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Effects of sediment-associated copper to the deposit-feeding snail, *Potamopyrgus antipodarum*: A comparison of Cu added in aqueous form or as nano- and micro-CuO particles

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Abstract

Increasing use of engineered nanoparticles (NPs) is likely to result in release of these particles to the aquatic environment where the NPs may eventually accumulate in sediment. However, little is known about the potential ecotoxicity of sediment-associated engineered NPs. We here consider the case of metal oxide NPs using CuO to understand if the effects of NPs differ from micron-sized particles of CuO and aqueous Cu ($CuCl_2$). To address this issue, we compared effects of copper added to the sediment as aqueous Cu, nano- (6 nm) and micro- (<5 μm) CuO particles on the deposit-feeding snail, *Potamopyrgus antipodarum*. Effects were assessed as mortality, specific growth rate, feeding rate, reproduction, and bioaccumulation after 8 weeks of exposure to nominal concentrations of 0, 30, 60, 120 and 240 μg Cu/g dry weight sediment. The results demonstrate that copper added to sediment as nano-CuO had greater effects on growth, feeding rate, and reproduction of *P. antipodarum* than copper added as micro-CuO or aqueous Cu. *P. antipodarum* accumulated more copper in the nano-CuO treatment than in aqueous Cu or micro-CuO treatments, indicating that consideration of metal form may be important when assessing risks of metals to the aquatic environment.

Keywords: sediment-associated copper oxide, nanoparticles, sediment, deposit feeder, bioaccumulation

1. Introduction

With the rapid development of nanotechnology, metal-based nanoparticles (metallo-NPs) are increasingly used in various consumer products such as cosmetics and sunscreens, dental fillings, solar-driven self-cleaning coatings and textiles (Royal Society, 2004). According to estimates, the number of consumer products on the market containing nanoparticles or nanofibers now exceeds 1300 and is growing rapidly (Project on Emerging Nanotechnologies, 2011). Nano-CuO has potentially wide industrial use in applications such as gas sensors, photovoltaic cells, antimicrobial coatings, in catalyst applications and in heat transfer nanofluids. Nanotechnology is used to modify material at the nano-scale (<100 nm) to create novel properties. Changes in the physicochemical and structural properties of materials caused by the decrease in particle size can lead to new and sometimes unexpected biological effects. Therefore, engineered NPs need to be evaluated in terms of their potential to pose risks to human health and the environment (Handy et al., 2008; Nowack, 2009).

Natural NPs, including nano-sized particles of metal oxides, exist in all ecosystems and play important roles in biogeochemical processes (Wigginton et al., 2007). As for other forms of metals, it can be expected that metallo-NPs will be released into the aquatic environment. Because many metallo-NPs interact when they come in contact with natural media such as freshwater, seawater and sediment, typically by dissolution and/or agglomeration, it is expected that they will preferentially partition to sediments. Until recently, most of the studies on the potential toxicity of NPs have focused on mammals (such as mice and rats) and/or on different types of cell lines. For example, Karlsson et al. (2008) showed that nano-CuO was highly toxic when compared to the bulk form of CuO, to other metal oxide nanoparticles as well as to carbon nanoparticles and carbon nanotubes in the human alveolar epithelial cell line A549 (Karlsson et al., 2008, 2009). Toxicity data for nano-sized CuO in aquatic organisms are rare, especially concerning effects of long-term exposure, and studies have mostly focused on bacteria, crustaceans (Heinlaan et al., 2008) and algae (Aruoja et al., 2009).

Deposit-feeding invertebrates may be exposed to deposited NPs through direct contact of body surfaces with sediment and by ingestion of sediment particles. Given that sediment is a relatively poor food source, deposit feeders have evolved to

ingest huge quantities of sediment (1–100 body weights daily), to preferentially select small, surface-rich particles for ingestion, and to be very good at solubilizing organic material associated with ingested sediment particles (Lopez and Levinton, 1987). For example, the snail, *Potamopyrgus antipodarum,* ingests approximately 6 mg sediment per mg body weight per day (Heywood and Edwards, 1962; Lopez and Levinton, 1987). For these reasons, deposit feeders may be at particular risk of exposure to metallo-NPs during the passage of sediment through the gut. There is evidence that uptake of metals such as Ag (Griscom and Fisher, 2002; Casado-Martinez et al., 2009) and Cd (Selck et al., 1999; Selck and Forbes, 2004; Baumann and Fisher, 2011) from the sediment-bound pool is the dominant route of uptake for deposit-feeding organisms. Most knowledge of the effects of bulk metals in aquatic systems is based on exposure via the water phase (despite the fact that bulk metals have long been shown to accumulate in high concentrations in sediment), and there is almost no information available on the bioavailability and toxicity of metallo-NPs via sediment exposure.

In the present study, the freshwater gastropod *P. antipodarum* was used because its feeding strategy includes processing large quantities of fine-grained sediment. European specimens of *P. antipodarum* have previously been referred to as *Hydrobia jenkinsi*, then *Potamopyrgus jenkinsi*, before it was shown that the European species was identical to *P. antipodarum* and was introduced from New Zealand to Europe in about 1859 (Benson and Kipp, 2011). It is an invasive parthenogenetic ovoviviparous deposit feeder (Robson, 1926; Winterbourn, 1970) that is found in running waters from small creeks to streams, lakes and estuaries (Winterbourn, 1970), in mud and sand, on rocks, gravel and aquatic plants (Michaut, 1968). This species has been recommended for use in the development of a reproduction test within the Organisation for Economic Co-operation and Development (OECD) guideline program (Duft et al., 2007).

