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# Differences in Soil Solution Chemistry between Soils Amended with Nanosized CuO or Cu Reference Materials: Implications for Nanotoxicity Tests

Heather V. A. McShane,*,[†] Geoffrey I. Sunahara,[‡] Joann K. Whalen,[†] and William H. Hendershot[†]

[†]Department of Natural Resource Sciences, McGill University, 21,111 Lakeshore Drive, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada

[‡]National Research Council—Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada

**S** *Supporting Information*

**ABSTRACT:** Soil toxicity tests for metal oxide nanoparticles often include micrometer-sized oxide and metal salt treatments to distinguish between toxicity from nanometer-sized particles, non-nanometer-sized particles, and dissolved ions. Test result will be confounded if each chemical form has different effects on soil solution chemistry. We report on changes in soil solution chemistry over 56 days—the duration of some standard soil toxicity tests—in three soils amended with 500 mg/kg Cu as nanometer-sized CuO (nano), micrometer-sized CuO (micrometer), or $Cu(NO_3)_2$ (salt). In the CuO-amended soils, the log $Cu^{2+}$ activity was initially low (minimum −9.48) and increased with time (maximum −5.20), whereas in the



salt-amended soils it was initially high (maximum −4.80) and decreased with time (minimum −6.10). The $Cu^{2+}$ activity in the nano-amended soils was higher than in the micrometer-amended soils for at least the first 11 days, and lower than in the salt-amended soils for at least 28 d. The pH, and dissolved Ca and Mg concentrations in the CuO-amended soils were similar, but the salt-amended soils had lower pH for at least 14 d, and higher Ca and Mg concentrations throughout the test. Soil pretreatments such as leaching and aging prior to toxicity tests are suggested.
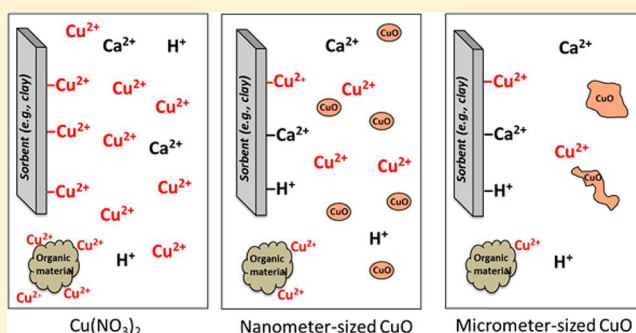
## INTRODUCTION

In terrestrial toxicity tests, organisms will be exposed to a suspected toxicant for a period of weeks or months (e.g., Organisation for Economic Cooperation and Development 1984, 2004),[1,2] In nanotoxicity tests, exposure to the nanometer-sized (nano) particles is often contrasted with that of exposure to the material in its micrometer-sized (micrometer) form.[3−5] If testing for the effects of soluble metal-based ENPs, then metal salt treatments are included to distinguish between toxic effects from released metal ions and those attributed to nanoparticles.[4,6,7] In these salt treatments, organisms are exposed to the concentration of metal ions expected to be released in the ENP-amended treatments during the experimental period. During the test period, the different amendments will begin to equilibrate with the test soils. Equilibration processes may modify the soil solution chemistry in the different treatments,[8−10] and resulting differences between exposure media could obscure nanoparticle-specific effects. Data on these changes will facilitate the design of robust terrestrial nanotoxicity tests.

Nano CuO is a sparingly soluble metal oxide nanomaterial. Its effects on organisms are investigated because of its present and potential use in commercial applications (e.g., Anita et al., 2011, Rastogi et al, 2011),[11,12] and the known toxicity of excess

Cu to organisms.[13,14] Most nano CuO toxicity tests to date have been conducted in aquatic media, and have included micrometer CuO and Cu salt treatments to distinguish between nanospecific and non-nanospecific effects (e.g., Dimkpa et al., 2011, Shi et al., 2011, Wang et al., 2012).[7,15,16] Measurements were taken during a relatively short exposure period—less than 3 days—and changes in solution chemistry during the tests were not considered. However, research suggests that in longer terrestrial tests, changes in properties thought to affect toxicity, such as $Cu^{2+}$ activity, pH, and dissolved cation concentrations,[17−19] may be not be comparable in soils amended with different sources of Cu.[8,20,21]

