0.33 ± 0.05	1.49 ± 0.74	4.08 ± 2.04	6.78 ± 0.73
		n = 3	n = 2	n = 2	n = 2	n = 2	n = 4
	Other	NA	0.88	NA	7.6 ± 1.2	NA	7.36 ± 1.71
			n = 1		n = 3		n = 2
	Hypericum humifusum			NA	0.01	NA	0.01

			n = 1		n = 1
1000	**Grass**	NA	0.08 n = 1	0.01 n = 1	NA
	Other	NA	0.01 n = 1	NA	NA

Not all species were present in each replicate, thus the number of replicates is given as n in each group. Data presented as mean ± SEM. Different letters denote statistically significantly different treatments ($P < 0.05$, Kruskal-Wallis, Dunn's test). Statistical analyses were not viable for all plant species due to very small sample numbers for each species. Grass species not identified.

To assess possible metal accumulation, the total copper concentration was assessed in the plant tissues (above ground parts, excluding roots). The control plants of different species showed Cu concentrations between 2.6 - 12.2 mg Cu kg^{-1} dw. The plants that grew in soils dosed with $CuSO_4$ had significantly higher internal Cu concentrations from 21 – 32.9 mg Cu kg^{-1} dw when compared to the controls (Table 6.1). In the CuO-core exposures, the total Cu concentration in plant tissues was also elevated and higher than the controls, although the range varied from 12 – 57.3 mg Cu kg^{-1} dw. The maximum concentration of Cu was observed in *Stellaria media*, which was not present in $CuSO_4$ exposures. In the coated CuO ENM exposures the range of Cu in plant tissues was similar to that of CuO-Core ad $CuSO_4$ (Table 6.2). The highest concentration of Cu was found in NH_4^+ exposures ~ 60 mg Cu kg^{-1} dw (in *Glechoma hederacea*).

As well as measuring the total copper concentration, essential and trace metals were also measured in the plant tissues to identify any effects on the ion balance (Table 6.3). There were no clear trends or changes in the elemental composition.

Table 6.2 Total concentration of Cu in CuO or $CuSO_4$ exposed plants in the soil after 25 weeks of exposure.

Nominal $[Cu]_{soil}$		**Total Cu in the plants, mg Cu kg^{-1} dw**					
mg Cu kg^{-1} dw	**Plant species**	**Control**	**$CuSO_4$**	**Core**	**PEG**	**COOH**	**NH_4^+**
200	***Hypericum humifusum***	5.79 ± 1.22a n = 4	22.38 ±7.51b n = 3	16.36 ± 7.77ab n = 4	10.37 ± 1.45ab n = 4	37.11 ± 11.74b n = 4	13.13 ± 2.63ab n = 4
	Glechoma hederacea	12.24 ± 4.43 n = 2	21.35 n = 1	29.20 ± 3.90 n = 3	27.18 ± 8.97 n = 3	24.83 ± 15.83 n = 3	69.39 n = 1
	Stellaria media	2.58 n = 1	NA	57.43 n = 1	NA	NA	25.87 n = 1
	Grass	9.28 ± 2.84 n = 3	n = 1	23.46 n = 1	<0.01, LOD n = 2	6.60 n = 1	27.18 ± 6.31 n = 4
	Other	NA	32.87 n = 1	NA	22.90 ± 2.94 n = 3	NA	19.49 ± 2.13 n = 2

Data expressed as mean ± SEM (*n* values are below the data). Asterisks denote statistically significant differences between treatments ($P < 0.05$, ANOVA).

Table 6.3 Total concentration of electrolytes in CuO or $CuSO_4$ exposed plants in the soil after 25 weeks of exposure.

Plant species	Measured concentration, mg kg^{-1}dw						
	Electrolytes	**Control**	**$CuSO_4$**	**Core**	**PEG**	**COOH**	**NH4+**
Hypericum humifusum	**Na**	77 ± 17	147 ± 57	101	274 ± 148	502 ± 349	139 ± 51
	K	13318 ± 3150	27447 ± 15085	22876 ± 289	11900 ± 279	42057 ± 14040	14059 ± 2571
	Ca	5375 ± 760	9836 ± 4488	5346 ± 172	5540 ± 745	9983 ± 3943	4690 ± 547
	Mg	2134 ± 214	2974 ± 1269	1942 ± 235	2209 ± 354	5745 ± 2104	2237 ± 225
	Fe	74 ± 6	132 ± 60	77 ± 33	120 ± 29	298 ± 112	77 ± 22
	Mn	80 ± 21	627 ± 265	114 ± 10	100 ± 18	229 ± 91	94 ± 13
	Zn	41 ± 6	69 ± 36	48 ± 4	40 ± 7	86 ± 26	39 ± 5
Glechoma hederacea	**Na**	328 ± 134	937 ± 229	267 ± 77	137 ± 50	374	476
	K	25500 ± 11103	30694 ± 17102	24309 ± 2412	16234 ± 4186	28903	18740
	Ca	17415 ± 2889	16006	18150 ± 1359	11916 ± 4828	23743	19732
	Mg	3735 ± 577	3129 ± 1868	4208 ± 304	2759 ± 1657	5223	4254
	Fe	187 ± 4	72 ± 31	79 ± 17	195 ± 46	195	265
	Mn	127 ± 90	434 ± 97	68 ± 1	61 ± 9	93	235
	Zn	115 ± 45	51 ± 27	50 ± 7	52 ± 36	77	132
Stellaria media	**Na**	153		197			600
	K	8335		27028			24613

	Ca	2505		8210			4615
	Mg	1054		3111			1893
	Fe	219		290			190
	Mn	70		109			160
	Zn	26		53			53
Grass	**Na**	189 ± 111	130	232 ± 82	545	43	684 ± 393
	K	21143 ± 4550	23474	31119 ± 9802	30408	10740	21903 ± 5989
	Ca	3784 ± 1675	6933	9394 ± 4024	9929	4264	5198 ± 1399
	Mg	2427 ± 427	2283	2035 ± 614	2481	1938	1810 ± 436
	Fe	273 ± 207	50	125 ± 43	122	55	137 ± 37
	Mn	105 ± 27	450	238 ± 75	684	93	139 ± 60
	Zn	39 ± 6	110	115 ± 46	76	36	87 ± 35
Other	**Na**		114		392 ± 259		953 ± 259
	K		38660		33069± 6935		18742 ± 4227
	Ca		8301		12637 ± 2161		5037 ± 863
	Mg		2314		4548 ± 808		1711 ± 293
	Fe		139		113 ± 7		154 ± 40
	Mn		706		84 ± 5		89 ± 8
	Zn		87		101 ± 20		27 ± 4

Data expressed as mean ± SEM (sample number is equal to that in Table 2). There were no significant differences between the treatments where n = 4, i.e., in the *Hypericum humifusum.* For clarity of the table, the results were rounded to the nearest mg.

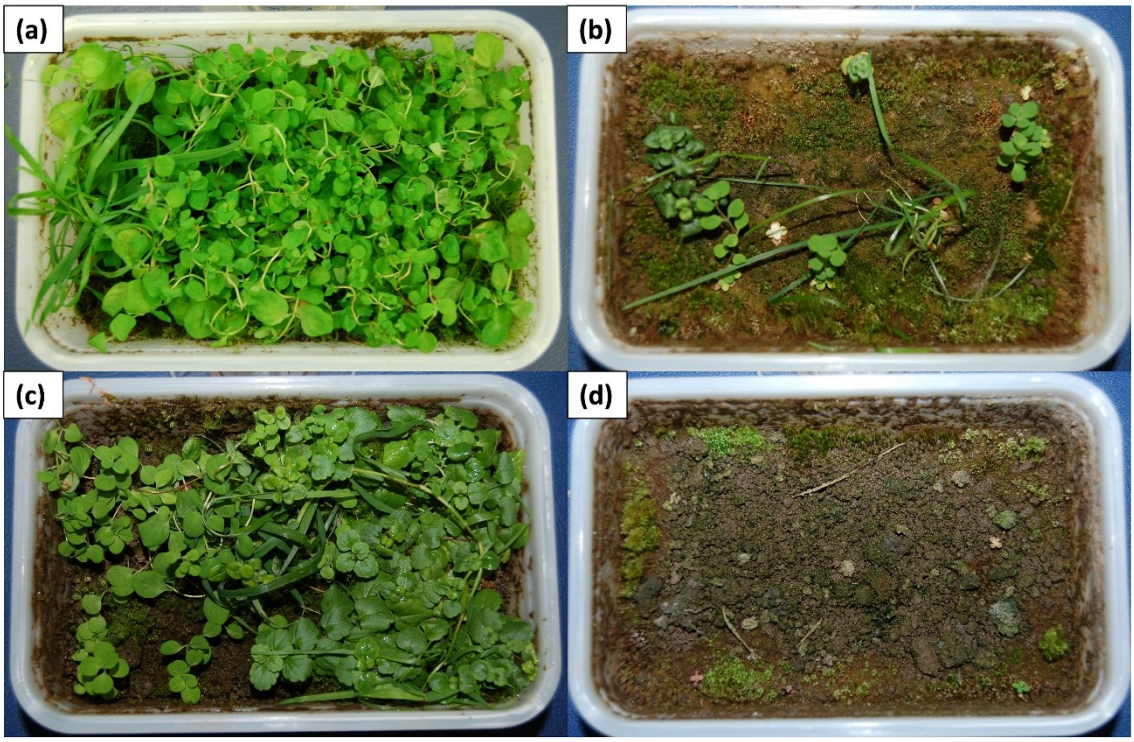

Figure 6.2 Example of vascular plant and moss cover in the (a) control, (b) $CuSO_4$ 200 mg Cu kg^{-1} dw, (c) CuO-core 200 mg Cu kg^{-1} dw and (d) CuO-core 1000 mg Cu kg^{-1} dw (all concentrations based on nominal). Note the abundance of plants in (a) and (c), while (b) has only a few plants.

Figure 6.3 Example of vascular plant and moss cover in the (a) control, (b) CuO-PEG 200 mg Cu kg^{-1}dw, (c) CuO-PEG 1000 mg Cu kg^{-1} dw (all concentrations based on nominal). Note the presence of different species in (b) and an almost 100 % cover of moss in (c).

Figure 6.4 Example of vascular plant and moss cover in the (a) control, (b) CuO-COOH 200 mg Cu kg^{-1}dw, (c) CuO-COOH 1000 mg Cu kg^{-1} dw (all concentrations based on nominal values).

Figure 6.5 Example of vascular plant and moss cover in the (a) control, (b) CuO-NH_4 200 mg Cu kg^{-1} dw, (c) CuO-NH_4^+ 1000 mg Cu kg^{-1} dw (all concentrations based on nominal values).

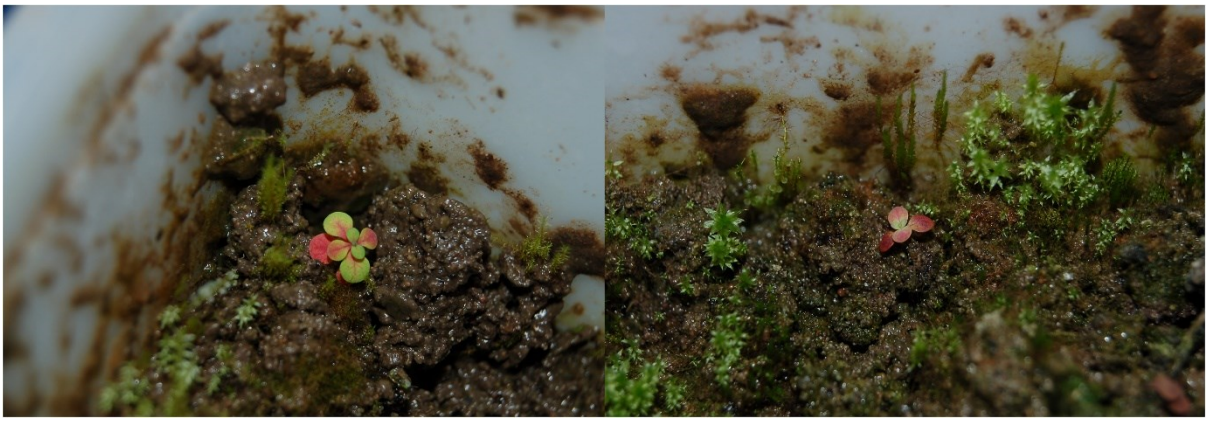

Figure 6.6 Example images of plants from the nominal 1000 mg Cu kg^{-1} dw as (a) CuO-core and (b) CuO-COOH. Note the discoloration of the leaves.

6.4 Results: CdTe QD experiment

A more selective analysis of plant growth was carried out within the CdTe QD experiment, where the percent cover was determined by visual analysis of the test containers, but the plants were not harvested for metal analysis. The vascular plant and moss cover in the controls was near 100 % and cover was similar in the nominal 50 and 500 mg CdTe kg^{-1} dw and CdTe-bulk exposures. In the top CdTe-bulk exposure, the vascular plant and moss cover was significantly lower (around 20 %, Figure 6.7). The CdTe-QD ENM exposures showed similar vascular plant and moss cover to controls and CdTe-bulk exposures at the nominal 50 mg CdTe kg^{-1} dw test concentration. Clear effects on plant cover were evident in the nominal 500 mg CdTe kg^{-1} dw CdTe QD ENM exposures where the PEG- and COOH-coated QDs showed the least amount of plants and moss, while the NH_4^+ coated exposure was similar to the CdTe-bulk and the controls. In the highest QD test concentrations there were no vascular plants or moss growth (Figure 6.7, Figures 6.8-10), except in the CdTe-PEG treated soils, which had around 10 % moss cover. The high ENM treatments with no plant matter had a biofilm-like layer and some mould, and the soil had dried in places (Figure 6.8).

