PAPER

Microbial response to copper oxide nanoparticles in soils is controlled by land use rather than copper fate†

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Copper (Cu) products, including copper oxide nanoparticles (nCuO), are critically important agricultural fungicides and algaecides. Foliar application onto crops and subsequent aerosol drift of these Cu products, especially nCuO, on to soil may alter nutrient cycling and microbial communities in both managed and unmanaged environments. We measured the influence of land use on soil microbial biomass and respiration in response to the addition of nCuO to an alluvial soil. Different land uses included grassland, forest and both organic and conventional managed row crops. Soil samples were amended with 1000 mg Cu per kg soil as $CuCl_2$, 16 nm CuO (16nCuO), 42 nm CuO (42nCuO), and larger than nanoparticle sized bulk CuO (bCuO). Copper availability immediately increased in all soils following Cu addition in the order of $CuCl_2$ > 16nCuO > 42nCuO > bCuO. After 70 days Cu availability was diminished across land uses and lowest in soils treated with bCuO. Using X-ray absorption near edge structure (XANES) spectroscopy, we determined that the relatively high availability of Cu after treatment with nanoparticle sized CuO was due to the dissolution of CuO particles and subsequent adsorption by soil materials. Respiration, an indicator of microbial activity, was suppressed by Cu additions, especially $CuCl_2$. Copper effects on soil microbial biomass were sensitive to land use. In agricultural soils, microbial biomass was unaltered by Cu form, regardless of concentration, whereas in unmanaged soils, it decreased following exposure to $CuCl_2$ and 42nCuO. Our results suggest that land use history has little impact on Cu chemical fate in soils, but strongly modulates microbial response to Cu exposure. These results are especially important for organic agricultural systems where copper fungicides are widely used but may suppress microbial mineralization of nutrients from soil organic matter.

Environmental significance

Copper based fungicides, including copper oxide nanoparticles, are widely used in agriculture and represent a direct and intentional release of nanoparticles into the plant and soil system. We present evidence that the microbial response to exposure to nanosized and larger CuO particles (16 nm, 42 nm, 430 nm) and Cu salts ($CuCl_2$) is unique to historical land management rather than copper form or concentration. Generalizing soil microbial community response to nanoparticle exposure across soils is therefore challenging as previous land management can be the primary influence on the observed soil microbial community response to nanoparticle treatment.

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Introduction

Since the adoption of the Bordeaux mixture ($CuSO_4$ and $Ca(OH)_2$) for fungal pathogen control in 19th century French vineyards, copper (Cu) has become a globally important tool for fungal and algal control.[1] In 2017 alone, 980 000 ha of horticultural land in California were treated with 1.55 Gg of Cu from Cu-based fungicides and algaecides.[2] In North America, South America, Europe, and Australia, Cu inputs to vineyards and orchards generally range between 1 and 11 kg Cu per ha per year, resulting in Cu accrual rates in excess of 5 mg Cu per kg soil per year in the surface 10 cm.[3–7] During foliar fungicide application, up to 50% of all Cu is immediately deposited on the soil surface through aerosol drift and/or foliar runoff, exposing surface soil to concentrated Cu solutions as high as 30 000 mg Cu per L.[8–11]

Use of highly available forms of Cu such as $CuSO_4$ in fungicidal sprays can induce leaf damage, whereas less available or 'slow release' forms like $Cu(OH)_2$ mitigate this risk, though often at the cost of prophylactic efficacy.[12] Decreasing the size of copper compounds to nanoparticle dimensions can alter the dissolution rate of these compounds, increasing the efficacy of the compounds.[13–15] Nano-sized copper formulations of $Cu(OH)_2$ are already being commercially adopted and used with success in agricultural systems.[16] Nano-sized copper(II) oxide particles (nCuO) were recently identified as a potential candidate for the control of *Verticillium* wilt fungus, a common pathogen that causes economic loss for high-value crops such as almonds, tomatoes, and pistachios.[13] Further, nCuO was measured to help control *Fusarium* wilt in tomatoes.[17] The use of nCuO may offer significant benefits over traditional slow-release Cu fungicides by controlling pathogens with lower total amounts of Cu applied.[13,15] However, nCuO may also negatively affect soil microbial communities performing essential nutrient cycling and other soil health functions.[18,19] Despite ubiquitous usage and accumulation in and around agricultural soils, the fate and influence of nCuO and Cu in general on soil microbial biomass and activity is still poorly understood.[3,14,18–20]

The effects of Cu and nCuO exposure on microbial biomass and activity are often negative, with reduction dependent upon Cu input amount, soil texture, pH, and soil organic carbon (SOC) content.[14,15,20–24] Soils with low pH, low organic carbon content, and coarse texture typically have more biologically available Cu than soils with high pH, high organic carbon content, and fine texture.[20–22,24] Regardless of soil properties, microbial communities in agricultural systems are particularly prone to Cu exposure through repeated application of Cu fungicides, potentially threatening microbial biomass and function.[25] Responses are dose dependent, with limited responses at low doses and non-linear perturbations experienced at high doses.[19,22,23,26] Therefore, repeated application of Cu fungicides and management practices that affect soil pH and SOC content may be expected to alter the response of soil microbial communities to Cu and nCuO exposure by altering Cu availability.[24] This may be particularly problematic for nCuO with greater solubility than larger particulate forms (*e.g.*, micron-sized), which may significantly increase Cu availability in soil compared to other forms of Cu, potentially further perturbing microbial biomass and function.[14,18,27–30]

Historically, potential metal availability to soil microbial communities is often measured using aqueous extractions, including deionized water or dilute solutions of salts, acids and/or bases.[31,32] However the speciation of ions in these extracts is different than what is in the soil and thus from that to which soil microbial communities are exposed. These limitations may be avoided by assessing the distribution and chemical speciation of Cu in soils directly using micro-X-ray fluorescence microscopy (XFM) and X-ray absorption near edge structure (XANES) spectroscopy, respectively. Unlike aqueous extraction approaches to Cu speciation, these techniques enable identification of discrete changes to Cu speciation and distribution in solid samples and can be related to changes in microbial biomass and function after exposure to various forms of Cu.[27,33–36]

Here we investigate how Cu form, size, and dose effect soil microbial biomass and activity across a soil carbon gradient arising from different land uses. This work bridges a causal gap between chemical and biological responses of soil to inputs of Cu. Specifically, the effect of 1000 mg Cu per kg soil from $CuCl_2$, 16 nm CuO (16nCuO), 42 nm CuO (42nCuO), and bulkier, 430 nm CuO (bCuO) on water extractable Cu, pH, and cumulative soil respiration from soils differing exclusively in historical land use were measured during a 70 day incubation. After 70 days, soil microbial biomass was then measured by phospholipid fatty acid analysis. The distribution and speciation of Cu in the soils treated with copper after 70 days was determined with XFM and XANES to allow direct correlation of Cu speciation on the microbial response to nanosized and larger bCuO particles. We hypothesized that exposure to high concentrations of soluble copper ($CuCl_2$) would alter soil respiration and soil microbial biomass to a greater extent than sparingly soluble forms of Cu, such as micron-sized bCuO. Further, we hypothesized that Cu speciation in soils after 70 days would be indicative of the degree of CuO dissolution, with nCuO dissolving to a greater extent than bCuO, and would account for any differences observed in available Cu after 1 hour and 70 days. Finally, we predicted that increasing SOC would increase soil microbial community resilience in the face of Cu exposure, with microbial biomass and soil respiration in high SOC soils being less responsive to Cu treatment than in low SOC soils.