In soils amended with nano CuO, micrometer CuO or Cu salts, differences in soil solution chemistry are likely to develop if equilibration processes or rates are dissimilar. Schematic 1 illustrates some of the major processes likely to occur as these CuO particles and Cu salts equilibrate with the soil. Soluble metal oxides in unsaturated solutions gradually dissolve, with an associated increase in metal ion activity.[20] In aquatic nano CuO
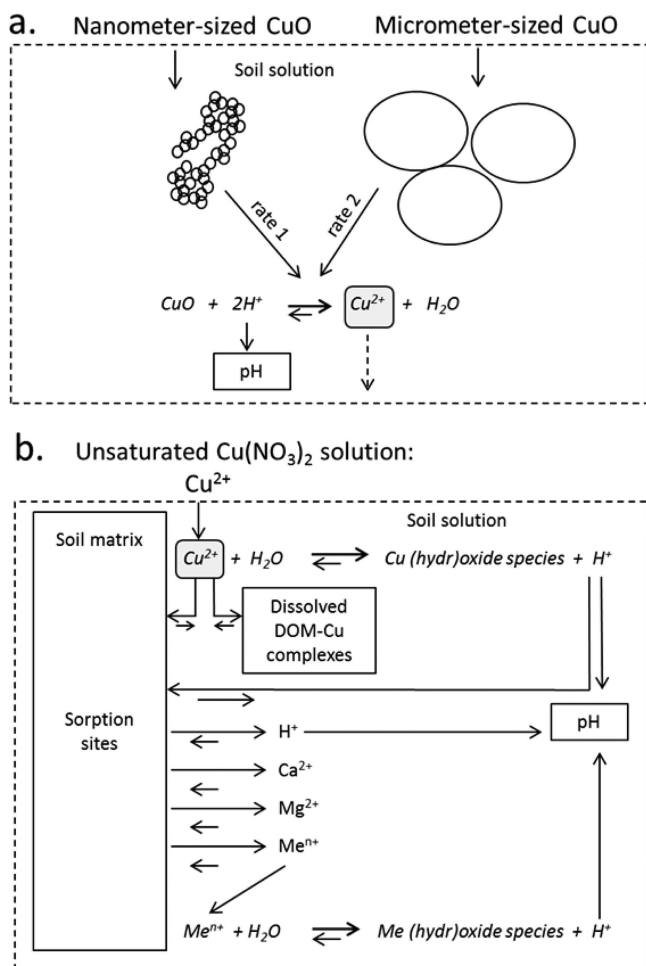
**Scheme 1. Major Processes Predicted for the Equilibration of Freshly Applied (a) CuO Powder, and (b) an Unsaturated Solution of $Cu(NO_3)_2$ with Soils[a]**



[a]Solid-line arrows indicate the proposed main direction of the reactions. Rates 1 and 2 refer to the rate of surface hydrolysis of CuO. Fixation processes such as the sorption-desorption reactions illustrated in Schematic 1b are expected to continue as the soils age, $Cu^{2+}$ ions penetrate into soil microsites, and equilibration proceeds. DOM = dissolved organic matter, Me = metal.

toxicity tests, the initially low dissolved Cu concentrations in nano CuO treatments had increased by the end of tests, with a greater increment in media amended with smaller particles,[7,15,22] and a similar increase in $Cu^{2+}$ activity could reasonably be anticipated in soils amended with nano or micrometer CuO. In contrast, studies demonstrated that $Cu^{2+}$ activity in soils amended with an undersaturated aqueous solution of Cu salts was initially high and declined with time,[21,23] observations explained by the high proportion of $Cu^{2+}$ ions in the amendment solution (Visual MINTEQ V2.53, 2008, http//www.lwr.kth.se/English/OurSoftware/vminteq), and the likelihood that the ions gradually penetrated into soil microsites, and underwent redistribution and sorption reactions.[21]

The pH and dissolved cation concentrations may also differ in soils freshly amended with CuO particles or Cu salt solutions. The dissolution of CuO in soils will likely be dominated by proton-consuming hydrolysis at the particle surface (Scheme 1a),[17] a hypothesis supported by the observed

increase in soil pH following amendment with CuO nano-particles.[6,24] Previous research with natural sesquioxides,[25] and recent research on ZnO nanoparticles in soils,[26] has suggested that proton complexation at the oxide surface may be a further mechanism contributing to an increase in pH if the metal oxide point of zero charge is higher than the soil pH. In contrast, the soil pH decreases and dissolved cation concentrations increases on addition of an undersaturated but concentrated solution of Cu salts.[8,9,27] It is thought that the changes in the salt-amended soils occur because the added $Cu^{2+}$ ions displace protons and cations from exchange sites, and metal ions entering the soil solution undergo hydrolysis reactions (Scheme 1b).

The objective of the current study was to determine whether $Cu^{2+}$ activity, pH, and dissolved cation concentrations differed significantly between soils amended with different Cu materials during a 56 day period, which is the length of the standard earthworm reproduction test.[2] Two natural soils were amended with a single concentration of Cu as nano CuO, micrometer CuO, or a Cu salt ($Cu(NO_3)_2$). A standard artificial soil was included to facilitate reproduction of these tests by other laboratories.[2]

## EXPERIMENTAL SECTION

**Copper Oxide Material Characterization.** Tests were performed on three CuO materials: "nano1" (nominal purity 99.5% and size 25–50 nm, U.S. Research Nanomaterials Inc., Houston, TX, U.S.A.), "micrometer1" (nominal purity 99% and size 40 nm, MKnano, Mississauga, ON, Canada) and "micrometer2" (nominal purity 98% and size <5 $\mu$m, MKnano, Table S-1, Supporting Information, SI). Particle size, shape, zeta potential, crystallinity, specific surface area, and purity were determined (SI Tables S-1 and S-2).