The aims of the present study were to compare the bioavailability and effects of Cu, added to sediment as aqueous Cu ($CuCl_2$), nano- or micro-CuO, on *P. antipodarum.* The long-term effects of the three forms of Cu on survival, growth rate, feeding rate and reproduction, as well as bioaccumulation were assessed. In the following, the different treatments will be referred to as the original copper form added to the sediment (i.e., aqueous Cu, nano- and micro-CuO).

2. Materials and methods

2.1. Chemicals

$CuCl_2 \cdot 2H_2O$, a source of aqueous Cu, and micro-CuO (<5 µm, cat. #20, 884-1, assay 98%) were purchased from Sigma–Aldrich (Broendby, Denmark). Copper acetate, sodium hydroxide and acetic acid were purchased from VWR for the synthesis of nano-CuO. The stock suspensions/solutions of the tested chemicals were prepared in deionized water (Millipore, resistivity < 18 MΩ cm^{-1}) for spiking sediment.

2.2. Nano-CuO and micro-CuO characterization

CuO nanoparticles (6 nm) were prepared by reacting Cu $(CH_3COO)_2 \cdot 2H_2O$ in the presence of NaOH and acetic acid (Hong et al., 2002). Crystal structure of nano- and micro-CuO powder was characterized by X-ray diffraction (XRD). Data were collected using a Nonius PDS 120 powder diffraction system equipped with a position sensitive detector. The characterization of zeta potential was carried out on the stock solutions using a Malvern Zetasiser Nano ZS. Stock suspensions of CuO nanoparticles were obtained by washing the samples three times with deionised water at the end of the synthesis. The final concentration of the suspension was 2.8 mg/mL (measured by ICP-AES). Stock micro-CuO suspensions were prepared by adding 9 mg in 7 mL deionised water and whirl mixing for 2 min. Particle size and morphology were assessed by transmission electron microscopy (TEM) using a Hitachi H-7100 operating at 100 kV and scanning electron microscopy (SEM) using a Philips XL30 at 5 kV.

2.3. Sediment spiking

Sediments were collected from Isefjord, Roskilde, Denmark. The top 5 cm of the surface sediment was collected and sieved (<250 µm for snail cultures; <125 µm for the experiment) using deionized water, rinsed three times using artificial freshwater (192 mg/L $NaHCO_3$, 8 mg/L KCl, 120 mg/L $MgSO_4$ and 120 mg/L $CaSO_4 \cdot 2H_2O$ in deionized water, pH 7.2) (USEPA, 2002), and then frozen (−20 °C) until use. The sieved sediment (<125 µm) had a water content of 37.7% (24 h at 105 °C; n = 3) and an organic content of 2.2% (6 h at 550 °C; n = 3). The background Cu concentration in the sediment (<125 µm) was 17.4 ± 0.74 (n = 3) µg Cu/g dry weight sediment. The nominal concentrations of each Cu form were 0, 30, 60, 120 and 240 µg Cu/g dry weight sediment, and the spiking efficiency was checked by flame atomic absorption spectrometry (FAAS, SpectrAA-220, VARIAN, Mulgrave, Australia). Sediments were spiked with Cu in a two step process. First, stock sediments (360 µg Cu/g dry weight sediment) of the different Cu forms were made by adding Cu dissolved (aqueous)/dispersed (nano-CuO, micro-CuO) in deionized water to wet sediment which was mixed for 24 h on a shaking table. Secondly, the Cu concentrations in the stock sediments were measured by FAAS, and the sediment was diluted with natural wet uncontaminated sediment to the desired concentrations and mixed for an additional 24 h on a shaking table.

2.4. Test species

P. antipodarum were collected from Salvadparken, Roskilde, Fjord, Denmark (Latitude: 55°38′31″N; Longitude: 12°5′16″E) in September 2009 and reared for 4 months in 10 L aquaria at 17 °C on natural pre-frozen sediment (<250 µm) and artificial freshwater. A food supplement of ground commercial fish food (Tubifex labiryn-basic, Tubifex Company, Częstochowa, Poland), baby cereal (Ekologisk Luomu Urtekram, Denmark) and dried spinach in equal ratios by weight was added to cultures twice a week. The overlying water was renewed every month, and every 4–6 weeks the cultures were given 3–4 spoonfuls of fresh pre-frozen sediment (Pedersen et al., 2009).

2.5. Experimental set up

P. antipodarum (shell length ≈ 4.1 mm) were exposed individually to each form of Cu with 10 replicates per treatment. Snails were exposed in 5 mL Nunclon multi-well dishes (Becton Dickinson Labware, Wilmington, North Carolina, USA) containing 0.3 g dry weight experimental sediment and 2.5 mL artificial freshwater. The overlying water was oxygenated before use and changed every 2 days to maintain adequate oxygen levels. Sediments were renewed every week to ensure adequate food levels and consistent metal exposure concentrations. The wells were covered with a lid and placed in an environmental chamber at 17 °C and a 12 h light:12 h dark photoperiod. The exposure period was 8 weeks. After the exposure, snails were transferred to clean wells containing oxygenated artificial freshwater in which they were held for 24 h to empty all remnants of sediment from their guts. Snails were rinsed in EDTA (1 mM) and milliQ water

three times, and transferred to glass containers, before storage at −20 °C until chemical analysis.

2.6. Specific growth rate

Individual shell length (L) was measured using an image analysis program (SigmaScanPro, version 5.0, Jandel, Erkrath, Germany) on day 0 and day 56. The Specific growth rates (SGRs) from day 0 to day 56 were estimated by the following equation (Kaufmann, 1981):

$$\text{Specific growth rate (SGR)}\% = \left[\frac{(\ln S_2 - \ln S_1)}{(t_2 - t_1)}\right] \times 100$$

where S_1 and S_2 are the shell lengths (mm) at time t_1 and t_2, respectively.

