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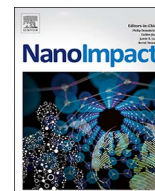
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Research paper

Comparative environmental fate and toxicity of copper nanomaterials



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ABSTRACT

Given increasing use of copper-based nanomaterials, particularly in applications with direct release, it is imperative to understand their human and ecological risks. A comprehensive and systematic approach was used to determine toxicity and fate of several Cu nanoparticles (Cu NPs). When used as pesticides in agriculture, Cu NPs effectively control pests. However, even at low (5–20 mg Cu/plant) doses, there are metabolic effects due to the accumulation of Cu and generation of reactive oxygen species (ROS). Embedded in antifouling paints, Cu NPs are released as dissolved Cu^{+2} and in nano- and micron-scale particles. Once released, Cu NPs can rapidly (hours to weeks) oxidize, dissolve, and form CuS and other insoluble Cu compounds, depending on water chemistry (e.g. salinity, alkalinity, organic matter content, presence of sulfide and other complexing ions). More than 95% of Cu released into the environment will enter soil and aquatic sediments, where it may accumulate to potentially toxic levels ($> 50\text{--}500 \mu\text{g/L}$). Toxicity of Cu compounds was generally ranked by high throughput assays as: $\text{Cu}^{+2} > \text{nano Cu(O)} > \text{nano Cu(OH)}_2 > \text{nano CuO} > \text{micron-scale Cu compounds}$. In addition to ROS generation, Cu NPs can damage DNA plasmids and affect embryo hatching enzymes. Toxic effects are observed at much lower concentrations for aquatic organisms, particularly freshwater daphnids and marine amphipods, than for terrestrial organisms. This knowledge will serve to predict environmental risks, assess impacts, and develop approaches to mitigate harm while promoting beneficial uses of Cu NPs.

1. Introduction

Worldwide annual production of copper (Cu) in 2015 was approximately 18.7 million metric tons (National Minerals Information Center, 2016), with an estimated 39% used in electrical and electronics applications, 30% in pipes, and the remainder in machinery, vehicles and consumer products. It is estimated that only a few hundred tons of the total production were converted to Cu-based nanoparticles (Cu NPs)

(Keller et al., 2013), despite there being many emerging applications for nano-Cu materials. Many applications involve the traditional role of Cu as a conductor, such as conductive dyes (Albrecht et al., 2016; Hokita et al., 2015; Tam and Ng, 2015; Kharisov and Kharissova, 2010; Tsai et al., 2015; Gopalan et al., 2016) or heat transfer fluids (Park et al., 2015; Montes et al., 2015; Azizi et al., 2016; Rizwan-ul-Haq et al., 2016), but the use of nano-Cu is rapidly expanding into novel applications such as catalysts in organic synthesis (Dugal and

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Table 1
Physicochemical characteristics of copper particles used in UC CEIN studies (adapted from Lin et al., 2015).

Property	Technique	Unit	Particles					
			nCu	nCuO	nCu(OH) ₂ -a	nCu(OH) ₂ -b	μCu	μCuO
Primary size	TEM	Nm	200–1000	20–100	~10	~50–1000 ^a	> 10,000	200–2000
Phase & structure	XRD		Cubic Cu, cubic Cu ₂ O	Monoclinic CuO	Orthorhombic Cu(OH) ₂ , impurities	Orthorhombic Cu(OH) ₂ , impurities	Cubic Cu	Monoclinic CuO
Shape/morphology	TEM		Irregular	Irregular	Irregular	Irregular	Dendritic	Irregular
Aggregate size in DI H ₂ O (50 μg/mL)	HT-DLS	Nm	1160 ± 202	420 ± 15	889 ± 156	1400 ± 143	69,100 ± 28,200	132 ± 176
Aggregate size in H buffer with alginate	HT-DLS	Nm	2710 ± 719	460 ± 4	953 ± 88	1170 ± 104	NA ^c	1350 ± 62
Zeta potential in DI H ₂ O (50 μg/mL)	ZetaPALS	mV	-46.3 ± 1.6	-16.5 ± 0.8	-45.1 ± 0.8	-53.8 ± 0.7	-32.5 ± 2.9	-28.5 ± 0.9
Zeta potential in H buffer with alginate	ZetaPALS	mV	-15.9 ± 1.4	-18.8 ± 0.9	-22.9 ± 0.6	-19.9 ± 0.8	-19.9 ± 0.8	-16.2 ± 1.5
Purity ^b	ICP-OES	wt%	84.8 ± 2.7	88.3 ± 1.3	47.1 ± 2.6	39.9 ± 1.4	94.9 ± 1.4	92.8 ± 1.1
Other elements/impurities ^a	ICP-OES, ICP-MS	wt%	Cu ₂ O, CuO	O, C	C, O, Na, Al, Si, P, Ca	C, O, Na, Al, Si, S, Zn	Cu ₂ O, CuO	O, C

^a From Adeleye et al. (2014).

^b Purity refers to weight percentage of each main component.

^c Hydrodynamic size could not be obtained because of rapid particle sedimentation.

Mascarenhas, 2015; Lennox et al., 2016; Barot et al., 2016), sensors (Albrecht et al., 2016; Tsai et al., 2015; Gopalan et al., 2016; Brahman et al., 2016; Pourbeyram and Mehdizadeh, 2016), solar cells (Yoon et al., 2010; Parveen et al., 2016; Shen et al., 2016), light-emitting diodes (Lee et al., 2015), hydrogen generation (Liu et al., 2015a; Liu et al., 2015b), and drug delivery (Woźniak-Budych et al., 2016). Based on the antifungal and antimicrobial properties of Cu⁺, Cu NPs are actively being developed for applications in agriculture and food preservation (Park et al., 2015; Montes et al., 2015; Dugal and Mascarenhas, 2015; Ray et al., 2015; Kalatehjari et al., 2015; Ponmurugan et al., 2016; ManiPrasad et al., 2015; Majumder and Neogi, 2016; Villanueva et al., 2016), textiles (Majumder and Neogi, 2016; Sedighi and Montazer, 2016), paints, coatings (e.g. lumber treatment) and water treatment (Ben-Sasson et al., 2016; Ma et al., 2016; Dankovich and Smith, 2014). The number of applications for regulatory approval of Cu-based nanopesticides has increased substantially in the past few years, highlighting the need for information about the likely exposure routes, doses and adverse effects on non-target organisms.

The speciation of copper (0, I, II) is used to design nanomaterials with vastly different properties. The highly conductive elemental copper (Cu(0) or nCu) can promote electron transfers (Azizi et al., 2016; Hussain et al., 2015; Yousef et al., 2015; Kind et al., 2012; Athawale et al., 2005). The unstable Cu(I), typically used as Cu₂O NPs, can cycle between Cu⁺ and Cu⁺² and efficiently catalyze a large number of reactions (White et al., 2006; Jiang et al., 2014; Tran et al., 2012), and has been studied for antimicrobial (Li et al., 2015; Abbasi et al., 2016) and antifouling activities. Cu(II), usually in the form of CuO, is used for energy storage (Qiu et al., 2013; Xu et al., 2015) and sensing (Albrecht et al., 2016; Pourbeyram and Mehdizadeh, 2016; Tian et al., 2015) applications. Cu⁺² can also be synthesized as Cu(OH)₂ NPs. In general, the toxicity of copper compounds has been exploited to protect agricultural crops from many pests, including those causing fungal infections (Montes et al., 2015; Kalatehjari et al., 2015; Bramhanwade et al., 2016; Epstein and Bassein, 2001; Begum et al., 2015; Fan et al., 2011; El-Habbaa et al., 2016; Wightwick et al., 2008; Stansly and Kostyk, 2015; Thind and Singh, 2015; Ferreira et al., 2007), and copper pesticides are now applied on some “organic” crops (Baker et al., 2002; Winter and Davis, 2006). Their use as an antimicrobial agent has also been considered in several applications (Emam et al., 2014; Shebl, 2014; Mjos et al., 2016; Congrádyová et al., 2014). Accordingly, there is a pressing need for a systematic evaluation of Cu NPs related to their environmental fate, bioavailability, release of

Cu⁺ and Cu⁺² ions, and toxicity. The University of California Center on the Environmental Implications of Nanotechnology (UC CEIN) (Godwin et al., 2009) developed and executed a plan to generate the information needed to develop risk assessments, comparing NPs to bulk materials, as well as to Cu salts. Here we present a comprehensive summary of findings from experiments that we conducted, along with a review of relevant literature on Cu NPs.