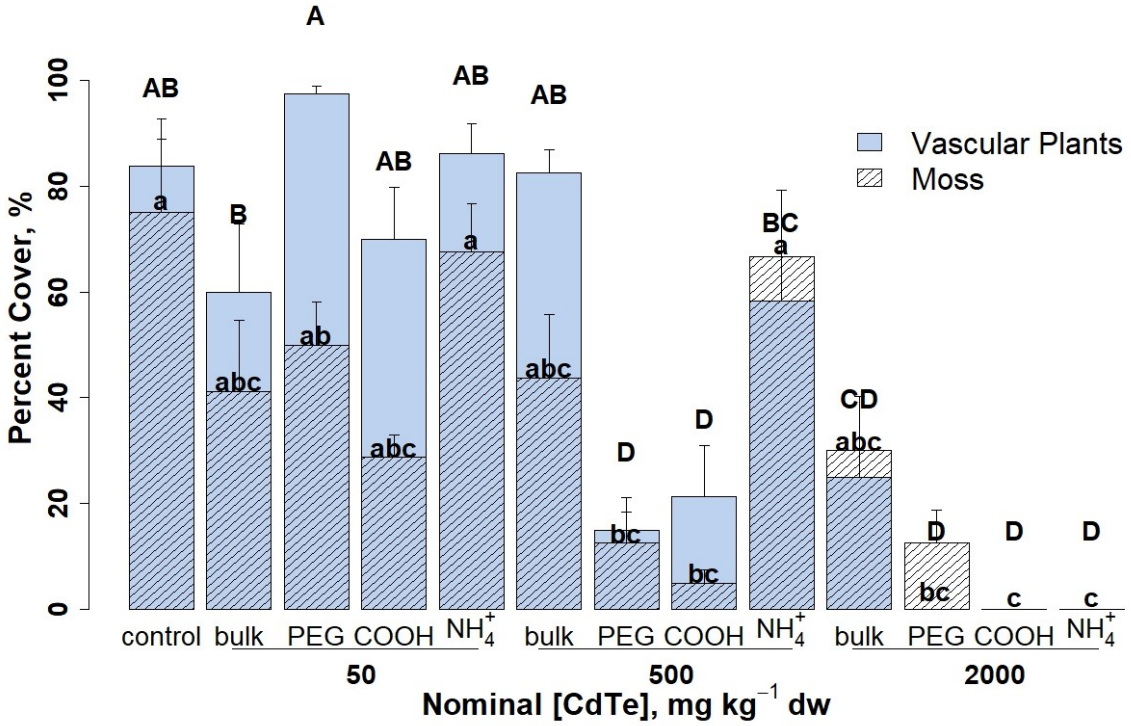

Figure 6.7 The total cover of vascular plants (solid bars) and moss (dashed bars) on the soil from exposure to controls, CdTe-bulk (as bulk), and differently coated CdTe QD ENMs, at 50, 500 and 2000 mg kg^{-1} dw nominal concentrations of CdTe QDs. Data shown as the percent cover of 4 replicate boxes of soil (mean ± SEM, n = 4). Different letters denote statistically significantly different treatments ($P < 0.05$, ANOVA). Capital letters represent differences between vascular plant cover and non-capital letters represent differences between moss cover.

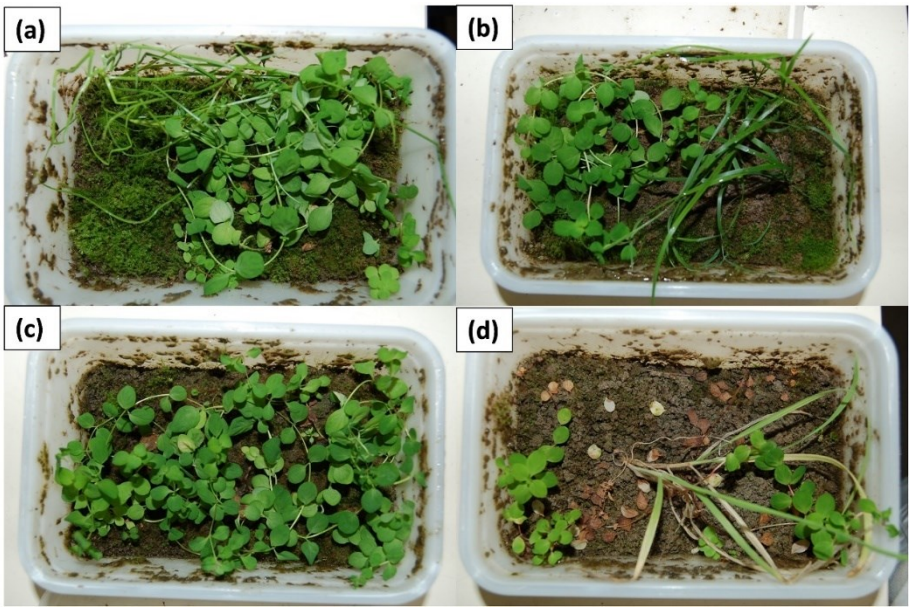

Figure 6.8 Example images of plants from the (a) control; and CdTe-bulk exposures at nominal (b) 50 mg CdTe kg^{-1} dw (c) 500 mg CdTe kg^{-1} dw and (d) 2000 mg CdTe kg^{-1} dw taken after 6 months since the QDs were mixed into the soil. Note the limited growth in the highest exposures (d).

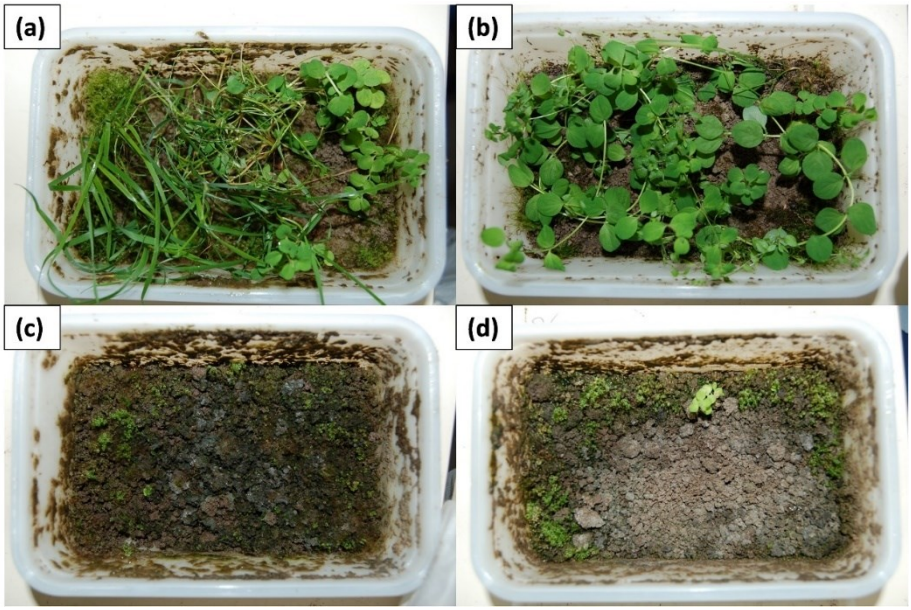

Figure 6.9 Example images of plants from the (a) control; and CdTe-PEG exposures at nominal (b) 50 mg CdTe kg^{-1} dw (c) 500 mg CdTe kg^{-1} dw and (d) 2000 mg CdTe kg^{-1} dw taken after 6 months since the QDs were mixed into the soil. Note the absence of vascular plants and presence of mould (potentially) (c,d).

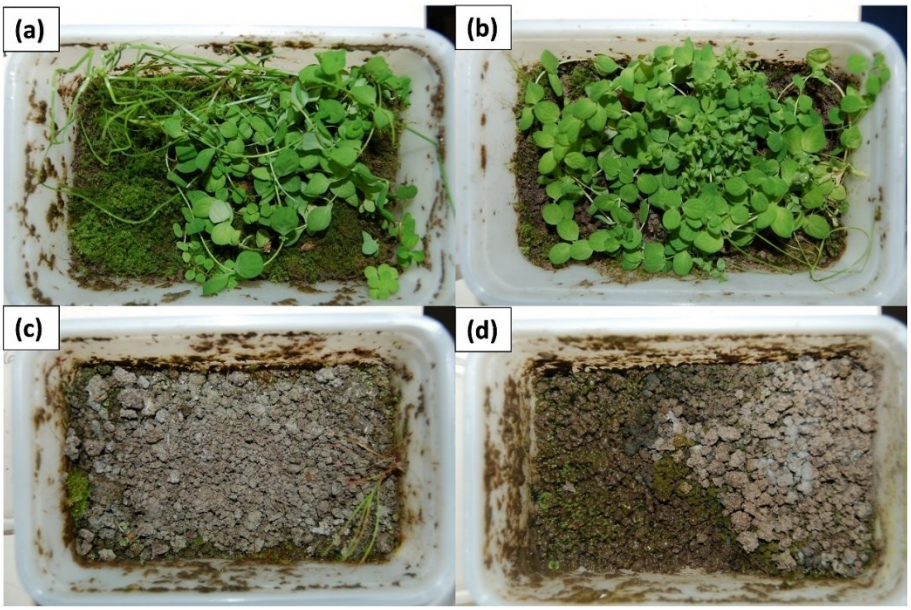

Figure 6.10 Example images of plants from the (a) control; and CdTe-COOH exposures at nominal (a) 50 mg CdTe kg^{-1} dw (b) 500 mg CdTe kg^{-1} dw and (c) 2000 mg CdTe kg^{-1} dw taken after 6 months since the QDs were mixed into the soil. Note the drier soil and presence of some mould (c), (d).

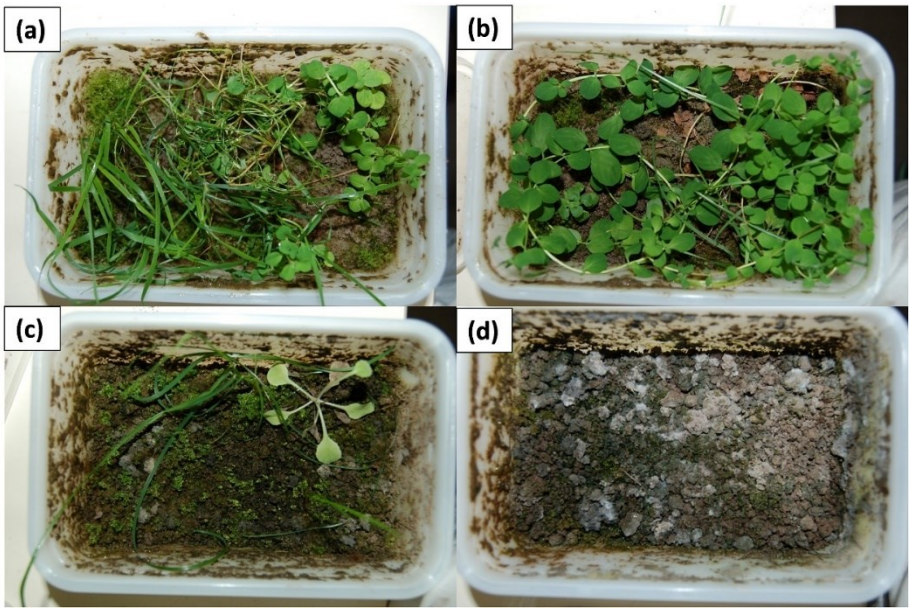

Figure 6.11 Example images of plants from the (a) control; and CdTe-NH_4^+ exposures at nominal (a) 50 mg CdTe kg^{-1} dw (b) 500 mg CdTe kg^{-1} dw and (c) 2000 mg CdTe kg^{-1} dw taken after 6 months since the QDs were mixed into the soil. Note the presence of mould (d) and limited plant growth (c, d).

6.5 Discussion

The results provide an insight into plant sensitivity to soil contaminated with ENMs as well as soil vitality. In the CuO ENM exposures, the ENMs were less toxic to plants than the $CuSO_4$ dosed soil, while in the CdTe QD exposures, the coated CdTe QDs were more toxic to plant growth than CdTe-bulk materials.

6.5.1 CuO ENM experiment

Plant cover was estimated to assess the potential effects of the contaminated soil on plant growth. It is likely the seeds originated from the added horse manure as well as the natural sandy loam soil. In the control soils, the vascular plants and moss covered almost the whole of the containers (Figures 6.1 and 6.2). Based on plant cover (%) and mass, $CuSO_4$ is a more potent herbicide than any of the CuO ENMs at the nominal exposure of 200 mg Cu kg^{-1} dw (Figure 6.1, Table 6.1). Dissolved copper is known to limit seedling development and plant growth (Muller et al., 2001), and the results here confirm this. But the data also suggests that dissolved Cu from the CuO ENMs did not result in a reduced plant growth. The reddish-purple coloured plants in CuO-core and CuO-COOH exposures (nominal 1000 mg Cu kg^{-1}, Figure 6) may be due to the increase in anthocyanins as a response to high Cu concentration, since pH was not more acidic (acidic pH can also result in an increase in anthocyanins in plants) in these exposures (Chapter 3). Chlorophyll loss and increase in anthocyanins has been observed before (*Arabidopsis thaliana* seedlings exposed to CuO ENMs in a liquid-agar, Nair and Chung, 2014). Moss cover was generally uniform throughout the treatments (Figure 6.1 – 5). Since moss does not require nutrients from the soil, it is more resistant to grow in contaminated areas,

although the least amount of moss was present in the nominal 1000 mg Cu kg^{-1} dw $CuO\text{-}NH_4^+$ exposure. This suggests mosses are more tolerant than vascular plants (which is generally expected, since they do not rely on moisture or nutrients from soil solution). However, the lower levels of moss may be an artefact of fewer moss spores being present in the batch of soil or horse manure. Alternatively, it could be related to the lower availability of moisture, due to the lack of plants that would aid the creation of condensation. The pierced lids were made uniform throughout treatments thus the drying could not be accounted to differences in the airflow.

Background copper concentration in *Hypericum* sp. in the control soils was in the range of one other report of this genus (~5.8 mg Cu kg^{-1} dw this study, 3.6 – 15 mg Cu kg^{-1} dw reported in Sherameti and Varma, 2015). In the $CuSO_4$ controls, the Cu was higher than the background Cu in this species. In other species, the concentration of Cu varied (Table 6.2), although with a trend of higher tissue Cu in the plants growing in $CuSO_4$ or CuO ENM soils, which gives evidence of Cu accumulation, although it is uncertain if this is in the nano or dissolved form. Exposure to both dissolved Cu and Cu ENMs has been found to influence ionic balance in plants (Fernandes and Henriques, 1991 and Trujillo-Reyes et al., 2016). Such evidence was not clear from the results in this study as the variation in data was relatively large, and no clear trends were apparent. This may be due to lower internalised Cu / CuO ENMs.