**Chemicals.** All reagents were ACS grade or higher. Ultrapure water (18 M$\Omega$ cm) was used in all experiments. Labware preparation is described in the SI.

**Test Soils.** Tests were conducted on two natural soils (FMT and BEN), and one artificial soil (ART). Selected phys-icochemical characteristics of the three soils are reported in SI Table S-3. The FMT soil was a sandy loam, pH 5.3, and the BEN soil was a sandy loam, pH 6.4. The soils had similar cation exchange capacities and organic matter content The ART soil, pH 6.4, was prepared to the specifications described by the Organization for Economic Cooperation and Development.[2]

**Soil Preparation and Aging.** Soils were amended with 500 mg Cu per kg soil because preliminary plant growth tests with barley (*Hordeum vulgare* L.) indicated that this concentration of a Cu salt in the BEN soil reduced root length by 50%. Details of the experimental procedure can be found in Section 1 of the SI. Briefly, three replicates were amended separately for each treatment. The CuO-amended soils were prepared by mixing CuO powder (nano1, micrometer1, or micrometer2) with air-dried soil in polypropylene bottles to produce a nominal concentration of 500 mg Cu per kg of dry soil. Soils were then moistened to 90 $\pm$ 1% field capacity, as described in Section 1 of the SI. The Cu salt-amended soils (salt1) were prepared by adding $Cu(NO_3)_2 \cdot 2.5H_2O$ (Fisher Scientific, Nepean, ON, Canada) solution to air-dried soil in sufficient water to reach 90 $\pm$ 1% field capacity. Unamended soil samples (negative control treatments) received water only. The final concentration of Cu in each replicate was determined by ICP-MS analysis following hot acid digestion[28] (SI Table S-4). Polypropylene bottles were stored at room temperature (22 °C) for the duration of the experiment, opened daily to
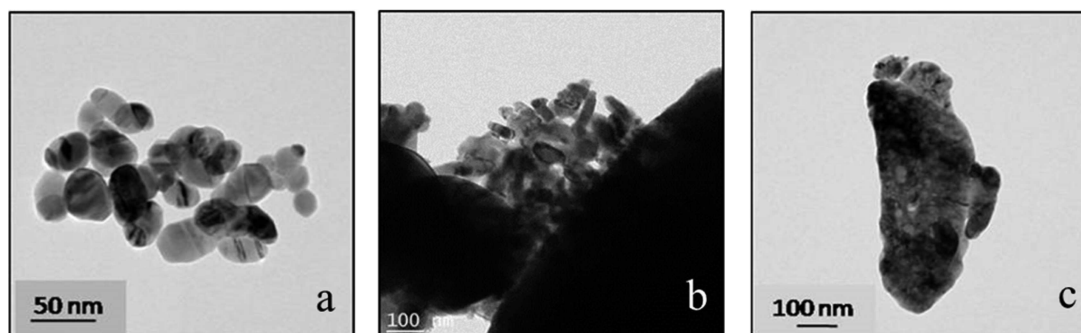
**Figure 1.** Transmission electron microscopy images of the three CuO powders. Panels show (a) nano1 material, (b) micrometer1 material, and (c) micrometer2 material. The scale bar is shown on each image.

facilitate gaseous exchange, and moisture replenished on a weekly basis. Measurements were made 1, 2, 4, 7, 11, 14, 27, and 56 d following soil amendment with Cu compounds. On each sampling date, a subsample of soil was extracted in 0.01 M $KNO_3$, which is commonly used as a proxy for the soil solution[29] and was appropriate because N-based compounds were not of interest in the present study. A separate test, described in the SI, determined that the extraction procedure did not cause a detectable change in $Cu^{2+}$ activity. Extracts were centrifuged, and the supernatant filtered through a 0.45 $\mu$m nylon filter (Mandel Scientific Company Inc., Guelph, ON, Canada). Dissolved Ca and Mg concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS), and $Cu^{2+}$ activity and pH were measured as described below.

**$Cu^{2+}$ Activity Measurement.** The $Cu^{2+}$ activity was measured using a Cu ion selective electrode (ISE) following the method described in Rachou et al., 2007.[30] This method was selected because the Cu ISE works on the principle of ion exchange through a Cu-specific membrane, so avoids the confounding effect of dissolved Cu complexes, and, potentially, suspended CuO nanoparticles, in soil extract solutions. Previous studies reported that interference from ions likely to be present in significant concentrations in the soil solution, such as $Ca^{2+}$ and $Al^{3+}$, is slight,[31] and although the Cu ISE is sensitive to both $Cu^+$ and $Cu^{2+}$ ions, the $Cu^+$ activity under aerobic conditions is a number of log units lower than that of $Cu^{2+}$ ions (Visual MINTEQ). In the present study, the test soils were regularly aerated and mixed to reduce the occurrence of anaerobic sites. Further methodological details are provided in Sections S-1 and S-3 of the SI.

