Supporting Information For

Copper Nanoparticle Induced Cytotoxicity to Nitrifying Bacteria in Wastewater Treatment: A Mechanistic Copper Speciation Study by X-ray Absorption Spectroscopy

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Supporting information comprises 12 pages of additional content including five tables, and nine figures showing details of enriched media cultivation and maintenance, characterization of CuNPs, model XAFS spectra, examples of LCF analysis, images of 10 kDa filters used in particle separation, and results of XAFS data analysis for all samples.

Nanoparticle Characterization and Suspension Preparation – After a 10:1 dilution with Milli-Q water, CuNP suspensions were characterized for hydrodynamic diameter (HDD) using a ZetaSizer Nano Series (Malvern, Worcestershire, UK). HDD measurements were completed using an average a 3 runs, with each run consisting of at least 10 measurements. (Table S1) Additionally, raw powders were mounted on carbon tape and characterized for particle size and morphology with a JEOL JSM-7600F (JEOL, Tokyo, Japan) Field Emission Scanning Electron Microscope (SEM). Collected images are shown in Figure S1. Particle size analysis for each powder was completed through manual examination of at least 400 particles using the Image-J software package. Histograms developed from particle size analysis are presented in Figure S2.

Table S1: Characterization Data of CuNPs used in this study

Particle Size (nm)		
	CuO	*Cu_2O*
Manufacturer	40	18
SEM	46.8 +/- 16.6	47.25 +/- 33.3
Hydrodynamic Diameter (nm)		
	CuO	*Cu_2O*
Milli-Q Water	1,596 +/- 68	720 +/- 15
1 wt % PVP	588 +/- 10	575 +/- 10

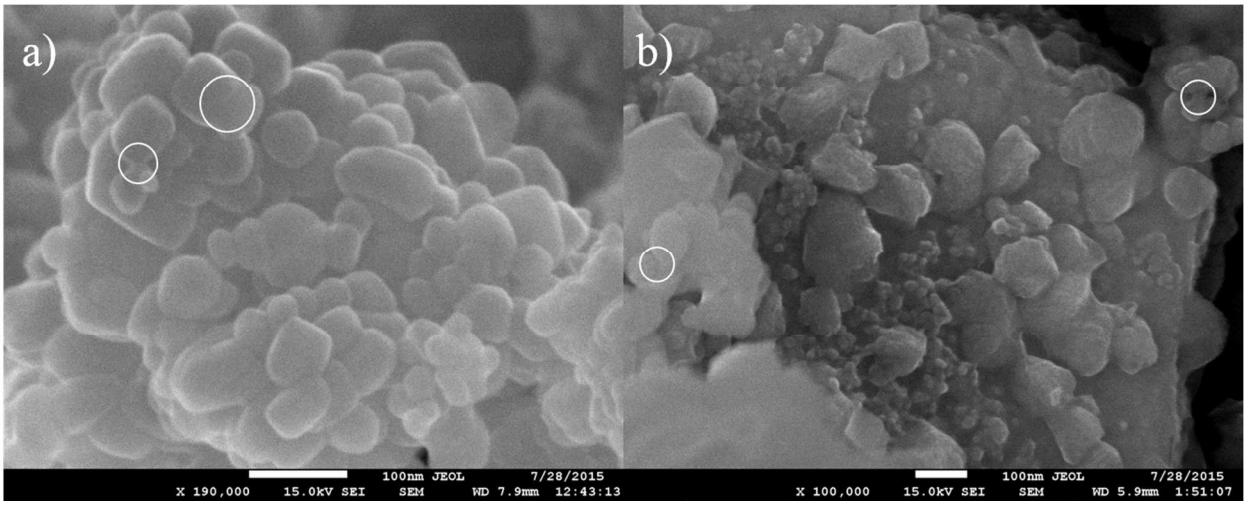

Figure S1: SEM images of (a) CuO and (b) Cu_2O nanoparticles used in this study. CuO particles are relatively consistent in size and morphology, while Cu_2O particles show the presence of very fine particles and larger aggregates. White circles indicate areas of particle sintering.