Materials and methods

Soils collection

Soils representing managed agricultural use and unmanaged forest and grassland systems were sampled in October 2016 at the Russell Ranch Sustainable Agriculture Facility at the University of California-Davis (Fig. S1†). The agricultural

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systems were tomato-corn rotations under either conventional (mineral fertilizer and synthetic pesticides) (Con) or organic management (compost and winter legume cover crop, no synthetic pesticides) (Org) for 23 years at the time of sampling. Neither agricultural soil was previously treated with copper-based fungicides. Unmanaged uses included a restored grassland with native grass species (Gra) and a riparian woodland dominated by valley oak (*Quercus lobata*) with an understory of native and invasive grasses (For). Soils developed on mixed mineralogy and carbonate-rich alluvium from nearby Putah Creek and are classified as Rincon silty clay loam (fine, smectitic, thermic Mollic Haploxeralfs) and Yolo loam silt loam (fine-silty, mixed, superactive, nonacid, thermic Mollic Xerofluvents) (Fig. S1†).[37] Though classified as different soil types, surface soils at the study site are similar in mineralogy, pH, and particle size distribution across all land uses, differing mainly in organic carbon (OC) content. Soils were sampled to a depth of 0–10 cm in triplicate and composited from 3 sampling sites separated by at least 50 m and gently crushed to pass through a 2 mm sieve before further air-drying prior to analysis of chemical properties (Table 1, Fig. S1†) and incubation experiments.

Soil physico-chemical characterization

Soil OC was determined by dry combustion after ball milling (ECS 4010 Costech Elemental Analyzer).[38] Soil pH and water extractable metals were determined after a 1 hour equilibration of 1 g of soil with 5 ml of water (>18.2 MΩ cm resistivity) at 28 °C.[39] After equilibration, the suspension pH was measured and a 1 ml aliquot was sub-sampled, centrifuged at 21 000g for 30 min, after which the supernatant was collected and diluted 1:10 in 1% nitric acid for elemental analysis by inductively coupled-plasma mass spectrometry (ICP-MS). For total Cu and total P analysis, 1 g of soil was added to 50 ml volumetric glass digestion vessels and acid digested with 7.0 ml of trace metal grade HCl (12.1 M) and 2.3 ml of HNO_3 (15.8 M) and allowed to equilibrate overnight. The following day, soils were digested at 120 °C for 4 h, vortexed repeatedly to ensure adequate mineralization, and then brought to volume. The resulting digestate was diluted 1:10 with 1% HNO_3 for trace metal analysis by ICP-MS. Calibration curve accuracy was confirmed by certified reference material (1640a). Digestion blanks and

a soil reference material (ERM CC141) were included in the digestion process for quality control and recovery assurance. Recovery of total Cu and total P in ERM 141 were >95%. Soil-available ammonium and nitrate extracted by 2 M KCl (10 g soil: 50 ml extractant) for 60 min were measured colorimetrically.[40,41] Soil-available P (Olsen P) was determined by extraction with 0.5 M $NaHCO_3$ at pH 8.5 (1 g of soil: 20 ml extractant) for 30 min and colorimetric determination as molybdate-reactive phosphate.[42,43]

Nanoparticle procurement and characterization

Micron size copper oxide particles (bCuO) and 42 nm copper oxide particles (42nCuO) were purchased from Acros Organics (USA) and US Research Nanomaterials, Inc. (USA), respectively. These particles were previously described in Rippner *et al.* 2020 and recharacterized for the current study, with good agreement between the two sets of data.[44] The smallest 16 nm copper oxide particles (16nCuO) were previously synthesized and further characterized for the current study.[28,45] Primary particle size and shape of all CuO particles was determined by transmission electron microscopy (TEM, Philips CM-12). Mean primary particle size ($n > 100$) for CuO samples was calculated from TEM images using Fiji.[46] Particle mineralogy was confirmed by X-ray diffraction (Rigaku Ultima IV, Japan).[28] Nanoparticle zetapotential and hydrodynamic diameter in 1 mM KCl was measured with a Zeta-Plus (Brookhaven Instruments Corp., USA).

Copper treatments

Prior to Cu exposure, 80 g of air-dried soil was added to 946 ml acid-washed glass jars. Soils were then exposed to 1000 mg Cu per kg soil in 3 forms: (1) ionic, as aqueous copper chloride ($CuCl_2$), (2) nanoparticle, as 16 nm and 42 nm diameter CuO (16nCuO and 42nCuO), and (3) larger, extending into microparticle CuO (bCuO). Cu treatments were implemented using the same volume of 18.2 MΩ cm water regardless of Cu source *via* pipette. Soils without copper amendment were also wetted with the same volume of 18.2 MΩ cm water, incubated, and included in the analysis at all time points. Each incubated soil treatment was established in triplicate.

Table 1 Select soil chemical properties for soils taken from the conventional (Con), organic (Org), grassland (Gra), and forest (For) systems at the Russell Ranch Sustainable Agricultural Facility near Winters, California, USA

Land use system	SOC (g kg^{-1})	pH (1:5 water)	Water-extractable Cu (mg kg^{-1})	Total Cu (mg kg^{-1})	Olsen P (mg kg^{-1})	Total P (mg kg^{-1})
Con	9.8 ± 0.4[a]	7.5 ± 0.02[b]	0.02 ± 0.02[a]	50.6 ± 0.8[b]	8 ± 0.2[a]	512 ± 12[a]
Org	12.7 ± 0.3[b]	7.6 ± 0.05[a]	0.18 ± 0.02[c]	62.5 ± 0.4[c]	30 ± 0.5[c]	891 ± 64[c]
Gra	14.3 ± 0.5[c]	7.2 ± 0.07[c]	0.09 ± 0.05[b]	52.1 ± 0.8[b]	8 ± 0.8[ab]	539 ± 13[b]
For	25.3 ± 1.6[d]	7.3 ± 0.03[c]	0.08 ± 0.01[b]	41.2 ± 0.1[a]	10 ± 1[b]	539 ± 9[b]

All data presented as mean ± standard deviation (SD), n = 3. Means separation was analyzed by Tukey's HSD with significance declared at $p \leq$ 0.05 and differences are denoted with lowercase letters.

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Incubation and soil respiration

After Cu exposure, all soils were brought to 70% of water holding capacity (WHC) using 18.2 MΩ cm water, capped with a Ball® jar lid with a butyl rubber septum, and incubated in the dark for 70 days at 28 °C.[40,47–49] Gas samples were collected by sampling 1 ml of gas from the headspace of 946 ml jars on days 1–5, 9, 14, 19, 24, 29, 35, 39, 44, 49, 54, 60, 64, and 70. The CO_2 content in the headspace was measured by an infrared gas analyzer (model S-151, Qubit Systems Inc., Kingston, Canada).[49] After each measurement, the jars were allowed to equilibrate with the atmosphere for 10 min under a fume hood to facilitate soil gas exchange.[48] To replenish water lost evaporatively during the equilibration period, the soils were adjusted to 70% WHC on days 9, 39, 60, and 70. WHC never decreased below 65%.

Gas flux calculations

Total soil respiration was calculated as the sum of soil CO_2 flux (μmol CO_2-C per kg soil) at each sampling time (eqn (1)).

$$f_i = \frac{1000(C_t[V_h + (V_w\alpha)])P}{RTW} \qquad (1)$$

where f is the gas flux (μmol CO_2-C per kg soil); C_t (μL CO_2-C per L) is the gas concentration in the gas phase at time t; V_h (ml) is the volume of the headspace; V_w (ml) is the volume of water in the soil during the incubation; α (ml gas per ml water) is the Bunsen absorption coefficient = 0.759 for CO_2; P is the standard atmospheric pressure (101.325 kPa); R is the universal gas constant (8.31451 L kPa mol^{-1} K^{-1}); T is temperature in Kelvin (301.15 K); and W is the oven dry mass of soil (kg). Gas flux emissions were corrected for ambient CO_2 concentration measured at each time point.[39]

Measuring copper availability and pH at 1 hour and 70 days

Soil pH and water extractable metals were determined 1 hour after treatment application and then again after the 70 day incubation period. Briefly, 1 g of soil was incubated with 5 ml of water (18.2 MΩ cm) for 1 hour at 28 °C.[39] After equilibration, the suspension pH was measured and a 1 ml aliquot was sub-sampled and centrifuged at 21 000g for 30 min. The supernatant was diluted 1 : 10 in 1% nitric acid for elemental analysis by ICP-MS using a certified reference material (natural water, NIST 1640a).