**Statistical Analyses.** Temporal changes in solution chemistry were analyzed by repeated measures analysis of variance (ANOVA), using the Greenhouse-Geisser corrected $p$ value. If the time* treatment effect was significant, then the differences between treatments were analyzed separately for each time period using a Tukey's HSD test. Results were deemed significantly different at $p \leq 0.05$. Statistical analyses were performed using SYSTAT for Windows, Version 13 (SYSTAT Software Inc., Chicago, IL, U.S.A.). Data are expressed as the mean ± standard deviation.

## ■ RESULTS AND DISCUSSION

**Nanoparticle Characteristics.** Results of characterization tests on the three CuO materials are shown in SI Table S-1. The nano1 particle diameter measured with transmission electron microscopy (TEM) ranged from 22 to 322 nm, with a median value of 43 nm (Figure 1).

The mean hydrodynamic diameter measured with dynamic light scattering (DLS) was 621 ± 210 nm, suggesting some agglomeration in the powder form. The two micrometer-CuO powders could not be successfully measured using either TEM or DLS due to the presence of numerous large particles. The specific surface areas of the nano1, micrometer1, and micrometer2 powders were 9.84, 1.28, and 0.77 $m^2$/g, respectively (SI Table S-2).

**Soil Cu Concentrations.** The Cu concentration in the CuO-amended soils ranged from 458 ± 11 mg Cu/kg soil in the micrometer2 ART soil to 522 ± 25 mg Cu/kg soil in the nano1 BEN soil (SI Table S-4). The Cu concentration in the salt1 soils ranged from 424 ± 24 to 452 ± 16 mg Cu/kg soil.

**Changes in $Cu^{2+}$ Activity in the Oxide-amended Soils.** The mean log $Cu^{2+}$ activities in the unamended soils ranged from −9.36 ± 0.15 in the BEN soils to −8.28 ± 0.41 in the FMT soils (Figure 2a−c).

Consistent with Scheme 1a, $Cu^{2+}$ activities in the nano1 soils increased with time, reaching a maximum of −5.35 ± 0.05 in the day 56 nano1 FMT soils. They were significantly higher than the $Cu^{2+}$ activities in the micrometer-amended soils for at least the first 11 days. The $Cu^{2+}$ activity continued to increase in all CuO-amended soils throughout the test, but the rate varied with the CuO material, soil type, and time. This is shown more clearly in Figures 2d−f, in which $Cu^{2+}$ activity is plotted on a linear scale. By the end of the test, $Cu^{2+}$ activities in all CuO-amended FMT soils were similar, but $Cu^{2+}$ activities in the nano-amended BEN and ART soils remained significantly higher than those in the equivalent micrometer1 soils. None of the CuO-amended soils had reached a steady state with respect to $Cu^{2+}$ activity within the first 28 d. The increase in log $Cu^{2+}$ activity in all soils was more gradual between days 28 and 56. However, Figure 2d−f demonstrates that $Cu^{2+}$ activities continued to rise in all FMT CuO-amended samples and in the BEN nano1 samples during this period. As soils were not sampled between days 28 and 56, it could not be established whether the $Cu^{2+}$ activity stabilized in any of the soils during this period.

