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Biodynamics of copper oxide nanoparticles and copper ions in an oligochaete - Part II: Subcellular distribution following sediment exposure

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ABSTRACT

The use and likely incidental release of metal nanoparticles (NPs) is steadily increasing. Despite the increasing amount of published literature on metal NP toxicity in the aquatic environment, very little is known about the biological fate of NPs after sediment exposures. Here, we compare the bioavailability and subcellular distribution of copper oxide (CuO) NPs and aqueous Cu (Cu-Aq) in the sediment-dwelling worm *Lumbriculus variegatus*. Ten days (d) sediment exposure resulted in marginal Cu bioaccumulation in *L. variegatus* for both forms of Cu. Bioaccumulation was detected because isotopically enriched ^{65}Cu was used as a tracer. Neither burrowing behavior or survival was affected by the exposure. Once incorporated into tissue, Cu loss was negligible over 10 d of elimination in clean sediment (Cu elimination rate constants were not different from zero). With the exception of day 10, differences in bioaccumulation and subcellular distribution between Cu forms were either not detectable or marginal. After 10 d of exposure to Cu-Aq, the accumulated Cu was primarily partitioned in the subcellular fraction containing metallothionein-like proteins (MTLP, ≈40%) and cellular debris (CD, ≈30%). Cu concentrations in these fractions were significantly higher than in controls. For worms exposed to CuO NPs for 10 d, most of the accumulated Cu was partitioned in the CD fraction (≈40%), which was the only subcellular fraction where the Cu concentration was significantly higher than for the control group. Our results indicate that *L. variegatus* handle the two Cu forms differently. However, longer-term exposures are suggested in order to clearly highlight differences in the subcellular distribution of these two Cu forms.

1. Introduction

Engineered metal nanoparticles (NPs), such as copper oxide (CuO) NPs, are being increasingly used in consumer products (e.g., inks, coatings and textiles for biocidal activity, lubricants, plastics, cosmetics, and electronics) (Buffet et al., 2013; Cioffi et al., 2005; Misra et al., 2012 Cioffi et al., 2005; Misra et al., 2012). As a result, environmental releases are likely to increase. Despite the growing number of nanotoxicological studies (Oberdorster et al., 2005; Kahru and Savolainen 2010; Klaine et al., 2012), the fate and concentrations of metal NPs in the environment, especially in sediments, remains vastly unknown (Moore, 2006; Gottschalk et al., 2013). Mesocosm and laboratory based studies have shown that Cu and CuO NPs aggregate/agglomerate in seawater, brackish water and freshwater (Griffitt et al., 2008; Buffet et al., 2013; Buffet et al., 2011; Gomes et al., 2011, 2011; Gomes et al., 2011), favoring their deposition onto sediment (Klaine et al., 2012; Thit et al., 2015a). Sediment-dwelling organisms are thus particularly at risk of exposure to these NPs (Croteau et al., 2011; Luoma and Rainbow, 2008; Pang et al., 2013). Yet, little is known on the ecotoxicity of metal NPs for sediment-dwelling organisms. In addition, uncertainties remain as to whether metal NPs are more or less bioavailable and toxic than their ionic counterparts (Buffet et al., 2011; Buffet et al., 2013; Cong et al., 2011; Ramskov et al., 2014; Pang et al., 2013 Cong et al., 2011; Ramskov et al., 2014; Pang et al., 2013).

Abbreviations: WSBB, weight-specific body burden; CD, cellular debris; d, day(s); DI, deionized water; DL, detection limit; dw, dry weight; h, hour(s); HDP, heat denatured proteins; LOEC, lowest observed effect concentration; min, minutes; MOD, synthetic moderately hard freshwater; MRG, metal rich granules; MTLP, metallothionein-like proteins; NOEC, no observed effect concentration; OECD, the organization for economic co-operation and development; Org, Organelles; t, time; US EPA, the United States environmental protection agency; ww, wet weight.

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Metal accumulation is governed by metal-specific and species-specific uptake and elimination processes (Rainbow, 2002). Once accumulated, metals can be present in metabolically available or detoxified forms. Metabolically available metals can interact with cells and damage physiologically sensitive molecules, proteins, DNA and organelles (e.g., mitochondria) (Aruoma et al., 1991; Banci et al., 2010a). Thus, toxicity is dependent on the concentration of metabolically available metals (Rainbow, 2002; Mouneyrac et al., 2003; Amiard et al., 2006). The subcellular distribution of metals and metal NPs can be characterized by fractionation of tissues into five operationally-defined subcellular fractions, i.e., metallothionein-like proteins (MTLP), metalrich granules (MRG), organelles (ORG), heat denatured proteins (HDP) and cellular debris (CD) (Wallace et al., 2003; Garcia-Alonso et al., 2011). MTLP and MRG are involved in the detoxification and storage of metals in excess. Metals sequestered in these fractions are presumably not available to cause cell damage (Rainbow, 2002; Wallace et al., 2003; Cain et al., 2004). In contrast, metals found in HDP and ORG are considered "metabolically available" and can damage cells (Roesijadi, 1992; Viarengo and Nott, 1993; Rainbow, 2002). Metals associated to CD have uncertain toxicological relevance since this fraction includes membranes, intact cells, and nuclei as well as metals associated with external body surfaces (Wallace et al., 2003; Cain et al., 2004). The subcellular distribution of detoxified metals in an organism affects the potential for metal transfer to higher trophic levels, as it affects the metal assimilation efficiency by the predator (Berthet et al., 2003; and the litterature cited therein). For example, MTLPs are easily degraded during digestion. As a result, Cu sequestered in MTLPs is more bioavailable to predators, compared to Cu sequestered in the insoluble MRGs.