We conducted a systematic empirical study of the potential risks of copper NPs by focusing on the six Cu-based materials: nano Cu (nCu), micro Cu (μCu), nano CuO (nCuO), micro CuO (μCuO), and two forms of Cu(OH)₂, which are formulated as commercial pesticides (CuPro and Kocide 3000, denominated nCu(OH)₂-a and nCu(OH)₂-b in this manuscript). The pesticides have primary particles in the nano-size range, but with a wide size distribution into the micron range. Most Kocide 3000 particles are micron-sized aggregates composed of nanosheets of Cu(OH)₂ that are bound together but dissociate in water (Adeleye et al., 2014) through the rapid dissolution of organic composites. For this reason the pesticide is considered “nano” (Adeleye et al., 2014), although it contains a mixture of nanoparticles and micron-sized particles. In addition, copper salts (e.g. CuCl₂, CuSO₄) were used in many of the experiments. The two nanopesticides had trace amounts of other elements, in addition to the polymer matrix, which may influence their toxicity. Except for the Cu(OH)₂ materials, the nano and micro materials were acquired uncoated, which affected their stability but minimized the potential toxicological influence of a coating. Although the primary particles of nCu, nCuO and nCu(OH)₂-a and nCu(OH)₂-b were in the nano range, once in suspension the particles agglomerated rapidly to hundreds of nm, and in some cases into the micron scale, depending on the aqueous media. Table 1 presents the most relevant characteristics of the various nano- and microscale particles.

Risk is a function of likelihood of exposure at a concentration or dose that may cause harm, and the type and level of harm (toxicity) that may result from exposure. To assess exposure, UC CEIN focused on potential applications that are most likely to result in environmental release of the Cu particles (e.g. Cu nanopesticides and Cu embedded in antifouling paints) (Fig. 1). Toxicity was measured with experiments at various biological levels using high throughput screening (HTS) with cells and embryos, bioassays with aquatic and terrestrial organisms, and studies with microorganisms.

We provide here a comprehensive view of the potential exposure and toxicity of these Cu NPs (nCu, nCuO and nCu(OH)) compared to their micron-size counterparts as well as Cu salts. Each of the individual studies has been previously published, where one can find considerably

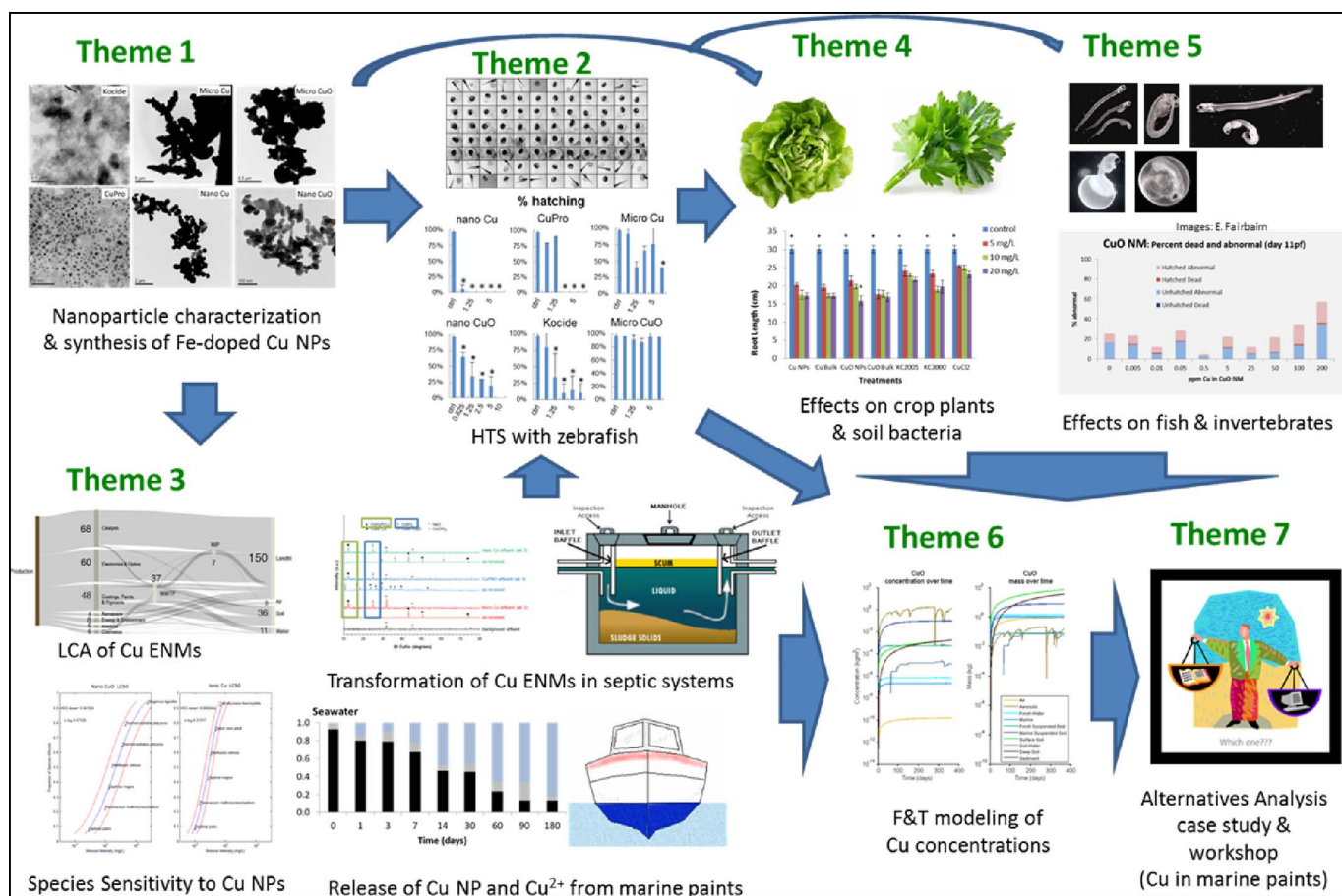


Fig. 1. UC CEIN Cu Working Group workflow: Theme 1: acquisition and characterization of the Cu particles, followed by distribution of the characterized materials to other themes; Theme 2: High throughput screening, which served to design and prioritize studies in Themes 4 (terrestrial toxicity) and Theme 5 (aquatic toxicity). In parallel, Theme 3 conducted life cycle material flow analyses to determine likely release estimates, exposure concentrations and doses for use in Themes 4 and 5. Exposure and toxicological data were transferred to Theme 6 to model risk based on expected concentrations/doses and hazards. Theme 7 conducted alternative analyses workshops for copper in paints. The project outcomes were (1) release estimates for various Cu NP applications; (2) assessment of likely exposure pathways and concentrations; and (3) ranking of toxicity of different species of Cu NPs, micron-scaled Cu particles and Cu salts.

more detail about the methods and conditions considered. This collection of results is intended to provide a broad perspective on the implications of these Cu NPs, as they are increasingly used in many applications.

2. Exposure assessment

2.1. Release and concentration estimates

Although Cu NPs can enter the environment via several applications, at present the majority of the releases are attributed to their use in marine antifouling paints (Adeleye et al., 2016) (which includes freshwater uses) and agriculture (Gardea-Torresdey et al., 2014), since there is a direct application of the product containing the nanomaterial to the receiving environmental medium. Cu-based nanomaterials are applied in agriculture directly as nanopesticides, or via biosolids from waste-water treatment (Lazareva and Keller, 2014a). However, the use of copper NPs in personal care products is still minimal (Keller et al., 2014), so their presence in biosolids is expected to be quite low, at low $\mu\text{g}/\text{kg}$ (Keller et al., 2014). Nevertheless, frequent application of biosolids may result in concentrations of copper NPs that may pose a concern over time.

The global market for paints is ~ 50 million metric tons per yr, but $< 10\%$ is used in specialty applications (The Freedonia Group, 2016a) such as marine antifouling paints, which represents only around 1% (Future Markets, Inc., 2016) (about 0.5 million tons). Since marine

paints can contain up to $\sim 50\%$ Cu, up to 0.25 million tons/yr of Cu may be released via this application. Thus, the amount of Cu potentially released in marinas, harbors, and shipyards is quite high. A recent study showed that the release from paints is ~ 3 to $27 \mu\text{g}/\text{cm}^2\text{-day}$, or in other terms, around 0.2 to 1.8% of the Cu NPs present in the paint were released in 180 days (Adeleye et al., 2016). Cu concentrations in water near the painted material can range from 40 to 630 mg/L; Cu can be dissolved, complexed with organics, or in nano- and micron-scale particles (Adeleye et al., 2016). While the objective of Cu in these paints is to maintain the surface free of specific target organisms, the above mentioned concentrations may have a negative impact on non-target organisms. However, depending on the exchange of the water surrounding the painted surfaces, the concentrations may decrease by 1–4 orders of magnitude within a few tens of cm from the surface.