6.5.2 CdTe QD ENM experiment.

The control soils in the CdTe QD exposures had an overall cover of > 85 % of plants and ~ 80 % of moss, which is lower than in the controls from the previous results

(CuO ENM exposure), but this may be due to the smaller amount of soil and horse manure used in the exposures (half of the quantity used in the fresh soil study, see Chapter 5 for details). Overall, effects on plant cover were evident only at the nominal 500 and 2000 mg CdTe kg^{-1} dw exposures (Figure 6.7). CdTe QD ENMs were more toxic to plant growth than the CdTe QD bulk counterpart. There were also differences in the effects in the CdTe QD ENM exposures, where CdTe-PEG and -COOH QDs were more toxic than the CdTe-NH_4^+ QDs. It is unclear if these effects are due to dissolved Cd / Te or the intact QDs. However, CdTe QDs have been found to impact seed germination and plant growth (Chen et al., 2014). Moss cover followed similar patterns to the vascular plants, which may be due to a variation in the presence of moss spores, or it may be due to the lack of moisture in the air due to lack of vascular plants (Figures 6.6 – 10). Furthermore, in the nominal 2000 mg CdTe kg^{-1} dw the presence of mouldy growth and some biofilm-like layer could have been preventing the moss growth. Based on the results, the presence of CdTe QDs in electrical components that eventually end up in landfills or in developing countries as e-waste, can be considered a cause for concern for plant growth, since the QDs are likely to degrade and inhibit seedling development (in case the QDs penetrate landfill liners).

6.6 Conclusion

Taken together, the results provide an insight to potential ENM toxicity in seedlings. In both experiments, effects on plant growth were evident in the highest nominal concentrations. In the case of CuO ENMs, plants were more sensitive to CuO ENMs than earthworms, since most earthworms survived in the nominal 1000 mg Cu kg^{-1}

dw exposures (Chapter 3), while no vascular pants grew in the soils. ENMs are less hazardous to plant growth than $CuSO_4$ suggesting existing copper hazard assessment will protect against CuO ENMs. In the case of CdTe QDs, plants are also more sensitive to CdTe-QDs than earthworms (Chapter 5), and coated CdTe QD ENMs are more hazardous to plants, suggesting the existing risk assessment on CdTe QD bulk materials does not cover CdTe QD ENMs. Even though the experiment was not completely controlled, it can be assumed equal amounts of seeds and moss spores would have been in the soils or horse manure, since all treatments came from the same batch. However, this batch was not uniformly mixed before application to the soil which may have led to some differences. Based on the results, mosses are more tolerant to growing on soil with higher metal and ENM contamination, which is likely due to limited contact with soil and the fact they do not take up water through roots. In summary, however, there were no clear effects of ENMs on plant growth / cover at environmentally relevant concentrations in any of the experiments.

Chapter 7. Reproductive effects of CuO ENMs in *Caenorhabditis elegans* exposed in liquid and soil media

7.1 Introduction

Caenorhabditis elegans is a soil-dwelling bacterivorous nematode. It has been extensively used as a model organism since the 1960s when Sydney Brenner and colleagues started the seminal work with the nematode and developed many of the protocols still used to study neurology and developmental biology (Brenner, 1974). To date nematodes have been extensively used in such research, as well as further afield in ecotoxicology. There are many benefits of using *C. elegans*, including their fast life-cycle, high reproductive output, ease of culturing, transparent body and most importantly the wealth of existing research, including a mapped genome (The *C. elegans* Sequencing Consortium, 1998; Antoshechkin et al., 2007). Furthermore, *C. elegans* offers a promising tool in the 3Rs framework of replacing, refining and reducing the use of laboratory animals in eco/-toxicological research (Hunt, 2017; Gonzalez-Moragas et al., 2015, 2017) which is being encouraged by regulators, including the REACH regulations (ECHA, 2015).

The ecotoxicity tests with nematodes have mainly been carried out in liquid media, e.g. a simple potassium-sodium medium (referred to as K-medium) or a moderately hard reconstituted water which is based on ISO guidelines (ISO, 2010). Testing in plain water has been discouraged due to the low ionic strength which can lead to osmotic stress in the nematodes (Donkin and Dusenbery, 1993). Tests have also been developed on agar plates (Williams and Dusenbery, 1998), on soil and sediments (Donkin and Dusenbery 1993, 1994); and more recently in simulated soil porewater (Tyne et al., 2013), since their natural habitat is soil.

The ecotoxicity of metals, particularly copper, is well established in *C. elegans.* It has been found acutely toxic with a 24 h lethal concentration to 50 % of the population

(LC_{50}) of 105 and 109 mg Cu l^{-1} in K-medium (Donkin and Dusenbery, 1993 and Boyd et al., 2003, respectively). The toxicity in sediments or soil is e.g. 24 h LC_{50} = ~ 1246 mg Cu l^{-1} or 534 mg Cu kg^{-1} dw in a sandy loam wetted with K-medium. Another study by Boyd et al. (2003) found the LC_{50} to increase depending on medium used, with the liquid medium being most toxic, followed by artificial soils, with natural soils having the highest LC_{50} values. Boyd et al. (2003b) found reproduction a more sensitive endpoint with an effect concentration to 50 % of the population (EC_{50}) to be 2 mg Cu l^{-1} in K-medium, while Harada et al. (2007) found the juvenile number to be reduced by 50 % on agar plates at concentrations of 63 mg Cu l^{-1}.

C. elegans has become a model organism for ENM research, where most of the work has been carried out either in liquid medium or agar plates (reviewed in Choi et al., 2014). So far there have been no experiments in soil, even though methods were developed decades ago (Donkin and Dusenbery, 1993) and established guidelines exist, e.g. ISO (2010). The tests carried out in liquid or agar plates have focussed on adverse effect endpoints such as survival, reproduction and growth of juveniles, biochemical and molecular responses (e.g., Ag ENMs Roh et al., 2009; Meyer et al., 2010; Jung et al., 2015), as well as uptake (e.g., CdTe QDs, Qu et al., 2011). It has been found that Ag ENMs can be internalised by the nematodes, reduce the growth of juveniles (Meyer et al., 2010) and significantly reduce reproduction at concentrations as low as 0.5 mg Ag ENM l^{-1} (Roh et al., 2009). CuO ENMs have been found to be internalised by *C. elegans* (Gao et al., 2008). A more recent study reported CuO ENMs interfering with nematode feeding and development and inducing neurological degeneration (Mashock et al., 2016). However, it seems that

currently no studies have been published with nematodes in soil or sediments using ENMs.

The present study was set out to determine the reproductive toxicity for differently coated CuO ENMs compared against a common CuO-core material, $CuSO_4$ and micron sized CuO (CuO-bulk) control. The main aim was to assess the reproductive toxicity of CuO ENMs and a secondary aim was to assess juvenile mobility and ENM adhesion to nematodes in the standard potassium medium. Having established the effects in the liquid medium, a more selective experiment was subsequently carried out with the same ENMs and controls in a standard Lufa 2.2 soil, to assess nematode reproduction. This more selective experiment also aimed to provide insight to how the dosing of ENMs influences the toxicity.

7.2 Methodology

Two nematode toxicity experiments were carried out based on Dhawan et al. (1999) and broadly the ISO 10872 guideline (ISO, 2010). Prior to the main experiments a small-scale range finding study was conducted. Adult egg-laying nematodes were placed on an agar plate and removed after 3-4 hours, the left eggs were left for approximately two days and then used in the experiments. Synchronised nematodes (approximately late L3 and early L4 stage) were used in all the experiments and both experiments were carried out at 19 ± 1 ºC, in constant dark, in 12-well microplates.

7.2.1 Engineered nanomaterial characterisation and stock solutions

The ENMs used in the nematode experiments are described in Chapter 2.2. In addition to using the $CuSO_4$ as a control, the micron size CuO material was used (CAS 1317-38-0, British Drug Houses Ltd, Analar grade) and is referred to as CuO-bulk in the text. The CuO ENM powders (core and coated) were black, while the CuO bulk powder had a brown hue. The initial ENM stocks were prepared by weighing out a desired amount of the dry ENM powder or CuO bulk, and dispersing it in Milli-Q water, followed by sonication 1 hour (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany). For the nematode experiments, the ENM stocks were prepared fresh on each day and sonicated for 1 hour prior to the preparation of the serial dilution series by continuously vortexing the solutions (1:1 volume: volume ratio), and dispensing the aliquots into respective wells or wetting the soil. Dry mixed soils were prepared only for 30 mg Cu kg^{-1} dw treatment, and dosing followed the same principle as described in Chapter 2.3. except the soils were wetted with the K-medium used in the liquid exposure, rather than deionised water (more details in section 7.2.8).

7.2.2 Total metal analysis

Total copper concentration was measured in each serial dilution used in the experiment (30, 15, 7.5,1.8, 0.45 and 0 mg Cu l^{-1}, nominal) by ICP-OES to confirm the exposure to copper (Chapter 2.6). In addition, total Cu concentration was measure in the soil samples collected on the day when the nematodes were introduced to the soil (i.e., after 1 day of dosing the soils as done in all of the earthworm experiments). The analysis was carried out as described in Chapter 2.6.

7.2.3 Extractable copper fraction

The first part of the two-step sequential extraction described in Chapter 2.7 was carried out on samples of the nematode soil experiment to investigate the accessible Cu fraction. The nematodes inhabit the porewater, thus any water-bound Cu / CuO particles could be in direct contact with the organisms.

7.2.4 Test species, test media and feed

The *Caenorhabditis elegans* (N2, Bristol strain) were obtained from CEH, Wallingford (originally from the *Caenorhabditis* Genetic Centre stock collection the University of Minnesota, St. Paul, MN, USA). The culturing and maintenance principles were based on Brenner (1974). Briefly, nematodes were grown on nematode growth medium or NGM-agar-plates, consisting of 2 % (w/v) agar, 51.3 mM $NaCl_2$, 0.25 % (w/v) bacteriological peptone, 12.93 µM cholesterol, 0.5 mM $CaCl_2$, 1 mM $MgSO_4 \times 7$ H_2O, NGM buffer (20 mM KH_2PO_4, 5.167 mM K_2HPO_4) with a pH 6.0 ± 0.1. The plates were seeded with *E. coli* (strain: OP50), and kept in a controlled temperature cabinet at 19 ± 0.5°C. The nematodes were transferred to a fresh NGM plate every week to keep the cultures healthy (Brenner, 1974).

Potassium medium (32mM KCl, 51mM NaCl, ionic strength 254), which was un-buffered and made up fresh on the day of use, was used in the experiments. The pH of the solution was 5.8 ± 0.2 (mean ± SEM, n = 3), measured with a glass combination electrode (Corning Instruments 420).

The same soil used in the earthworm experiments (Chapters 2.5 and 3-5), Lufa 2.2, was used in the *C. elegans* tests as it is a good representative of a standard soil

used in nematode experiments (Hoss et al., 2009). In addition, the use of the same soil allowed a comparison between the earthworm and nematode tests. As nematodes have been found to require a high ambient moisture content - higher than recommended in the ISO (2010) TG (Huguier et al., 2013) - the soil was wetted with K-medium to ~ 70 % of the WHC with K-medium and feed mixture combined (ISO, 2010). Soil pH was measured as described in Chapter 2.5. The pH of both media after ENM or Cu-salt addition was between 4.5 and 5.5, which is in the tolerable range of 3 - 12 for *C. elegans* survival and reproduction (Khanna et al., 1997).

The uracil deficient strain of *E. coli* (OP50) was used as feed in all the experiments. The solution containing the *E. coli* was prepared by the method in ISO 10872 (ISO, 2010). Briefly, *E. coli* was cultured in TB-medium for 48 hours at 37°C. Following this, a sub-sample of 50 ml was taken and centrifuged at 750 g for 10 minutes (swing out rotor, Harrier 18/80 HSE). The supernatant was removed and the pellet was re-suspended in 10 ml of K-medium, followed by centrifugation and resuspension twice. A serial dilution of the suspension was prepared to obtain an absorbance of 0.71 at 600 nm (7315 spectrophotometer, Jenway Ltd, UK). The OD_{600} = 0.71 = 1000 formazine absorption units (FAU) and the final concentration in each test well is required as ~ 500 FAU in the liquid exposure and ~ 1000 FAU in soil, due to different conditions (optimised method for reduced number of nematodes based on ISO, 2010 and Tyne et al., 2013). To this end, 5 µl of the bacterial stock was added to 1 ml liquid test solution and 10 µl into the soil exposures. A cholesterol solution of 5 mg ml^{-1} in absolute ethanol was prepared to be used in experiments and added to test vessels with the food (0.2 % cholesterol w/v in the food mixture) making the final amount 0.001 % of in 1 ml of test solution and 0.002 % in the soil medium (ISO, 2010).

7.2.5 ENM characterisation in liquid test medium: dissolution and nanoparticle size.

Dissolution of Cu^{+} from the CuO ENMs was assessed by a dialysis experiment in nematode liquid test media. The experiment was carried out based on the method described in Besinis et al. (2014). The dialysis tubing and preparation was identical to that described in Besinis et al. (2014). Briefly, dialysis tubing cellulose membrane with molecular weight cut off at 12,000 Da (product code: D9777, Sigma-Aldrich Ltd, Dorset, UK), with an approximate exclusion size of 2.5 nm, was used to make 70 mm long tubes. The final tube measurements were 70 mm long and 25 mm wide. Stocks of 100 mg Cu l^{-1} (nominal) of the CuO ENM variants and micron size CuO were prepared in Milli Q water, and 8 ml of each stock was placed into the tubing. The tubes were secured with a knot and a medi-clip at each end to prevent leaking, and tied to a stand above the beakers (Besinis et al., 2014). The filled bags were immediately placed in the 600 ml beakers containing 492 ml of Milli-Q water (making a total volume of 500 ml). Control beakers, in triplicate, containing only Milli-Q water in the tubing were set up with the CuO ENM containing beakers. The beakers were covered with cling film and gently stirred with a multipoint magnetic stirrer (RO 15P power, Ika-Werke GmbH & Co. KG, Staufen, Germany). Samples were collected from each beaker at T = 0, 0.5, 1, 2, 3, 4, 6, 8 and 24 h and acidified with 2 % HNO_3 prior to analysis for Cu concentration by ICP-MS (Chapter 2.5). The remaining contents of the dialysis bags were collected after 24 h, acidified as above, and total Cu concentration was measured by ICP-OES. The pH and temperature of the media were measured at the beginning and end of the 24 h period.

Nanoparticle tracking analysis (NTA) was carried out using the NanoSight LM10 (Salisbury, UK) as according to Besinis et al., (2014). The particles were sized in the stock solutions used in the experiments (K-medium, 30 mg Cu l^{-1}). Measurements were made on three subsamples of each solution. The laser output was set at 30 mW at 640 nm. This method is based on light scattering to visualise individual particles and track their Brownian motion. The software uses the rates of diffusion to calculate the hydrodynamic radius based on Stokes-Einstein equation (Besinis et al., 2014).