Here, we assess the bioavailability and subcellular distribution of Cu after sediment exposures to CuO NPs and aqueous Cu (Cu-Aq) in the sediment-dwelling freshwater oligochaete *Lumbriculus variegatus*. We also investigate whether burrowing behavior and survival are impacted by exposure to these forms of Cu. We chose *L. variegatus* as a model organism due to its widespread distribution throughout Europe and Northern America and its importance in the ecosystem (e.g., prey for a range of species) (Phipps et al., 1993). *L. variegatus* has also been recommended for toxicity testing (Chapman et al., 1999) and bioaccumulation studies (Phipps et al., 1993). This species is widely used as a standard US EPA and OECD test organism (e.g., OECD guideline 225 and 315). *L. variegatus* can be synchronized to reduce biological variation among individuals, thereby minimizing variability in test results. *L. variegatus* ingests sediment, from which it extracts the organic constituents as nutrient source (Phipps et al., 1993; Ankley et al., 1994). Since contaminants are also bound to sediments, sediment uptake is likely an important exposure route for metals (Phipps et al., 1993; Selck and Forbes, 1998; Camusso et al., 2012).

To circumvent the confounding influence of background Cu (in sediment and worms) and increase detection sensitivity (Croteau et al., 2014), isotopically enriched Cu was used in the exposures (^{65}CuO NPs and ^{65}Cu-Aq). The use of enriched stable isotopes further allowed us to conduct experiments at environmentally realistic Cu concentrations (Roman et al., 2007). In addition to bioaccumulation dynamics (Ramskov et al., 2015), knowledge of subcellular Cu distribution will help understand how sediment-dwellers, such as *L. variegatus*, respond to exposure to aqueous Cu and CuO NPs and handle the accumulated Cu.

2. Materials and methods

2.1. General experimental design

L. variegatus were exposed for up to 10 days (d) to sediment spiked with either isotopically enriched Cu (^{65}Cu-Aq) or isotopically modified ^{65}CuO NPs (see overview in Tables S1-S2, as well as Section 2.4). Additional worms were exposed to either clean sediment (controls) or sediment spiked with trace amounts of HNO_3 (pH control) to mimic the amounts of HNO_3 used in the ^{65}Cu-Aq treatments. An example of the exposure setting is shown in Fig. 1a. After 5 d of exposure, a subsample of worms from each treatment was transferred to clean sediment and allowed to eliminate the accumulated ^{65}Cu for up to 10 d. Mortality and burrowing activity were recorded throughout the exposure period (Table S2). At selected time points during uptake and elimination, *L. variegatus* were collected to measure weight-specific bodyburden (WSBB) and/or characterize the subcellular distribution of newly accumulated ^{65}Cu (Table S2). To minimize potential Cu contamination, all laboratory-ware was soaked for at least 24 hours (h) in 15% HNO_3 and 5% HCl (Baker), except for Teflon vials, which were soaked for 5 d in 30% HNO_3. Subsequently, all laboratory-ware was rinsed thoroughly with deionized and subsequently with Ultrapure (MilliQ) water and dried in a laminar flow hood before use.

2.2. Sediment preparation

Sediment was collected from San Francisco Bay, California, USA, one kilometer southeast of the discharge point of a Regional Water Quality Control Plant (Dyke et al., 2012), as described in Ramskov et al. (2015). According to Dyke et al. (2012), Cu concentration at the site varies between 30 and 60 μg Cu g^{-1} dw sed. Sediment was sieved with synthetic freshwater (MOD, 0.06 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.096 g L^{-1} $NaHCO_3$, 0.004 g L^{-1} KCl and 0.06 g L^{-1} $CaSO_4 \cdot 2H_2O$, USEPA 2002) to <63 μm (experimental sediment) or to <345 μm (for cultures) and left to settle overnight. The overlaying water was then removed, and the sediment was frozen at −20 °C. Prior to an experiment, sediment was thawed, rinsed with MOD water twice and homogenized thoroughly by hand mixing. The water content (28.1%) was determined after drying aliquots (n = 6) of sediment for 24 h at 105 °C. The organic content (7.6%) was determined as loss on ignition (6 h at 550 °C) on six sediment aliquots. The ^{65}Cu concentration of clean sediment (< 63 μm) was 73 ± 2 μg ^{65}Cu g^{-1} dry weight (dw) sediment (Ramskov et al., 2015). Total Cu concentration in the sediment was 220 μg Cu g^{-1} (Supplementary information, SI).

2.3. Experimental organisms

L. variegatus was purchased from Aquatic Foods (Fresno, California) and reared in the laboratory in glass aquaria filled with MOD water. Unbleached shredded paper tissues (Careness Nature 6103, Aabenraa, Denmark) were added as a substrate. The culture was kept at 15 °C in the dark. Aeration was provided using a pump, silicon tubes and glass pipette tips. The MOD water was changed weekly. Worms were fed finely ground TetraMin fish food weekly. Two weeks prior to the onset of the experiment, worms were transferred to an aquarium containing clean sediment (< 345 μm) and MOD water. One week prior to the onset of the experiment, large adult worms were selected and the physiological status of the worms was synchronized, as described in Ramskov et al. (2015). The anterior parts of *L. variegatus* were then transferred to an aquarium containing MOD water until use.

2.4. Test chemicals

Isotopically modified ^{65}CuO NPs were synthesized as described in Misra et al. (2012) using enriched $^{65}CuCl_2 \cdot 2H_2O$ (99% enrichment, Trace Sciences International, USA). The ^{65}CuO NPs were spherical with a diameter of 7 ± 1 nm and a hydrodynamic diameter in deionized (DI) water of 77 ± 5 nm. Cu concentration in the NP suspension was 3800 mg Cu L^{-1}. More information on the NP