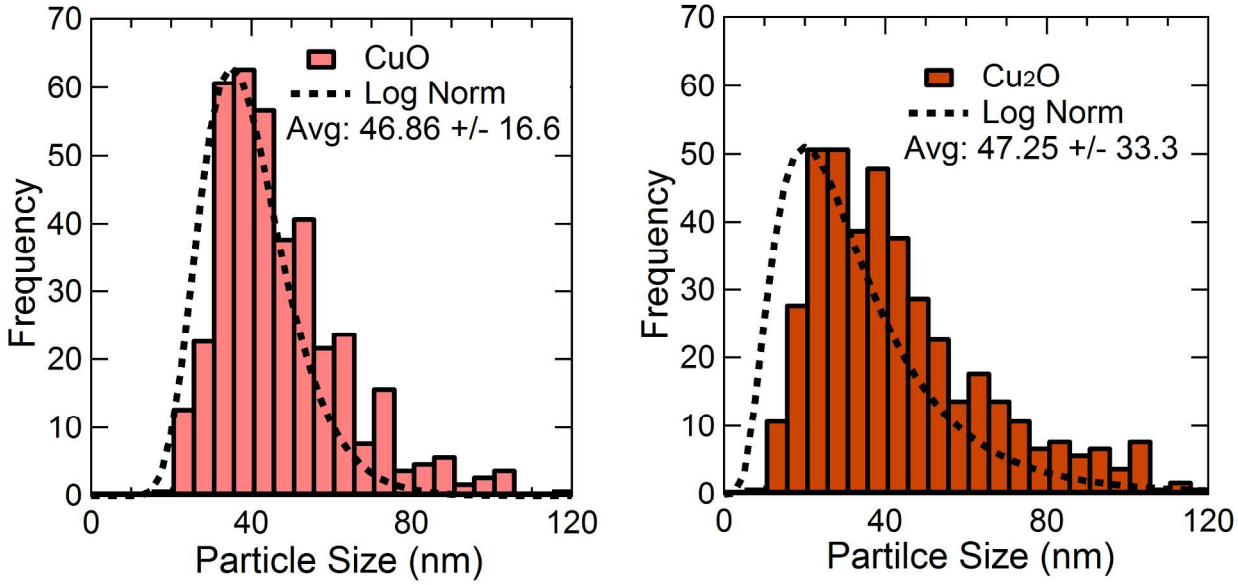

Figure S2: Particle Size distribution of CuO and Cu_2O particles utilized throughout this study. Histograms produced through manual analysis of at least 400 particles for both CuNP powders using Image-J software package.

Nitrifying Enrichment Cultivation –A continuously stirred tank reactor (CSTR) with a 7 L reacting compartment and a 3 L settling compartment was used for nitrification enrichments. The reactor was fed an inorganic medium devoid of organic carbon, with ammonium (500 mg of NH_4-N/L, $(NH_4)_2SO_4$) as the sole energy source with additional macro- and micro-nutrients. (Table S2) Filtered laboratory air was provided at 1 L/min to ensure adequate aeration. Mixing was provided by magnetic stirring at 200 RPM. All the chemicals used to prepare stock solutions for metal partitioning and inhibition studies were certified ACS grade or above. The components of the inorganic medium feeding the nitrifying reactor was prepared using in-house distilled water and outlined in Table S2. Other stock solutions and their dilutions were prepared with Milli-Q deionized water.

Table S2: Composition of Reactor Influent

Compound	Concentration (mg/L)	Compound	Concentration (mg/L)
$MgSO_4 \cdot 7H_2O$	252	$MnSO_4 \cdot H_2O$	3.3
KH_2PO_4	102	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.7
K_2HPO_4	261	$CuCl_2 \cdot 2H_2O$	0.8
$NaHCO_3$	350	$ZnSO_4 \cdot 7H_2O$	3.0
$FeSO_4 \cdot 7H_2O$	3.3	$NiSO_4 \cdot 6H_2O$	0.6

Metal Partitioning Analysis – Cu partitioning and dissolution was tracked through careful analysis of Cu concentration in both sample supernatant and biomass. After equilibration, samples were centrifuged for 5 min at 4000g (C & A Scientific, Manassas VA, Bio-Lion XC-L5) to isolate biomass. A portion of the supernatant was extracted and analyzed through microwave assisted digestion (EPA method 3015A) followed by inductively coupled plasma-optical emission spectrometry (ICP-OES, Thermo Fisher iCAP 6000 Series) to determine total Cu concentration. Additionally, 12 mL aliquots of supernatant were placed in 10 kDa (3 nm) Amicon Ultrafiltration filters (Millipore, Billerica MA) and centrifuged at 4,500 g for 20 min using a fixed angle rotor. Thermo Scientific, Sorvall RC 6+) This filter size retains any remaining CuNPs and large extracelluar polymeric substances remaining in solution. The resulting filtrate was analyzed for ionic Cu concentration using our previously described digestion procedures. After removing supernatant, biomass samples were washed twice with Milli-Q Water to remove any weakly adsorbed Cu from the biomass surface. Washed biomass was collected through filtration on a 0.45 μm Nylon Membrane filter (Whatman) and digested prior to ICP-OES analysis by EPA method 3051A. Both aqueous and adsorbed Cu concentrations were calculated after correction for the background values from control samples. (No Cu exposure) Initial testing demonstrated that increased incubation times (i.e., 12 h) did not alter Cu ion partitioning (Figure S3). Metal partitioning analysis was used to generate isotherms for both ionic Cu and CuNPs. Isotherms using CuNPs (Figure S4) do not reach a plateau, while isotherms using ionic Cu show a clear saturation of adsorption sites. (Figure 1b Main Text)