2.7. Mortality, reproduction, feeding rate, and bioaccumulation

The number of surviving offspring and adult mortality in each experimental well were recorded every week by examining snails under a dissecting microscope (at 10×).

Because embryos take approximately 30–35 days (at 15 °C) to form inside the brood pouch, we disregarded offspring production during weeks 1–4, and instead quantified reproductive output during weeks 5–8 so that we could be sure that all offspring were exposed from the start of their development. Average feeding rate (from week 5 to week 8) was measured as the dry weight of fecal pellets produced per day. Each week the fecal pellets from individual adult snails were sieved from the sediment with a 150 µm sieve and rinsed with deionized water. The pellets were dried at 105 °C for 24 h and subsequently weighed. The concentrations of Cu in fecal pellets were measured by FAAS. In order to measure body burden with the analytical detection limits for Cu it was necessary to pool all 10 snails per treatment. After 8 weeks, the concentration of Cu in snails was measured by FAAS. Copper in the sediment at the end of the 4th week was measured by FAAS to compare with the measured concentrations at the start of the experiment.

In the procedure of FAAS, all samples (snail, sediment, or fecal pellets) were lyophilized (Christ Alpha 1–2, Osterode, Germany) at around −50 °C overnight before digestion. A pool of 10 snails was ground into powder using a mortar and pestle. The lyophilized sediment, fecal pellets or resulting snail powder were transferred to a weflon tube, weighed and digested with 65% HNO_3 in a microwave oven (Milestone MLS-1200 Mega, Leutenkirch, Germany). The digestion program included 6 min each at 250 W, 400 W, 650 W and 250 W. After cooling, the digestion solution was filtered and measured by FAAS.

2.8. Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey's Honestly-Significant-Difference Test was used to determine significant differences among Cu forms for all of the exposed groups (excluding the control). One-way ANOVA followed by Dunnett's test was used to compare exposed versus control treatments for each Cu form separately. Analysis of covariance (ANCOVA), with Cu form as the factor and body burden as the covariate, was used to determine whether differences among Cu forms in effects on snail feeding rate, SGR and reproduction were the result of differences in bioavailability, toxicity or both. The differences were considered significant when $p \leq 0.05$ and marginally significant when $0.1 \geq p > 0.05$. All statistical analyses were conducted using SYSTAT version 13.

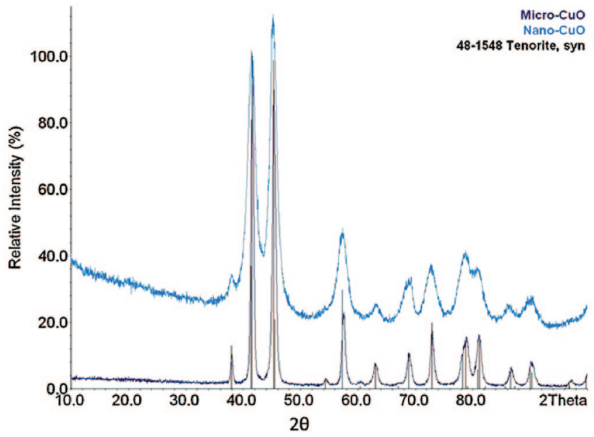

Figure 1. Powder XRD patterns of nano and micro CuO samples corresponding to tenorite (ICDD 48-1548).

3. Results

3.1. Measured sediment concentrations

The start sediment concentrations were close to the nominal concentrations with an added background concentration of ca. 17 µg Cu/g dry weight sediment. There was not much change in sediment Cu concentration between the start and end of the weekly sediment change. However, the concentration of Cu in fecal pellets was substantially higher (between 67 and 856 times higher) than the concentration of Cu in sediment (Table 1).

3.2. Characterization of nano- and micro-CuO

Both powders were identified as tenorite copper oxide by XRD and no other material was detected (Figure 1). Synthesised CuO NPs were monodispersed while the commercial micro-CuO sample was polydispersed with the presence of both micro- and nano-sized particles, as shown in Figure 2. The average size of CuO NPs was 6 ± 1 nm. Nanoparticles ranging from 10 to 50 nm were present in the micro-CuO (Figure 2). The suspensions prepared as described above were both visually clear and no sedimentation was observed. These observations were confirmed by zeta potential measurements in deionized water, especially for nano-CuO (44.0 mV) compared to micro-CuO (−16.9 mV). Zeta potential measurements indicate the relative stability of a suspension. However, the zeta potential drops significantly in fresh water and stabilizes at approximately 9 and 0 mV, respectively. The drop in zeta potential indicates the destabilization of the suspensions and sedimentation can be visually assessed for both samples. In such cases the effective surface area exposed by the agglomerates is much lower than the nanoparticles that are well suspended, and surface area is a key aspect that can affect dissolution.

3.3. Mortality, growth, feeding rate, and reproduction

There was no or minimal mortality of Cu in all treatments (data not shown). There was no interaction effect of copper form and copper concentration on SGR (Cu form × Cu Concentration: $p = 0.428$). Copper form significantly affected SGR ($p = 0.007$), as did copper concentration ($p = 0.028$). Comparison among the three copper forms showed that SGR was significantly lower for nano-CuO than for aqueous Cu ($p = 0.007$; Figure 3). There was no significant difference between micro-CuO and nano-CuO ($p = 0.673$), but SGR was marginally lower for micro-CuO than for aqueous Cu ($p = 0.062$). There was no

Table 1. The nominal and actual (measured) concentrations of Cu in start sediment, end sediment with fecal pellets, fecal pellets alone (FP), and calculated end sediment (mean ± SD, n = 3) (units: µg Cu/g dry weight sediment).