X-ray fluorescence microscopy and X-ray absorption spectroscopy

Soils from the conventional and forest systems that represented the lowest and highest SOC content were selected for X-ray fluorescence microprobe (XFM) analyses. Soils were air dried and ground to 500 μm prior to measurement. Soils from the conventional plots were imaged at −20 °C on the XFM Beamline 10.3.2 of the Advanced Light Source (ALS) at the Lawrence Berkeley National Laboratory, Berkeley, CA, USA.[50–52] All data were collected in fluorescence

mode using a Canberra 7-element Ge solid state detector. Samples were mounted onto a Peltier stage (−20 °C) and scanned in 2D. Elemental distribution mapping was carried out at 9080 eV (100 eV above the Cu K-edge) with 7 × 7 μm pixels.

Soils from the forest plots were imaged on the VESPERS XFM beamline at the Canadian Light Source (CLS), Saskatoon, SK, CA.[53] Samples were mounted onto a stage (25 °C) and scanned in 2D. All data were collected in fluorescence mode using a Hitachi 4-element Si solid state detector. Mapping was carried out at 9080 eV (100 eV above the Cu K-edge) with image resolution limit of 2 × 2 μm pixels.[50,54]

X-ray adsorption near edge structures (XANES) data were collected on one sample from every Cu× land use treatment from $CuCl_2$, 16nCuO, and 42nCuO on the HXMA beamline at the CLS.[55] Due to time and equipment constraints, XANES data was only collected for two soil samples treated with bCuO (Con and For). To prevent Cu reduction, soil XANES data were collected at 63 K.[56] Standards for Cu adsorbed to silicate minerals, Cu complexed by Suwanee River fulvic acid, Suwanee River natural organic matter, a sodium salt of humic acid from lignite, or phytate, and CuO were prepared following published methods.[57–59]

Specifically, Cu-silicate standards were prepared following Furnare *et al.* 2005. Copper solutions (4.0 × 10^{-5} M Cu) were made using copper chloride in 18.2 MΩ cm water that was boiled and cooled by purging with N_2 gas. One gram of ground albite, chlorite, illite, montmorillonite, orthoclase, quartz, serpentine, and vermiculite were individually suspended in 30 ml of copper solution inside of 3500 Dalton SpectraPor 3 dialysis bags (Rancho Dominguez, CA). Each bag was then suspended inside of 1 L bottles containing 970 ml of copper solution continuously purged with N_2 gas. The solution in the bottles was changed every 12 hours over a 36 hour equilibration period. After 36 hours, the dialysis bags were removed, and the suspended minerals were decanted into centrifuge tubes and pelleted out by centrifugation. After centrifugation, the supernatant was decanted, and the mineral solids were gently dried under a N_2 atmosphere then stored in a desiccator at room temperature until analysis.

Standards for copper complexed by Suwanee River fulvic acid, Suwanee River natural organic matter, and a sodium salt of humic acid from lignite (Sigma Aldrich) were prepared following Karlsson *et al.* 2006. Specifically, 100 mg of each material was weighed into 2 ml centrifuge tubes with $CuCl_2$ dissolved in N_2 purged 18.2 MΩ cm water to yield a Cu concentration of 10 mg g^{-1} on a dry mass basis. Solutions were shaken for 72 hours and then dried under a N_2 atmosphere and stored in a desiccator at room temperature until analysis.

The copper phytate standard was prepared following He *et al.* 2006.[59] Briefly, 30 ml of a N_2 purged solution of 0.05 M sodium phytate ($C_6H_6O_{24}P_6Na_{12}$) was added to 100 ml of a N_2 purged solution containing 0.2 M of HCl and 0.09 M of $CuCl_2$. The pH of the solution was slowly raised to pH 6 over

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the course of an hour using 1 M NaOH while purging with N_2 gas. The precipitate was collected by bottle top filtration (0.22 μm filter) and washed with 60 ml of N_2 purged 18.2 MΩ cm water. The filtrate was dried under a N_2 atmosphere and stored in a desiccator at room temperature until analysis.

The 16nCuO particles were made following Siddiqui *et al.* 2013.[45] Briefly, 300 ml of 0.2 M copper acetate monohydrate [Cu(CH$_3$COO)$_2$·H$_2$O)] and 1 ml of glacial acetic acid was heated and mixed vigorously. Upon boil, 15 ml of 6 M NaOH solution was rapidly added to the vigorously mixed solution, forming copper oxide nanoparticles. The resulting precipitate was concentrated by centrifugation, washed repeatedly with 18.2 MΩ cm water, and dried at 60 °C for 6 hours.

The remaining standards, other than the 42nCuO particles, were purchased from Sigma Aldrich and were of ACS purity or better (Table S1†). Copper K-edge XANES measurements of the Cu-standards were taken on either the HXMA or XFM 10.3.2 beamlines. Standard spectra of Cu complexed by organic matter were collected at 63 K to limit organic matter oxidation and subsequent Cu reduction.[56] The remaining standards were collected at room temperature. Three individual spectra were collected for each standard and were deadtime-corrected, deglitched, calibrated using a Cu foil (1st derivative maximum value set at 8980.48 eV) pre-edge background subtracted, and post-edge normalized using custom LabVIEW software available at the beamline.[60] No evidence of Cu reduction was observed when evaluating spectral quality from each of the three scans per standard or sample. Soil Cu speciation was determined by least-square linear combination fitting using custom LabVIEW software available at ALS beamline 10.3.2 and using Athena (Demeter, Ver. 0.9.26).[61–63]

PLFA analysis

After completion of the incubation experiment, soils were homogenized and a sub-sample from each jar was flash frozen in liquid nitrogen and lyophilized for PLFA analysis by Microbial ID, INC (Newark, DE). Total soil microbial biomass was calculated as the sum of 37 individual PLFA biomarkers that were present across all soils (μmol PLFA kg^{-1} soil) (Table S2†).[64–69]

Statistical methods

Data were tested for assumptions of analysis of variance using the Shapiro–Wilk test for normality of residuals and Levene's test for homogeneity of variance. Data that did not meet these criteria were log transformed to meet the assumptions of ANOVA prior to final analysis. Multiple comparisons were made using Tukey's honestly significant difference test (Tukey's HSD) with significance declared at $p \leq 0.05$ and denoted with differing lower-case letters. Standard least squares linear regression was performed to analyze the effect of Cu form and use on soil response variables after Cu treatment. Any significant interactions were interpreted using least-squares means interaction plots and

Tukey's HSD. The standardized effect size of each factor to the results was estimated using the F-statistic and reported as $F_{i,j} = F$-statistic, where i = specific treatment degrees of freedom-1 and j = total degrees of freedom – all treatment degrees of freedom-1. The F-statistic comes from the ratio of between treatment variance to within treatment variance; by comparing the F-statistic between two treatment groups such as copper form and land use, the relative contribution of each treatment group to modeled results can be estimated. All statistical analyses were performed with JMP 15 Pro (2020; SAS Institute, Cary, NC) and figures were made in Python (Version 3.6) using the Pandas, Matplotlib, and Seaborn libraries.[70–72]

Results and discussion

Soil characterization

Long term differences in land use altered the chemical properties of the soils at Russell Ranch (Table 1, Fig. S1†). SOC was highest in the For and Gra soils compared to the agricultural soils. This is because plant residues are not removed in these systems leading to an accumulation of soil carbon over time. After twenty-three years, Org management led to increased SOC relative to soils under Con management. This is due to the growth of cover crops and inputs of compost that offset carbon loss due to plant residue removal during cropping.[38] Soil pH was higher in the agricultural systems (Con and Org) than Gra or For systems. Available Cu was highest in Org systems and lowest in Con systems. Soil Cu accumulation (>20%) under Org compared to the other soils was attributed to the relatively high Cu content (200–600 mg kg^{-1}) of the poultry litter compost applied at 9 Mg ha^{-1} annually for 26 years.[73] The Con and Gra soils had similar total Cu content, whereas the For soil had less total Cu, potentially due to its unique location on a lower alluvial terrace than the other soils. Though elevated SOC and pH are often associated with decreased soil Cu bioavailability, these results suggest that even relatively modest metal inputs delivered *via* organic matter amendments can drive soil metal availability (Table 1).[74]