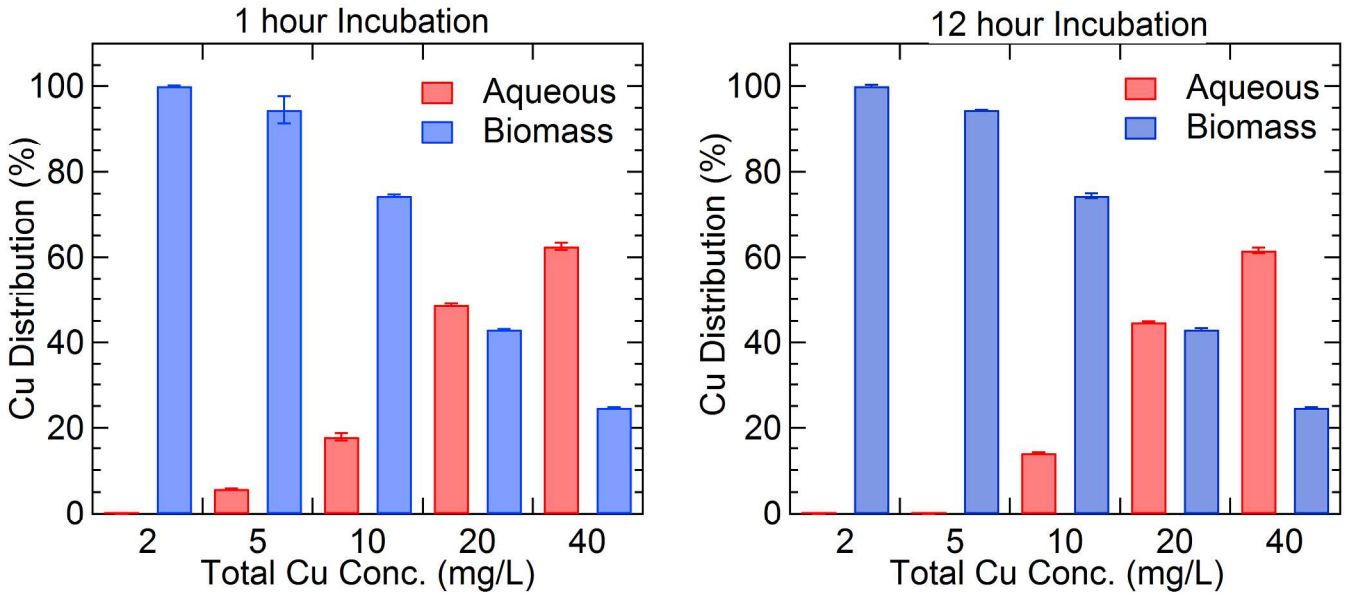

Figure S3: Results of ionic copper ($CuSO_4$) partitioning after 1 h or 12 h incubation with enriched media. After removing supernatant, the collected biomass was washed twice with Milli-Q Water to remove any weakly adsorbed Cu from the biomass surface. Any Cu unaccounted for is assumed lost during this washing procedure. Partitioning results show no statistical differences between 1 h and 12 h incubation periods.