Form	NC[a]	Start sed	End sed with FP	Fecal pellets	End sed[b]
Aqueous Cu	0	17.4 ± 0.74	18.1 ± 2.09	82.0 ± 34.61	14.9
	30	46.4 ± 2.11	45.1 ± 0.83	166.4 ± 11.23	37.2
	60	76.1 ± 0.62	74.4 ± 8.50	290.6 ± 27.98	62.4
	120	127.2 ± 4.27	120.1 ± 6.90	426.1 ± 43.62	103.5
	240	238.4 ± 13.08	226.1 ± 10.94	1050.5 ± 168.02	193.6
Nano-CuO	0	17.4 ± 0.74	18.7 ± 0.73	82.0 ± 34.61	15.5
	30	44.0 ± 1.31	37.63 ± 3.59	188.8 ± 48.78	32.4
	60	71.9 ± 3.48	65.8 ± 3.94	290.4 ± 96.33	56.4
	120	122.5 ± 5.31	116.3 ± 10.48	387.5 ± 155.25	102.1
	240	231.8 ± 10.21	227.7 ± 9.56	444.6 ± 151.19	216.4
Micro-CuO	0	17.4 ± 0.74	17.5 ± 0.88	82.0 ± 34.61	14.3
	30	45.7 ± 1.53	45.2 ± 4.78	139.04 ± 34.61	40.6
	60	72.5 ± 5.88	72.5 ± 3.29	239.6 ± 53.14	61.4
	120	123.3 ± 9.72	129.7 ± 9.25	512.9 ± 62.69	114.1
	240	251.8 ± 4.02	248.0 ± 13.89	606.9 ± 149.00	229.5

a. NC-nominal concentration.
b. Calculated concentration of Cu in experimental week 8 (Mean value of the total amount of Cu in sediment at the end of the experiment minus the total amount of Cu measured in FP, divided by the total amount of sediment in the treatment).

significant effect of aqueous Cu on SGR at any exposure concentration (p = 0.918), whereas micro-CuO had a marginal effect on SGR (p = 0.085). In contrast, SGR was significantly lower at nano-CuO exposure concentrations of 60, 120, 240 µg Cu/g dry weight sediment treatments compared to the control (Conc-60: p = 0.001; Conc-120: p = 0.024; Conc-240, p = 0.029; Figure 3).

Overall, feeding rate decreased with increasing Cu concentration for all copper forms (Figure 4). There was no interaction effect of copper form and copper concentration on feeding rate (Cu form × Cu concentration: p = 0.935). Copper form significantly affected feeding rate (p = 0.01), and copper concentration marginally affected feeding rate (p = 0.083). Comparison among the three copper forms showed that feeding rate was significantly lower for nano-CuO than for aqueous Cu (p = 0.001; Figure 4). There was no significant difference between micro-CuO and nano-CuO (p = 0.423) or aqueous Cu (p = 0.158). Average feeding rate was significantly lower at all exposure concentrations compared to the control for nano-CuO (all p < 0.02) and micro-CuO (all p < 0.03). For aqueous Cu, the 240 µg Cu/g dry weight sediment treatment differed significantly from the control (p = 0.005) and the 120 µg Cu/g dry weight sediment treatment differed marginally from the control (p = 0.064).

There was no interaction effect of copper form and copper concentration on reproduction (p = 0.867). Copper form