World demand for fungicides is over 660,000 tons/yr (The Freedonia Group, 2016b), of which only a fraction are copper based. In California alone, 7300 metric tons of copper pesticides are sold annually. In Cu-based pesticides, organic copper compounds represent 56% by weight, copper sulfate 34%, cupric oxide 4%, and cuprous oxide 6% (California Department Of Pesticide Regulation, 2014). At present there is no separate tracking of copper nanopesticides. Given the benefit of slower release of Cu^{2+} from copper nanopesticides, compared to copper sulfate, NPs are likely to increase their share of the market. Manufacturer recommendations for the use of $\text{Cu}(\text{OH})_2$ nanopesticide indicate that it can be used for a wide range of crops, including vegetables (e.g. lettuce, spinach, corn, and wheat),

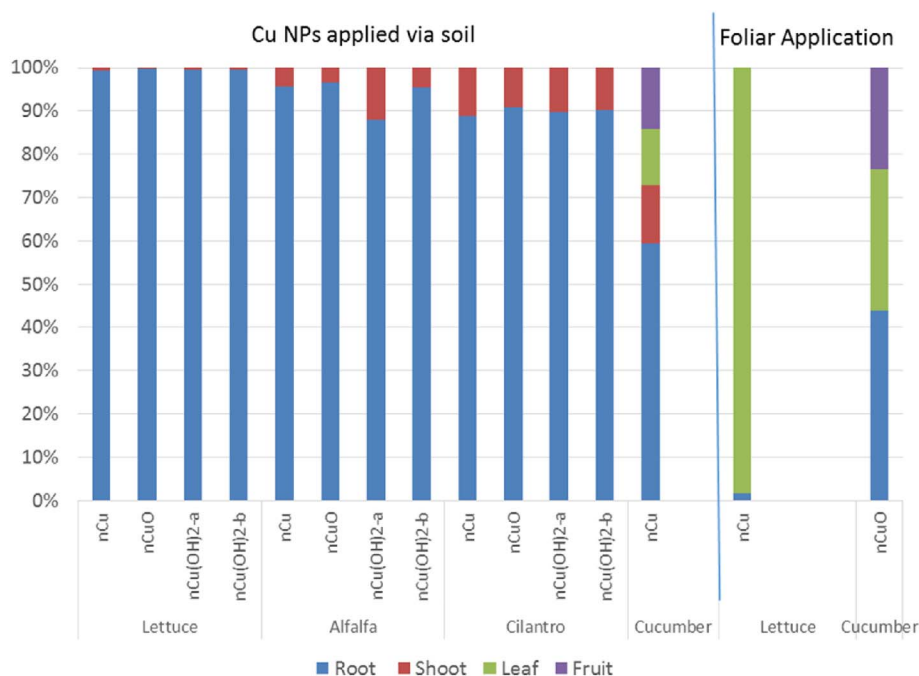


Fig. 2. Distribution of Cu taken up by different crops: lettuce (Hong et al., 2014), alfalfa (Hong et al., 2014), cilantro (Zuverza-Mena et al., 2015), and cucumber (Zhao et al., 2016a). Plant species on the left were exposed via soil, and the two plant species on the right had foliar application.

fruits (berries, peaches, and papaya) as well as trees (oak, sycamore, pine, and fir). The $\text{Cu}(\text{OH})_2$ nanopesticides are typically applied at initiation of new growth and repeated at 2–4 week intervals to control a wide range of fungal and bacterial diseases. They may be applied at rates of 0.05 to 0.8 g/m^2 (or 0.5–8 kg/ha) per event (Certis USA, Kocide 3000, n.d.). This corresponds to around 10–50 mg per plant, depending on application amount and planting density.

Uptake by crop plants exposed to Cu NPs varies depending on plant species, Cu NP, mode of application, and growth media (e.g. soil vs. hydroponics). For example, for lettuces (Hong et al., 2014), alfalfa (Hong et al., 2014) and cilantro (Zuverza-Mena et al., 2015) exposed via soil, uptake of nCu, nCuO, and the two nCu(OH)₂ nanopesticides resulted in accumulation of Cu mostly (> 87% and some cases > 99%) in the roots, with little translocation to the stems, and almost none to leaves (Fig. 2). Cu NPs may accumulate in the outer parts of root tissues (Fig. 3), as shown in micro-XRF studies (Servin et al., 2017). However, cucumbers exposed to nCu via the soil translocated it readily to upper plant tissues, including stems, leaves and fruits (Zhao et al., 2016a). When the application of Cu NPs is foliar, a much larger fraction of the Cu taken up by the plant remains in leaves or fruits, although some plants do exhibit translocation from upper tissues to roots by phloem. For example, lettuces exposed to nCu(OH)₂-b, following the recommended application amount, accumulated around 1350–2010 mg Cu/kg dry weight (DW) after 30 days of foliar exposure. The accumulated Cu was mostly inside the tissues, since the lettuces were washed thoroughly (Zhao et al., 2016b). While most of the copper (97–99%) was sequestered in the leaves, a small fraction (1–3%, 17.5–56.9 mg/kg) was translocated to root tissues through phloem loading (Zhao et al., 2016b). A fraction was in the form of nano- and microparticulates, although not necessarily as $\text{Cu}(\text{OH})_2$. Considering typical US daily lettuce consumption, the Cu content in the leaves would represent an additional 2.2–3.3 mg Cu/person-day, which is within recommended intake guidelines of 0.7–10 mg Cu/person-day (Trumbo et al., 2001). However, a diet rich in fruits and vegetables protected with Cu nanopesticides, in addition to other sources of Cu, may result in elevated Cu intake for some individuals.

2.2. Environmental fate of Cu NPs

Once released into the environment, NPs immediately begin to undergo a number of transformations (Garner and Keller, 2014). Homo- and hetero-aggregation, coating with natural organic matter, sedimentation, dissolution, oxidation in oxic environments, reduction or sulfidation in anoxic waters all initiate from the moment the dry ENM powder is placed in an aqueous medium. However, different processes dominate at various stages, depending on ENM composition and environmental parameters.

Aggregation of Cu NPs in natural waters depends on ENM speciation, aqueous media characteristics, specifically ionic strength (IS), natural organic matter (NOM) concentration, and pH (Adeleye et al., 2014; Conway et al., 2015a; Miao et al., 2015; Torres-Duarte et al., 2015; Rispoli et al., 2010; Gomes et al., 2011). Given their high density (8.96 g/cm^3 for nCu, 6.31 g/cm^3 for nCuO), Cu NPs settle out rapidly once they reach micron scale. nCu, with an isoelectric point (IEP) of pH 2.1, rapidly forms highly polydisperse micron-scale aggregates in simple salt solutions (Adeleye et al., 2014) and natural waters (Conway et al., 2015a), both in the absence and presence of organisms (Griffitt et al., 2007). Rapid aggregation of nCu leads to fast sedimentation; for instance, only 20% of initial nCu mass was detected after 6 h in 10 mM NaCl at pH 7 (Adeleye et al., 2014). However, in the presence of zebrafish and moderately hard freshwater, ~40% of the initial mass of nCu remained suspended after 48 h, indicating a fraction of the nanoparticles may have been stabilized by NOM released by the fish (Griffitt et al., 2007).

In contrast, nCuO is relatively stable in freshwater, with a critical coagulation concentration (CCC) of 40 mM NaCl at pH 7 (Adeleye et al., 2014). However, stability of nCuO is strongly influenced by salinity and pH (Conway et al., 2015a; Torres-Duarte et al., 2015; Gomes et al., 2011; Buffet et al., 2013). Rapid aggregation and sedimentation of nCuO occurs at high IS (e.g. lagoon and seawater) due to complete screening of electrostatic charges on particle surfaces (Conway et al., 2015a). Sedimentation of nCuO is also pH dependent; it reached values of 64%, 40%, and 39% at pH 4, 7, and 11, respectively in 10 mM NaCl (Adeleye et al., 2014). Particle size also influences sedimentation. Sedimentation of 50 nm CuO in seawater was faster, compared with