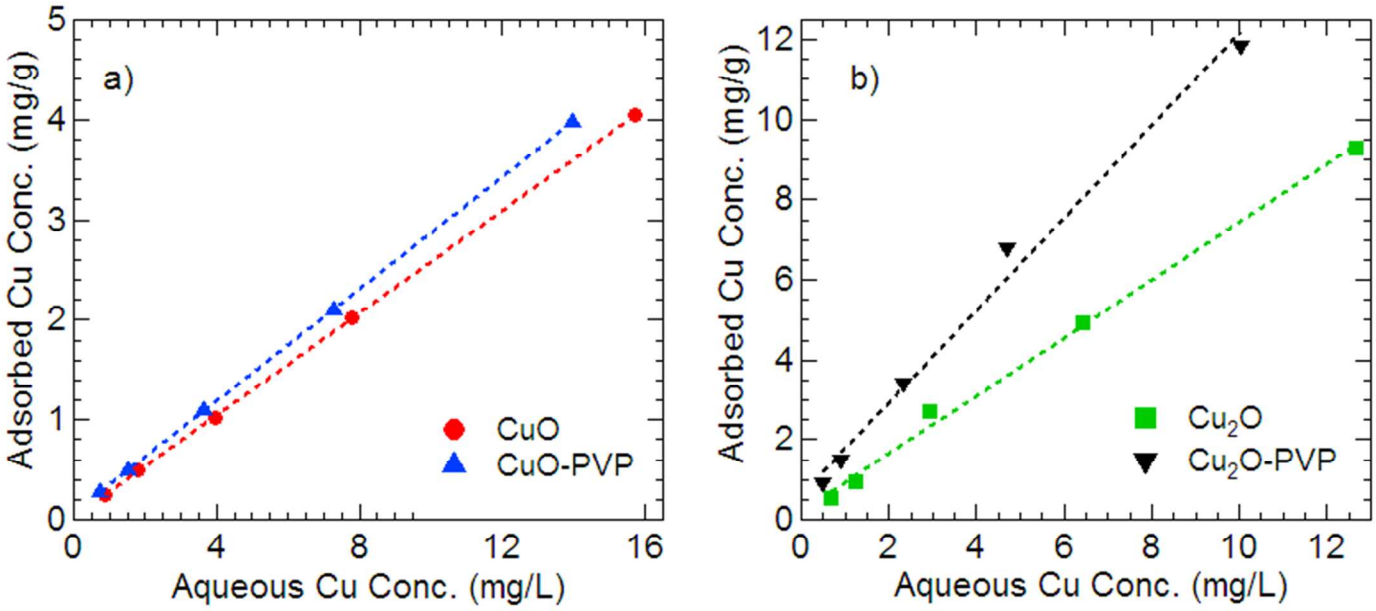

Figure S4: Absorption isotherms showing CuNP distribution between aqueous phase and biomass of uncoated and PVP coated a) CuO and b) Cu_2O particles after 3 h incubation. No plateau is reached in these systems at the mixing time and TSS level utilized.

X-ray Absorption Spectroscopy Analysis (XAFS) – XAFS spectra were collected in transmission and florescence mode simultaneously. The incident beam was monochromatized by using a Si (111) fixed-exit, double-crystal monochromator. The beam size was focused to an area of 500×500 μm. A minimum of three scans were conducted to achieve an adequate signal/noise ratio. The beam energy was calibrated by assigning the first inflection of the absorption edge of Cu foil to 8979 eV for the Cu-K edge. X-ray absorption near edge structure (XANES) data reduction, including averaging, background removal by spline fitting, normalization and derivatization followed standard methods using the IFEFFIT software package.[1] Linear combination fitting (LCF) was conducted on normalized and first derivative spectra of the samples using IFEFFET to quantify Cu speciation. The fitting range was -20 to 30 eV relative to the Cu K-edge for both normalized and first derivative spectra. The initial distribution of Cu species present in the unexposed biomass was determined through linear combination fitting (LCF) of the normalized and first derivative of the normalized spectra. To model the Cu speciation a suite of Cu-organic complexes were used. The Cu-organic complexes included carboxylate (Cu-Acetate), amine (Cu-Histidine), and sulfhydryl (Cu-Cysteine) functional groups common to those associated with enzymes, proteins, and extracellular membranes. Pristine powders of both CuO and Cu_2O were diluted with PVP and pressed into self-supported 13 mm pellets sealed in Kapton Tape for and also used as standard references during sample analysis. Cu_2O powders were stored and prepared under anerobic conditions to ensure stability of the Cu(I) oxidation state and minimize oxidation through contact with air. XAFS spectra of all references samples, as well as the first derivative of normalized spectra are presented in Figure S5. Additionally, XAFS spectra of biomass samples that had been exposed to identical concentration of ionic Cu and CuNPs are also displayed in Figure S5. A representative example LCF results is shown in Figure S6. A complete breakdown of LCF analysis for all exposed biomass samples used in this study can be found in Tables S3 and S4.