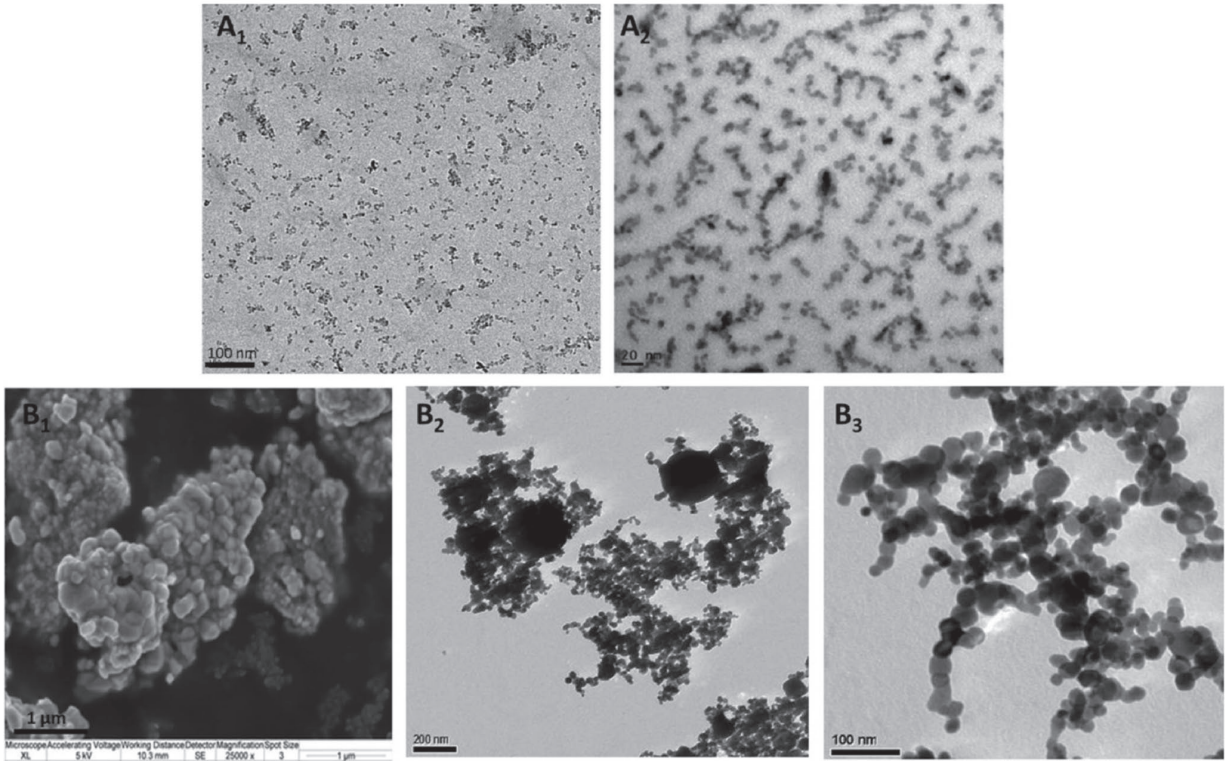

Figure 2. TEM images of A) nano-CuO, B_2–B_3) micro-CuO particles and SEM image of B_1) mirco-CuO showing monodispersed CuO nanoparticles in the synthesised sample *vs.* polydispersed micro and nanoparticles in the commercial micro-CuO sample.

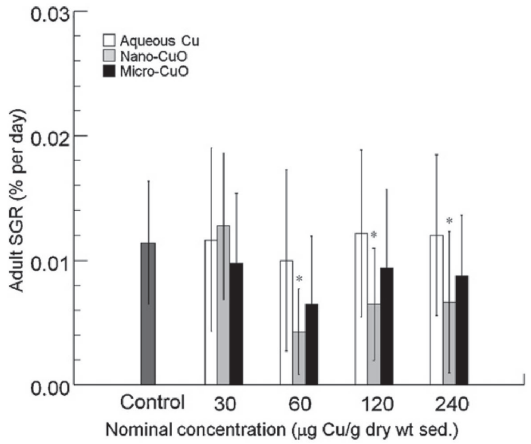

Figure 3. Specific growth rate (SGR). Effects of three Cu forms (sediment spiked with aqueous Cu, nano-CuO or micro-CuO) and different copper concentrations on *P. antipodarum* from day 0 to day 56 (mean ± SD, n = 10). Asterisks represent significantly different effects of concentrations on SGR in each Cu-treated group compared to the control ($p \leq 0.05$).

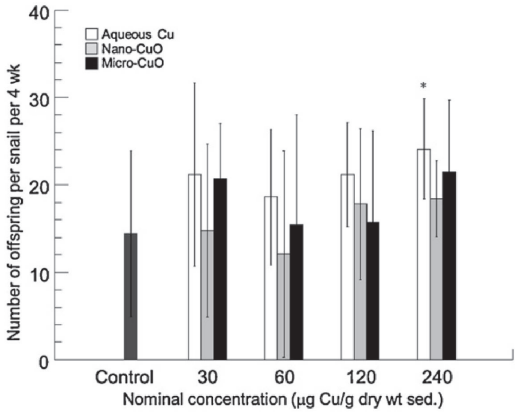

Figure 5. Reproduction effects of three Cu forms (sediment spiked with aqueous Cu, nano-CuO or micro-CuO) on *P. antipo*darum. Reproduction was determined as total number of offspring produced during 4 weeks (weeks 5–8; mean ± SD, n = 10). Asterisks represent significantly different effects of concentrations on reproduction in each Cu-treated group compared to the control ($p \leq 0.05$).

significantly affected reproduction (p = 0.016). Copper concentration marginally affected reproduction (p = 0.058). Comparison among the three copper forms showed that reproduction was significantly lower for nano-CuO than for aqueous Cu (p = 0.011). There was no significant difference between micro-CuO and nano-CuO (p = 0.366) or aqueous Cu (p = 0.262). For all three forms, reproduction did not differ significantly from the control except in the 240 µg Cu/g dry weight sediment aqueous Cu treatment (p = 0.014; Figure 5).

3.4. Body burden

Generally, copper body burden (BB) increased with increasing exposure concentration for all copper forms (Figure 6). Copper BB in snails appeared to be higher for nano-CuO than for aqueous Cu or micro-CuO in three of the four exposure concentrations, though the data could not be tested statistically since all snails in each treatment were pooled for analysis (i.e., one data point per treatment).

4. Discussion

Based on published toxicity studies (Oberdörster et al., 1994; Smith et al., 2007; Aruoja et al., 2009; Schiestl et al., 2009), concerns about the risks of NPs to the environment have been raised. More and more toxicity investigations of NPs show that the environmental fate and ecotoxicity of NPs are influenced by more factors than particle composition, such as particle size/size distribution, solubility and agglomeration, shape and crystal structure, surface area, mass and number concentrations, charge and chemistry and the presence of impurities (Tiede et al., 2008). NPs are potentially more toxic than bulk materials with the same composition mostly due to the increased specific surface area and reactivity, which may lead to increased bioavailability and toxicity. For example, Bello et al. (2009) showed that ferric reducing ability of serum (FRAS)-measured biological oxidative damage to be strongly correlated with NP specific surface area and total content of selected transition metals (especially Fe, Cr, Co, Mo and Mn). Some studies have demonstrated that nano-CuO is up to 50-fold more toxic than bulk CuO towards crustaceans (Heinlaan et al., 2008), algae (Aruoja et al., 2009), protozoa (Mortimer et al., 2009) and yeast (Kasemets et al., 2009). In all of these studies nanoparticulate CuO with an average size of 30 nm was used along with bulk CuO which however was not characterized. All of these studies were also conducted in water. In our study, we did not see such a large difference in toxicity among Cu forms. The reduced difference observed in the present