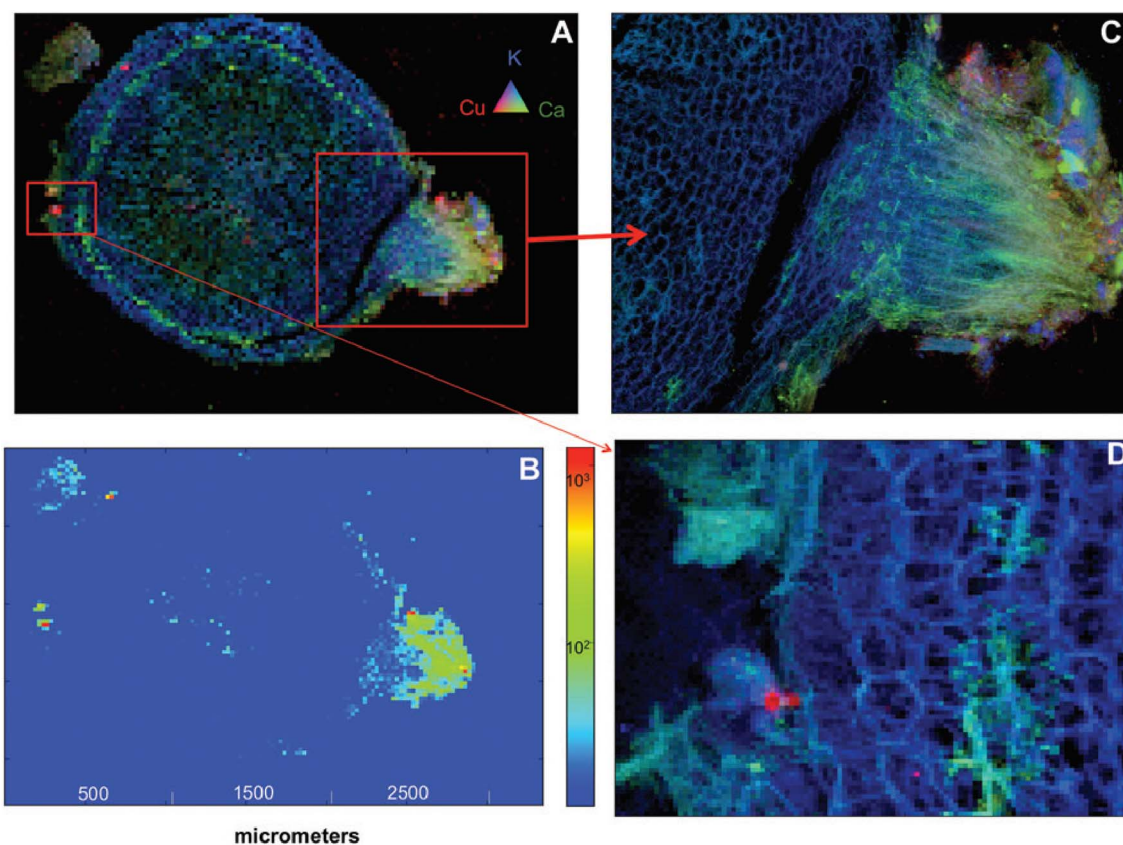


Fig. 3. Accumulation of nCuO and Cu in lettuce roots as seen using micro-XRF: (A) root cross-section showing Cu in red; (B) Cu concentration (raw intensity units); (C) magnification of secondary root; and (D) magnification of primary root (adapted from ref. [Servin et al., 2017](#), with permission). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

10 nm CuO ([Torres-Duarte et al., 2015](#)), indicating that smaller particles have longer resident time in suspension. Adsorption of ions in natural waters may strongly influence nanoparticle stability. nCuO is stabilized by the presence of phosphate ions ([Conway et al., 2015a](#)), which reverse NP surface charge polarity at concentrations as low as $0.1 \text{ mg PO}_4^{3-}/\text{L}$.

NOM, surfactants, and polymers stabilize Cu NPs via electrostatic and/or steric influences ([Adeleye et al., 2014](#); [Miao et al., 2015](#); [Worthington et al., 2013](#); [Perreault et al., 2012](#)). The critical coagulation concentration of nCuO increased from 40 to 75 mM NaCl in the presence of 0.25 mg/L Suwanee River NOM (SRNOM) ([Adeleye et al., 2014](#)). Similarly, the aggregation and sedimentation of Cu NPs were suppressed by extracellular polymeric substances (EPS) from a marine phytoplankton ([Adeleye et al., 2014](#)) and activated sludge ([Miao et al., 2015](#)). When 10 mg/L nCuO was suspended in 10 mM NaCl for seven days, suspended Cu increased from 0.10 mg/L in the absence to 0.32 mg/L in the presence of SRNOM, and to 0.48 mg/L in the presence of algal EPS ([Adeleye et al., 2014](#)). Bovine serum albumin (BSA) stabilized nCuO more than alginate and activated sludge EPS due to a stronger steric repulsive energy ([Miao et al., 2015](#)). Due to the abundance of polymeric stabilizer and very negative surface charge, $\text{nCu}(\text{OH})_2\text{-b}$ was much more stable than nCu and nCuO in freshwater and up to 100 mM NaCl, sedimenting very slowly (days) ([Adeleye et al., 2014](#); [Conway et al., 2015a](#)).

Homoaggregation is important very early on, or when there are few natural colloids present. Once the Cu NPs reach natural waters, the concentration of suspended particles will be 4–6 orders of magnitude greater than that of the NPs. Under these conditions, heteroaggregation is likely to overwhelmingly dominate the fate of the Cu NPs ([Wang et al., 2015](#); [Zhou et al., 2012](#); [Praetorius et al., 2012](#)).

Dissolution of Cu NPs in natural waters over a 90-day period

generally correlates with ENM aggregation and oxidation state, pH, and NOM, although in saline waters the formation of insoluble complexes also drives dissolution ([Adeleye et al., 2014](#); [Conway et al., 2015a](#); [Gomes et al., 2011](#); [Kent and Vikesland, 2016](#)). Highly aggregated Cu NPs have a reduced surface area, which decreases the dissolution rate. In simple salt solutions, dissolution rate was $\text{nCu}(\text{OH})_2\text{-b} > \text{nCu} \gg \text{nCuO}$, and in all cases, the dissolution rate decreased as pH increased from 4 to 11 ([Adeleye et al., 2014](#); [Wang et al., 2013](#)). Dissolution of Cu NPs was enhanced at high IS in the presence of NOM due to additional complexation. For example, dissolution of $\text{Cu}(\text{OH})_2\text{-b}$ after 90 days was 7.0%, 10.9%, and 17.4% at 1, 10, and 100 mM NaCl, respectively ([Adeleye et al., 2014](#)). nCu underwent rapid dissolution followed by complex formation in waters with moderate to high salinity, likely as a result of the ENM being in a non-oxidized state ([Conway et al., 2015a](#)). EPS and other NOM can coat the Cu ENM surfaces, in some cases reducing the initial dissolution rate ([Adeleye et al., 2014](#); [Conway et al., 2015a](#)). However, the released Cu^{2+} may be bound by negatively charged functional groups in NOM, driving dissolution. Dissolution of $\text{Cu}(\text{OH})_2\text{-b}$ after 90 days increased at 1, 10, and 100 mM NaCl to 12.7%, 13.5%, and 20.7% when 5 mg of carbon/L from phytoplankton EPS was present; and to 8.4%, 13.2%, and 18.8% with 5 mg/L SRNOM ([Adeleye et al., 2014](#)). The overall rates of dissolution depend on aggregation, NOM coating, and other ions present ([Adeleye et al., 2014](#); [Conway et al., 2015a](#)).

The dissolution of nCuO is very slow: on the order of weeks in freshwater, and in seawater dissolution is $\leq 1\%$ after months, over a wide range of initial nanoparticle concentrations (10 $\mu\text{g/L}$ –10 mg/L) ([Adeleye et al., 2014](#); [Conway et al., 2015a](#); [Gomes et al., 2011](#); [Buffet et al., 2013](#); [Buffet et al., 2011](#); [Hanna et al., 2013](#); [Atha et al., 2012](#)). However, these studies were performed under almost saturated conditions, where the maximum solubility of CuO may be reached. In

unsaturated and somewhat idealized conditions, the dissolution of nCuO may occur in a matter of hours to days (Kent and Vikesland, 2016). Surface water renewal and immobilization of NPs on a substrate can lead to accelerated dissolution, even for these relatively insoluble NPs.

The transformation of Cu in the environment is controlled by the chemistry of both the particles and the environment (Flemming and Trevors, 1989). Cu is commonly partitioned into aqueous (free and complexed ions), solid (particulate), and biological (adsorbed and incorporated) media (Flemming and Trevors, 1989). Dissolved Cu^{2+} released by some Cu NPs is readily oxidized to Cu^{2+} and then complexed in the environment (Adeleye et al., 2014; Lin et al., 2015; Conway et al., 2015a; Flemming and Trevors, 1989). As a result, inorganic Cu ions in natural waters exist mostly as complexes of carbonate, hydroxide, and NOM (Lin et al., 2015; Flemming and Trevors, 1989; Berg, 1984). The fraction of free Cu ions decreases by increasing IS and pH in the presence of EPS (Adeleye et al., 2014). In fact, free Cu ions were non-detectable at pH 11 (Adeleye et al., 2014). Moreover, under low redox conditions and high S^{2-} , CuS will form relatively insoluble compounds, even at the nanoscale (Kent and Vikesland, 2016).

Bioaccumulation of Cu and/or Cu NPs has been observed in many studies, from cell membranes in single-cell organisms (Bielmyer-Fraser et al., 2014), to aquatic filter feeders (e.g. mussels) that pack and excrete Cu (ion and NPs) in pseudofeces (Hanna et al., 2013; Montes et al., 2012), in fish (Abdel-Khalek et al., 2016), marine invertebrates (Torres-Duarte et al., 2015), and in terrestrial plants (Hong et al., 2014; Zhao et al., 2016a; Zhao et al., 2016b; Zhao et al., 2016c). While it is likely that Cu is internalized as Cu^{2+} or in organic complexes, in some cases Cu NPs are ingested or taken up from soil into the organisms, where they likely dissolve. In terrestrial plants, translocation of Cu was observed from roots to above ground tissues when the exposure was via soil or hydroponic media, and from leaves to stems and roots when the exposure was foliar. In many cases bioaccumulation factors (Cu in tissue/Cu in surrounding medium) are 2 to 4 orders of magnitude.

The accumulated knowledge on NP fate and transport, as reflected in the nanoFate model (Garner et al., 2017), indicates that Cu-based NPs would enter the environment mostly via treated effluent from wastewater treatment plants (WWTPs), biosolids from WWTPs applied to agriculture, and Cu-based nanopesticides. Assuming a continuous input of Cu NPs, and the dissolution and transformation of the NPs once released, the concentrations of dissolved Cu^{2+} in the freshwater would increase by $< 0.1 \mu\text{g/L}$ relative to background, and the concentrations of small aggregates of Cu NPs in the water column would be $< 1 \text{ ng/L}$ (Garner et al., 2017). Most of the Cu (dissolved and Cu NPs hetero-aggregated with sediment particles) would be accumulated in the sediment beds of freshwater and marine environments. Agricultural soils receiving WWTP biosolids would accumulate Cu (dissolved and Cu NPs hetero-aggregated with soil particles) at concentrations ranging from 1 to $10 \mu\text{g/kg}$ above the background Cu concentrations (Garner et al., 2017).