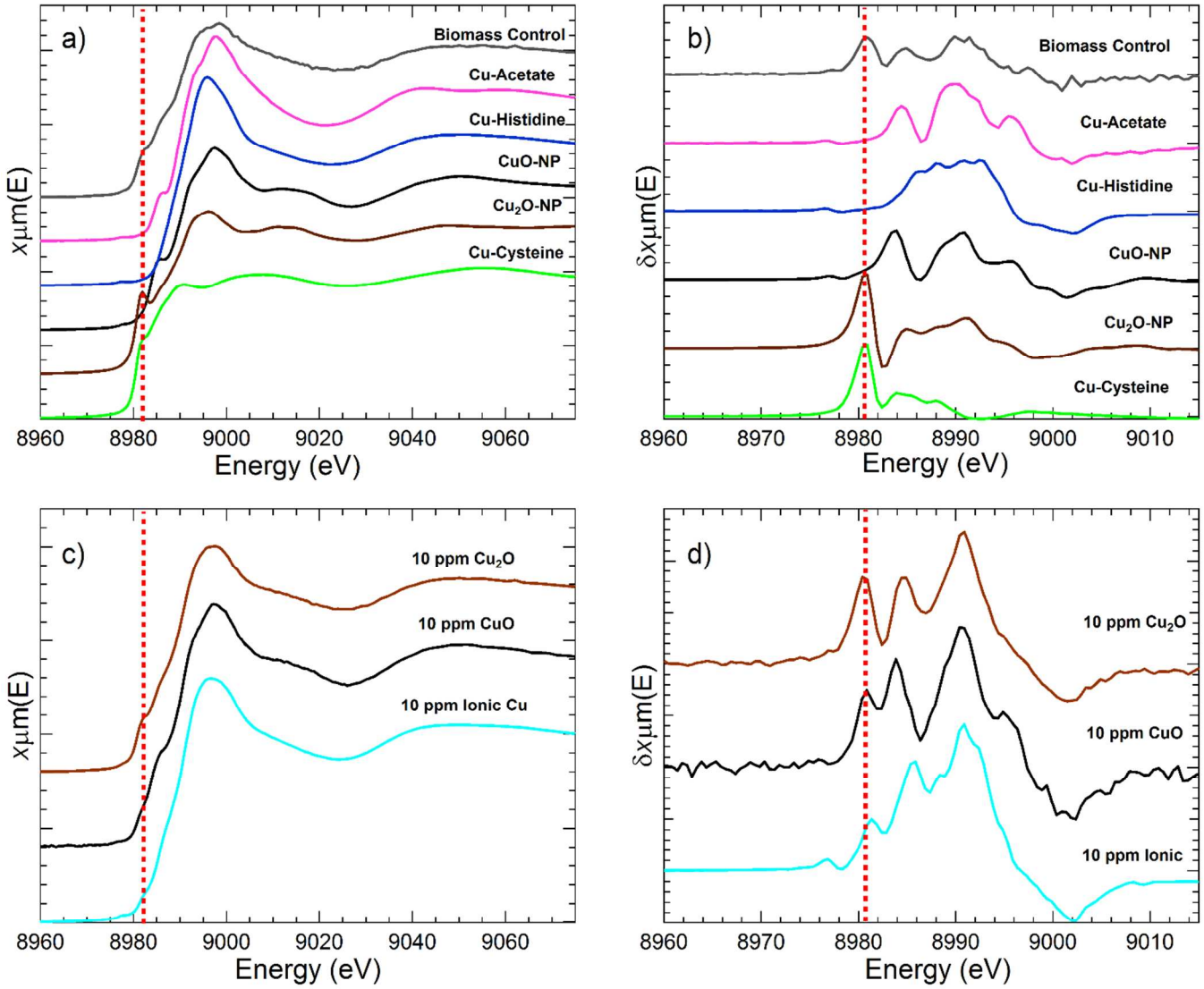

Figure S5: Cu K-edge XAFS spectra for reference materials and representative ionic Cu and CuNP exposed sample (10 mg/L). Data is presented as both normalized and first derivate of normalized to highlight spectra features. Red dotted line indicates the location of the Cu(I) oxidation state. All spectra have been offset for visual clarity. a) Normalized XAFS spectra of reference samples, b) first derivate XAFS spectra of reference samples, c) normalized XAFS spectra of freeze dried biomass samples after incubation with 10mg/L of ionic Cu or CuNPs, d) first derivative XAFS spectra of freeze dried biomass samples after incubation with 10mg/L of ionic Cu or CuNPs. All data collected at the Advanced Photon Source, Argonne National Laboratory, Sectors 10BM and 20ID.

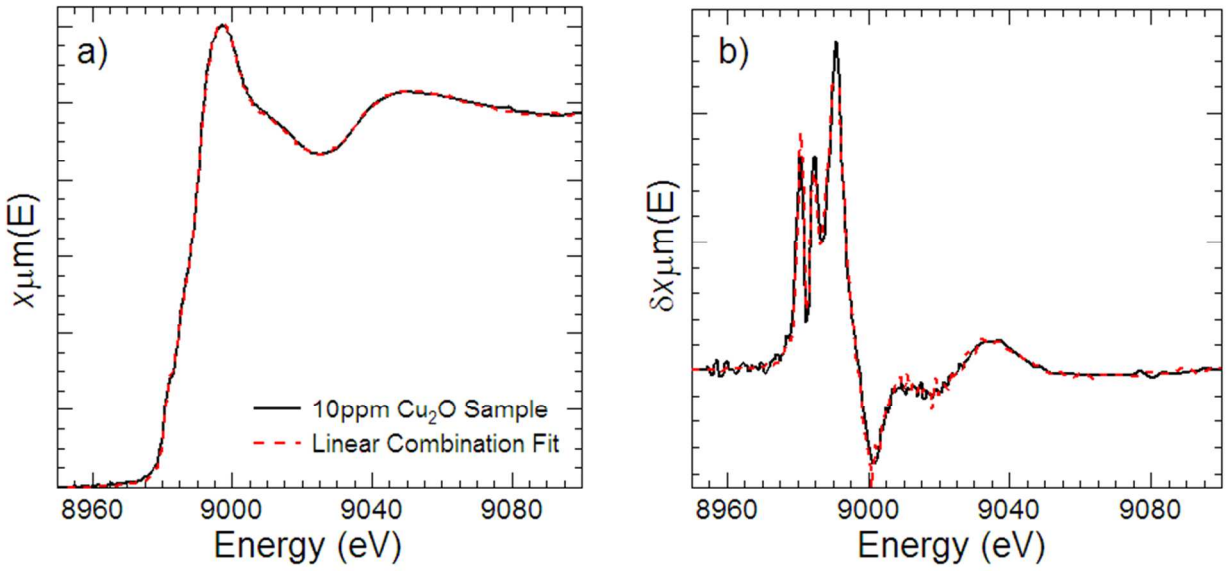

Figure S6: An LCF result for a representative biomass sample (10 mg/L Cu_2O, 3 h) using both a)Normalized data and b) the First Derivative of Normalized spectra.