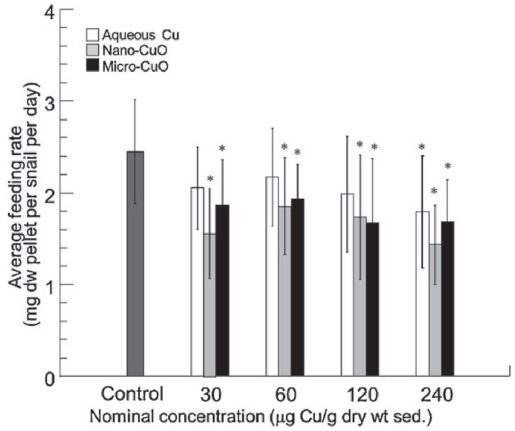

Figure 4. Average feeding rate. Effects of three Cu forms (sediment spiked with aqueous Cu, nano-CuO or micro-CuO) on *P. antipodarum* (weeks 5–8; mean ± SD, n = 10). Asterisks represent significantly different effects of concentrations on average feeding rate in each Cu-treated group compared to the control ($p \leq 0.05$).

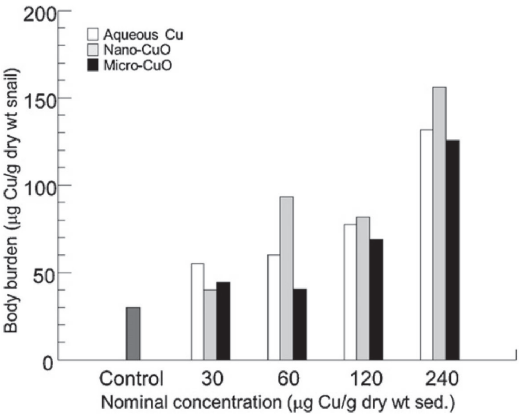

Figure 6. Copper body burden in whole snails after exposure for 8 weeks. Snails were pooled within treatments (n = 1 containing up to 10 snails).

Table 2. Copper in different compartments after exposure for 8 weeks at the higher concentration (60, 120, 240 µg Cu/g dry weight sediment treatments).

Treatment		Aqueous Cu	Nano-CuO	Micro-CuO
Compartment	NC[a]			
The Cu amount passed through snails (µg) (amount in snails + amount in fecal pellets)	60	29.14	22.82	26.89
	120	40.10	34.39	37.76
	240	78.62	27.70	44.96
Cu amount in snails (µg)	60	0.23	0.31	0.16
	120	0.28	0.31	0.28
	240	0.50	0.58	0.48
Percent accumulated Cu (%)	60	0.79	1.36	0.60
	120	0.70	0.90	0.74
	240	0.64	2.09	1.07

[a] NC-nominal concentration, units: µg Cu/g dry weight sediment.

study may, at least partly, be related to a difference in metal speciation when mixed into the sediment compartment. However, how the association of nano-metals with sediment components changes over time, and whether metallo-NPs added to sediment would become more or less bioavailable compared to aqueous forms of metal remains to be determined.

In the present study, adult SGR, feeding rate and reproduction were significantly lower for nano-CuO than for aqueous Cu. This suggests a physical component to the toxicity of the nano-CuO that was not simply caused by dissolution of the NPs releasing soluble copper ions, which is consistent with the results of Buffet et al. (2011). We also found that Cu body burden was somewhat higher for snails exposed to nano-CuO compared to aqueous Cu and micro-CuO. Currently, little is known about relative bioavailability of metallo-NPs compared to bulk metals in invertebrates; however studies of mammals have shown that NPs are more easily taken up by cells compared to metal ions. For example, Limbach et al. (2007) found that iron-, cobalt-, manganese-, and titania-containing silica nanoparticles efficiently entered lung epithelia cells (A549) compared to reference cultures exposed to aqueous solutions of the same metals. The study suggests that the metallo-NPs were taken up by a Trojan-horse type mechanism and that the cell membrane of lung epithelia cells seems to have improved barrier capacities for metal ions compared to metal particles. The pathway and extent of uptake of insoluble particles through the digestive tract are known to be size-dependent (Hodges et al., 1995; Donaldson et al., 1998). Chen et al. (2006) compared small size copper nanoparticles (23.5 nm) and copper microparticles (17 µm) administered by oral gavage to mice and found that nanoparticles induced substantial toxicological effects and heavy injuries to kidney, liver and spleen of experimental mice which was not the case for copper microparticles. Moreover, NPs can promote phagocytosis at gastrointestinal mucosa and cause antigen-mediated immune responses (Lomer et al., 2002).

It has been demonstrated that CuO nanoparticles are much more cytotoxic and genotoxic and show a much higher ability to cause mitochondrial depolarization compared to micron-sized particles of CuO (Karlsson et al., 2009). CuO nanoparticles have also been shown to cause oxidative stress, measured as an increase in oxidative DNA damage, whereas this could not be seen for micron-sized particles of CuO (Karlsson et al., 2005). In particle-ingesting organisms, such as *Daphnia*, accumulation of NPs in the digestive tract has been observed (Baun et al., 2008; Kahru et al., 2008). In our study, there were no significant differences in toxicity between nano- and micro-CuO forms added to sediment. This may have been due to the presence of nanoparticles in the micro-CuO sample, which also underlines the importance of including characterization data when conducting toxicity tests (Cong et al., 2011).