7.2.6 Range finding experiment

The range finding experiment was carried out on juveniles in a duplicate test design. The test concentrations were 0, 25, 50, 100 and 200 mg Cu l^{-1} (nominal) as $CuSO_4$, CuO-core, -PEG, -COOH, -NH_4^+ ENMs. These were prepared by dilution series of the top concentration (1:1 dilution). Ten juveniles were exposed in 1 ml of the solution in sterile 24-well plates (GBO, UK) for 24 hours in constant dark at 20^oC. After 24 hours, the number of mobile (alive) juveniles were recorded.

7.2.7 Experimental design, method and test material dosing: liquid medium

The experiments were based broadly on the ISO 10872 (2010) guideline, with modifications based on Dhawan et al. (1999). The nominal test concentrations were 0, 0.46, 1.8, 7.5, 15, 30 mg Cu l^{-1} as $CuSO_4$ and each of the CuO ENM or micron size CuO variants in six replicates. The dilution series were prepared in bulk, vortexing each dilution prior to further diluting it. The test treatments were split over

two 12-well plates, including three wells of controls on each plate. One late L3 to early L4 stage hermaphrodite (n = 6 per $CuSO_4$, CuO ENM or bulk treatment, n = 60 per control) from a synchronous culture was exposed in 1 ml of the test solution in 6-well plates for 72 hours, with food and cholesterol as recommended in the ISO 10872 (2010). The exposure was carried out in a constant temperature cabinet at 19 ± 0.5 ºC, in the dark. After 72 hours, the endpoints analysed were reproduction (juvenile production), juvenile locomotion and ENM adhesion to the nematodes. In addition, from each of the 30 mg Cu l^{-1} treatment, one adult was removed after 72 hours for imaging. The images were taken of the live nematodes using digital micro-imaging device Leica DMD 108 (Leica, UK). These images were used to provide preliminary evidence of the ENM adhesion to the nematodes. The experiment was terminated after 72 h by adding 250 µl of 1 g l^{-1} Rose Bengal into each well to stain the nematode cuticle and incubating the plates at 80°C for 10 minutes. These steps result in straightened and easily counted nematodes (ISO, 2010).

7.2.8 Experimental design, method and test material dosing: soil medium

A modified version of the soil test method given in the ISO10872 (2010) was used. Modifications where similar to the K-medium experiment described above and the test soil was wetted to 100 % of its water holding capacity. The experimental design included nominal concentrations of the different Cu, CuO ENM and CuO bulk variants at 0, 15 and 30 mg Cu kg^{-1} added wet to the soil (15 and 30 mg Cu l^{-1} stock solutions), and 30 mg Cu kg^{-1} dw applied dry to the soil. The experiment included six replicates of each test concentration split over 12-well plates, including controls and K-medium controls. The experimental conditions were identical to the liquid

experiment described above. The number of juveniles produced were measured after 72 hours.

The stock solutions of ENMs were added to the soils and are hereafter referred to as wet mixed soils in text. The amount of soil required for six replicates (3500 mg dw including an additional 500 mg to be used for pH and metal analyses) was wetted with 2000 µl of the 15 or 30 mg Cu l^{-1} $CuSO_4$, CuO ENM or CuO-bulk solutions. This mixture was then aliquoted into twelve well plates with ~ 501 mg (to take account for the added liquid) in each well. Five µl of the bacterial food and cholesterol mixture was added to the wells, followed by the introduction of an adult hermaphrodite. The experiment was terminated as described in the previous section. To recover the nematodes from the soil a colloidal silica suspension with a density of 1.13 ± 0.005 g cm^{-3} (Ludox™, CAS 7631-86-9) was used, as described in ISO 10872 (2010). Briefly, the soil was washed with tap water into centrifuge tubes, and centrifuged at 800 g for 5 minutes (swing out rotor, Harrier 18/80 HSE). The supernatant was decanted into a labelled Petri dish. Two ml of the described Ludox suspension was added to the tube followed by mixing for 5 minutes and centrifugation at 800 g for 5 minutes. After centrifugation, the tubes were left to stand ~ 5 minutes to allow the nematodes to float to the supernatant, which was then poured into the labelled Petri dish. Most of the worms can be found in the first Ludox suspension supernatant, but the steps of mixing with 2 ml Ludox suspension and centrifugation were repeated twice. The offspring were counted in the Petri dishes.

The dry powders were mixed in the soil followed by wetting, and are referred to as dry mixed soils in text. The $CuSO_4$, CuO ENMs and micron sized CuO powder was applied to the soil dry as described in Chapter 2.2, to reach a nominal concentration

of 30 mg Cu kg^{-1} dw. Here the volume of the initial amount of soil was smaller respective to the total amount of soil required for the experiment (3000 mg dw). The soil was then wetted to 100 % WHC with K-medium, and the soil was aliquoted into 6 replicates over two 12-well plates. The experiment was carried out and terminated as described in the previous section.

7.2.9 Statistical analyses and data presentation

All statistical analyses were carried out as described in Chapter 2.9. In addition to this SigmaPlot v 13.0 (Systat Software Inc.) was used where the raw data points from the dialysis experiment were fitted with a curve, applying the legal binding one site saturation equation (single rectangular hyperbola, two parameter y = a * x /(b + x). Maximum release rates were estimated manually from the steepest part of the curve (maximum release rate = y / x) (methodology based on Besinis et al., 2014). In addition, reproduction data was plotted in and a sigmoid curve was fitted to estimate an EC_{50} value. In all figures “Cu” represents the $CuSO_4$ treatments, ‘bulk’ to represent the micron size CuO particles, ‘core’ as the uncoated and ‘PEG’, ‘COOH’ and ‘NH_4^+’ to refer the coated CuO ENMs. Since soil, as well as K-medium experimental solutions, were prepared in a batch and then divided between the exposure replicates, pH was measured in the original batch solution / soil, due to small volume of the exposures, resulting in only one measurement for the pH for each treatment.

7.3 Results

7.3.1 ENM characterisation in K-media

The ENMs were characterised in the potassium medium in the top concentration 30 mg Cu l^{-1} (nominal) used in the experiments. The stock solutions showed visible aggregates in all treatments. A more uniform dispersion, with minimal visible aggregates, was only achieved after sonication in the CuO-core and CuO-NH_4^+ exposures, and resulted in a black hue to the stocks. The CuO-PEG and -COOH stocks showed more aggregates and a less uniform dispersion and no visible hue. The CuO-bulk material showed moderate dispersion of visible aggregates, as well as dispersion that gave the K-medium a brown hue. To confirm the aggregate size and presence of nanoscale particles, NTA was carried out. The NTA results support the presence of large aggregates, yet also the presence of particles in the nanoscale (Table 7.1). The data show the aggregate hydrodynamic diameters from particle size distribution measurements made by NTA (using Nanosight LM10). The maximum (Max) size is the largest diameter detected while the mode is the particle size that occurred most often, and the D50 value represents that 50 % of the detected particles had a diameter smaller than the value shown. The concentration shows the number of particles per millilitre analysed. Tracks shown by *n* is the number of completed measurements from 3 replicates of the dispersions. The results indicate that CuO-core, -NH_4^+ and –bulk materials had most particles in the nanoscale, while CuO-PEG and –COOH materials resulted in large aggregates (Table 7.1).

Table 7.1 Characterisation results of the different CuO ENM variants and CuO-bulk particles in the potassium (K) medium at a nominal concentration of 30 mg Cu l^{-1}.

Treatment	**Max**	**Mean ± SD** (nm)	**Mode**	**D50**	**Concentration**, (x 10^8 particles ml^{-1})	**Tracks (n)**
Core	211	119 ± 63	14	102	0.91	123
PEG	287	174 ± 75	183	176	0.87	128
COOH	501	246 ± 172	157	162	0.47	110
NH_4^+	280	148 ± 100	21	124	1.31	163
Bulk	255	91 ± 89	34	49	4.75	525

Mean is based on completed tracks. SD –standard deviation; * Concentration based on the instrument value. D50 value represents that 50 % of the detected particles had a diameter smaller than the value shown.

7.3.2 Release of dissolved Cu in nematode culture medium

To assess if the CuO ENMs / CuO-bulk released dissolved Cu into the liquid exposure media, a dialysis experiment was conducted. The control medium remained below the LOD (< 0.1 µg Cu l^{-1}) throughout the dialysis experiment, while an increase in dissolved Cu was evident in all CuO treatments (Figure 7.1). The CuO bulk materials were found to release very small amounts of Cu over a 24 h period, although the amount was more than from the CuO-core ENMs, which released the lowest amount of Cu, at the slowest rate. The PEG-coated CuO followed by CuO-COOH ENMs released Cu rapidly, while CuO-NH_4^+ released Cu at a slower rate than the former two. In all cases, the amount of dissolved Cu saturated after 4 hours, except CuO-core, where saturation was clearer after 8 hours.

The pH in the dialysis beakers did not significantly differ between different treatments or between the beginning and end of the dialysis ($P > 0.05$, Kruskal-Wallis). The pH of the control media was 6.06 ± 0.01 (mean ± SEM, $n = 4$). The pH was 6.08 ± 0.02, 6.03 ± 0.01, 6.09 ± 0.01, 6.05 ± 0.01 and 6.04 ± 0.02 in the CuO-core, -PEG, -COOH, -NH_4^+ and -bulk treatments (mean ± SEM, $n = 6$).

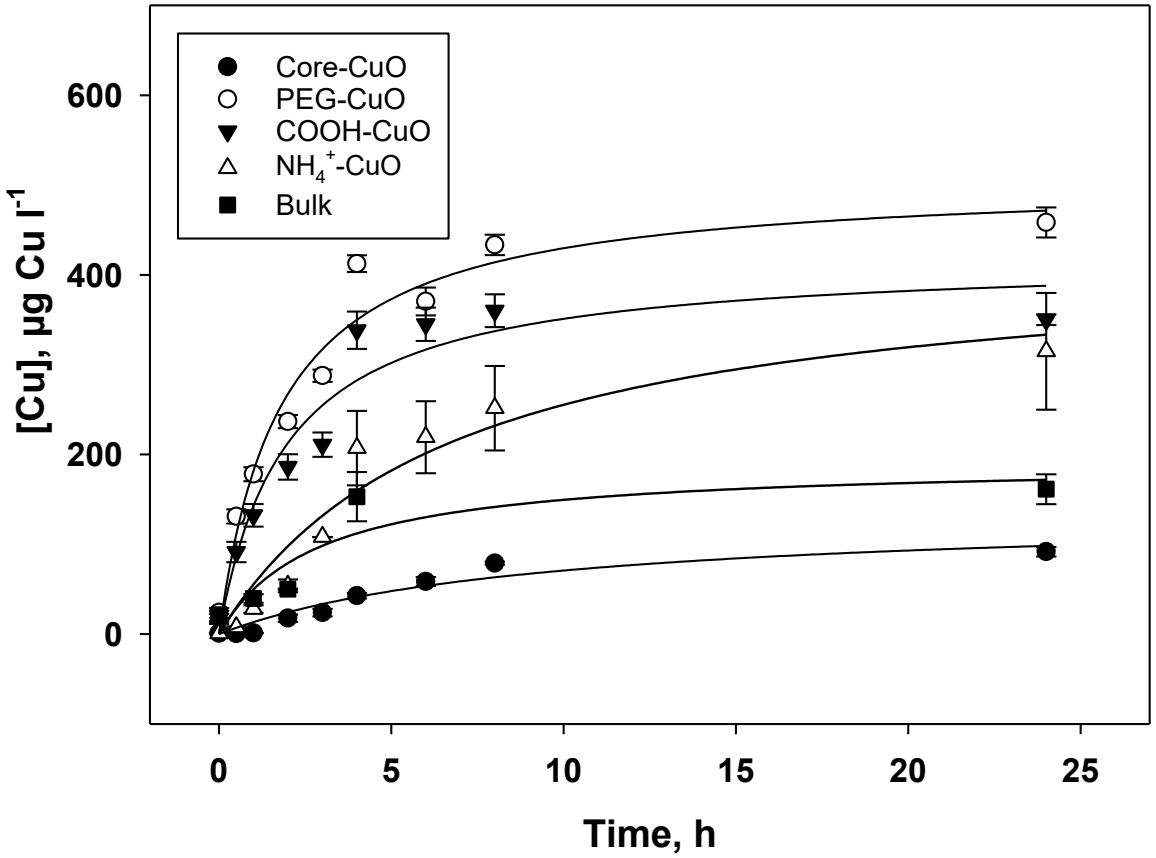

Figure 7.1 Dissolution of Cu from CuO ENM variants in K-medium (data as mean ± SEM, $n = 3$; curves were fitted to raw data). All r^2 values showed a good hyperbolic curve fit > 0.8 and the line equations are shown in Table 7.2.

The maximum release rate of Cu was calculated for the maximum slope, when the dissolution of Cu was highest and not at equilibrium (t = 1 h), while the average

release rate was calculated using the formula of the curve (Table 7.2). The maximum and average release of dissolved Cu were similar in the CuO-bulk treatment, while there was an almost ten-fold difference between the average and maximum release rate in the CuO-core treatment (Table 7.2). The CuO-PEG and –COOH treatments had a higher maximum release rate than the average release rate, while it was the opposite in the case of CuO-NH_4^+.

Table 7.2 The equations to support Figure 7.1 and respective r^2 values for the curve fits, along with the calculated average and maximum release rate of dissolved Cu from the CuO ENMs / CuO bulk material.

Treatment	**Release of dissolved Cu**	r^2	***Average,*** ***μg Cu l^{-1} h^{-1}***	***Max,*** ***μg Cu l^{-1} h^{-1}***
Core	$y = 135.82 * x / (9.18 + x)$	0.9	13.3	1.4
PEG	$y = 506.74 * x / (1.79 + x)$	0.9	181.6	190
COOH	$y = 424.09 * x / (6.55 + x)$	0.8	56.2	146
NH_4^+	$y = 419.28 * x / (1.94 + x)$	0.9	142.6	60.7
Bulk	$y = 187.34 * x / (3.9 + x)$	0.8	38.2	39.1

7.3.3 K-medium and soil pH in the experiments

The pH of the control exposures in K-medium were as expected, at a value of around 6. In the control soil it was less, which was also anticipated, since the Lufa 2.2 soil has a pH of 5.5 ± 0.2 measured in Milli-Q water (Chapter 3). In the K-medium, the pH increased with increasing concentrations of CuO-Core, CuO-NH_4^+

and CuO-bulk exposures, although it remained unchanged in the CuO-PEG and CuO-COOH exposures across the different concentrations. In soil, the pH did not follow similar trends as in the K-medium, since there was no evident trend in pH with increasing amount of ENMs or CuO-bulk in the soil. This suggests the soil pH was dominantly impacted by the soil chemistry, buffer capacity, and not the addition of ENMs. Overall, both K-medium and soil pH was not found as a co-factor in the reproduction output of *C. elegans* ($P > 0.05$, ANCOVA). This was based on a simulated replication of the data assuming that the pH did not change in exposures once it was divided between the replicates.