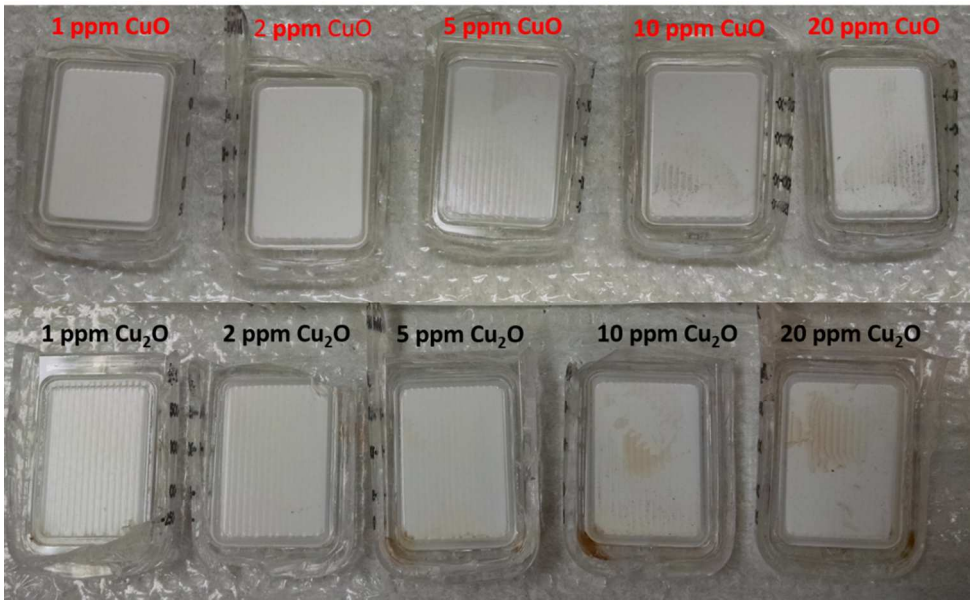

Figure S7: 10 kDa Amicon Filter Insert used to separate particulate CuNPs from ionic Cu after 3 hour incubation with biomass. Visual inspection clearly indicates CuNPs remaining in the supernatant. Filters were removed from inserts and analyzed through both SEM and XAFS for additional information on the form and speciation of Cu.