In the present study, feeding rate decreased with increasing exposure concentration for all copper forms. Adult specific growth rate decreased with increasing exposure concentration for nano-CuO, but not for the other Cu forms. Metal exposure may decrease feeding rate of organisms, which can sometimes be caused by avoidance of highly contaminated sediment or by impairment of digestive processes, which prevents gut transit of the food (Luoma and Rainbow, 2008). Although no effects on mortality occurred for any Cu form or concentration in our study, it is possible that mortality would have occurred over a longer exposure period, particularly in those treatments in which feeding was reduced.

The number of surviving offspring increased with increasing exposure concentration for all Cu forms. This result was unexpected, and we did not follow the longer-term survival of offspring. However, even though there was no difference in reproduction between the control and any of the copper forms, the number of offspring was significantly lower for snails exposed to nano-CuO than to aqueous Cu. This may indicate that the observed reproductive effects are more due to physical effects of the particles than to aqueous Cu toxicity. Further analyses would be needed to confirm this hypothesis.

In the present study, copper body burdens increased with increasing exposure concentration for all copper forms. A number of studies indicate that freshwater snails accumulate metals, including Cu, from three routes of exposure: water, sediment, and diet (Laskowski and Hopkin, 1996; Gomot and Pihan, 1997; Gomot-de Vaufleury and Pihan, 2002; Heng et al., 2004; Notten et al., 2005). In the present study, the primary route of copper exposure to *P. antipodarum* was via dietary intake of sediment. Most organisms have evolved strategies for

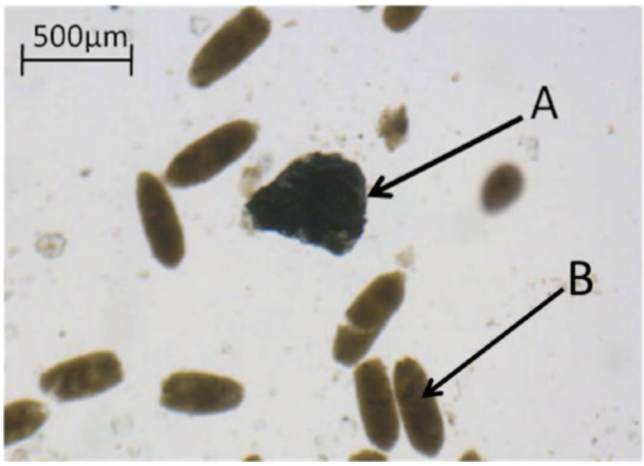

Figure 7. Picture of a large black micro-CuO particle (A) in the 240 µg/g micro-CuO treatment. B: fecal pellets.

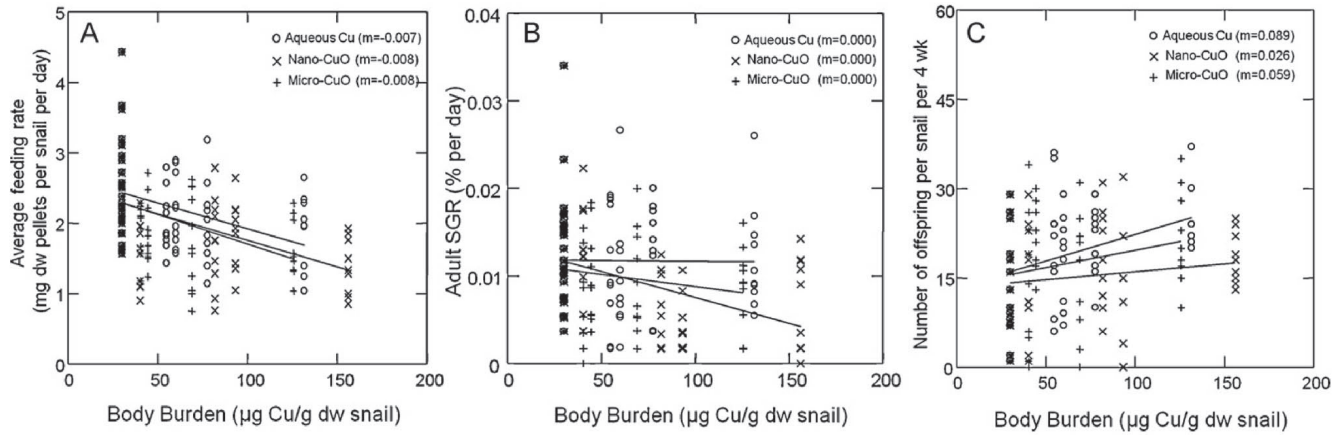

Figure 8. Relationship between Cu body burden in snails and feeding rate, SGR, and reproduction of *P. antipodarum* in sediment spiked with aqueous Cu, nano-CuO or micro-CuO (mean ± SD, $n = 10$; The slopes (m) for the regressions of the response parameters versus BB are shown in the figure legend). Feeding rate (A): ANCOVA, BB: $p < 0.01$, Form: $p > 0.1$; SGR (B): ANCOVA, BB: $p < 0.01$, Form: $p > 0.1$; Reproduction (C): ANCOVA, BB: $p < 0.01$, Form: $p \leq 0.05$. None of the interaction terms between BB and form were significant.

regulating internal copper concentrations, since copper is an essential metal. Some species store excess copper by sequestering the metal in forms that are either metabolically available (e.g., as metallothionein) or unavailable (e.g., phosphate granules) (Phillips and Rainbow, 1989; Simkiss, 1981; Viarengo, 1989; Depledge and Rainbow, 1990; Mason and Nott, 1981).