Table 7.3 K-medium and soil pH measured at the beginning of the experiments (day 0).

Nominal Cu concentration			**Treatment**				
mg Cu kg^{-1} dw **mg Cu l^{-1}**	**Control**	**$CuSO_4$**	**Core**	**PEG**	**COOH**	**NH_4^+**	**Bulk**
K-medium							
0.46	6.07	5.39	6.13	6.03	5.95	5.98	6.11
1.8		5.5	6.35	6.09	6.1	6.10	6.47
7.5		5.11	6.64	6.05	6.06	6.54	6.63
15		5.2	6.7	5.86	5.91	6.35	6.58
30		5.06	6.72	5.83	5.87	6.54	6.5
Soil							
15	5.88	5.8	6.15	5.96	5.55	5.71	5.76
30		5.45	6.01	5.90	5.70	5.81	5.17
30*		5.63	6.04	6.05	5.50	5.79	5.5

Data represents one measurement of the prepared medium that was then divided between the exposure replicates.

7.3.4 Total copper concentration K-medium

Further to the ENM characterisation in the liquid K-medium, total copper concentration was measured. The control medium had no background copper concentration (< LOD, 0.1 µg Cu l^{-1}), while the $CuSO_4$ exposures resulted in an expected increase of total Cu, which was within 80 – 100 % of the nominal concentration (Figure 7.2). In the CuO bulk exposures, the measured concentration of Cu was close to nominal in the 30 mg Cu l^{-1}, but dropped to around 50 % of the nominal in the rest of the exposures. This is likely due to the increased aggregation, which results in lower concentration of CuO-bulk particles in subsequent dilutions. The latter is also likely the case for all the ENM exposures. The concentration of Cu was ~ 80 - 100 % of the nominal in the 1.8 -30 mg Cu l^{-1} CuO-core ENMs exposures (Figures 7.2b-e), while it significantly dropped in the lowest concentration (Figure 7.2a). The known differences in the stoichiometry of the coating resulted in an expected lower measured concentration of Cu concentration in all of the coated CuO ENMs (Chapter 7.2 and Chapter 2.5). However, it was lower than expected in the lowest nominal concentrations due to dilution time resulting in aggregation (Figure 7.2). Further to the total copper concentration, the mean aggregate size is shown as a comparison on Figure 7.2f.

7.3.5 Total and extractable concentration of copper in soil

The total copper concentration was also confirmed in the soil exposures. Since nematodes live in the soil porewater, the water available fraction was also assessed for total copper concentration (Figure 7.3). The control soil contained a background Cu concentration of (mean ± SEM, *n* = 8): 2.18 ± 0.04 mg Cu kg^{-1} dw dw, and of this

total Cu, 5.37 ± 1.8 % was water extractable. Exposure to $CuSO_4$ resulted in an expected increase in total soil Cu concentrations that were close to the nominal values (> 80 %, Figure 7.3). The soils treated with CuO-bulk and CuO-core were also close to nominal (> 80 %, Figure 7.3). The soils treated with the coated CuO ENMs did not show the same measured total Cu concentrations. Instead, the amount of Cu measured in the soil varied depending on the type of ENM coating, as a result of coatings having different masses (Figure 3.1, discussed in detail in Chapter 3 and Chapter 2.5). Despite the differences, the amount of Cu in all cases was at least > 50 % of the nominal. Further to Figure 7.3, Table 7.4 presents the proportion of the total soil Cu concentration that was water extractable. The method did not allow the determination of intact particles in the extracts, but does show a likely substantially bioavailable fraction. There were differences between the amount of water extractable Cu (mg Cu kg^{-1} dw), which was related to the total Cu in the soil (Figure 7.3, Table 7.4). The percent water available fractions were similar between the different ENM exposures, although some differences remained, mainly CuO-COOH ENMs being most available in the water compared to the rest of the treatments in wet dosed soils (Table 7.4). In the dry dosed soils, however, the CuO-bulk was found to be most water available compared to the rest of the treatments (excluding the control). There was a strong positive correlation between total copper in soil and water extractable copper (r_s = 0.8, P < 0.05, Spearman's).

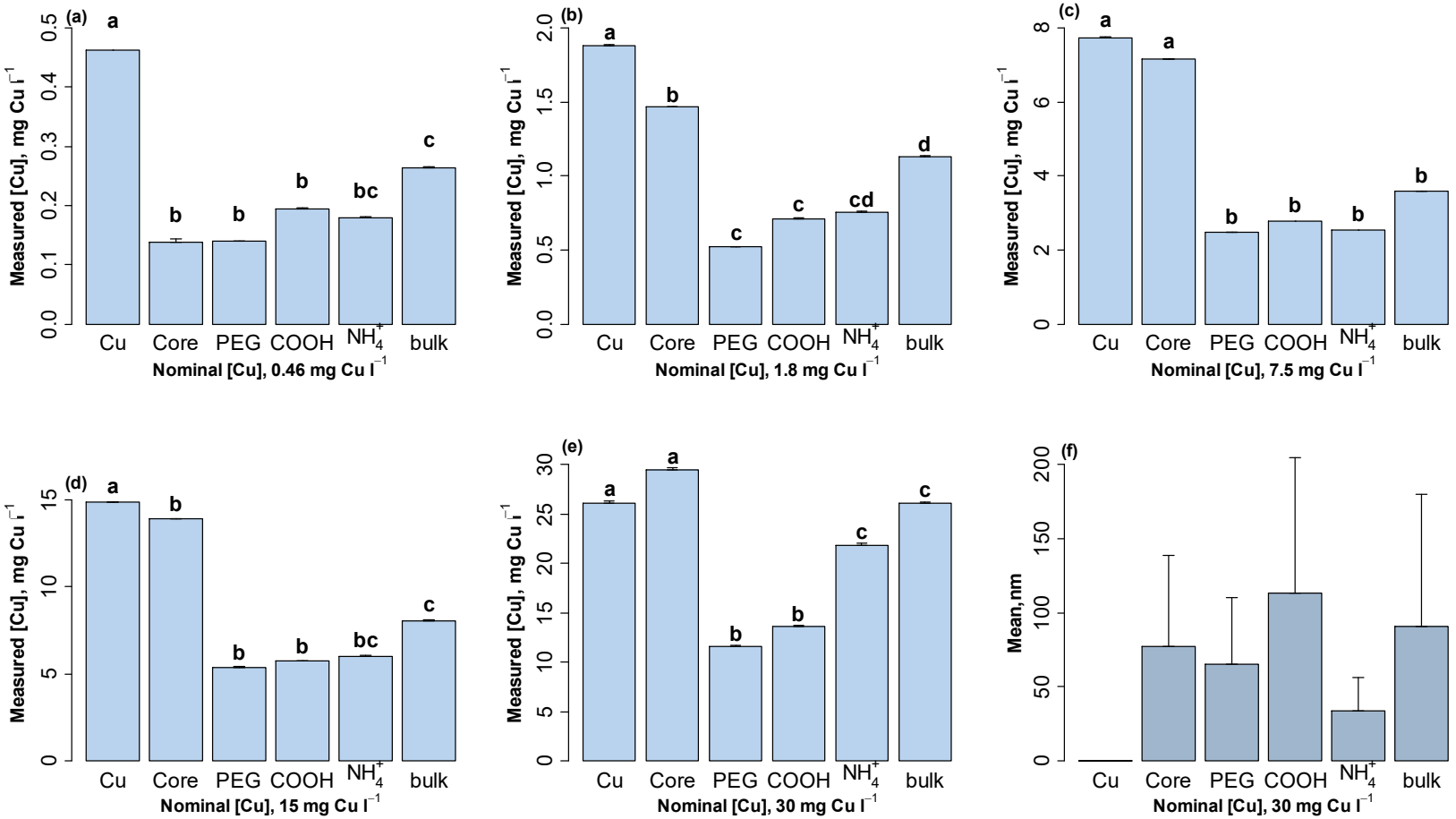

Figure 7.2 (a) – (e) Total measured [Cu] in all K-medium exposures at nominal concentrations 0.46, 1.8, 7.5, 15, 30 mg Cu l^{-1}, data presented as mean + SEM (n = 2) in mg Cu l^{-1}. (f) Mean nanoparticle diameter as measured by NTA in the 30 mg Cu l^{-1} exposures, data presented as mean + SD (instrument default). Different letters denote statistically significant treatments ($P < 0.05$, ANOVA, Tukey HSD).

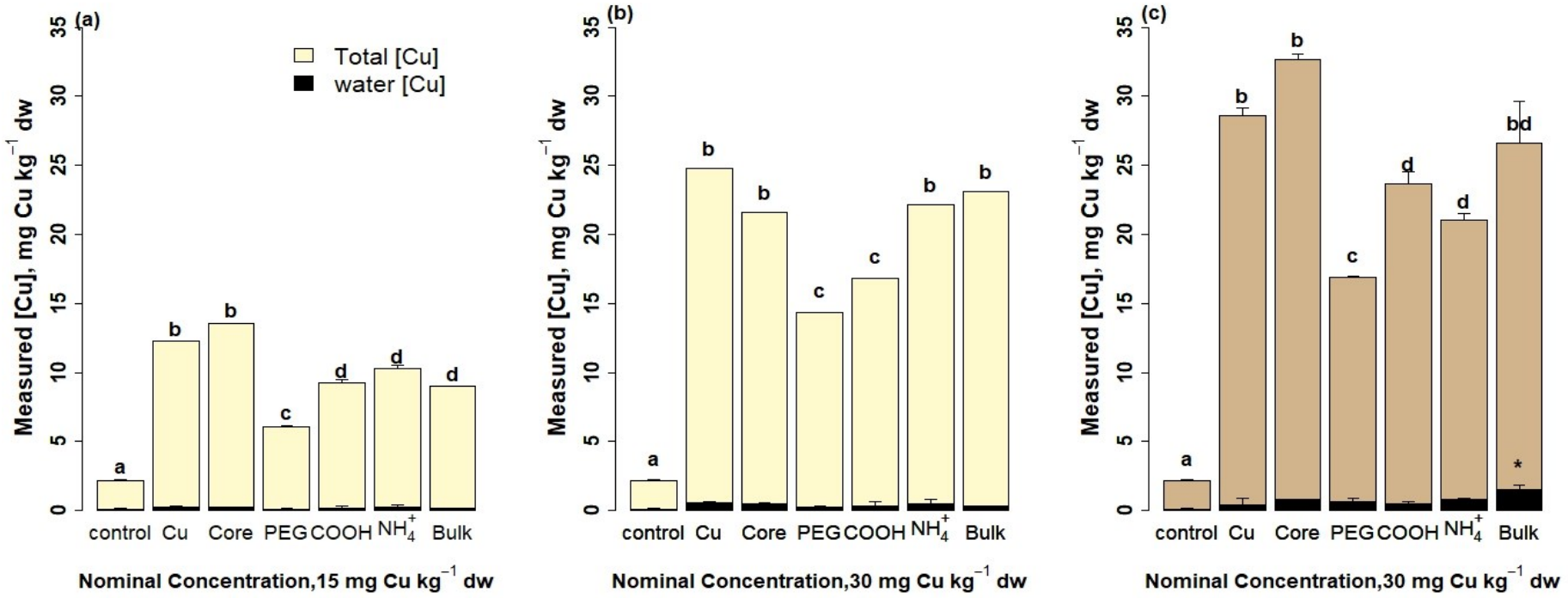

Figure 7.3 Total and water extractable [Cu] in the experimental soil at a nominal concentration of (a) 15 mg Cu kg^{-1} dw and (b) 30 mg Cu kg^{-1} dw wet mixed into the soil and (c) 30 mg Cu kg^{-1} dw dry mixed into the soil. Data presented as mean ± SEM (n = 2, duplicate technical replicates of each replicate). Different letters denote statistically significant differences between the total [Cu] in the soil ($P < 0.05$, ANOVA, Tukey HSD). Asterisks denotes a statistically significant difference between the treatment and the rest ($P < 0.05$, ANOVA, Tukey HSD).

Table 7.4 Water extractable [Cu] in experimental soils with percent water extractable [Cu] of the measured total [Cu] (Figure 3) in the *C. elegans* experiment.

Treatment	Nominal [Cu]					
	15 mg Cu kg^{-1} dw		30 mg Cu kg^{-1} dw		30 mg Cu kg^{-1} dw*	
	Water extractable [Cu]	Water extract-able [Cu] % of measured	Water extractable [Cu]	Water extract-able [Cu] % of measured	Water extractable [Cu]	Water extract-able [Cu] % of measured
	mg Cu kg^{-1} dw	***%***	***mg Cu kg^{-1} dw***	***%***	***mg Cu kg^{-1} dw***	***%***
Control	0.1 ± 0.0.^{a}	5.37 ± 1.8^{a}				
$CuSO_4$	0.27 ± 0.05^{b}	2 ± 0.43^{b}	0.53 ± 0.1^{b}	2 ± 0.48^{b}	0.89 ± 0^{b}	3.16 ± 0^{b}
Core	0.26 ± 0.01^{b}	1.89 ± 0.05^{b}	0.51 ± 0.01^{b}	2.38 ± 0.07^{b}	0.82 ± 0.01^{b}	2.49 ± 0.01^{c}
PEG	0.13 ± 0.03^{c}	2.08 ± 0.28^{b}	0.25 ± 0.03^{c}	1.77 ± 0.24^{b}	0.68 ± 0.22^{b}	3.98 ± 1.25^{bcd}
COOH	0.32 ± 0^{d}	3.35 ± 0^{d}	0.63 ± 0^{d}	3.76 ± 0^{c}	0.65 ± 0.03^{b}	2.75 ± 0.03^{c}
NH_4^+	0.26 ± 0.13^{bcd}	2.46 ± 1.19^{bd}	0.52 ± 0.25^{bcd}	2.33 ± 1.33^{bc}	0.82 ± 0.05^{b}	3.92 ± 0.34^{d}
Bulk	0.17 ± 0^{c}	1.91 ± 0^{b}	0.32 ± 0^{c}	1.48 ± 0^{d}	1.5 ± 0.32^{c}	5.55 ± 0.55^{e}

All total or extractable [Cu] data presented as mean ± SEM (n = 3, triplicate analysis of the batch soil sample). Different letters denote groups that are statistically significantly different ($P < 0.05$, ANOVA, Tukey HSD).