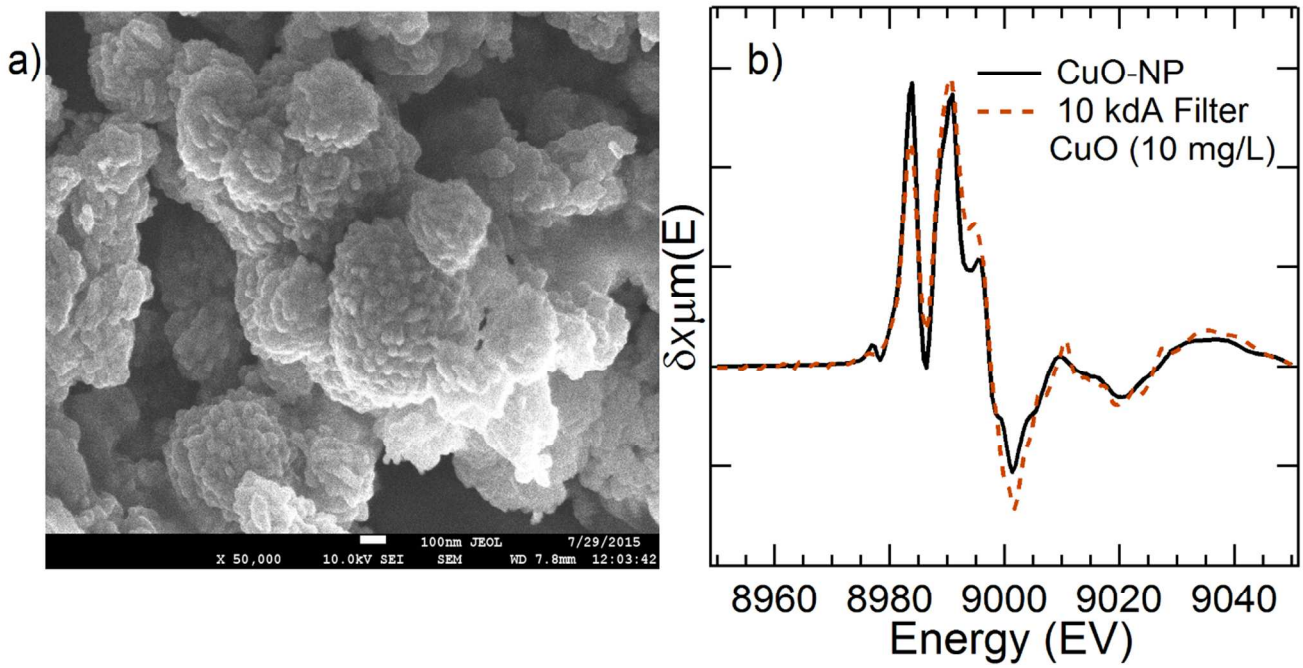

Figure S8: Analysis of 10 kDa filters used to separate particulate copper from ionic Cu after 3 h incubation of CuO samples with biomass. a) SEM images of extracted filters show evidence of CuNPs retained by the filter. b) XAFS analysis indicates that the retained material is primarily CuO.

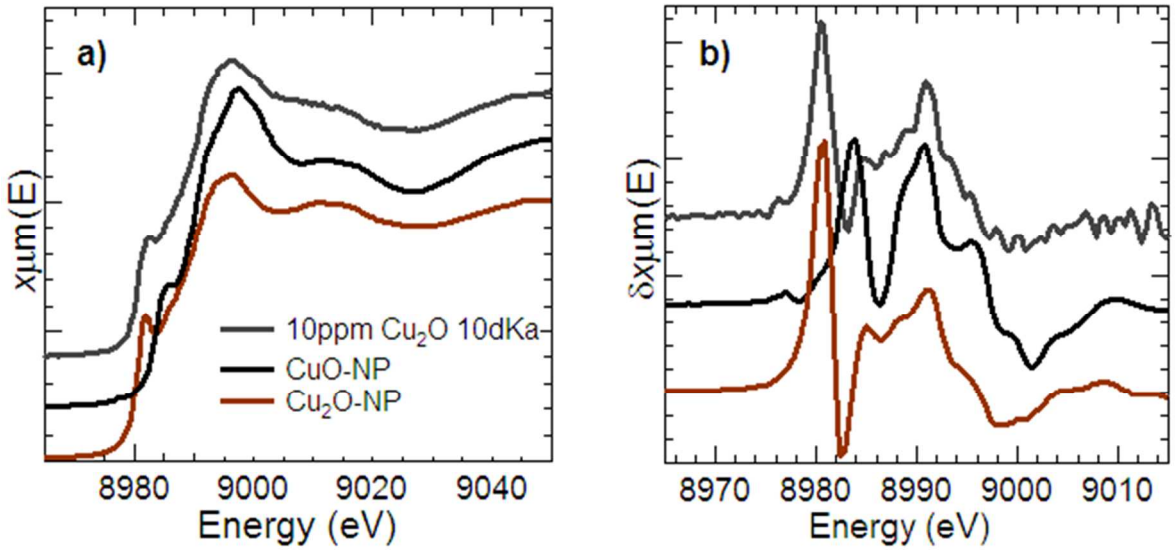

Figure S9: Cu K edge XAFS analysis of 10 kDa filters used to separate particulate copper from ionic Cu after 3 h incubation of Cu_2O samples with biomass. Analysis demonstrates that CuNPs remain stable in suspension, with a portion of the Cu_2O oxidizing to CuO.

Table S3: Best fit of Cu speciation by liner combination fitting (LCF) of Cu k-edge XAFS spectra for biomass samples exposed to ionic Cu and uncoated CuNPs. The inherent error in LCF analysis is +/- 5-10%[2]

Total Cu concentration		2 mg/L		5 mg/L		10 mg/L		20 mg/L		40 mg/L	
		Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative
Ionic Cu	Cu-Acetate	27.14	26.88	30.97	30.96	28.39	28.79	27.46	27.77	26.61	27.77
	Cu-Histidine	36.40	37.02	43.00	44.44	48.83	51.46	52.92	55.95	54.13	56.90
	Cu-Cysteine	36.45	36.10	26.02	24.60	22.77	19.75	19.62	16.28	19.26	15.33
	Reduced χ^2	0.0000171	0.0000448	0.0000193	0.0000273	0.0000388	0.0000342	0.0002435	0.0000363	0.0000520	0.0000403
Total Cu concentration		1 mg/L		2 mg/L		5 mg/L		10 mg/L		20 mg/L	
		Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative
CuO	Cu-Acetate	32.75	27.68	32.44	26.12	28.93	25.91	26.97	23.09	22.02	20.93
	Cu-Histidine	15.20	14.23	14.90	13.43	11.48	10.75	7.34	7.76	3.22	4.87
	Cu-Cysteine	39.71	37.17	38.92	35.07	29.99	28.07	19.16	20.26	8.41	12.73
	CuO-NP	12.33	20.90	13.72	25.36	29.58	34.64	46.51	48.87	66.34	61.45
	Reduced χ^2	0.0000700	0.0000737	0.0000734	0.0000574	0.0000463	0.0000410	0.0000439	0.0000311	0.0000546	0.0000188
Cu_2O	Cu-Acetate	29.68	22.99	28.70	21.26	22.51	17.00	18.29	13.53	15.67	10.96
	Cu-Histidine	20.58	19.84	21.68	20.67	23.73	23.20	24.58	25.68	29.23	28.62
	Cu-Cysteine	39.85	30.87	38.54	28.56	30.23	22.83	24.57	18.17	21.04	14.72
	CuO-NP	5.88	14.49	8.17	19.07	15.61	23.39	15.17	22.57	16.87	23.28
	Cu_2O-NP	3.98	11.78	2.88	10.42	7.90	13.55	16.46	20.04	17.17	22.39
	Reduced χ^2	0.0000340	0.0000508	0.0000373	0.0000442	0.0000409	0.0000318	0.0000371	0.0000258	0.0000373	0.0000240