Available and total P were greatest in the Org and lowest in the Con and Gra soils (Table 1). This is due to the low ratio of N:P in composts relative to plant needs; when composts are applied to meet the N requirements of crops, P is almost always applied in excess of crop nutrient needs.[75] Over time, this excess P builds up due to the limited mobility of P in soils. Transition metals such has Cu have a high affinity to form insoluble P complexes, potentially leading to Cu immobilization in soils with excess available and total P from long term compost application.[76,77]

CuO particle characterization

Mean particle size was 16 ± 10 nm for 16nCuO, 42 ± 26 nm for 42nCuO, and 430 nm ± 640 nm for bCuO (Fig. 1A–C). The large variance in particle size for the bCuO particles was due to a large fraction of very large particles >1000 nm. Despite
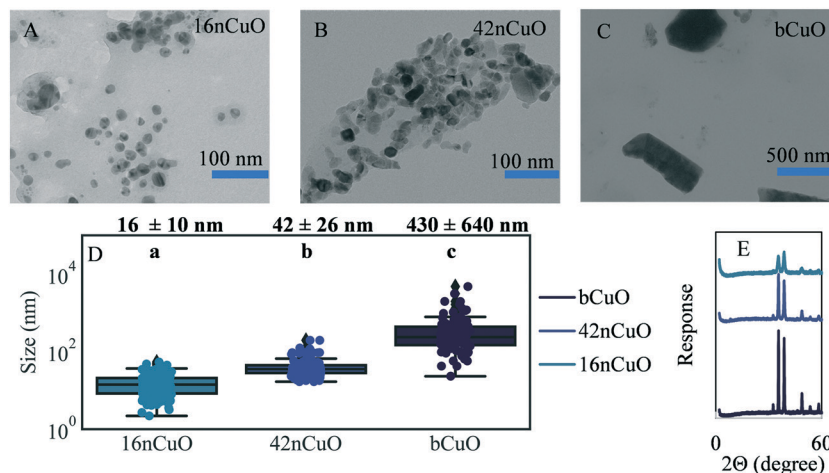
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Fig. 1 Copper oxide particle characterization: A) 16nCuO, B) 42nCuO, C) bCuO, D) mean particle size distribution for 16nCuO (n = 153), 42nCuO (n = 154), and bCuO (n = 101); E) diffractograms for 16nCuO, 42nCuO, and bCuO; mean size comparisons were analyzed by Tukey's HSD with significance declared at $p \leq 0.05$ and denoted with lower case letters.

some overlap in the particle size for each of the CuO particle size classes, mean particle diameters were different (Fig. 1D). For all particles, CuO speciation was confirmed by bulk X-ray diffraction, and for 16nCuO and 42nCuO by bulk X-ray absorption near edge structure (XANES) spectroscopy (Fig. 1E and S2†). X-ray peak broadening in both the diffractograms and XANES spectra were observed with decreasing particles size (Fig. 1E), due to the smaller crystalline domain size of the nanoparticles increasing signal scattering.[78] An increasing proportion of surface defects per unit mass for the

nCuO can decrease the degree of crystallinity per unit mass of CuO, manifesting in the decreased diffractogram intensities observed here. Such defects could lead to enhanced dissolution of nCuO compared to bCuO.[79,80]

In 1 mM KCl, the zetapotential (average surface charge) and hydrodynamic radius (size when dispersed in solution) of the 16nCuO particles was 43 ± 1 mV and 310 ± 13 nm, respectively, and for the 42nCuO particles, 24 ± 1 mV and 2500 ± 55 nm, respectively. The zetapotential and hydrodynamic radius of the bCuO particles were not recorded
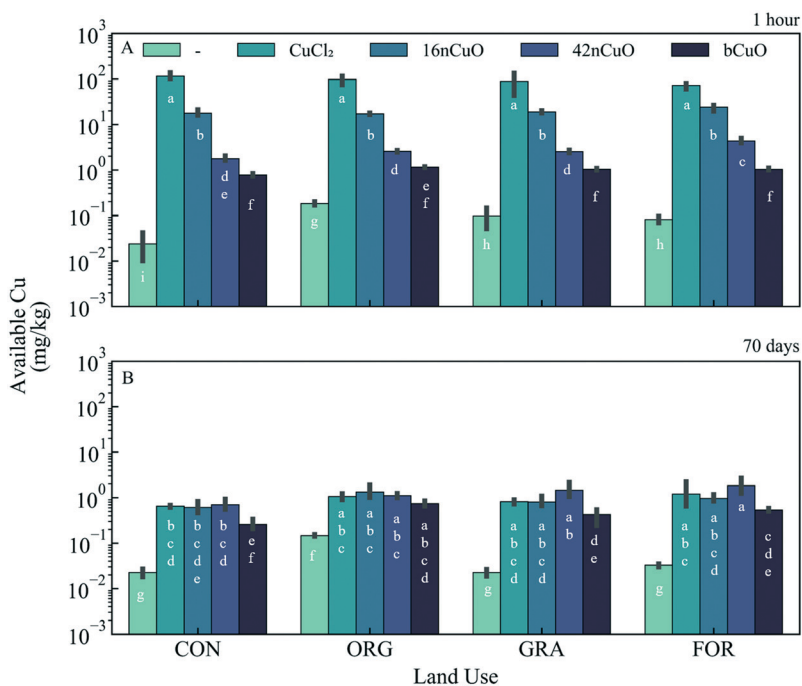

Fig. 2 Available copper (Cu) at A) 1 hour and B) 70 days in soils from conventional (Con), organic (Org), grassland (Gra), and riparian forest (For) systems untreated (—) or treated with 1000 mg copper Cu per kg soil in the form of CuCl₂, 16nCuO, 42nCuO, or bCuO. Data are presented as mean ± standard deviation (n = 3). Means separation was analyzed by Tukey's HSD with significance declared at $p \leq 0.05$ and differences are denoted with differing lower-case letters.

due to rapid sedimentation of the bCuO particles when suspended. Positive zetapotentials for nCuO particles in dilute salt solutions were previously measured by others and are expected in neutral pH solutions given the range of isoelectric points (pH 6.9–9.5) for CuO found in the literature.[81–84] Nanoparticles with zeta potentials >30 mV or ≤30 mV are generally considered to be stable in suspension due to electrostatic repulsion, while particles with zeta potentials closer to 0 mV are prone to aggregation. Both 16nCuO and, to a greater extent, 42nCuO, aggregated in 1 mM KCl as evidenced by the relatively large hydrodynamic radii of the 2 particle size classes compared to their mean particle size as determined by TEM (Fig. 1A, B, and D). Differences in the respective hydrodynamic radii of the two sizes of CuO likely reflect differences in surface charge.[85] The molarity of the soil solution is likely significantly greater than 1 mM, leading to enhanced aggregation due to diminished electrostatic repulsion tempered by the presence of naturally occurring ligands in solution such as HSO_4^-, SO_4^{2-}, HPO_4^{2-}, HCO_3^-, and acid functional groups of dissolved organic matter.[23,86–89] These ligands can alter the surface charge of nanoparticles leading to aggregation.[90,91]

Influence of CuCl₂, nCuO, and bCuO on available Cu and pH over time

Background soil Cu availability in untreated soils was greatest in the Org soil and lowest in the Con soil (Fig. 2A, Table S2†). Among untreated soils, soil pH was higher in the

agricultural soils compared to the unmanaged systems after 1 hour (Fig. 3A, Table S5†). Under aerobic conditions, soil Cu availability is controlled by soil pH, with decreases in pH mobilizing Cu and increases in pH immobilizing Cu.[1,4,7,58,89,92–94]