Future studies should address questions regarding the effect of Cu NP coatings, the nature of the released Cu species from paints and coatings, and the potential accumulation of Cu and Cu NPs in agricultural applications where application rates will likely be continuous and at higher concentrations than other releases.

3. Toxicity assessment

Although Cu is an essential element for many biological processes, doses of Cu above the required level can be toxic to many organisms. As such, the concentration and bioavailability of Cu in natural environments is very important when considering ecosystem health (Flemming and Trevors, 1989). The bioavailability of Cu depends on speciation (ionic, complexed, or nano, oxidation states) and environmental factors including pH, redox potential, water, soil and sediment type, water

hardness, and organic content (Garner and Keller, 2014; Flemming and Trevors, 1989; Cornelis et al., 2014). In comparison to terrestrial organisms, Cu tends to be quite toxic to aquatic biota, whose sensitivity to Cu and Cu NPs depends on factors such as surface-area-to-volume ratio, respiratory rates, and, for fish, flow rate over gill surfaces, among others (Flemming and Trevors, 1989). Cu is more bioavailable in aquatic than in terrestrial systems, where it can be bound in minerals. In addition to the known toxic effect of exposure to non-nano Cu, there is the potential for additional nano-related toxicity resulting from exposure to Cu NPs in the environment (Garner and Keller, 2014; Perreault et al., 2012; Baun et al., 2008; Manusadźianas et al., 2012; Shaw et al., 2012). The following studies present a hierarchical assessment of Cu NP toxicity.

3.1. High throughput/content screening studies

At the first level of screening, a zebrafish embryo HTS assay for hatching interference ranked the particle hazard as CuCl_2 (0.1 mg/L) $>$ nCu (0.25 mg/L) $>$ $\text{nCu(OH)}_2\text{-a}$ = $\text{nCu(OH)}_2\text{-ab}$ (0.3 mg/L) $>$ nCuO (0.5 mg/L) $>$ μCu = μCuO ($\geq 1 \text{ mg/L}$) (Lin et al., 2015). The differences are statistically significant ($p < 0.05$), except where an equal sign is noted. Although all the particles were stabilized with a natural dispersal agent (alginate), there was significant ($p < 0.05$) aggregation in Holtfreter's medium (Table 1) (Lin et al., 2015). Therefore, the observed toxicity of a given particle also reflects its bioavailability within the water column. Cu from Cu NPs interfered with the hatching enzyme, ZHE1, through a mechanism that involve binding of Cu ions to histidines in the enzyme center (Lin et al., 2013). There was a strong correlation between hatching interference and particle dissolution (weight % dissolved in 48 h) in Holtfreter's medium (Lin et al., 2015). However, if the nano- and micron-scale Cu particles were first passed through a model septic system, the particles transformed to water-insoluble inorganic $\text{Cu(H}_2\text{PO}_4)_2$ and CuSO_4 and to a lesser extent Cu bound to the organic matter in the septic system, reducing the bioavailability of Cu^{2+} and minimizing hatching interference (Lin et al., 2015).

Bacterial HTS growth inhibition assays performed on *Escherichia coli* dispersed in minimal medium ranked their toxicity (Cu concentration yielding 50% inhibition, in mg/L) as CuCl_2 (38 ± 8) $>$ nCu (120 ± 14) = CuSO_4 (140 ± 23) = nCuO (160 ± 17) $>$ nCu(OH)₂-a, nCu(OH)₂-b, μCu , μCuO (all > 250) (Kaweeteerawat et al., 2015). Similar assays performed on *Lactobacillus brevis* dispersed in Lactobacilli MRS broth ranked the various Cu species (in mg/L) as nCuO (3.6 ± 0.1) = nCu(OH)₂-a (4.0 ± 0.1) $>$ nCu (5.7 ± 0.2) = nCu(OH)₂-b (6.2 ± 0.9) $>$ CuCl_2 (7.8 ± 0.5) $>$ μCu = μCuO (≥ 120) (Kaweeteerawat et al., 2015). Aggregation of the particles in the different media was significant ($p < 0.05$), with the nanoscale particles ranging from 250 to 1600 nm, and the microscale particles from 1300 to 1500 nm. Thus, the observed toxicity reflects the bioavailability of the particles in the water column. These bacteria tolerate the various Cu species at significantly ($p < 0.05$) higher concentrations than the zebrafish embryos. Generally, CuCl_2 was the most toxic due to higher release of bioavailable Cu, followed by nCu. Commercial Cu nanopesticides were less toxic than nCu and nCuO, and toxicity was lowest for micron-scale Cu particles. Sub-lethal effect assays qualitatively measuring membrane potential, membrane damage, cellular ROS generation, and electron transport activity revealed that Cu ions and nanosized Cu resulted in significant ($p < 0.05$) membrane damage and a decrease in electron transport activity in *E. coli*, while micron sized particles had no effect (Kaweeteerawat et al., 2015). Although *L. brevis* showed similar trends, there was a stronger response to the Cu NPs and significant ($p < 0.05$) membrane damage from exposure to both nCu(OH)₂ and the copper salts (Kaweeteerawat et al., 2015). Exposure to Cu NPs resulted in oxidative stress (measured by biotic reactive oxygen species (ROS) generation) for *E. coli* and *L. brevis*, but there was no effect from CuCl_2 , CuSO_4 , μCu or μCuO at the

concentrations studied ([Cu] up to 250 mg/L) (Kaweeteerawat et al., 2015). These results are in line with previous work indicating that exposure to Cu NPs caused cellular oxidative stress in bacterial cells (Li et al., 2013), yeast cells (Kasemets et al., 2013), and mammalian cells (Liu et al., 2014). Exposure to ionic and nano- and micro-scale Cu particles resulted in DNA damage (using an in-vitro plasmid assay), but while nCu and μ Cu induced complete degradation of plasmid DNA, both n-Cu(OH)₂ converted the supercoiled plasmid to open circular (single-strand breaks) and linearized plasmid (double-strand breaks) (Kaweeteerawat et al., 2015). Ionic Cu, nCuO and μ CuO only partially converted the supercoiled DNA (Kaweeteerawat et al., 2015). An abiotic study confirmed that only nCu and μ Cu generated significant ($p < 0.05$) in-situ oxidants. Cellular internalization of nCu, nCuO, nCu(OH)₂-a and nCu(OH)₂-b by *E. coli* and *L. brevis* was also observed, but there was no internalization of μ Cu or μ CuO (Kaweeteerawat et al., 2015). Thus, it appears that toxicity is not only due to membrane damage and ROS generation by Cu²⁺, but also due to direct DNA damage, particularly from internalized nCu.

High content screening using sea urchin embryos indicated that toxicity of the various Cu species, based on 96 h exposure and in terms of their developmental effects concentration affecting 50% of exposed organisms (EC50, in μ g/L), was ranked as follows: CuSO₄ (33) > nCu (50) = nCu(OH)₂-b (60) > nCu(OH)₂-a (80) > μ Cu (177) > μ CuO (> 500), nCuO (5395). The differences are statistically significant ($p < 0.05$). The slow dissolution of commercial nCuO was an important factor; a high-purity lab-synthesized version of nCuO had an EC50 of 450 μ g/L (Torres-Duarte et al., 2016). The toxicity ranking was similar to the zebrafish embryos, but the sea urchin embryos were more sensitive to these Cu compounds in general. Although there was no significant ($p < 0.05$) oxidative damage to proteins, there was a decrease in total antioxidant capacity (Torres-Duarte et al., 2016). Changes in the redox environment caused by internalized Cu resulted in specific developmental abnormalities in sea urchin embryos including disruption of the aboral-oral axis, even in the case of nCuO. Abnormal skeleton formation and delayed development also occurred (Torres-Duarte et al., 2016). In a related study, early exposure to nCuO (same as was used in previous CEIN studies) caused a significant ($p < 0.05$) increase in intracellular ROS even at 500 ppb. However, CuSO₄ did not result in increased ROS for Cu concentrations from 500 μ g/L to 10 mg/L. nCuO was shown to be internalized, and may thus play a direct role in ROS generation. Conversely, while nCuO did not cause changes in mitochondrial membrane potential (MMP), CuSO₄ significantly ($p < 0.05$) decreased MMP even at 1 mg/L. Interestingly, nCuO (≥ 500 μ g/L) and CuSO₄ (≥ 200 μ g/L) acted as chemosensitizers and increased the developmental toxicity of vinblastine, a cell division inhibitor (Torres-Duarte et al., 2016; Wu et al., 2015).