7.3.6 Range finding experiment

The control juveniles survived the 24 h period of the range finding experiment, while the exposure to $CuSO_4$, CuO-bulk and all CuO ENMs resulted in a 100 % mortality in all exposure concentrations. This data was used to design an experiment that would reach a 100 % mortality, thus no reproduction, as well as provide sublethal concentrations.

7.3.7 Reproduction of *C. elegans* in K-medium

Having established the results from the range finding experiment, the reproduction experiment was carried out with a range of concentrations. The controls produced an expected number of juveniles: > 30 per nematode (Figure 7.4a) with a coefficient of variation < 10 %. The nematodes exposed to $CuSO_4$ also produced ≥ 30 juveniles per nematode in the nominal 0.46, 1.8 and 7.5 mg Cu l^{-1} exposures. The reproduction was significantly reduced in the nominal 15 and 30 mg Cu l^{-1} exposures, but there was no difference between the number of juveniles in the two exposures (P < 0.05, Kruskal-Wallis, Dunn's test). The CuO bulk exposures followed a similar pattern to $CuSO_4$ (Figure 7.4). In the CuO ENM exposures, the effects were only evident in the nominal 15 and 30 mg Cu l^{-1} exposures, with no coating specific effects (Figure 7.4e and f). Differences between the ENMs, $CuSO_4$ and CuO-bulk were clearer at the nominal 30 mg Cu l^{-1}. Although there was no clear dose response in all of the treatments, the EC_{50} values were estimated from plotting the raw data and fitting it with a sigmoid curve, the EC_{50} values were estimated to be 25, 20.5, 16.2, 20.2, 19.5, 19.2 mg Cu l^{-1} as $CuSO_4$, core, PEG, COOH, NH_4^+, bulk CuO ENMs.

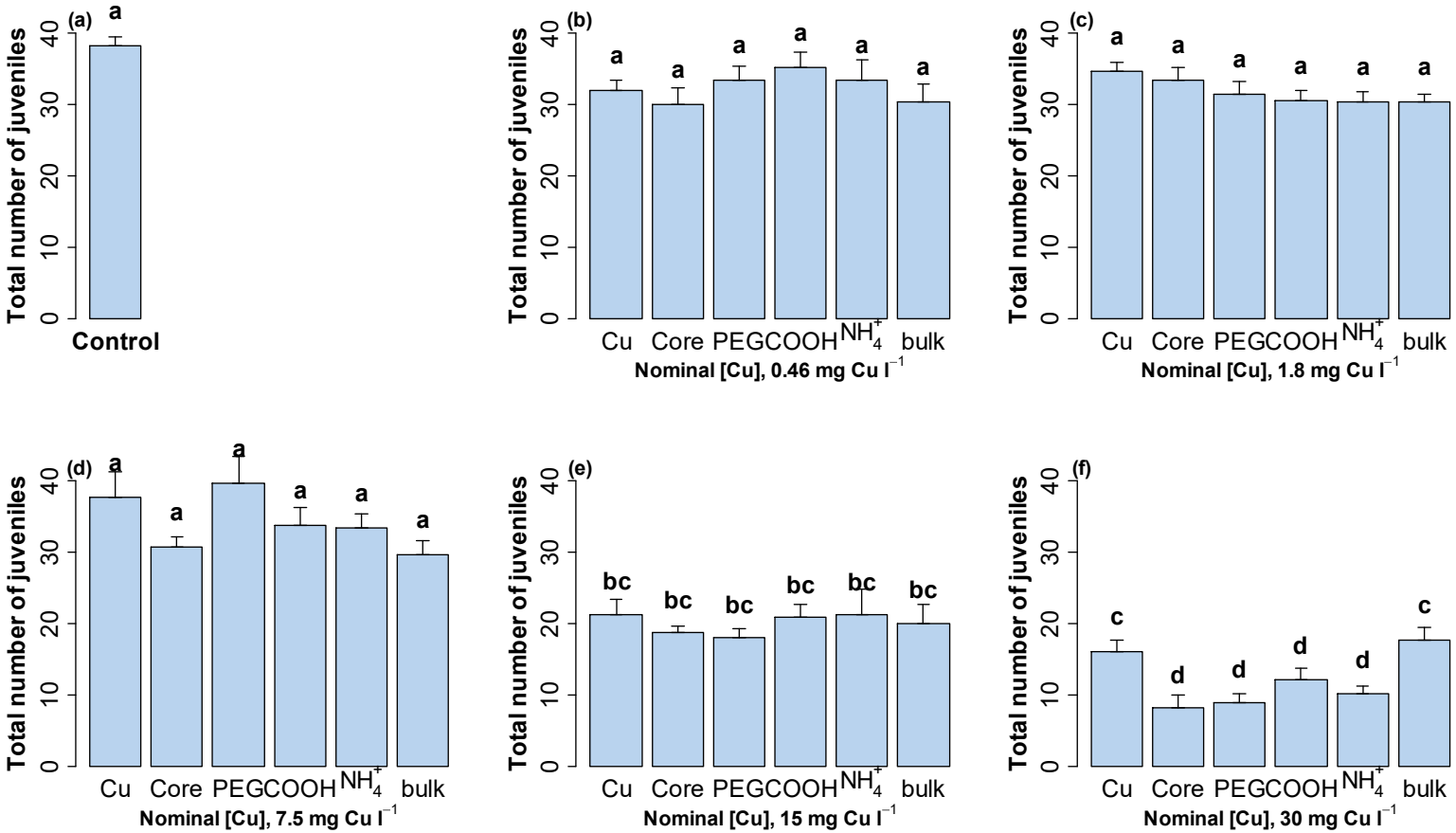

Figure 7.4 Total number of juveniles (*C. elegans*) produced by one adult hermaphodite in a 72 h reproduction test. (a) Control treatment is the only bar in the figure and contained <0.01 (LOD) mg Cu l^{-1} (b)-(f) Total number of juveniles produced per individual nematode in each treatment after 72 h period in different treatments at nominal concentrations of Cu. Results presented as mean + SEM (n = 60, controls and n = 6, Cu or CuO ENM exposures). Different letters across the panels denote statistically significant treatments (P < 0.05, Kruskal-Wallis, Dunn's test).

7.3.8 Juvenile locomotion and apparent adhesion of CuO ENM and bulk particles.

Furthermore, to assess the viability of the juveniles produced, their locomotion was noted throughout the experiment. The controls and most ENM and bulk exposures, showed no difference in juvenile swimming. However, the juveniles were observed to be immobilised (not responding to stimuli) in $CuSO_4$ and Core-CuO treatments at 30 mg Cu l^{-1}after the 72-h period (Table 7.5). Moreover, upon visual inspection the adhesion of ENM aggregates to the nematode surface was scored. The nematodes were almost immediately covered in numerous aggregates after being introduced to the particle treatments. However, over time they could release themselves from some, creating more aggregates with their movement. Out of the exposures, all ENMs and bulk CuO particle aggregates were found to be stuck on juvenile or adult surface. It was not possible to clearly image this phenomenon but some investigative images are shown in Figure 7.5 of the control, CuO-COOH and CuO-bulk exposed nematodes at the nominal 30 mg Cu l^{-1} concentration. The images also show the potential of ENM or bulk uptake by nematodes.

Table 7.5 The scores of juvenile *C. elegans* locomotion / swimming and apparent particle/aggregate adhesion to the adult nematodes and hatched juveniles exposures to $CuSO_4$, CuO ENMs and CuO-bulk at different nominal concentrations.

Nominal concentration	**Control**	**$CuSO_4$**	**Core**		**PEG**		**COOH**		**NH_4^+**		**Bulk**	
	JS	JS	JS	PA	JS	PA	JS	PA	JS	PA	JS	PA
0.46	✓	✓	✓	×	✓	×	✓	×	✓	×	✓	×
1.8	✓	✓	✓	×	✓	×	✓	×	✓	×	✓	×
7.5	✓	✓	✓	×	✓	×	✓	✓	✓	✓	✓	✓
15	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓
30	✓	×	×	✓	✓	✓	✓	✓	✓	✓	✓	✓

JS – juvenile swimming; PA – apparent particle (either ENM or bulk aggregate) adhesion to juveniles and/or adult. ✓ yes; × no.

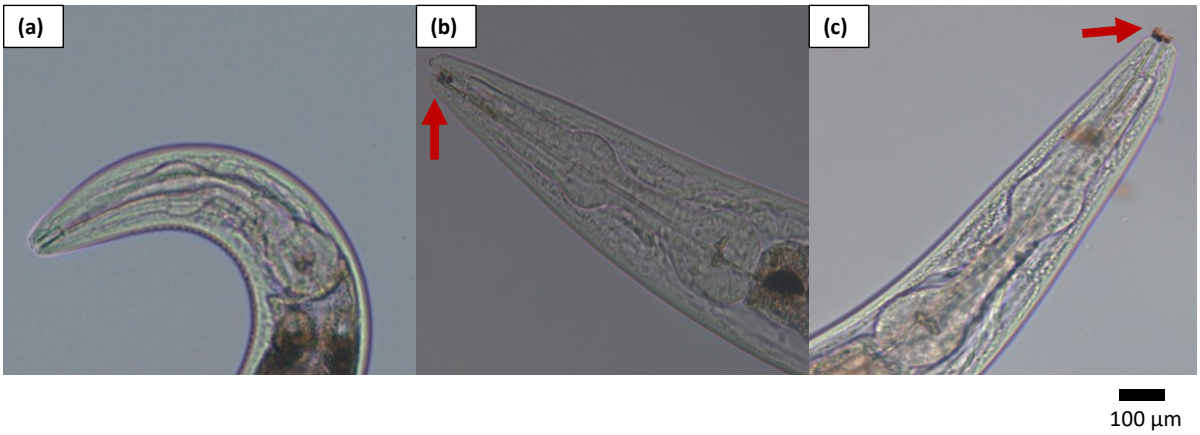

Figure 7.5 Example images of adult nematodes (*C. elegans*) from (a) controls, (b) CuO COOH and (c) CuO-bulk exposures at nominal 30 mg Cu l^{-1} concentration after approximately 24 - 30 h of exposure. Note, the arrows point to what potentially was an ENM or bulk particle aggregate.

7.3.9 Reproduction of *C. elegans* in Lufa 2.2 soil: wet and dry spiked

In general, reproduction was higher in the Lufa 2.2 soil when compared to the potassium medium controls above, as well as the additional K-medium control included in the soil experiment (Figure 7.6a; the coefficient of variation of reproduction in controls < 10 %). There were no effects on reproduction in the nominal 15 mg Cu kg^{-1} dw exposures (dosed as 15 mg Cu l^{-1} stocks). Reproduction was only decreased in the CuO-COOH and -NH_4 wet mixed soils at 30 mg Cu kg^{-1}dw (dosed as 30 mg Cu l^{-1} stocks). Reproduction was reduced in all dry mixed soils (nominal concentration at 30 mg Cu kg^{-1} dw) of the ENMs and CuO-bulk treatments, although not statistically significantly in the $CuSO_4$ treated soils. There was a statistically significant negative correlation between water extractable Cu / CuO particles and reproduction (r_s = -0.7, P < 0.05 Spearman's), but no relationship between the total amount of measured Cu and reproduction.

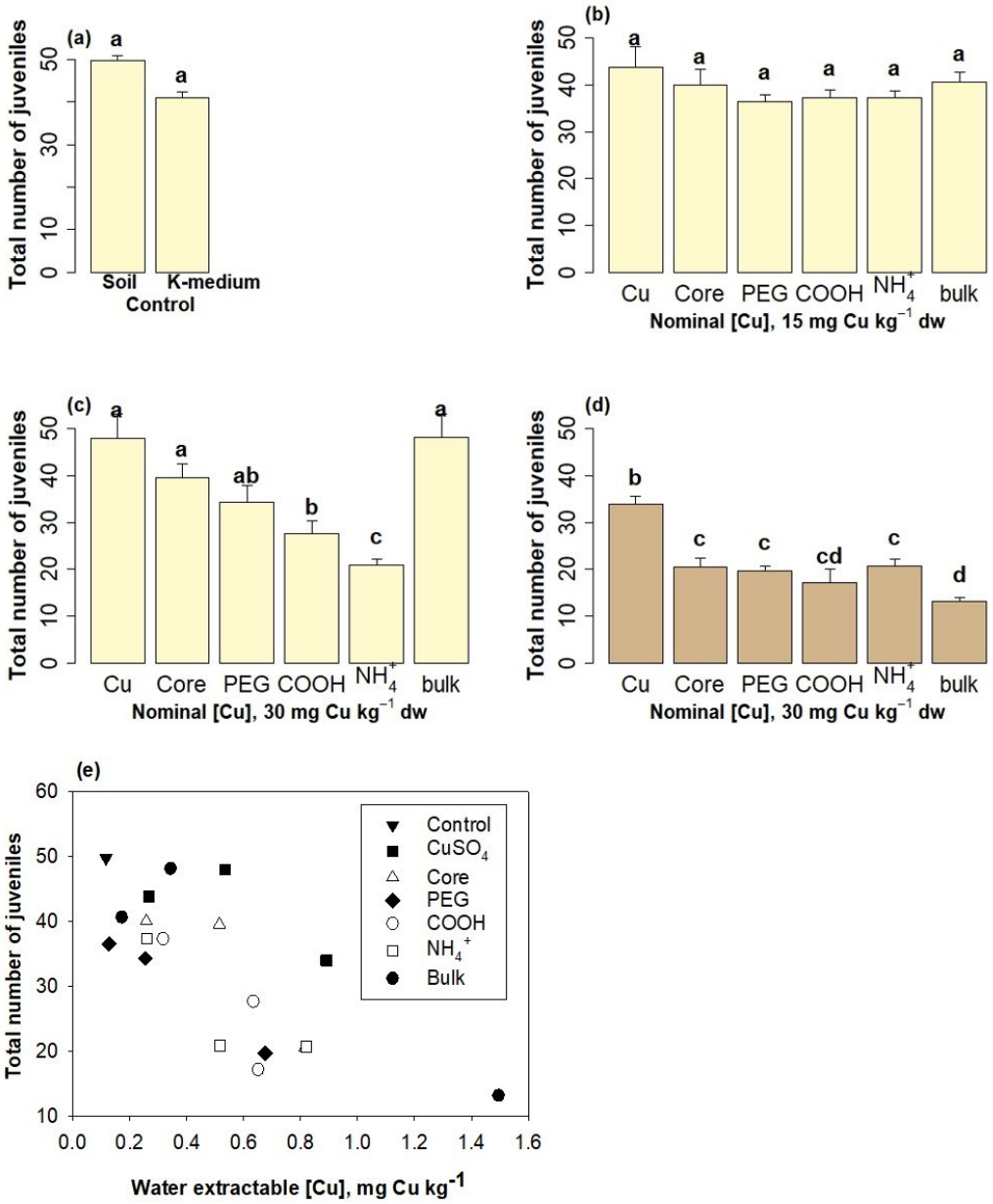

Figure 7.6 Total number of juveniles (*C. elegans*) produced inthe soil experiment. (a) Total number of juveniles in the controls, control treatment is the first bar and contained 2 mg Cu kg^{-1}, K-medium control is the second bar and contained <0.01 (LOD) mg Cu l^{-1}. (b) Total number of juveniles in the nominal 15 mg Cu kg^{-1} dw wet spiked soils. (c) 30 mg Cu kg^{-1} dw wet spiked soils; (d) 30 mg Cu kg^{-1} dw dry spiked soils. All results presented as mean + SEM (*n* = 6 Cu or CuO ENM treatments, *n* = 48 controls). Different letters denote statistically significantly different treatments across panels ($P < 0.05$, Kruskal-Wallis, Dunn's test). Total number of *C. elegans* juveniles produced in relation to water extractable [Cu] in soils (e).