One hour after exposure to copper, soil Cu availability was dependent on the interaction between Cu form and use ($F_{9,31}$ = 11, $p < 0.0001$), though Cu form ($F_{3,31}$ = 2830, $p < 0.0001$) had a stronger effect on Cu availability than land use ($F_{3,31}$ = 8, $p = 0.0004$) (Fig. 2A, Table S3†). Soil pH at 1 hour after treatment with Cu was specific to the interaction between land use and Cu form ($F_{9,31}$ = 40, $p < 0.0001$) (Fig. 3A, Table S5†). However, Cu form ($F_{3,31}$ = 11 000, $p < 0.0001$) had a much stronger effect on soil pH than land use ($F_{3,31}$ = 160, $p < 0.0001$) (Fig. 3A, Table S4†). The addition of CuO, regardless of size, elevated soil pH, though the 16nCuO particles increased pH the least (Fig. 3A, Table S5†). The buffering capacity of colloid solutions is well known and is controlled by the isoelectric point of the minerals in suspension.[71–73,95–97] After 1 hour equilibrium, the pH of the stock suspension for 16nCuO (pH 5.5 ± 0.3) was significantly lower than the pH of 42nCuO suspensions (pH 8.1 ± 0.2), and bCuO (pH 7.9 ± 0.2). Particle size-dependent increases in soil pH at 1 hour after application were likely due to differences in the surface charge and buffering capacity of the differently sized particles, rather than by CuO dissolution associated condensation reactions.[20,29,98–101] Treatment with CuCl₂ resulted in soil acidification, likely *via* Cu²⁺ hydrolysis, which further increased Cu availability from the highly
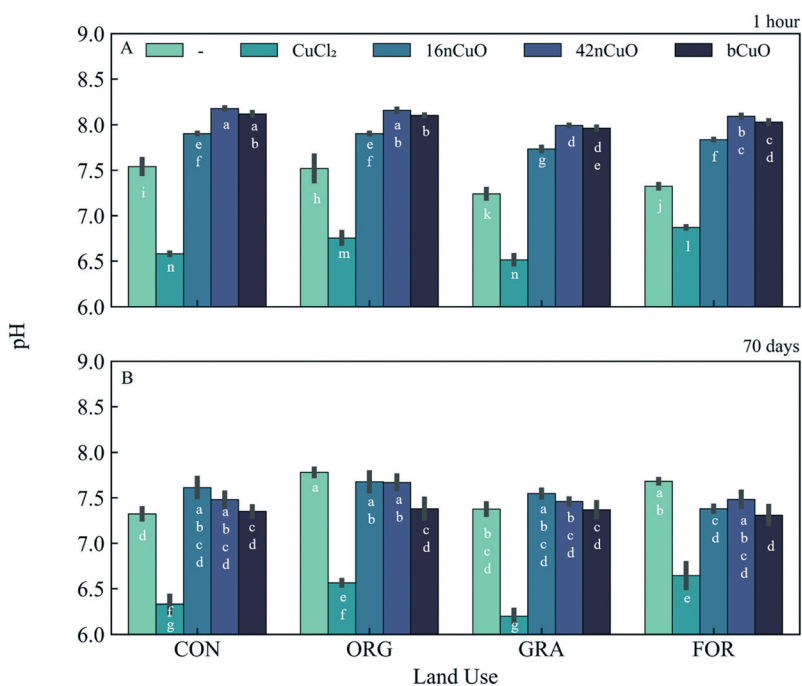

Fig. 3 Soil pH at A) 1 hour and B) 70 days in soils from conventional (Con), organic (Org), grassland (Gra), and forest (For) systems untreated (—) or treated with 1000 mg copper Cu per kg soil in the form of CuCl₂, 16nCuO, 42nCuO, or bCuO. Data are presented as mean ± standard deviation (n = 3). Means separation was analyzed by Tukey's HSD with significance declared at $p \leq 0.05$ and differences are denoted with differing lower-case letters.

soluble salt (Fig. 3A, Table S4†). Copper availability after 1 hour was inversely related to particle size ($CuCl_2$ > 16nCuO > 42nCuO > bCuO) with pH playing little role in the observed differences in Cu availability among the CuO treatments (Fig. 2A, Tables S3 and S5†). Specifically, soil pH after 1 hour was the same after treatment with 42nCuO and bCuO, but Cu availability was significantly greater after treatment with 42nCuO compared to bCuO (Fig. 2A and 3A, Tables S3 and S5†). Similar size-controlled particle dissolution kinetics were previously reported in both water and soils.[79,80,102]

Over time, absolute soil Cu availability decreased across all Cu treatments. Copper availability after 70 days in soils exposed to copper was driven by Cu form ($F_{3,32}$ = 21, p < 0.0001) to a greater extent than by land use ($F_{3,32}$ = 13, p < 0.0001), with no interaction between the two ($F_{9,32}$ = 1.2, p = 0.32) (Fig. 2B, Table S4†). Cu availability after 70 days was lowest in soils treated with bCuO (Fig. 2B, Table S4†). Soils under Con management had the lowest Cu availability after 70 days regardless of Cu treatment source (Fig. 2B, Table S4†). However, after 70 days, soil pH after treatment with copper was specific to the interaction between land use and Cu form ($F_{9,32}$ = 4.9, p = 0.0004), though Cu form ($F_{3,32}$ = 360, p < 0.0001) had a stronger effect on soil pH than land use ($F_{3,32}$ = 7.3, p = 0.0007) (Fig. 3B, Table S6†). In soil from the Con system, treatment with 16nCuO increased soil pH (7.61 ± 0.12) while treatment with $CuCl_2$ (pH 6.33 ± 0.11) decreased pH relative to the untreated control (pH 7.29 ± 0.06). These results are consistent with water condensation during CuO dissolution and water hydrolysis by Cu^{2+} during $CuCl_2$ dissolution.[98,103] In soil from the Org system (Fig. 3B), treatment with 1000 mg kg^{-1} bCuO (pH 7.38 ± 0.13) and $CuCl_2$ (pH 7.49 ± 0.22) decreased pH compared to the untreated control (pH 7.79 ± 0.06). In the Gra system, soil pH decreased only with $CuCl_2$ (pH 6.20 ± 0.08) relative to the untreated control (pH 7.40 ± 0.08) whereas in the forest soil, pH decreased after treatment with 16nCuO (pH 7.38 ± 0.04), bCuO (pH 7.31 ± 0.12), and $CuCl_2$ (pH 6.64 ± 0.16) relative to the untreated control (pH 7.68 ± 0.03) (Fig. 3B, Table S6†). It is unclear why the treatment with bCuO in the organic system and both 16nCuO and bCuO in the forest system decreased soil pH (Fig. 3B, Table S6†). Both nCuO and bCuO have been previously shown to increase soil pH over short time periods, and increase or decrease pH over longer time periods.[29,36,104]

The relatively high pH (>7.2) of the soils in the current study likely led to rapid Cu immobilization after dissolution, limiting Cu availability.[85,105] Regardless of treatment form or concentration, Cu availability decreased over time with the largest absolute change associated with $CuCl_2$ and 16nCuO compared to 42nCuO and bCuO (Fig. 2A and B, Tables S3 and S4†). However, changes in soil pH were specific to Cu form. Treatment with bCuO decreased soil pH over time compared to treatment with 42nCuO and both decreased pH over time more than $CuCl_2$, 16nCuO, or "no Cu" treatment (Table S7†). Other investigators have reported Cu availability

to increase over time in acidic soils amended with CuO particles due to CuO dissolution under acid soil conditions.[80–84] Under the alkaline soil conditions of the current study, immobilization would be favored due to adsorption by silicates or complexation by soil organic matter.[105,106]

Despite the limited availability of Cu in alkaline soils, long term application of Cu based fungicides, including nCuO, can significantly increase total soil Cu, leading to Cu saturation of the cation exchange complex and altering plant growth.[3,4,6] Total soil Cu concentrations of 1500–3000 mg Cu per kg soil have been observed in agricultural soils due to long-term (>100 year) use of Cu fungicides, and in some cases even necessitating changes in land use.[4–7]

Cu speciation 70 days after treatment with $CuCl_2$, nCuO, and bCuO

Bulk copper XANES spectroscopy was employed to speciate Cu in treated soils. Cu added as $CuCl_2$ was primarily bound by soil materials including adsorbed to silicates and complexed by soil organic matter (Tables 2 and S8†).[107] In XFM maps of both the Con and For soils treated with $CuCl_2$, individual soil particles were visible in both maps with Cu, Ca, and Fe fluorescence channels (Fig. 4A and I) as well as the Cu channel alone (Fig. 4E and M). This indicated that the copper from the $CuCl_2$ treatment was bound by the soil materials, in agreement with the XANES results.