Desouky (2006) found that the number of granules in the digestive gland of the garden snail increased after 10 days of exposure to metals (Al, Zn, Cd). In granule cells, metals can bind to ligands such as P and S and become insoluble and immobile. This mechanism may account for the high Cu concentration found in *P. antipodarum*. In addition, copper body burden in snails appeared to be higher for nano-CuO than for aqueous Cu and micro-CuO. Although the result could not be tested statistically due to the need to pool snails for analysis, it is consistent with unpublished pilot studies in our laboratory. One hypothesis to explain such a result could be a more efficient binding of aqueous Cu to metallothioneins and other low-molecular-weight proteins or uptake into granules. If nano-CuO enters cells as particles, it may not readily bind to metallothioneins or be incorporated into granules and thus be more difficult to regulate.

Bioavailability of contaminants in food is a function of many factors. The size of a food particle can determine bioavailability as well as the chemical form of the associated contaminants (Gibaldi, 1991). Generally, smaller food particles can contain more contaminant than large particles with the same mass because particle surface areas increase with decreasing particle size. Many deposit feeders, such as *P. antipodarum* (Hylleberg and Gallucci, 1975; Taghon, 1982; Lopez and Kofoed, 1980), preferentially ingest small particles, and this very likely explains our observation of a much higher concentration of copper in fecal pellets compared to sediment for all copper forms and concentrations (Table 1). Furthermore, gastropods have complex sorting mechanisms in the gut and digestive diverticula that are strongly affected by particle size. Particles ingested by gastropods are ground in the gizzard, sorted by a crystalline style, and only particles ≤4 µm pass into the digestive gland for intracellular and/or extracellular digestion, whereas larger particles pass directly to the intestine for egestion (Dillon, 2000). Therefore, it would be expected that particle size of food passing through the gut can modify bioavailability of contaminants associated with it.

Table 2 shows copper in different compartments after exposure for 8 weeks at the higher exposure concentrations. Particularly at the two highest exposure concentrations, the amount of nano-CuO passing through the guts of snails was lowest but the percent of accumulated Cu was highest (Table 2). Thus Cu added to sediment as nano-CuO was more bioavailable to *P. antipodarum* than Cu added in micro form. Thus, our results suggest that particle selection by deposit feeders may be an important factor that could increase the bioavailability and toxicity of metallo-NPs compared to metals in bulk form and contribute to differences among different size classes of NPs.

The presence of large black particles in the sediment of the micro-CuO treatment at the highest concentration suggests that micro-CuO aggregated (Figure 7). These aggregates were not observed at the lower concentrations or in the control, aqueous Cu, or nano- treatments. The aggregates of micro-CuO did not seem to reduce the amount of micro-CuO passing through snail guts. In fact the amount of micro-CuO passing through the guts of snails was higher than the amount of nano-CuO at the highest exposure concentration. Consistent with this observation was that feeding rate in the micro-CuO treatment was higher than in the nano-CuO treatment (Figure 4).

In the present study, snails passed more sediment-associated aqueous Cu through their guts than nano-CuO, but accumulated less aqueous Cu than nano-CuO at the same exposure concentration (Table 2). Similarly, we found that snails passed more aqueous Cu through their guts than micro-CuO, but accumulated less aqueous Cu than micro-CuO at the highest exposure concentration (Table 2). This could suggest that aqueous Cu is associated more strongly to sediment particles than nano-CuO and micro-CuO. Several processes will influence whether a metal in sediment becomes bioavailable, such as physical, chemical and biochemical phenomena that bind, unbind, expose or solubilize a metal, or movement of a released metal to the membranes of an organism, etc. (Ehlers and Luthy, 2003; Luoma and Rainbow, 2008). The different physicochemical properties of aqueous Cu, micro-CuO and nano-CuO may result in differences in the way the different forms of Cu are bound in sediment with consequences for bioavailability. In addition, since the micro-CuO was also found to contain nanoparticles (10–50 nm), this may explain the minimal differences that we observed between the nano- and micro-CuO treatments.

In order to determine whether differences in effects on snail feeding rate, SGR and reproduction were the result of differences in bioavailability, toxicity or both, we performed an ANCOVA with form as the categorical variable and body burden as the covariate. The results are shown in Figure 8. For feeding rate and SGR, the analyses show that body burden

influenced snail responses, but there is no additional effect of form when adjusted for body burden. This suggests that the difference among forms is due to differences in bioavailability and not to differences in reactivity when normalized to body burden. For reproduction, both body burden and form influenced reproductive output suggesting that both bioavailability and reactivity of different Cu forms influenced snail reproduction (Figure 8).

5. Conclusions

Few ecotoxicity studies have focused on the effects of long-term exposure to sediment-associated metal oxide nanoparticles on deposit feeders. Here, we reported the first comparative study of the chronic effects of sediment spiked with three copper forms on *P. antipodarum*. Our results demonstrated that the three forms of Cu differed in bioavailability and that this difference alone could explain differences in effects on snail feeding rate and growth rate. There was a greater negative effect of nano-CuO than the other forms of Cu on snail reproduction, even when differences in bioavailability were accounted for, suggesting that differences in reactivity, as well as bioavailability, contributed to effects on this endpoint. The different physicochemical properties of aqueous Cu and nano-CuO may result in differences in the way the different forms of Cu are bound in sediment with consequences for bioavailability. Our results suggest that for the same total metal sediment concentrations, nano-CuO was more bioavailable than aqueous Cu to *P. antipodarum*. Based on our study, it seems that the environmental fate and effects of nano-CuO may not be accurately predicted from studies of other forms of Cu. However, whether such differences among Cu forms are large enough to be of practical importance for ecological risk assessment remains to be determined.

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