The higher initial $Cu^{2+}$ activities in the nano1 soils are consistent with results of aquatic nanotoxicity tests in which dissolved Cu concentrations in solutions amended with nano CuO were up to 141 times higher than those in solutions containing a similar mass of micrometer CuO after 72 h or less.[7,22] The increase in dissolved Cu concentrations in these aquatic studies was attributed to particle dissolution. Although the nanoparticles were not tracked in the current study, the increase in $Cu^{2+}$ activity in the CuO-amended soils, and the more rapid initial increase in treatments amended with smaller
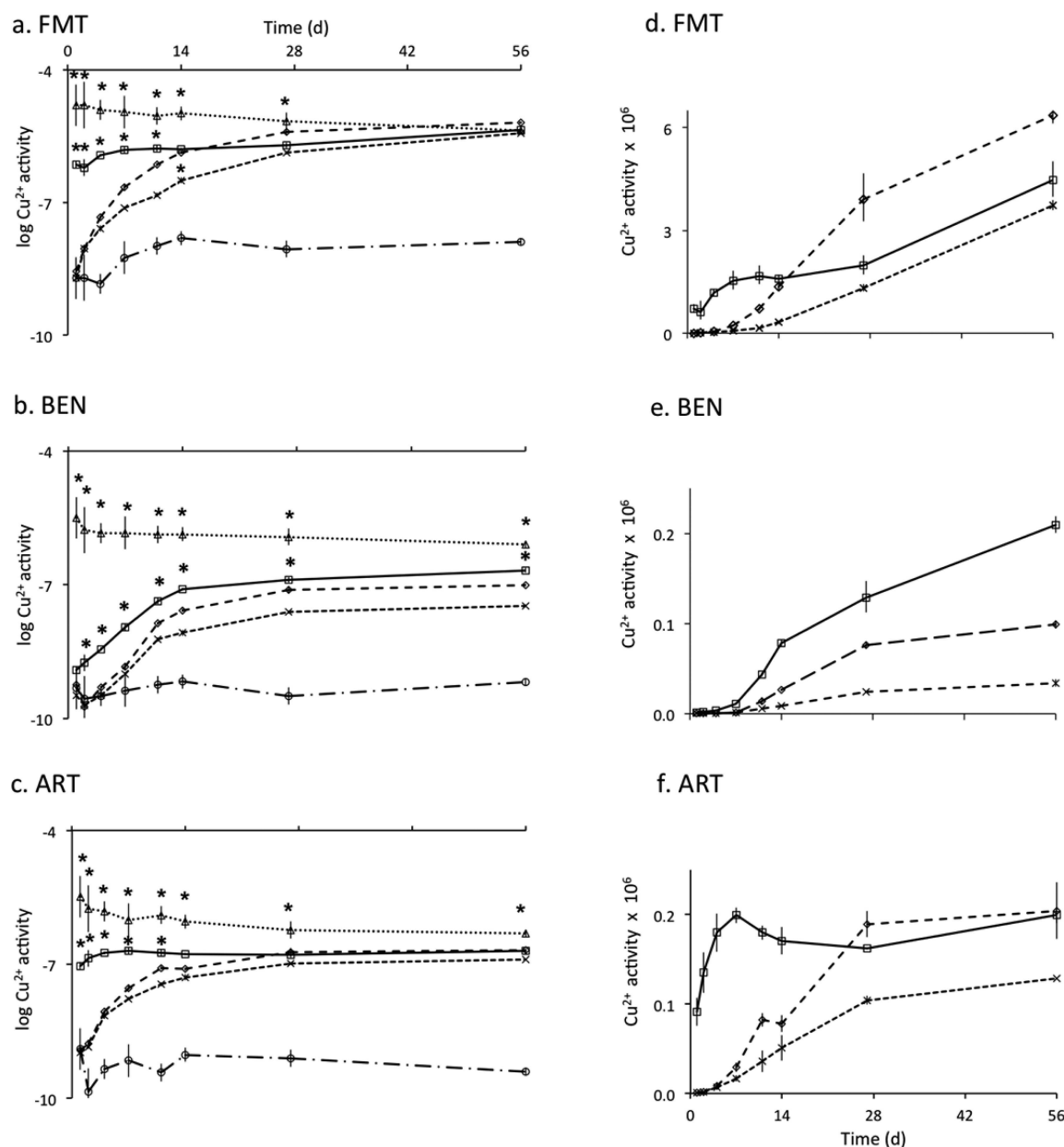
**Figure 2.** Temporal changes in log Cu$^{2+}$ activity in three soils amended with different Cu materials. a–c: log Cu$^{2+}$ activity for all Cu sources, d–f: Cu$^{2+}$ activity on a linear scale in CuO-amended soils. Treatments: □, nano1 CuO; ×, micrometer1 CuO; ◊, micrometer2 CuO; △, Cu salt1; and ○, unamended soil. Vertical lines indicate standard deviations ($n = 3$). Small standard deviations are obscured by symbols. Significant differences ($p \leq 0.05$, shown on log scale graphs only) between nano1 and salt1 soils are indicated by an asterisk (*) above salt symbols, between nano1 and both micrometer-amended soils by an asterisk above the nano1 symbols, and between the nano1 and micrometer1 soils by an asterisk above the micrometer1 symbols.

particles, suggests that dissolution was also occurring in the CuO-amended soils. By the end of the test, the Cu$^{2+}$ activities in the nano1- and salt1-amended FMT soils were not significantly different (Figure 2a). The rate of CuO dissolution is inversely correlated with pH,[7,32] so these results suggest that the nanoparticles were dissolving more rapidly in the more acidic FMT soils, although this needs to be confirmed by investigating the relationship between soil pH and CuO particle dissolution.