Copper speciation in soils treated with 16nCuO was similar to that of soils treated with $CuCl_2$, with no copper remaining as CuO after 70 days (Tables 2 and S8†). Rather, the copper was bound by soil materials, primarily adsorbed by silicates, and complexed by soil organic matter. In XFM maps of both the Con and For soils treated with 16nCuO, individual soil particles were visible in both maps using the Cu, Ca, and Fe fluorescence channels (Fig. 4B and J) as well as the Cu channel alone (Fig. 4F and N). Few aggregates of Cu appear to remain as bright Cu points in the map, with copper evenly distributed among the individual soil particles. Given the XANES results for the 16nCuO treatment and the fact that ligands in the soil solution would promote nanoparticle aggregation, the mapping results provide further evidence of 16nCuO dissolution.[91]

After treatment with 42nCuO, Cu was measured by Cu XANES to be primarily bound by soil materials, with some Cu remaining as CuO (Table S8†). The amount of CuO remaining after the 70 day incubation was significantly greater than was measured after treatment with 16nCuO, but significantly less than was measured after treatment with bCuO. Soil particles are visible in tricolor Cu, Ca, Fe X-ray fluorescence distribution maps (Fig. 4C and K) as well as the Cu map (Fig. 4G and O) in both the Con and For soils, evidence of some CuO dissolution and subsequent adsorption/complexation by soil materials. Scattered

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Table 2 Copper speciation determined by X-ray absorption near edge structure (XANES) spectroscopy after 70 days in soils treated with 1000 mg Cu per kg soil using $CuCl_2$ ($n = 4$), 16nCuO ($n = 4$), 42nCuO ($n = 4$), and bCuO (bCuO, $n = 2$). Soils (0–10 cm depth) were sampled from the conventional (Con) and organic (Org) agricultural treatment plots, and from adjacent restored grassland (Gra) and riparian forest (For) at the Russell Ranch Sustainable Agricultural Facility

	Normalized sum of squares (10^{-5})	CuO %	Soil bound Cu %	Sum %
$CuCl_2$	2.5 ± 0.3	0 ± 0^a	101 ± 1^a	100 ± 1
16nCuO	3.1 ± 1.8	0 ± 0^a	100 ± 1^a	100 ± 2
42nCuO	3.4 ± 1.8	46 ± 5^b	53 ± 5^b	99 ± 1
bCuO	2.5 ± 1.8	60 ± 0.4^c	41 ± 7^c	101 ± 0

Means separation for copper speciation was analyzed by Tukey's HSD with significance declared at $p \leq 0.05$ and differences among treatments within the same column are denoted with lowercase letters.
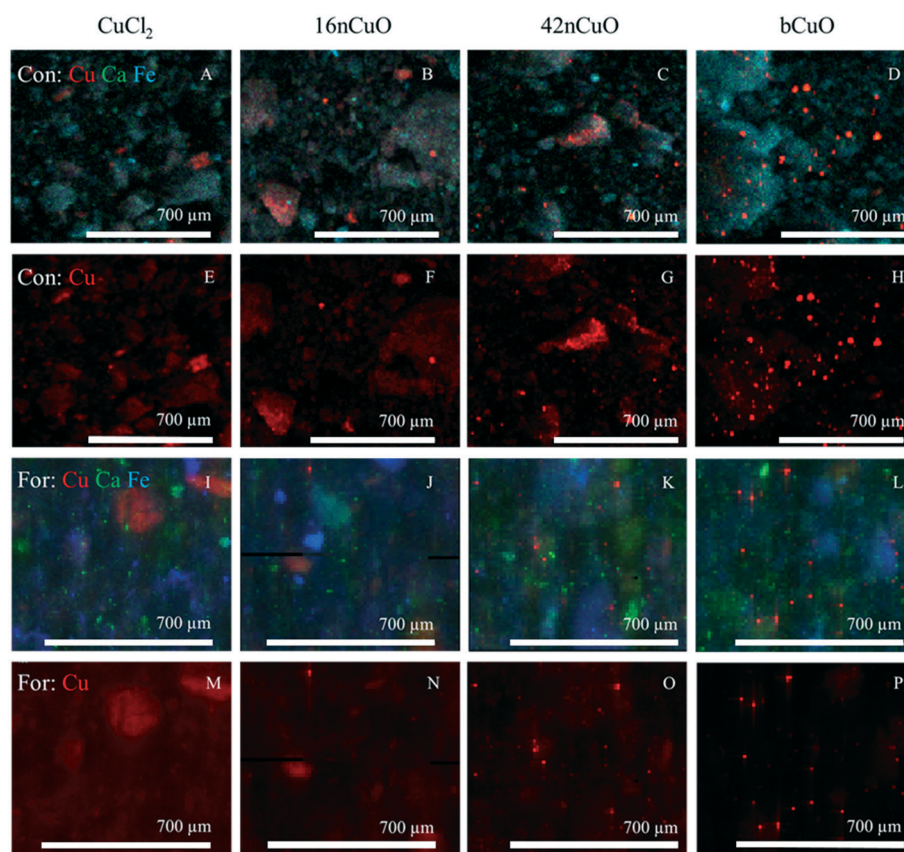

Fig. 4 X-ray fluorescence distribution maps of Cu, Ca, and Fe in soil under the conventional treatment (Con) treated with 1000 mg copper (Cu) per kg soil from A) $CuCl_2$, B) 16nCuO, C) 42nCuO, D) bCuO; same maps of Con soil with only Cu visible from E) $CuCl_2$, F) 16nCuO, G) 42nCuO, H) bCuO taken on XFM 10.3.2 at the ALS; X-ray fluorescence distribution maps of Cu, Ca, and Fe from the forest soil (For) treated with 1000 mg copper (Cu) per kg soil from I) $CuCl_2$, J) 16nCuO, K) 42nCuO, or L) bCuO; same maps of For soil with only Cu visible from M) $CuCl_2$, N) 16nCuO, O) 42nCuO, or P) bCuO collected on XFM VESPERS at the CLS; increased red saturation is indicative of increased localized Cu content.

aggregates of Cu are clearly visible in both soils, indicative of incomplete dissolution.

As measured by Cu XANES, copper in soils treated with bCuO remained primarily as CuO with some dissolution having occurred. Significantly more CuO was measured after 70 days in soils treated with bCuO than after treatment with 16nCuO or 42nCuO (Tables 2 and S8†). Soil particles in both the Con and For soils are visible in tricolor Cu, Ca, Fe X-ray

fluorescence distribution maps (Fig. 4D and L), but barely visible in Cu maps (Fig. 4H and P), evidence of limited Cu adsorption/complexation by soil particles. Rather, most of the copper appears as aggregates of copper in the maps.

Regardless of soil type, CuO particle dissolution was controlled by particle size. After 70 days, soils treated with 16nCuO had no remaining CuO. Rather, all Cu spectra could be modeled either as Cu adsorbed by silicates and complexed

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by organic matter. Soils treated with 42nCuO had more remaining CuO than those receiving the 16nCuO treatment, but less than those receiving the bCuO treatment (Tables 2 and S8,† Fig. S3†).