In an evaluation of 24 different metal oxides, nCuO was found to rank among the top most toxic within this group of NPs (Zhang et al., 2012). Human bronchial epithelial (BEAS-2B) and murine myeloid (RAW 264.7) cell lines were used to assess oxidative stress and acute pulmonary inflammation. Exposure to nCuO resulted in a marked decline in cell viability even at 0.4 μ g/L and a dose-dependent decline in the cellular energy levels (Zhang et al., 2012). Additionally, nCuO also affected ROS production, intracellular calcium flux, mitochondrial membrane potential, and surface membrane permeability in BEAS-2B and RAW 264.7 cells (Zhang et al., 2012). To corroborate the in vitro assays, in vivo toxicity assays of acute pulmonary inflammation were conducted with C57 BL/6 mice. nCuO induced statistically significant ($p < 0.05$) increases in neutrophil cell counts, monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) levels, compared to less toxic NPs, indicating a strong immune system defense response (Zhang et al., 2012). In addition, at least 10 cytokines and chemokines were significantly ($p < 0.05$) increased in response to nCuO exposure. Even though nCuO was associated with significant ($p < 0.05$) cellular and lung toxicity, nCuO does not fit the hypothesis that transition metal oxides are toxic due to conduction band energy overlap with the

cellular redox potential (Zhang et al., 2012). nCuO Ec (− 5.17 eV) falls outside the overlap region (− 4.12 to − 4.84 eV) (Zhang et al., 2012). nCuO dissolved by 17.9 \pm 0.2% in bronchial epithelial cell growth medium and 9.4 \pm 0.8% in Dulbecco's modified Eagle medium within the experimental time frame (24 h) (Zhang et al., 2012). Thus, it is likely that the main toxicity mechanism is Cu²⁺ dissolution. To evaluate whether nCuO can generate ROS, a representative redox couple contributing to the cellular redox potential, cytochrome c-Fe³⁺/cytochrome c-Fe²⁺ was chosen. Cytochrome c is an essential component of the mitochondrial electron transport chain, which can lead to ROS generation. CuO converted the heme moiety in the cytochrome c, Fe²⁺, to Fe³⁺. CuCl₂ is also capable of oxidizing cytochrome c, thus it is most likely that released Cu²⁺ from nCuO is responsible for oxidizing cytochrome c (Zhang et al., 2012). These results highlight the need to handle dry powder Cu NPs with extreme caution, and to avoid inhalation of suspensions during the application of nanopesticides.

3.2. Aquatic toxicity studies

Different classes of aquatic organisms process Cu NPs differently. For a marine diatom, *Thalassiosira weissflogii*, the lowest observable effect concentrations (LOECs) were 78 μ g/L for nCuO and 173 μ g/L for CuCl₂ (Bielmyer-Fraser et al., 2014). After a 7 days exposure to nCuO or CuCl₂, *T. weissflogii* had higher proportions (1–3 orders of magnitude) of Cu in cell wall fractions, compared with organelle fractions (Bielmyer-Fraser et al., 2014). In contrast, higher Cu concentration was observed in the organelle fraction after exposure to Cu²⁺ compared to nCuO, indicating nCuO remained attached to the cell wall, while Cu²⁺ was internalized. For a coastal marine benthic amphipod, *Leptocheirus plumulosus*, exposed for 10 days to nCuO the median lethal concentration in sediment (LC50) was 868 \pm 89 μ g Cu/g dry weight (DW) (Hanna et al., 2013). At the highest exposure concentration the sediment contained 1098 \pm 37 μ g Cu/g, while sediment pore water contained 0.37 \pm 0.1 μ g Cu/L and the overlying water 0.32 \pm 0.1 μ g Cu/L, thus indicating very slow dissolution of nCuO. However, while amphipods in control groups stored 148 \pm 10 μ g Cu/g, exposed amphipods accumulated 585 \pm 9 μ g Cu/g (Hanna et al., 2013), suggesting that Cu bioaccumulation is mainly from sediments, not from pore water. Marine mussels (*Mytilus galloprovincialis*), exposed to 3000 μ g/L nCuO for 30 days accumulated 79.1 \pm 12.5 μ g Cu/g DW, which was 60 times more Cu than in control animals (Hanna et al., 2014). The feeding rate of exposed mussels decreased by 48% and growth was 68% lower, compared to control animals. Mussels excreted pseudofeces containing as much as 110,000 μ g Cu/g (Hanna et al., 2014). Pseudofeces can be ingested by other organisms in the water column or sediments, potentially resulting in Cu bioavailability and biomagnification.

Given the likelihood that a fraction of Cu NPs will pass through WWTPs (Lazareva and Keller, 2014b), the microbial community in a decentralized wastewater treatment system (septic tank) was subjected to 3 individual experiments lasting 3 weeks to a low uniform dose (10 mg/L Cu) of different Cu particles: nCu, nCu(OH)₂-b, and μ Cu (Taylor and Walker, 2016). The three particles disrupted septic tank function, which was unable to meet treatment objectives in terms of 5-day biochemical oxygen demand and pH (Taylor and Walker, 2016). There were also noticeable fluctuations in microbial community structure and phenotypes. However, 3 weeks after the end of exposure, the treatment system returned to baseline conditions (Taylor and Walker, 2016). This indicates that while a regular low dose of Cu from the NPs can result in sporadic system disruption, overall the treatment system maintains proper function. The Cu NPs were transformed to copper phosphate and sulfurized compounds, reducing the toxicity of the effluent by 95% or more (Lin et al., 2015).

To more broadly assess the nanotoxicity of Cu NPs, dozens of studies with different organisms, including many in the USEPA ECOTOX

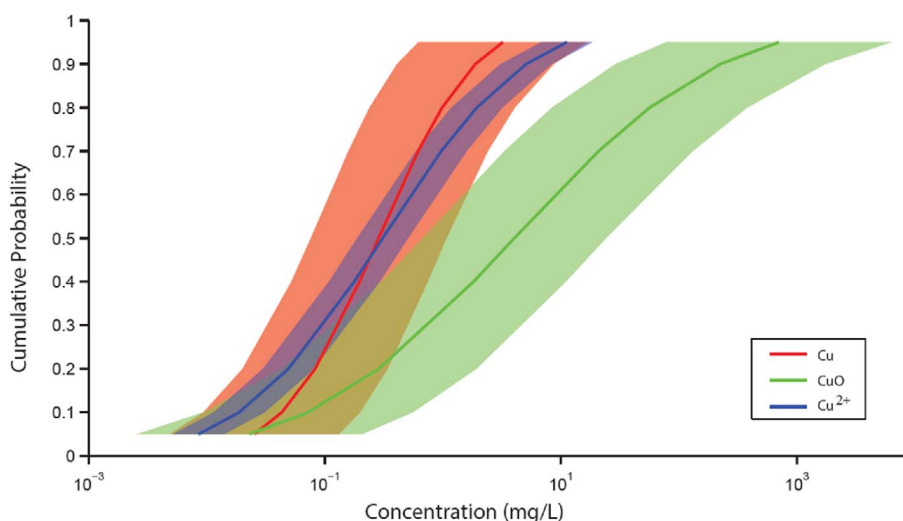


Fig. 4. Copper species sensitivity distributions for nCu, nCuO, and Cu^{2+} . The 95% CI is depicted as the shaded region in color corresponding to each curve. Adapted from ref. Garner et al. (2015).

database, were evaluated (ECOTOX Database, 2014). Only acute toxicity in freshwater was considered, given the paucity in marine in general and freshwater chronic toxicity data. For Cu^{2+} , 24-h LC_{50} ranges from 0.086 $\mu\text{g/L}$ to 282 mg/L for a wide range of organisms, with a median of 5.8 mg/L. (ECOTOX Database, 2014) Although there are only a few studies on the toxicity of nCu, 48-h LC_{50} ranges from 47 $\mu\text{g/L}$ to 419 $\mu\text{g/L}$ for *Ceriodaphnia dubia* (Gao et al., 2009; Griffith et al., 2008) and from 700 $\mu\text{g/L}$ to 1500 $\mu\text{g/L}$ for *Danio rerio* juveniles and adults (Griffitt et al., 2007; Griffith et al., 2008). For nCuO, 24-h LC_{50} ranges from 470 $\mu\text{g/L}$ to 217 mg/L (Manusadzianas et al., 2012; Heinlaan et al., 2008; Blinova et al., 2010; Gallego et al., 2007), while 24-h EC_{50} ranges from 30 $\mu\text{g/L}$ to 126 mg/L across several organisms (Blinova et al., 2010; Mortimer et al., 2011; Kasemets et al., 2009; Mortimer et al., 2010; Jo et al., 2012). This information was used to construct species sensitivity distributions (SSDs) for freshwater organisms (Garner et al., 2015). The toxicity threshold was much higher for nCuO relative to nCu or Cu^{2+} ions (Fig. 4). The SSDs for nCu and Cu^{2+} almost overlapped, indicating similar toxicity thresholds (Garner et al., 2015). Of the species used to develop the nCuO and nCu SSDs, the LC_{50} values tend to be lowest for various daphnia species (e.g. *C. dubia*, *Daphnia pulex*, and *Daphnia magna*), indicating high sensitivity, while crustaceans and fish tolerated higher concentrations of nCuO and nCu (Garner et al., 2015).