The increase in Cu$^{2+}$ activity in the CuO-amended soils varied with time and between the different materials and, over the course of the test, was not related to the initial BET-measured specific surface area of the CuO powders (Figure 2,

SI Table S-1). A weak correlation between specific surface area and increase in Cu$^{2+}$ activity was also reported in cell media.[33] Dissolution is a complex process, dependent on characteristics of both the medium and the particle.[34] As well as surface area and solution pH, the properties affecting the rate of dissolution of metal oxide surfaces include particle microporosity, surface curvature, and proportion of crystal discontinuities.[34−36] These properties are likely to be important in determining the rate of nanoparticle dissolution,[34,37] but their routine measurement in complex media such as soils is not yet technically feasible. The rate of nanoparticle dissolution in soils is also pertinent to risk assessment; once nanoparticles are completely dissolved, existing soil quality standards for Cu can be applied. However,
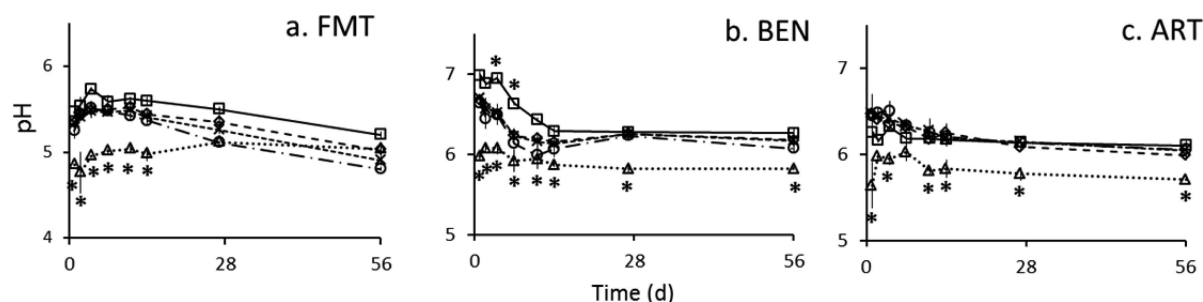
**Figure 3.** Temporal changes in pH in three soils amended with three different Cu materials. Treatments: □, nano1 CuO; ×, micrometer1 CuO; ◊, micrometer2 CuO; △, Cu salt1; and ○, unamended soil. Vertical lines indicate standard deviations ($n$ = 3). Small standard deviations are obscured by symbols. Significant differences ($p < 0.05$) between nano1- and salt1 soils are indicated by a lower asterisk (*), and between the nano1- and micrometer-amended soils by an upper asterisk.
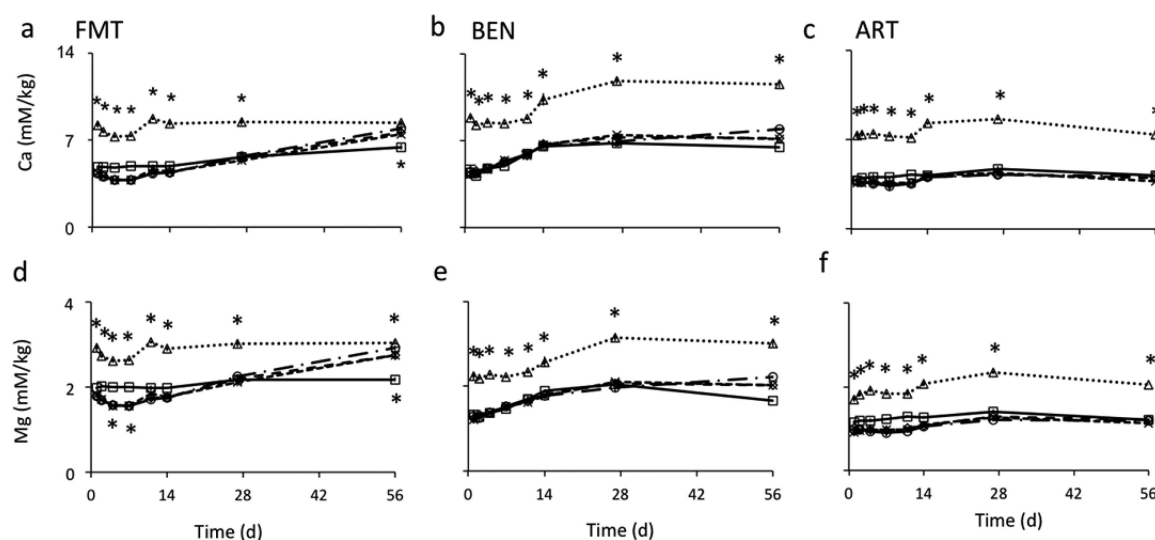


**Figure 4.** Temporal changes in dissolved Ca (a−c) and Mg (d−f) concentrations in the three Cu-amended soils. Treatments: □, nano1 CuO; ×, micrometer1 CuO, ◊, micrometer2 CuO; △, Cu salt1; and ○, unamended soil. Vertical lines indicate standard deviations ($n$ = 3). Small standard deviations are obscured by symbols. Significant differences ($p < 0.05$) between nano1 and salt1 soils are indicated by an upper asterisk (*), and between nano1 and micrometer-amended soils by a lower asterisk.

until techniques are developed to track nanoparticles in complex media, predicting the rate of dissolution and consequent changes in $Cu^{2+}$ activity in soils freshly amended with particulate Cu materials remains challenging, particularly if based solely on commonly available pretest characterization techniques.[38] As yet, the fate of metal-based nanomaterials in soils must therefore be largely determined through indirect rather than direct measurements.