Most studies do not include Cu adsorbed to phyllosilicate minerals commonly found in soils from arid or Mediterranean climates or those that have undergone little pedogenisis.[27,33,103,108–116] Rather, Cu from CuO nanoparticles is often modeled as complexed by SOM, neoformed as Cu_xS, or adsorbed by iron or aluminum oxides.[27,33,109,116] However, reducing conditions such as those that lead to Cu_xS formation are less likely to occur in soils from arid or Mediterranean climates.[107,113] Further, soils in these climates, and more generally, soils that have undergone minimal mineral weathering, are dominated by phyllosilicate minerals and are unlikely to contain significant amounts of iron or aluminum oxides.[107,113,114] As a result, the general reporting and understanding of soil organic matter complexation, sulfide formation, or adsorption by iron and aluminum oxides as the main fate of Cu added to soils may be incomplete.[27,103,108–110] Our results suggest that adsorption by silicates is the primary sink for Cu in alkaline soils. Further, our results imply that size-dependent dissolution rates may entail distinct kinetic and thus thermodynamic fates of CuO fungicides compared to larger particulate formulations. These results are important given the wide use of copper fungicides in agriculture and high probability that they end up in contact with soils after application.

Influence of CuCl₂, nCuO and bCuO on soil respiration, microbial biomass, and biomass normalized respiration

Land use exerted a strong control over soil organic carbon and therefore soil microbial biomass (Fig. S4†).[117–119] The unmanaged soils had the greatest total microbial biomass, with greater biomass in the For system than the Gra system (Fig. 5A, Table S9†). Among the agricultural soils, the Org soil had greater microbial biomass than the Con soil (Fig. 5A, Table S9†). This is due to the long-term addition of compost and cover crops grown in the Org soil which act to increase soil carbon, the main substrate for microbial growth in soils.[38] While total microbial biomass was well correlated with soil organic carbon (SOC), the Gra system exhibited statistically higher biomass per unit SOC (0.33 ± 0.003 μmol PLFA mol⁻¹ SOC-C) compared to the Con (0.28 ± 0.003 μmol PLFA mol⁻¹ SOC-C), Org (0.28 ± 0.008 μmol PLFA mol⁻¹ SOC-C), or For (0.26 ± 0.007 μmol PLFA mol⁻¹ SOC-C) systems (Table 1, Fig. 5A, Table S9,† Fig. S4†).

The response of soil microbial biomass to treatment with copper depended on the combination of land use and Cu form ($F_{9,30}$ = 9.2, p < 0.0001) (Fig. 5A, Table S9†). Land use had a larger effect on soil microbial biomass ($F_{3,30}$ = 1400, p < 0.0001) than Cu form ($F_{3,30}$ = 3.9, p = 0.0192). The 42nCuO and CuCl₂ treatments significantly decreased total PLFAs in the For and Gra soils compared to their respective untreated controls (Fig. 5A, Table S9†). Total PLFA content of Con and Org soils was not significantly altered 70 days after copper treatment, regardless of form (Fig. 5A, Table S9†). Though
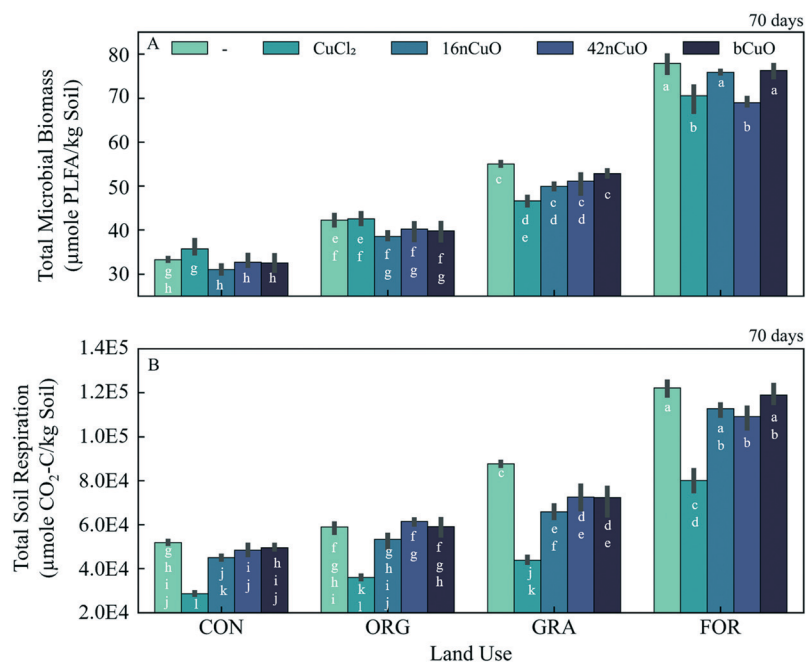

Fig. 5 A) Total soil PLFAs after 70 day, B) cumulative (70 day) soil respiration in soils from conventional (Con), organic (Org), grassland (Gra), and forest (For) systems untreated (—) or treated with 1000 mg copper Cu per kg soil in the form of CuCl₂, 16nCuO, 42nCuO, or bCuO. Data are presented as mean ± standard deviation (n = 3). Means separation was analyzed by Tukey's HSD with significance declared at p ≤ 0.05 and differences are denoted with differing lower-case letters.

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unexpected, the overriding effects of previous land use on soil microbial biomass to Cu exposure help explain the diverse microbial responses to various nanomaterials, including nCuO observed in this and other studies.[18,20,22,26,120] Specifically, Collins *et al.*, 2012, found no change to the fatty acid profile of soil microbial communities after treatment with Cu nanoparticles, but this may have been because the soil they used came from an agricultural system which our results suggest are insensitive to Cu treatment in general and CuO nanoparticle treatment in particular. Ge *et al.*, 2011, measured significant changes in soil microbial community as estimated by total extractable DNA in grassland soils treated with TiO_2 and ZnO nanoparticles, but this may have been due to the selection of a grassland soil for experimentation which our results suggest are sensitive to nanoparticle treatment.[26] Further, Simonin *et al.*, 2015, noted that in their experiment to evaluate the effects of TiO_2 nanoparticles on soil microbial communities in soils of contrasting textures that the only soil that was responsive to nanoparticle exposure was a silty-clay-loam, a result they found surprising. However, this soil was taken from a pasture, one of only two soils (out of six) in their study that was not cropped. Our results suggest that the response they measured may be attributed to historical use as pasture, which is more similar to unmanaged systems compared to cropped systems, rather than an intrinsic property of the soil itself.[19] Similarly, Simonin *et al.*, 2018, measured limited effects of CuO NPs on microbial abundance in agricultural soils treated with $CuSO_4$ and CuO

nanoparticles, but did observe a decrease in soil microbial activity in some soils, in agreement with the results of the current study.[20]

Soil respiration increased with SOC content, and the unmanaged soils accordingly had greater total respiration compared to the agricultural soils (Fig. 5B and S10†). Similar to total microbial biomass, total respiration per unit SOC was uniquely high for Gra soils (510 ± 6.8 μmol CO_2-C per mol SOC-C) compared to the Con (440 ± 4 μmol CO_2-C per mol SOC-C), For (400 ± 10 μmol CO_2-C per mol SOC-C), or Org (380 ± 18 μmol CO_2-C per mol SOC-C) systems (Table 1, Fig. 5B, Table S10†).