The lower toxicity of nCuO in freshwater may reflect its slower dissolution at low ionic strengths and Cu^{2+} complexation in the presence of organic matter (Adeleye et al., 2014; Garner and Keller, 2014; Griffith et al., 2007; Blinova et al., 2010; Mortimer et al., 2010; Garner et al., 2015; Wang et al., 2011). Other studies indicate that the toxic effect observed from nCuO strongly correlates with the fraction of NP dissolved in aquatic media (Garner and Keller, 2014; Blinova et al., 2010; Aruoja et al., 2009). In freshwater and marine systems, nCu and nCuO were found to cause some toxicity across a range of toxicological endpoints at concentration < 1 mg/L (Griffitt et al., 2007; Griffith et al., 2008; Heinlaan et al., 2008; Aruoja et al., 2009; Garcia et al., 2011), while others only found toxicity at greater exposure concentrations (Blinova et al., 2010; Baek and An, 2011).

3.3. Terrestrial toxicity studies

The effects of exposure to the Cu particles was studied for several plants. Cilantro (*Coriandrum sativum*) was cultivated in soil contaminated with each of the six Cu particles (nCu, nCuO, nCu(OH)₂-a, nCu(OH)₂-b, μCu and μCuO) and CuCl_2 from germination to harvesting after 30 days (Zuverza-Mena et al., 2015). Statistically significant

($p < 0.05$) decreases in seed germination were observed at ≥ 20 mg Cu/kg soil for nCuO (48%), nCu (45%), μCuO (40%), and ionic Cu (35%). nCu(OH)₂-b decreased germination by 30% at 80 mg Cu/kg, but nCu(OH)₂-a had no statistically significant ($p < 0.05$) effect on germination, relative to controls (Zuverza-Mena et al., 2015). Although the plants accumulated Cu in shoots in a dose-dependent manner, the source of Cu (different particles or CuCl_2) had no impact (Zuverza-Mena et al., 2015). Root elongation was not affected by any treatment, and only nCu at 80 mg/kg and μCuO at 20 mg/kg and 80 mg/kg reduced shoot elongation by 11 to 12% (Zuverza-Mena et al., 2015). In addition, μCuO at 20 mg/kg reduced the relative chlorophyll content, and at 80 mg/kg significantly ($p < 0.05$) plant biomass (Zuverza-Mena et al., 2015). All treatments reduced P accumulation, except nCu(OH)₂-a at 20 mg/kg. B, Zn, Mn, Ca, Mg, P, and S were significantly ($p < 0.05$) reduced in shoots (Zuverza-Mena et al., 2015). Thus, Cu-based particles may affect the nutritional value of cilantro. In contrast with aquatic organisms, nCuO resulted in more negative effects than nCu or the two nCu(OH)₂ nanopesticides at comparable Cu concentrations. Similarly, seedling studies found inhibited growth resulting from exposure to nCuO, such as Syrian barley at 0.5 mM nCuO (Shaw et al., 2014), soybeans and chickpeas at 500 ppm Cu (Adhikari et al., 2012), mung beans and wheat at 335 and 570 mg nCu L⁻¹ respectively (Lee et al., 2008), radish seedlings at 10 mg/L (Atha et al., 2012), and lettuce seedlings EC_{50} at 0.1 mg/L (Liu et al., 2016).

Lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*) grown in hydroponic media were exposed for 15 d, after 10 d of germination, to the six particles (nCu, nCuO, nCu(OH)₂-a, nCu(OH)₂-b, μCu and μCuO) and CuCl_2 at Cu concentrations from 0 to 20 mg/L (Hong et al., 2014). All Cu particles and dissolved Cu^{2+} significantly ($p < 0.05$) reduced root length in both plant species at ≥ 5 mg/L (Hong et al., 2014). Exposure to 20 mg/L nCuO or nCu resulted in the shortest root lengths in both plants. Considerable bioaccumulation of Cu occurred in roots (5000 to 9000 mg/kg plant DW) for all lettuce plant treatments with little differentiation with regards to type of particle, but there was noticeable more accumulation when exposed to Cu^{2+} (5000 to 20,000 mg/kg DW) (Hong et al., 2014). Significant ($p < 0.05$) translocation of Cu to lettuce leaves was only observed for nCu at 10 mg/L and 20 mg/L. In contrast, alfalfa shoots exhibited more translocation from roots to leaves for all treatments and followed a dose-dependent pattern (Hong et al., 2014). There was no effect on uptake of Ca, Mg, Mo, Mn, Zn, and Na from the various treatment, but P, S and Fe uptake was altered (Hong et al., 2014). Released Cu^{2+} likely bound phosphate in the media, markedly reducing [P] in roots of lettuce and alfalfa, although the effect was less significant ($p < 0.05$) in the shoots of

either plant (Hong et al., 2014). While lettuces did not exhibit any reduction in catalase activity, indicating no increased formation of ROS, alfalfa tissues exhibited a considerable change at 10 mg/L nCuO, and in alfalfa roots at 5 mg/L nCu(OH)₂-a and μCuO (Hong et al., 2014). Surprisingly, ascorbate peroxidase activity in lettuce roots increased in all treatments except μCuO, and it also increased in alfalfa roots in all treatments except the two nCu(OH)₂ nanopesticides. These early growth stage results indicate some noticeable effects from all Cu NPs, although no clear pattern was exhibited with regards to toxicity ranking based on physiological or biochemical indicators.

Lettuce grown from seed to maturity in soil, and exposed through foliar spray to nCu(OH)₂-b during the last 4 weeks before harvesting at 1050 mg/L to 2100 mg/L, exhibited no visible damage (Zhao et al., 2016b). In fact, leaf biomass, which is the harvestable product, increased significantly ($p < 0.05$). However, metabolomics analysis revealed that nCu(OH)₂-b altered metabolite levels in the lettuce leaves (Zhao et al., 2016b). The tricarboxylic (TCA) cycle and several amino acid-related biological pathways were disturbed. Some antioxidant levels (cis-cafeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid, dehydroascorbic acid) were significantly ($p < 0.05$) decreased (by 17 to 46%) compared to the control, indicating that oxidative stress and a defense response occurred. Nicotianamine, a chelator, increased by 12 to 27 times compared to the control, which may represent a detoxification mechanism. The up-regulation of polyamines (spermidine and putrescine) and increased potassium may mitigate oxidative stress and enhance tolerance (Zhao et al., 2016b). These changes in antioxidants within the leaves may alter their nutritional value.

Hydroponically grown lettuce seedlings (18-day-old) were treated for 15 days with a core-shell Cu@CuO nanomaterial at 10 and 20 mg/L, and CuSO₄·5H₂O at 10 mg/L (Trujillo-Reyes et al., 2014). Both Cu²⁺ ions and nCu@CuO significantly ($p < 0.05$) reduced water content, root length, and dry biomass of the lettuce plants (Trujillo-Reyes et al., 2014). Exposure to nCu@CuO resulted in significant ($p < 0.05$) accumulation of Cu in roots compared to CuSO₄. Both Cu treatments increased catalase activity but decreased ascorbate peroxidase activity in the roots (Trujillo-Reyes et al., 2014). In addition, relative to the control, nCu@CuO altered the nutritional quality of lettuce leaves, since the treated plants had significantly ($p < 0.05$) more Cu, Al and S but less Mn, P, Ca, and Mg (Trujillo-Reyes et al., 2014). Thus, in addition to the similarity in response, the effects of Cu exposure were, generally, more noticeable in the intake tissues (i.e. roots or leaves). Exposure to Cu alters nutritional value by modifying levels of metabolites and minerals.

Foliar application of nCuO and μCuO at 50, 100 and 200 mg/L to cucumber plants (*Cucumis sativus*) from 3 weeks old until fruit harvest, resulted in Cu accumulation in leaves (Hong et al., 2016). However, Cu did not increase in roots and fruits. Cucumber yield, length, and diameter of fruits was unaffected by nCuO or μCuO at all treatment levels (Hong et al., 2016). At the highest exposure level (200 mg/L), nCuO impacted cucumber photosynthesis in seedlings but the plants recovered at the adult age (Hong et al., 2016). There were no visible signs of toxicity in the leaves. In contrast, for nCu applied to *C. sativus*, significant ($p < 0.05$) translocation of Cu was observed. When plants were exposed to 10 mg/L to 20 mg/L nCu in hydroponic media, Cu uptake in the roots was greater (> 1000 mg Cu/kg DW) than in stems (100–175 mg Cu/kg DW) and leaves (3–4 mg Cu/kg DW), but in all cases there was significant ($p < 0.05$) increase relative to unexposed controls (Zhao et al., 2016c). When plants were exposed to 200–800 mg nCu/kg in soil, Cu was also highest in the roots, but there was significant ($p < 0.05$) translocation to stems, leaves and even cucumber fruits (Zhao et al., 2016a). However, the translocation factor (Cu in shoots/Cu in roots) was lower in plants exposed to nCu (0.22) and those only exposed to bioavailable Cu in the soil (0.33), suggesting that not all the Cu taken up in the roots was available for translocation when it was supplied as nCu (Zhao et al., 2016a). Although the plants showed no visible damage, exposure to nCu reduced photosynthetic

rate (similar to previous work with nCuO), and increased transpiration rate (Zhao et al., 2016a). The concentrations of Na, P, S, K, Mo, Fe and Zn decreased in all cucumber tissues in exposed plants (Zhao et al., 2016c). Formation of Cu-phosphate complexes at the root surface decreased P uptake. In addition, metabolomics demonstrated that *C. sativa* engages an active defense mechanism in the root zone against nCu stress: up-regulation of amino acids to sequester/exclude Cu/nCu; down-regulation of citric acid to reduce the mobilization of Cu ions; ascorbic acid up-regulation to combat reactive oxygen species; and up-regulation of phenolic compounds to increase antioxidant activity (Zhao et al., 2016c). nCu spiked in soil also up- or down-regulated 15 metabolic pathways in cucumber fruits: carbohydrate metabolism (5 pathways), amino acid synthesis and metabolism (6 pathways), and pathways related to lipid metabolism, biosynthesis of other secondary metabolites, and energy metabolism (Zhao et al., 2016a). Despite these metabolic changes, root, stem, leaf and fruit biomasses were not impacted by exposure to nCu, so the effect may be only on fruit quality and needs further exploration (Zhao et al., 2016a).