Table S4: Best fit of Cu speciation by liner combination fitting (LCF) of Cu k-edge XAFS spectra for biomass samples exposed to CuNPs dispersed in 1 wt% PVP solution. The inherent error in LCF analysis is +/- 5-10%[2]

Total Cu concentration		1mg/L		2 mg/L		5 mg/L		10 mg/L		20 mg/L	
		Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative	Normalized	1st derivative
CuO-PVP	Cu-Acetate	28.06	28.88	36.59	25.46	21.92	18.82	25.18	15.33	22.02	10.47

	Cu-Histidine	14.43	14.85	12.88	13.09	20.37	18.09	15.15	16.99	13.23	16.19
	Cu-Cysteine	37.68	38.78	33.64	34.19	29.44	25.27	23.20	20.59	15.08	14.06
	CuO-NP	19.82	17.47	16.88	27.23	28.25	37.81	36.45	47.07	49.65	59.26
	Reduced χ^2	0.0000360	0.0000624	0.0000310	0.0000487	0.0000584	0.0000374	0.0000356	0.0000251	0.0000252	0.0000134
Cu_2O-PVP	Cu-Acetate	29.83	19.58	27.00	16.54	23.48	19.49	23.10	16.53	19.89	14.28
	Cu-Histidine	21.42	17.01	22.13	17.61	26.76	25.87	24.90	32.17	37.62	37.58
	Cu-Cysteine	40.05	26.29	36.26	22.22	31.53	26.18	31.02	22.20	26.71	19.18
	CuO-NP	8.68	20.03	14.59	27.39	14.00	18.99	13.02	20.71	15.76	19.96
	Cu_2O-NP	0	17.06	0	16.22	4.21	9.44	0	8.38	0	8.99
	Reduced χ^2	0.0000334	0.0000360	0.0000385	0.0000382	0.0000315	0.0000301	0.0000418	0.0000307	0.0000570	0.0000305

Cu2O Dissolution – 20 mg/L samples of uncoated and 1 wt % PVP coated Cu2O were dispersed in either Milli-Q water, or Biomass media, both buffered to pH 7.5, and allowed me rotary mix for 3 h. Samples were centrifuged at as previously described to separate biomass and aggregated solids from the supernatant. 12 mL aliquots of supernatant were removed and processed through 10 kDa Amicon Filters (See *Metal Partitioning Analysis*) to isolate ionic Cu. Table S5 clearly indicated that samples incubated in Milli-Q water release approximately 2.25 time the amount of ionic Cu as sample incubated with biomass liquor. This different demonstrates the amount of Cu likely internalized by the biomass causing the observed inhibition.

Table S5: Summary of ionic Cu concentrations after injection of 20 mg/L uncoated and PVP coated Cu_2O samples and subsequent 3 h mixing with either Milli-Q water, or mixed biomass media, both buffered to pH 7.5. The concentration of ionic Cu in samples mixed in Milli-Q water is at least 2.25 times higher than the amount of ionic Cu found in samples incubated with the biomass media. This difference can be explained by the uptake of Cu ions by the biomass, resulting in the observed inhibition.

Incubation Solution	*Cu_2O (mg/L)*	*PVP-Cu_2O (mg/L)*
Milli-Q Water (pH = 7.5)	0.659 +/- 0.004	0.945 +/- 0.003
Biomass Liquior (pH = 7.5)	0.29 +/- 0.005	0.4 +/- 0.15
Milli-Q Water / Biomass	2.27	2.36

References

(1) Ravel, B.; Newville, M. *J. Synchrotron Radiat.* **2005**, *12*, 537.

(2) Gräfe, M.; Donner, E.; Collins, R. N.; Lombi, E. *Analytica Chimica Acta* **2014**, *822*, 1.