7.4 Discussion

No clear coating effect on nematode reproduction in the liquid medium was evident, although the results indicate that CuO ENMs are more hazardous to nematodes than $CuSO_4$ and CuO-bulk. In the soil experiment, a coating effect is apparent in the wet dosed soils, while in the dry mixed soils the coating effect is less clear, although a trend is present, and CuO ENMs are more hazardous than $CuSO_4$, but less hazardous than CuO-bulk.

7.4.1 ENM behaviour and total Cu concentration in exposure media (K-medium)

The ENMs were visibly forming aggregates in the potassium medium, which was also confirmed by NTA analysis (Table 7.1). The high salt concentration in the exposure medium was likely the cause of the large aggregates, as reported in previous studies (Meyer et al., 2010; Tsyusko et al., 2012b). While aggregation is not usually favourable in nanotoxicology, and a plethora of methods have been developed to maintain the materials in solution, their most likely fate in the environment is that the ENMs will aggregate. In the present study, the aggregates settled in the bottom of the exposure wells, where the nematodes were also settling. This created a direct exposure and interaction between the particle aggregates, feed (bacteria) and the test organisms, such as described in previous studies (Meyer et al., 2010). Dissolution of Cu from the CuO ENMs /bulk was coating dependent (Figure 7.1).

7.4.2 Total and extractable Cu in the exposure soil

The control soil contained an expected background concentration of Cu of which 5 % was water extractable, in line with the previous studies (Chapters 3 – 5). Furthermore, the $CuSO_4$ dosing was > 80 % of the nominal in all cases (Figure 7.3a and b), but the water extractable fraction was higher than previous work (Chapters 3-4). This may be because the K-medium contains cations that may have been competing with dissolved Cu for sorption sites on soil particles (Donkin and Dusenbury, 1993), which may lead to higher bioavailability of Cu. Interestingly, this higher availability was not evident when ENMs were applied wet to the soil, but dry mixed soils have ~1 - 2 % more Cu / CuO ENMs water extractable, which is also coating dependent (e.g., CuO-COOH materials do not follow this trend, Table 7.4). The most Cu/ CuO particles were water extractable in the dry mixed soils dosed with CuO-bulk, which was around more than double than in the wet dosed soils (Table 7.4). This may potentially be due to the increased aggregation in the stock solutions that aggregated further when mixed in with the soils. It is likely that the water extracts here presented a large quantity of intact particles, since the dissolution of Cu was very low from the CuO bulk materials. The ENMs also follow a similar phenomenon, although, it is expected a mixture of dissolved Cu and intact particles were present, at least in the coated variants since dissolution was higher in K-medium (Figure 7.1).

7.4.3 ENM effects on *C. elegans* in K-medium

The reproduction in the controls met the ISO10872 (2010) criteria of 30 > juveniles per adult. However, this is a lower number of total juveniles than other studies, since an older stage of the nematode (>48 h) was used than in the ISO TG. But multiple

repeats of controls (*n* = 60) and several preliminary tests in K-medium resulted in a similar reproductive output in the liquid medium (~40 juveniles by 72 hours). This may be due to an inherent developed difference in the in-house culture, which may have led to a lower reproductive output of all nematodes, as well as the K-medium being a more stressful environment thus reducing the number of offspring produced. Nonetheless, the nematodes in the controls and lower exposures looked healthy, developed into egg laying adults, and were consistently moving around laying eggs that developed into juveniles by 72 hours. Overall, the results do not indicate a clear dose response as expected based on the studies in the literature of $CuSO_4$ (Boyd et al., 2003), since dissolved Cu was not found as toxic in the current study (effects seen at > 15 mg Cu l^{-1}), which may be accounted to natural variation. Another difference between this study and Boyd et al. (2003), was that cholesterol was added into the K-medium in this study, since it is vital for *C. elegans,* and they are not able to synthesise it (Lagido et al., 2009; Matyash et al., 2001).

There was no clear coating effect on the reproductive success of the nematodes (Figure 7.4), but overall, the ENMs were equally (15 mg Cu l^{-1}) or slightly more toxic (30 mg Cu l^{-1}) than the $CuSO_4$ and CuO-bulk to nematode's reproductive output (number of juveniles produced), regardless of differences in total Cu concentration in the media, as well as the differences in dissolution (Figure 7.1 and Figure 7.2d and e). The latter may indicate an effect not related to amount of Cu or Cu release in the ENM and bulk exposures, but related to the presence of large aggregates (discussed below), which may have caused further stress. Compared to studies with other ENMs in the same exposure media, CuO ENMs used in this study are less toxic than Ag ENMs (Roh et al., 2009) and differently sized ZnO ENMs (Khare et al., 2015).

To assess the fitness of the juveniles, their locomotion was monitored. Juveniles in the controls were actively swimming throughout the exposure. Out of all the exposures, juveniles were only immobilised in the $CuSO_4$ and CuO-core exposures at the 30 mg Cu l^{-1} concentrations (Table 7.5). Although the number of juveniles was reduced in all Cu and CuO ENM/ bulk exposures, the Cu-salt and core ENMs were more potent in toxicity (survival effects) than the rest of the materials to juveniles. This may be due to the higher Cu stress in the media, although CuO-core ENMs were shown not to release much dissolved Cu (Figure 7.1). Also, these treatments did not immediately induce mortality / immobility in the adult nematodes, suggesting the juveniles were more sensitive to $CuSO_4$ and CuO-core ENMs. Recently, Bicho et al. (2017) found juveniles of *Enchytraeus crypticus* were more sensitive to CuO ENMs than $CuCl_2$. The higher toxicity of CuO-core materials compared to the coated and bulk CuO may be due to the presence of smaller aggregates, as well as more particles in the nano-scale (Table 7.1).

In the beginning of the experiment, as soon as the nematode was added to the exposures, it was clear that the aggregates of all CuO ENM and CuO-bulk materials were adhering to the nematode surface, which continued until the end of the experiment (Table 7.5). This was less evident in the CuO-core exposures, where in general the ENMs did not form as large aggregates, and more particles were expected to be in the nanoscale (NTA CuO-core mode = 14 nm, Table 7.1 and 7.5). The coated materials and CuO bulk had a higher tendency to adhere to nematodes at lower concentrations, and it also appeared that nematodes were ingesting the particles (Figure 7.5), with no difference between the positively or negatively charged coatings. Uptake of ENMs has been seen before with CuO ENMs (Gao et al., 2008) and others, e.g. Au and Ag ENMs, and CdTe QDs (Gonzalez-Moragas et al., 2017;

Meyer et al., 2010; Qu et al., 2015). However, in this study, the results are only preliminary and the accumulation of ENMs via ingestion requires further study.

7.4.4 ENM effects on *C. elegans* reproduction in soil: different effects respective of dosing method

In the soil experiment, the reproduction in the control animals met the ISO 10872 (2010) criteria of 30 > juveniles per adult. Furthermore, similar numbers have been reported in Hoss et al. (2012) in soil experiments with nematodes. The overall reproduction in the soil was higher than in the K-medium, which may be due to a more ion rich medium for nematode health. $CuSO_4$ was less hazardous to nematodes in the wet mixed soils than in the dry mixed soil which may be related to the higher concentration of water extractable Cu. The ENMs, except NH_4^+ coated, and CuO-bulk followed the same trend as $CuSO_4$ exposures which potentially is related to the higher number of Cu / CuO particles in the water extracts. A coating specific effect was apparent in the CuO-NH_4^+ exposures, since despite the differences in the water extractable fractions of Cu / CuO particles between the wet and dry dosed soils, the reproductive effects were very similar (Table 7.4, Figure 7.6). The dosing of the soils and the difference in the reproductive effects requires further study, since the results here suggest the outcomes are coating and material specific (CuO-bulk vs nano). This may lead to an over / underestimation of the risks posed.

7.4.5 Differences between K-medium vs soil

Overall, these results are in line with other studies that have indicated that the soil medium is more protective to nematodes based on a comparison of the nominal and measured total concentrations and that the wet dosed soils were spiked with stock solutions of 15 and 30 mg Cu l^{-1}, the same as were used in the liquid experiments (Figure 3 vs Figure 4; Donkin and Dusenbery, 1993; Boyd et al., 2003a). However, when the water extractable concentration of Cu is considered the soil media induced effects at a lower concertation of mg Cu l^{-1}, In K-medium the ENMs are readily in contact with the nematodes even as aggregates, and dissolved Cu from the particles would be freely available in the media. If dissolution occurred in soils, the dissolved Cu can be adsorbed onto organic matter or other soil particles making is less bioavailable. Most of the experimental work with ENMs and nematodes have been done in liquid medium, as it allows the full characterisation of the ENMs, and a multitude of endpoints can be analysed. However, if the first tier of acute and reproductive toxicity data were required the tests in soils would be more realistic, and less waste would be produced compared to the earthworm toxicity tests. Advances are being made in improving the soil toxicity test with nematodes (Huguier et al., 2013; Hoss et al., 2012) and new methods for nematode separation are being developed to increase the number of endpoints assessed (Kim et al., 2015).

7.4.6 Conclusions

Taken together, the results suggest that $CuSO_4$ and ENMs are more toxic in liquid medium than in soil based on total measured concentrations and furthermore the toxicity in soil depends on the dosing method and coating. The ranking of toxicity for

$CuSO_4$ and CuO ENMs in different media is wet mixed soils < dry mixed soils < liquid medium. Interestingly, the CuO bulk material does not follow this trend, since the ranking is wet mixed soils < liquid medium < dry mixed soils. The results of this study raise two issues: first, testing in liquid media might overestimate risks, and second, the method of dosing may influence the outcomes in the soil tests. The first problem may be solved by estimating the factor by which the soil is more protective, although for this, the second issue, i.e., method of dosing, needs to be standardised to represent the most likely route of exposure, and more studies need to be carried out.

Chapter 8. General Discussion and Conclusions

"*The game of science is, in principle, without end. He who decides one day that scientific statements do not call for any further test, and that they can be regarded as finally verified, retires from the game*."

-Karl Popper (2008)

8.1 Key findings

The results presented in Chapters 3 – 7 provide new insight into the different toxic effects of the various coated CuO and CdTe QD ENMs. The main hypothesis of the research project was that the coating will determine the toxicity because it is what the organism “sees” first. This was shown in the confines of this thesis; but the overall results suggest the reason for coating dependent toxicity is that it allows access to the toxic core of the ENMs and / or increases its environmental persistence. Broadly, the COOH-coated materials in both the CuO ENM (Chapter 3) and CdTe QD (Chapter 5) exposures were the most toxic out of the materials tested in earthworms in fresh soil experiments, though, concerning different endpoints. This changed after a period of ageing, where the NH_4^+ coated materials were more toxic. In general, CuO ENMs were less hazardous than $CuSO_4$ to earthworms (Chapter 3). While in contrast, CdTe QDs were more hazardous than CdTe QD bulk in earthworms.

The CuO ENMs did not bioaccumulate in earthworms (Chapter 4) and the bioaccumulation factors were on similar with those for $CuSO_4$ (~ 0.3), except in the case of PEG-coated ENMs. The latter, however, may have been due to the overall lower concentration of Cu in soils, though, an ENM specific effect cannot be excluded (Chapter 5).

The incidental plant growth that occurred whilst ageing the soils for CuO ENM and CdTe QD exposures, provided information on ENM hazard on seed germination (Chapter 6). With the incidental nature of the experiment in mind, plants showed a greater sensitivity to $CuSO_4$ than CuO ENMs (coated and uncoated) suggesting the current hazard assessment in place for dissolved Cu would cover the risks of ENMs.

In contrast, plants were more sensitive to CdTe QD ENMs, than the CdTe-bulk QDs, suggesting the ENMs form is more hazardous to plants.

In exposures with *C. elegans* (Chapter 7), there was no specific coating effect in the liquid exposures, although, coating effect was clear in soil exposures which depended on the method of dosing (wet *vs* dry). This gives reason to suggest the toxicity was influenced by how the coating reacted in different types of media it supports the importance of considering the media in toxicological studies. The hazard ranking varied in *C. elegans* media, where ENMs were only more hazardous than $CuSO_4$ or CuO-bulk in wet dosed soils (ENMs added to the soil as stock solutions) and slightly more hazardous in liquid media.

8.2 Confirming the exposure in soil and earthworms

Total concentration of metal(loid)s were measured by ICP-MS or -OES to confirm the exposure. This method is useful to assess the effects of ENMs on a total metal basis that also enables the comparison with results on metal toxicity in organisms. Additionally, it is a requirement to confirm the exposure in regulatory tests, where methods exist, (OECD, 2010). Based on traditional metal toxicology, total metal concentrations can be considered poor predictors of toxicity, since it is acknowledged that only a fraction of the total concentration is bioavailable (OECD, 2016). Therefore, water and 0.1M HCl acid extractable fractions as well as the total concentrations in earthworms were measured. However, the limitation with these measurements were, clearly, the lack of evidence whether earthworms were in contact or, indeed, accumulated intact ENMs.