The response of total soil respiration to copper treatment was unique to the combination of land use and Cu form ($F_{9,30} = 3.9$, $p = 0.0024$), but was more strongly influenced by land use ($F_{3,30} = 700$, $p < 0.0001$) than Cu form ($F_{3,30} = 160$, $p < 0.0001$) (Fig. 5B, Table S10†). Treatment with 1000 mg Cu per kg soil from $CuCl_2$ induced a decrease in total soil respiration across all treatments (Fig. 5B, Table S10†). Treatment with 42nCuO decreased total respiration in soils from the For and Gra systems, but not in soils under agricultural use. Both 16nCuO and bCuO inhibited respiration from the Gra soil, but not from soils in any of the other systems (Fig. 5B, Table S10†). Treatment with >100 mg CuO NPs per kg soil was previously observed to alter soil microbial activity and substrate induced respiration, but the effects of nCuO on continuous respiration processes were not previously measured.[18–20] Interestingly, Simonin *et al.*, 2018 measured that in agricultural soils, exposure to Cu from
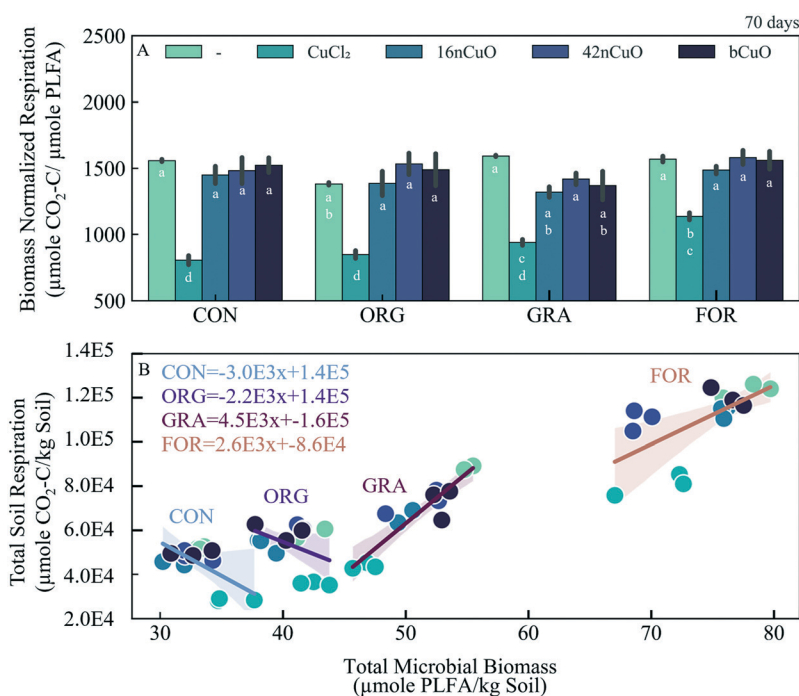

Fig. 6 A) Biomass normalized respiration (μmol CO_2-C/μmol PLFA) for untreated soils (—) or after treatment with 1000 mg Cu per kg soil in the form of $CuCl_2$, 16nCuO, 42nCuO, or bCuO. Data are presented as mean ± standard deviation ($n = 3$). Means separation was analyzed by Tukey's HSD with significance declared at $p \leq 0.05$ and differences are denoted with differing lower-case letters; B) total soil respiration plotted against total PLFAs and analyzed by linear regression.

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CuSO$_4$ and CuO nanoparticles caused inconsistent alterations to substrate induced respiration after 7 and 90 days. Out of six soils, only one soil exhibited altered substrate induced respiration from nanoparticle exposure after 90 days and 2 soils from exposure to CuSO$_4$. This contrasts with the current study in which CuCl$_2$ inhibited respiration in all soils over the course of 70 days. This difference may be due to the relatively high concentration of Cu used in the current study (1000 mg Cu per kg soil) compared to that used by Simonin *et al.*, 2018 (100 mg Cu per kg soil).

Biomass normalized respiration (BNR) was used to evaluate SOC conversion efficiency, a proxy for system stress/activity similar to the specific respiration rate or respiratory quotient (Fig. 6A, Table S11†).[121,122] Among the untreated controls, soil BNR was lowest for Org and similar for the other three land uses (Fig. 6A, Table S10†). In soils treated with copper, soil BNR response was primarily influenced by Cu form ($F_{3,30} = 120$, $p < 0.0001$) and to a lesser extent, land use ($F_{3,30} = 9.5$, $p < 0.0001$), with little interaction ($F_{9,30} = 2.1$, $p = 0.0586$) (Fig. 6A, Table S11†). Specifically, BNR was suppressed by treatment with CuCl$_2$, indicative of stress (Fig. 6A, Table S11†).[121,122] Sereni *et al.* 2021 reported contradictory results in their recent meta-analysis. Specifically, they found that Cu contamination was associated with increased biomass normalized respiration rates, but this was in acidic soils.[123] In alkaline soils, like those used in the current study, they did not find a linkage between biomass normalized respiration and Cu contamination.[123] However, Badía and Martí, 2003, previously observed that stress inhibits biomass normalized respiration in alkaline soils.[122] Given that treatment with CuO greatly increased soil pH compared to treatment with CuCl$_2$ and increased available Cu less than CuCl$_2$, it would be expected that treatment with CuO, regardless of size, would have minimal effect on biomass normalized respiration compared to treatment with CuCl$_2$.[122,123]

Interestingly, divergent responses to general Cu treatment were observed among the soils. Specifically, respiration and microbial biomass were coupled in the For soil and decoupled in the Con and Org soils. These distinct relationships between respiration and microbial biomass by land use are particularly apparent when respiration was plotted against microbial biomass for all Cu treatments. In soils from both the For ($r^2 = 0.42$, $p = 0.0088$) and Gra systems ($r^2 = 0.86$, $p \leq 0.0001$), increasing Cu content is associated with both decreased respiration and microbial biomass, leading to positive linear regression fit slopes (Fig. 6B). In soils from the Org ($r^2 = 0.2$, $p = 0.1104$) and Con systems ($r^2 = 0.4$, $p = 0.0155$), increasing Cu content was associated with lower respiration but little or positive change in microbial biomass, leading to negative linear regression fit slopes, and indicative of greater metal stress tolerance (Fig. 6B).[124]

Surprisingly, we failed to reject the null hypothesis that increasing SOC would confer greater protection to soil microbial communities from copper exposure as was previously observed by Rousk *et al.*, 2012. Rather our results were similar to Sominin *et al.*, 2018, who measured no clear linkage between increasing SOC and microbial resilience to copper exposure. In the current study, land use history was seemingly more important than SOC given the divergent response of the microbial communities in Org and Gra systems to Cu exposure despite similarities in SOC content. A practical implication of this for nanoparticle toxicity assays is that the effects of nCuO on microbial communities may be underestimated if only agricultural soils are used as the test media.

Conclusions

The fate of copper in soil was dependent upon the form and size of the Cu involved. As revealed by XFM and XANES spectroscopy, CuO underwent size dependent dissolution, corroborated by greater increases in available Cu for soils treated with smaller nanoparticulate CuO. Addition of CuCl$_2$ acidified the soil through precipitation and hydrolysis reactions. In contrast, additions of CuO raised the soil pH within 1 h, but after 70 days decreased the soil pH, potentially due to respiration related acidification.

Though Cu form strongly affected chemical properties such as soil pH and available soil-Cu, land use dominated the response of microbial response to Cu treatment, often resulting in responses unique to the combination of land use and Cu form. Microbial biomass in the unmanaged systems was sensitive to Cu amendment, especially in the Gra soil that had the greatest respiration (activity) and microbial biomass per unit SOC. Microbial biomass in conventional and organic systems was unaltered by Cu exposure; but respiration was inhibited in all soils by CuCl$_2$ at 1000 mg Cu per kg soil. This suggests that microbially mediated nutrient cycling, important for facilitating nutrient availability, particularly in organic agricultural systems, is vulnerable to copper exposure even with minor alteration of microbial biomass size. However, our study demonstrates that novel nCuO biocides have fewer deleterious effects on soil microbial communities compared to salt-based Cu treatments, at least in the short-term (70 days), due to comparatively slower dissolution and minimal soil pH disturbance.

Additionally, the unique relationship between soil respiration and microbial biomass after copper treatment within each land use underscores that microbial responses to Cu amendment are often more sensitive to historical use than Cu treatment rate or form. Thus, it is important to consider historical land use when interpreting soil Cu nanoparticle toxicity testing results and potentially, metal and nanoparticle toxicity results in general.

Conflicts of interest

There are no conflicts to declare.

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