While crop plants are the intended recipients of nanopesticides and are more likely to be exposed to Cu NPs in biosolids, impacts to surrounding wild plants were also considered. A study compared the effects of nCu(OH)₂-b on a wild herbaceous annual plant (*Clarkia unguiculata*), radish (*Raphanus sativus*), and wheat (*Triticum aestivum*) under the same conditions. The uptake and toxicity of nCu(OH)₂-b grown in potting, grassland, or agricultural soils were found to be dependent on plant species, soil type, soil nutrient levels, and illumination intensity (Conway et al., 2015b; Conway and Fate, 2015). Exposure to soil nCu(OH)₂-b concentrations as low as 10 mg/L caused stunted growth in *C. unguiculata* and resulted in a complete cessation of photosynthesis during the period of peak growth in individuals under high stress growth conditions (high light levels and limited soil nutrients). These plants also had elevated fractions of oxidized photosystem II (PsII) reaction centers, which is consistent with known mechanisms of photosynthetic disruption caused by ionic Cu (Xiong et al., 2006; Janik et al., 2010; Peng et al., 2013). Uptake and translocation patterns also suggest that nCu(OH)₂-b underwent partial or total dissolution in the potting soil used (pH 5.7), and were being taken up as ionic Cu rather than as particulate Cu(OH)₂ (Conway et al., 2015b). However, *C. unguiculata*, radish, and wheat grown in grassland or agricultural soils had leaf Cu concentrations 1 to 2 orders of magnitude lower than plants grown in potting soil, with the highest Cu concentrations in roots. Few physiological impacts due to nCu(OH)₂-b exposure were seen in these plants, possibly as a result of decreased translocation to leaves. One exception was seen in wheat grown in grassland soil under low illumination levels during the 8th week of exposure to 25 mg nCu(OH)₂-b/L-week, which had decreased PsII quantum yield efficiencies, photochemical quenching, electron transport rates, and ratios of oxidized PsII reaction centers, compared to control plants. Additionally, radish plants grown under high light conditions and exposed to nCu(OH)₂-b had significantly ($p < 0.05$) larger hypocotyls than control plants, but there was significantly ($p < 0.05$) lower grain yield in wheat plants grown under the same conditions.

In summary, although effects were observed in seedlings and young plants when exposed to the various Cu particles and dissolved Cu²⁺, mature plants generally exhibited fewer apparent symptoms, and in several cases produced more biomass, particularly in tissues with commercial value (e.g. lettuce leaves and cucumber fruits). The terrestrial plants tolerated the exposure to Cu NPs better than aquatic organisms, with higher levels needed to produce measurable effects. This may be due to lower dissolution (and thus bioavailability) of Cu in terrestrial environments due to interactions with soil and natural organic matter (NOM), and the need for translocation through the root systems. In general, exposure to Cu compounds resulted in alteration of metabolite profiles, inducing anti-oxidant response, potential defense mechanisms (e.g. down-regulation of citric acid in root exudate to

minimize Cu dissolution, up-regulation of chelators and other metabolites that serve to protect the plants), reduced photosynthetic rates and increased transpiration rates in some species. There was no clear trend in toxicity ranking, in some cases nCu being the most toxic, but in many others nCuO was as toxic or more.

Only a few studies have been conducted that directly test the toxicity of terrestrial organisms to Cu NPs in soil media. Generally, growth inhibition does not occur until relatively high exposure concentrations, indicating that other toxic endpoints would not occur at likely environmental concentrations for either nCu or nCuO. Rousk et al. (2012) found an acute toxic response to soil bacteria from nCuO that did not occur upon exposure to bulk CuO. The growth inhibition EC₅₀ was found to be around 2254 mg/kg for soil bacteria (Rousk et al., 2012). The principal mechanism of toxicity was found to be dissolution of the metal oxide. Dimkpa et al. investigated the effects of nCuO on sand grown wheat and beans and found both reduced root and shoot growth at 500 mg/kg, with again a greater effect seen from the nano scale rather than the bulk particles (Dimkpa et al., 2013; Dimkpa et al., 2015). These effects were not observed until relatively high exposure concentrations (100 mg/kg) were reached (Dimkpa et al., 2015). Unrine et al. found no adverse effect to *E. fedita* to nCu at exposure concentrations as high as 65 mg/kg across a range of ecologically relevant endpoints (Unrine et al., 2010). A number of studies have been conducted on the sensitivity and toxicity of microbes and plants to nCuO, however the majority of these were tested in growth media and not in soil. For example, Baek and An found the 24-hr microbial growth inhibition EC₅₀ to fall between 28.6 and 65.9 mg/L (Baek and An, 2011).

Future studies should consider the effects of Cu NP speciation and transformation, once they are released from the product (e.g. painted surfaces, coatings, nanopesticides) into different environmental matrices, on toxicity. For example, the toxicity of most Cu NPs was decreased by > 95% after passing through the WWTP, and the speciation was very different from the input NPs. Studies are needed that go beyond the original, pristine NPs. Further work is needed to understand Cu NP toxicity in soils and in particular microbial communities.

4. Conclusions

Increasing use of Cu NPs, particularly in applications where they are directly released into the environment, such as in antifouling paints and pesticides, will result in increasing organismal exposure. Given that inhalation of Cu NPs may result in pulmonary inflammation and a strong immune system response, even at low concentrations, occupational exposure to paints and pesticides containing Cu NPs must be minimized by using appropriate personal protective equipment, particularly when handling dry powders of Cu NPs (e.g. during formulation) or aerosolized pesticide formulations.

For those Cu NPs released from consumer products, passing through waste water treatment plants, they are likely to affect treatment process even at the mg/L level. Furthermore, the presence of organic matter in wastewater treatment systems increases the likelihood of Cu transformation/complexation with organics into less toxic forms of Cu. Cu NPs were transformed to phosphate and sulfurized Cu compounds which precipitate out in the biosolids.

Once released, Cu NPs are expected to rapidly homoaggregate initially, but in the presence of natural colloids, heteroaggregation will dominate. Once in the micron scale, sedimentation is relatively fast (hours), removing most of the aggregated NPs from the water column, unless they are stabilized by EPS or other NOM. Dissolution rate is fastest for nCu(OH)₂, followed by nCu, but both are on the order of days. nCuO dissolves much more slowly and, in some cases, dissolution may take weeks to months.

Toxicity ranking based on HTS studies was generally Cu²⁺ > nCu > nCuO ≈ nCu(OH)₂ > μCu ≈ μCuO, although there were some cases where nCuO toxicity was greater than nCu. The assays

consistently indicated that ROS production was an important toxicity mechanism, although for some organisms other toxicity mechanisms arose such as membrane damage, decreased electron transport activity, degradation of plasmid DNA, decreased total antioxidant capacity, and developmental abnormalities.

Terrestrial plants exposed to pesticides based on Cu NPs may receive only a few mg per spray event, but even after washing, there could still be residual Cu in harvested products (e.g. fruits, leaves, roots). Cu concentration in these tissues can increase by several orders of magnitude, and is a strong function of point of exposure: plants translocate some of the Cu from roots to above ground tissues when the exposure is via soils, and vice versa when the exposure is foliar. While terrestrial plants appear to be more tolerant to Cu²⁺ and Cu NPs in general, if Cu NPs are applied to agricultural soils, either directly or in biosolids, they may have effects on seed germination and initial growth rates. Released Cu also sequesters available phosphate, which can result in nutrient imbalance. Exposure to Cu NPs also alters the levels of several elements and metabolites, potentially affecting the nutritional value of crops.

At present, the environmental risks of Cu NPs are generally low, given that average exposure concentrations are expected to be in the ppb range. The slow release of Cu from Cu NPs in paints and pesticides serves to minimize short-term impacts, but the increasing accumulation of Cu in sediments and soils may eventually reach and surpass the lowest observable effect concentrations. Localized hot spots in locations where Cu NP-based products are heavily used may result in ecological damage. Although Cu can be eventually sequestered via sulfidation, as it is buried in deeper sediments and soils, it is important to keep in mind that these processes are very slow.

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