It may be speculated, based on the known information on dissolution of the CuO ENMs (low µg Cu l^{-1} range, Table 2.1), that earthworms faced a mixture of dissolved Cu and intact / aggregated CuO ENMs in soils. Since if dissolution occurred in soil, some of the released copper was potentially readily adsorbed onto soil particles. However, dissolution may have also occurred in the earthworm gut which would have resulted in a direct exposure. Furthermore, if the ENMs were taken up by endocytosis (such phenomena have been noted in Diez-Ortiz et al., 2015 with Ag ENMs), ENMs could have dissolved within the tissues.

In CdTe QD studies, the results for concentration ratios (Cd to Te) aided assessment of accumulation (Chapter 5). It is possible, CdTe-bulk QDs were taken up as intact QDS (or indeed, Cd and Te were accumulated at the same rate by earthworms). In contrast, mostly Cd was accumulated in coated CdTe-QD ENM exposures. This may suggest the either: a) Te from nano-QDs was less bioavailable, or b) it was excreted more rapidly (than Te from CdTe-bulk QDs). Other studies with QDs have generally shown higher accumulation of Cd (in mussels exposed to uncoated CdTe, Peyrot et al., 2009).

8.3 Does the coating of the engineered nanomaterial determine biological effects?

There were some clear differences in effects between the different coated ENMs in all experiments with the earthworms and nematodes in soils that was not explained by the differences in total concentration of Cu / Cd and Te. Overall, the CuO-COOH material was the most acutely toxic to earthworms in fresh soil, and part of this toxicity may be due to faster dissolved metal release from the ENM (Table 2.1; Figure 3.2). For long term, sub-lethal effects on earthworms, the CdTe QD-COOH

had the most impact on reproduction in the fresh soil, while it did not induce the adverse avoidance behaviour as seen in the CuO-COOH exposures (Chapter 3). This implies the -COOH coating on different cores does not lead to the same biological effects. Moreover, it does not fit the idea of chemoreceptors sensing the coating first (-COOH in this case). It was rather the leaching metal (Cu) from the core that dominated the chemoreceptor responses in the earthworm. In keeping with this notion, sodium pump activity in the earthworms was negatively correlated with the Cu accumulation in the earthworms, suggesting ionoregulatory toxicity rather than oxidative stress as an important mechanism of toxicity. This provides further evidence in the effects being mostly due to dissolved Cu.

In contrast to the CuO ENM exposures, the dissolution as well as water extractability of Cd or Te from were highest in the CdTe-NH_4^+ QDs (Table 5.1, Figure 5.2.). This does not fit the observations with CuO ENMs. Although the exact details of the chemistry of the coatings were not provided, hypothetically the ENM synthesis could have resulted in a more stable -COOH ligand on the surface of CdTe QDs than on the surface of CuO ENMs, or, indeed, there may have another confounding actor that determined the stability.

Based on the results from the *C. elegans* exposures in different media (Chapter 7) another possible speculation may be suggested with regards to the coating effect. The coating determined the persistence or reactivity of intact ENMs in solution. The reactivity in that when ENMs were dispersed on liquid first some dissolution may have occurred (Figure 7.1, Chapter 7). Therefore, if the stocks were then added to the soils, the dissolved Cu may have more readily been adsorbed to soil particles rendering it less bioavailable. In the dry mixed soils (ENMs added to the soil as dry powder), the results were similar to earthworm exposures, where CuO-COOH was

the most toxic of the ENMs tested, although toxicity was close to the other ENMs and, interestingly, CuO-bulk (micron-size CuO ENM analogue) was most potent of all chemicals tested. It was not expected the CuO-bulk would be acutely toxic, and a preliminary experiment with CuO-bulk did not result in acute effects in earthworms (data not shown, based on a single replicate of soil dosed with CuO ENMs, carried out in the same conditions as Chapter 3).

On reflection, a pure coating control in each experiment would have increased the robustness of the results. Although based on the literature findings of the coatings, it is unlikely the toxic effects were accounted to the functional groups. Ammonia (NH_3) is known to be highly toxic, however, ammonium is of low toxicity to earthworms (NH_4^+ acute toxicity in concentrations > 2 g kg^{-1}, Hughes et al., 2008). Additionally, carboxyl groups (COOH) are produced naturally in the environment (e.g. by plants and microorganisms) as well as carboxyl groups are at the end of amino acids, known as the “C-terminus”. Carboxylic acids have been shown toxic to bacteria (Vázquez et al., 2011), although it depends on the pKa value (i.e. dissociation constant). However, in experiments presented here (Chapter 3, 5) it is unlikely COOH exerted toxic effects since there were no similar biological effects between, CuO-COOH and CdTe-COOH exposed earthworms. Polyethylene glycol has not been considered toxic (in mammals, MAK, 2012), although PEG on its own has been shown to cause osmotic pressure in cells, leading to a reduction in the Na^+/K^+-ATPase (sodium pump) activity (*in vitro* tests, Esmann et al., 2008). This was not clear in earthworm studies, since there was no concentration dependent effects of PEG on the sodium pump activity, suggesting the effects were not related to the coating of the ENMs (Figure 3.5 and Figure 5.5).

8.4 Does ageing the soil decrease toxic effects?

Interestingly, the effects of exposure to aged soils were different to that of ENMs in fresh soils. Notably, the toxicity ranking of the ENM coatings was different in aged soils, with the CuO-NH_4^+ coating being the most acutely toxic for the CuO ENM and CdTe QD exposures. Consequently, in the reproduction test, the CdTe QDs-NH_4^+ was more toxic in aged soil, compared to the CdTe QDs-COOH in fresh soil. The explanation of this change in the order of the coating toxicity with soil age needs further investigation. It could be related to the stability of the coatings in soil (discussed above), and effects of the coatings on some detail of soil quality or nutrient availability. Crucially, the overall hazard from the soil with soil age was material specific. After a period of aging (12 months), the CuO ENMs were less toxic to earthworms and less Cu was taken up by the worms. This suggests the Cu bioavailability from the ENMs in lower in aged soil. However, the opposite was true for the CdTe QDs which were generally more toxic and with more Cd and Te accumulation from the (6 month) aged soil. Thus, for environmental risk assessment purposes, the hazard and the ranking of materials may change with time in the soil. It also offers a complication when determining maximum loads for metals in sewage sludge, since the ENMs may initially be regarded as “inert” and not bioavailable, this can change over time as shown by this study. Ageing effects have also been shown by increasing number of researches, e.g., toxicity of Ag ENMs increases age in earthworms (Diez-Ortiz et al., 2015) as well as in springtails (McKee et al., 2017), while toxicity of ZnO ENMs in springtails decreased over time (Waalewijn-Kool et al., 2013).

8.5 The *C. elegans* test compared to the earthworm test

The earthworm test for sub-lethal effects such as reproduction is time-consuming as well as it produces a lot of waste (high volume of contaminated soil), and there may be some advantages in regulatory testing to using smaller organisms, such as nematodes instead. The *C. elegans* test in liquid K-media was readily performed in 12-well plates, and demonstrated reproductive effects, although there were no clear coating effects and a possible suggestion for effects was the mechanical and physical stress of high particulate aggregates in the media (Chapter 7). Exposure in liquid media enabled some physico-chemical characterisation of the ENMs, but it showed higher toxicity compared to natural soil to *C. elegans*. The toxicity is likely associated with more direct contact with ENMs as well as metal dissolution in the liquid compared to soil. From a hazard view point, the *C. elegans* test may give a margin of extra safety compared to the earthworm test, but this needs to be balanced against concerns about “over regulation” when using more sensitive tests (such as in liquid media). There might be scope for using the *C. elegans* in liquid media and soil as screening tool which can be followed by more select studies with earthworms. Also, a safety factor could be estimated from tests with more sensitive species, however, species sensitivity is highly variable and often the same species is not always the most sensitive to all chemicals (van Straalen and van Leeuwen, 2001). However, many researchers are pointing towards the feasibility of using *C. elegans* for such screening of ENMs (Choi et al., 2014; Gonzales-Moragas et al., 2015; Hanna et al., 2016). Choosing a single species to perform initial screening would require more research, justification and inter-laboratory testing (Höss et al., 2012). And as raised in Chapter 7, the method of dosing should be evaluated and standardised for regulatory use.

8.6 Conclusions

Taken together, the big questions asked in the beginning of the thesis were broadly answered (compare Figure 1.2 and Figure 8.2). Although the hypothesis of “the coating determines toxicity” was shown only to be partly true with many caveats to it. A revised hypothesis, based on the findings, could be “the coating influences the reactivity of the ENMs which in turn impact the toxicity”. In conclusion, the thesis has contributed novel information on the toxicity of pristine and aged CuO and CdTe QD ENM variants. Furthermore, a less studied mechanism of toxicity, ion regulation, has been explored in earthworms and shown to be influenced by exposure to metal-based ENMs. Additionally, the *C. elegans* tests offered an insight to effects in liquid and solid media and a comparison between wet and dry mixed soils. With all the above in mind, more research is still needed to develop clear conclusions if the current risk assessment of metals in soils will also be protective of metal(loid)-based ENMs.

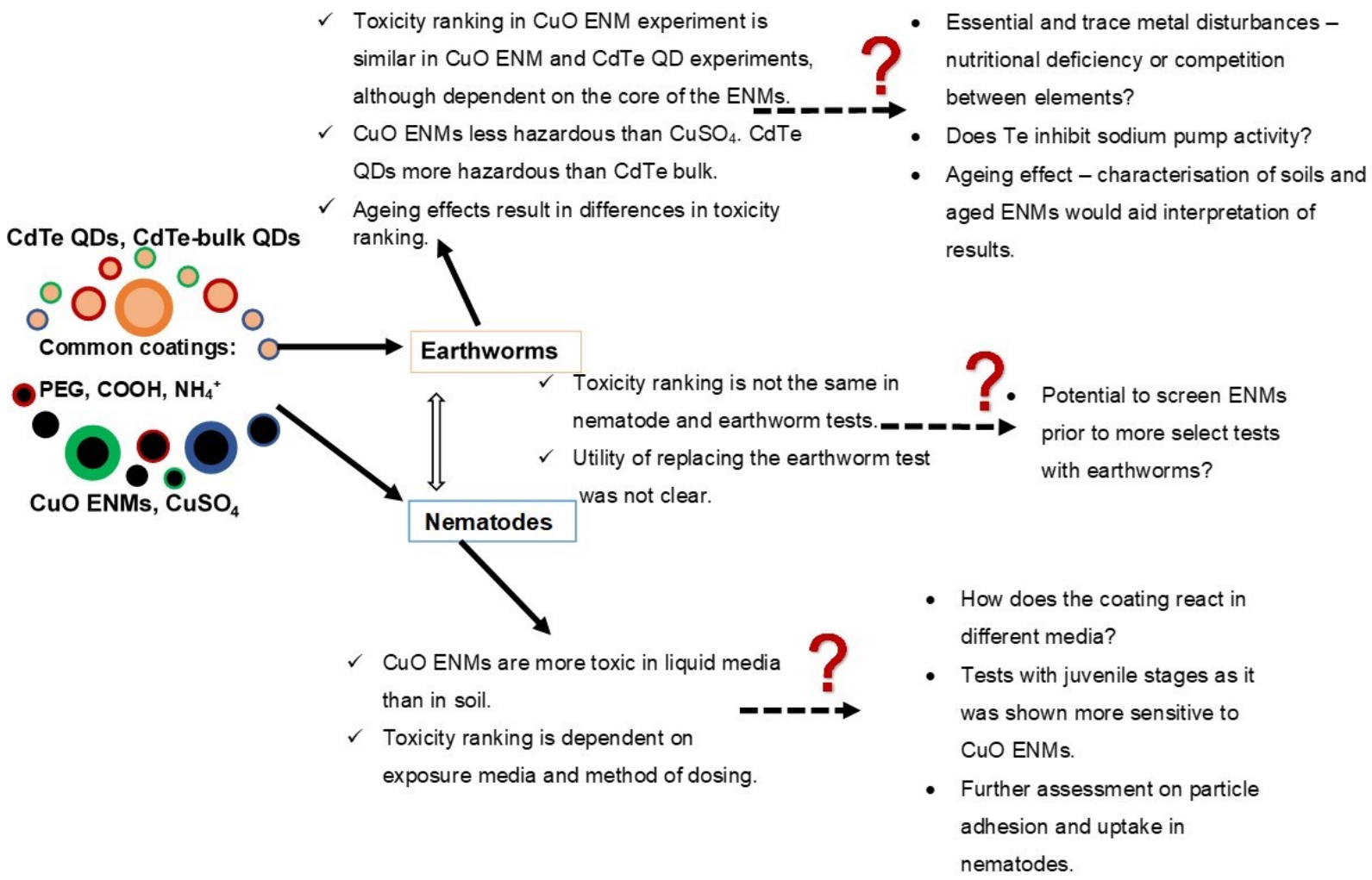

Figure 8.1 Answers to to the questions raised in the beginning of the thesis presented in Figure 1.1 and new questions and ideas that were formulated based on the results.

8.7 Future work and recommendations

The new findings have also raised several new questions and problems that warrant future research as well as potential recommendations (Figure 8.2). In addition to future research, the work presented ideas on maximising scientific value of one batch of soil (where possible), e.g., assessing plant growth (if relevant) and using the soils for aged experiments, since the latter provides more realistic scenario than soil aged without the presence of organisms. The areas of future work (in addition to those listed in Figure 8.1) and recommendations are as follows:

1. One of the main limitation of the work (this thesis) was the inability to measure presence of intact particles in soil and earthworm tissues, therefore, research in particle uptake in organisms is essential to understand ENM behaviour in organisms. Methods such as spICP-MS should be further developed and utilised where possible (Navratilova et al., 2015)
2. Essential and trace metals should be measured alongside the total metal of concern (where possible). This can provide a useful profile of the metal balance within the organism that can offer signs to what may be causing the toxic effects.
3. The modes of action investigated here were oxidative stress and ion regulation. Other endpoints should be explored that may be more sensitive as than the endpoints tested, e.g. genotoxicity, as CdTe QDs have shown to cause DNA damage in other organisms (Lopes-Rocha et al., 2017).
4. In this thesis, only ageing effects on ENM toxicity were assessed, however, effects of varying abiotic factors such as temperature, moisture, soil organic

matter *etc.* should be investigated. Specifically, temperature and moisture due to the global problem of climate change.

5. The general approach to this thesis was to establish a proof of principle for ENM bioavailability and subsequent effects in the laboratory, therefore the concentrations used were higher than those likely to occur in the environment. Future studies should assess the long-term effects of lower concentrations as well as more realistic exposure scenarios, e.g. application of sewage sludge Lahive et al. (2017).

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