In contrast to the oxide-amended soils, $Cu^{2+}$ activities in the salt1 soils were initially high and decreased with time (Figure 2a−c). After 24 h, the $Cu^{2+}$ activities in the salt1 soils were 1.35 to 3.42 log units higher than those in the equivalent nano1 soils, with a maximum activity of −4.80 ± 0.02 in the FMT soil. Although $Cu^{2+}$ activities subsequently declined, they were significantly higher in the BEN and ART salt1 soils than in the equivalent nano1 soils to the end of this study. The initially high $Cu^{2+}$ activities in the salt-amended soils were expected; modeling software indicated that more than 99% of Cu was added as free $Cu^{2+}$ ions (Visual MINTEQ), and high initial $Cu^{2+}$ activities have been reported in other soils freshly amended with Cu salts.[9,21] The subsequent decline in $Cu^{2+}$ activity as the salt-amended soils aged was also previously reported,[21,39] and is thought be the result of ongoing fixation processes such as the complexation and adsorption reactions

illustrated in Scheme 1b that occur as the soils age and the added $Cu^{2+}$ ions equilibrate with the soil. Even though $Cu^{2+}$ activities increased in the CuO-amended soils and decreased in the salt-amended soils, they remained significantly higher in both the BEN and ART salt-amended soils at the end of the test. The implications of potential differences in $Cu^{2+}$ activity between treatments amended with different sources of Cu for nanotoxicity tests are discussed below.

**Changes in pH and Dissolved Cation Concentrations.** The observed changes in soil solution chemistry in the salt-amended soils are consistent with the processes illustrated in Scheme 1b. The pH of the nano1 soils were not significantly different from those of the equivalent micrometer-amended and unamended soils on most sampling days. The only exception was the nano1-amended BEN soil, in which the pH was significantly higher on days 4 and d 7 (Figure 3). Interestingly, an increase in soil pH was also observed following 7 h incubation with nano CuO,[24] or 28 d incubation with ZnO.[6] In contrast, the pH of the salt1 soils was initially up to one pH unit lower (i.e., 10-fold higher $H^+$ activity) than those of the nano1 soils. Although the pH of the FMT salt1 and nano1 soils were similar by day 28, the pH of the BEN and ART salt1 soils remained significantly higher than those of the equivalent nano1 soils throughout the test. In the absence of other reactive

sites, each unit decrease in pH theoretically increases the $Cu^{2+}$ activity 100-fold (eq 1).[17]

$$Cu^{2+} + 2H_2O \leftrightarrows Cu(OH)_2 + 2H^+ \qquad (1)$$
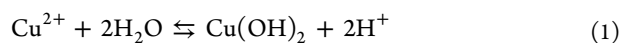
Although in soils, this quantitative relationship will be modified by pH-dependent sorption of $Cu^{2+}$ ions to reactive sites such as organic ligands and oxide surfaces, the higher $Cu^{2+}$ activities in the salt-amended soils may be partly explained by their lower pH.

The dissolved Ca and Mg concentrations in the nano1 and micrometer-amended soils were similar to those in the unamended soils (Figure 4), suggesting that the soil buffered the slow changes in soil solution chemistry that occurred as the CuO particles equilibrated with the soils. No other studies were found in the available peer-reviewed literature that reported on changes in cation concentration in soils amended with particulate metal oxides. In contrast, the dissolved Ca and Mg concentrations were significantly higher in the salt1 soils than in the other treatments throughout the present study, and are in agreement with the decrease in soil pH and increase in dissolved cation concentrations following amendment with Cu salts reported in other studies.[8,9]

**Measuring Soil Free Ion Activity.** Measuring free metal ion activity during exposure tests presents a challenge for nanotoxicologists. Techniques recently used to measure $Cu^{2+}$ ion activity in test media amended with nano CuO have included the Cu ISE (Kakinen et al., 2011[40] and the present study), Cu biosensors,[40] Cu chelation,[15,41] ultrafiltration,[4] centrifugation,[33] and diffusive gradient in thin films.[42] Factors determining the technique selected include the test medium (e.g., aqueous solution, sediment, soil), the expected metal ion concentration, and the technique available (an ISE sufficiently accurate for most environmental studies is only available for Cu, and under certain conditions for Ag). However, as measured ion activities and dissolved metal concentrations are operationally defined,[29] results obtained using different techniques may not be comparable. The situation is further complicated by the potential for interactions between the nanoparticles and the equipment used to measure ion activity, which makes it difficult to validate any technique in nanoparticle-amended soils. For this reason, in the current study the differences between $Cu^{2+}$ activities in the different treatments, identified using the Cu ISE, were discussed in relative rather than absolute terms. Further research on the relative effectiveness of the different methods to predict uptake and toxicity from non-nano sources of Cu in soils amended with Cu-containing nanoparticles is required so that, where possible, methods to measure metal ion activity in different media during nanotoxicity tests are standardized, thus facilitating comparison of results from different studies.[43]

**Considerations for Nanotoxicity Study Design.** The challenge of conducting terrestrial toxicity studies on actively transforming materials has previously been discussed in the context of volatiles.[44] However, although there is an increasing awareness that nanoparticle transformations will modify their interactions with organisms,[10,45−47] little attention has been paid to the implications for nanotoxicological studies of changes in test media resulting from material transformations during nanotoxicity tests. The present study provides an example of these transformations in soils. In previous nano-CuO toxicity studies, the treatments to control for effects of ionic Cu were based on the $Cu^{2+}$ activity or dissolved Cu concentration in the nano-CuO treatment at a certain point in time.[4,7,48] The present results suggest that the initial similarity in $Cu^{2+}$ activities in soils freshly amended with different Cu materials will be ephemeral because of differences in material equilibration rates and processes. Consequently, test organisms in the different treatments may be responding to dissimilar concentrations of $Cu^{2+}$ ions in each experimental treatment.

Given the major influence of pH on metal solubility and speciation,[29] differences in soil pH in the oxide and salt treatments may further confound interpretation of results. It may be difficult to unravel the effects on toxicity of the different $Cu^{2+}$ activities and pH values in the treatments. Taking the results of the current study as an example, it may be anticipated that toxicity in the salt-amended soils would be greater than in the oxide-amended soils because of higher $Cu^{2+}$ activity, but it is also possible that higher concentrations of protons, $Ca^{2+}$, and $Mg^{2+}$ ions could reduce uptake of $Cu^{2+}$ ions by blocking sites on biological tissue surfaces.[19,49] A further consideration in soils with different pH values is the potential for differences in vigor if the test species is pH-sensitive.

Although the scope of the present study was constrained, the results suggest that unless material transformations are taken into consideration, nano-CuO toxicity tests may not accurately reflect the nanoparticle-specific effect on organisms. Some techniques to reduce or modulate differences between the test and reference treatments are described below.

**Recommendations.** A number of actions can be taken to reduce or mitigate differences between soil solution chemistry (specifically, $Cu^{2+}$ activity, pH, and dissolved cation concentrations) in the different treatments during nano-CuO toxicity tests. To reduce the difference between the solution chemistry in the salt- and oxide-amended soils, the pH of salt-amended soil can be buffered by applying lime, and the dissolved cation concentrations reduced by leaching soils prior to the toxicity test.[9,50] Once limed and leached, the salt-amended soils can be air-dried and stored until required. Salts such as Cu-acetate that can buffer changes in soil pH may also be considered. To adequately encompass the range of $Cu^{2+}$ activities in the nanoamended soils, preliminary tests can determine their range, and micrometer-Cu and Cu salt treatments that encompass this range included in the toxicity test. It is also important that the $Cu^{2+}$ activity is measured in all treatments during the test, at least at both the start and end of the toxicity tests.

The inclusion of adequate reference treatments in terrestrial nanotoxicity tests is necessary to correctly determine whether nanoparticles pose a threat to soil organisms. This study demonstrates that care must be exercised when designing nanotoxicity tests for soluble nanoparticles such as CuO, to ensure that artifacts due to the temporal changes in soil solution chemistry during equilibration of the different treatments do not obscure the effects of the nanoparticles on organisms. The increase in dissolved metal ion concentrations in ZnO-amended media[6,51,52] and the decrease in soil pH and increase in dissolved cation concentrations following the addition of Zn, Cd, or Ni salts to soils,[8,9] observed in previous stueies, suggest that similar considerations are required for other soluble metal-based nanoparticles.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information
Section S-1: Experimental details (Labware preparation, experimental procedure, $Cu^{2+}$ activity measurement); Figure S-1: Cu ISE calibration curves; Section S-2: Quality control data for ICP-MS analysis; Table S-1: CuO material character-

istics; Table S-2: Metal contaminants in CuO materials; Table S-3: Soil characteristics; Table S-4: Total Cu concentrations in the test soils; Section S-3: Effect of extraction process on $Cu^{2+}$ activity; and Table S-5: Electrical potential in unextracted and extracted CuO samples in water or 0.01 M $KNO_3$. This material is available free of charge via the Internet at http://pubs.acs.org.

■ **AUTHOR INFORMATION**

**Corresponding Author**
*Phone: +1 514 398 7890; fax: +1 514 398 7990; e-mail: heather.mcshane@mail.mcgill.ca.

**Notes**
The authors declare no competing financial interest.

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