## Title

Copper status of exposed microorganisms influences susceptibility to metallic nanoparticles



## Journal

Environmental Toxicology and Chemistry, 35(5)

## ISSN

0730-7268

## Authors

Reyes, Vincent C
Spitzmiller, Melissa R
Hong-Hermesdorf, Anne
et al.

## Publication Date

2015-09-21

## DOI

10.1002/etc.3254

Peer reviewed

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 35, No. 5, pp. 1148–1158, 2016

Printed in the USA

# COPPER STATUS OF EXPOSED MICROORGANISMS INFLUENCES SUSCEPTIBILITY TO METALLIC NANOPARTICLES

VINCENT C. REYES,† MELISSA R. SPITZMILLER,† ANNE HONG-HERMESDORF,‡ JANETTE KROPAT,‡ ROBERT D. DAMOISEAUX,§ SABEEHA S. MERCHANT,‡ and SHAILY MAHENDRA*†

†Department of Civil and Environmental Engineering, University of California, Los Angeles, California, USA
‡Department of Chemistry and Biochemistry, University of California, Los Angeles, California, USA
§Molecular Screening Shared Resource, Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, California, USA

*(Submitted 11 September 2014; Returned for Revision 6 January 2015; Accepted 16 September 2015)*

**Abstract:** Although interactions of metallic nanoparticles (NPs) with various microorganisms have been previously explored, few studies have examined how metal sensitivity impacts NP toxicity. The present study investigated the effects of copper NPs (Cu-NP) exposure on the model alga *Chlamydomonas reinhardtii* in the presence and absence of the essential micronutrient copper. The toxic ranges for Cu-NPs and the ionic control, $CuCl_2$, were determined using a high-throughput adenosine triphosphate (ATP)-based fluorescence assay. The Cu-NPs caused similar mortality in copper-replete and copper-deplete cells (median inhibitory concentration [IC50]: 14–16 mg/L) but were less toxic than the ionic control, $CuCl_2$ (IC50: 7 mg/L). Using this concentration range, the Cu-NP impacts on cell morphology, copper accumulation, chlorophyll content, and expression of stress genes under both copper supply states were assessed. Osmotic swelling, membrane damage, and chloroplast and organelle disintegration were observed by transmission electron microscopy at both conditions. Despite these similarities, copper-deplete cells showed greater accumulation of loosely bound and tightly bound copper after exposure to Cu-NPs. Furthermore, copper-replete cells experienced greater loss of chlorophyll content, 19% for Cu-NPs, compared with only an 11% net decrease in copper-deplete cells. The tightly bound copper was bioavailable as assessed by reverse-transcriptase quantitative polymerase chain reaction analysis of *CYC6*, a biomarker for Cu deficiency. The increased resistance of copper-deplete cells to Cu-NPs suggests that these cells potentially metabolize excess Cu-NPs or better manage sudden influxes of ions. The results suggest that toxicity assessments must account for the nutritional status of impacted organisms and use toxicity models based on estimations of the bioavailable fractions. *Environ Toxicol Chem* 2016;35:1148–1158. © 2015 SETAC

**Keywords:** Environment Ecotoxicology Nanotoxicology Nanomaterials Microbe Reactive oxygen species Algae Photosynthesis

## INTRODUCTION

Metallic nanoparticles (NPs) constitute the most widely used class of NPs [1]. Copper-containing NPs (Cu-NPs) are used in antimicrobial coatings, steel alloys, lubricants, electronics, ceramics, and polymers because of their low cost, availability, and unique properties [2]. Studies that examine the characteristics of synthesized NPs and NP-containing products are needed to develop safe and sustainable applications of NPs. It is particularly crucial to examine how Cu-NPs interact with microorganisms under environmentally relevant exposure conditions. Studies suggest that algae are among the most metal-sensitive organisms likely to be exposed to NP-containing wastes washed into aquatic environments [3]. Photosynthetic algae are critical to global carbon sequestration, as some estimates suggest that microalgae are responsible for as much as 25.6 $kg\,C/m^3/yr$ of carbon uptake and possess a greater theoretical photosynthesis efficiency than higher plants (9% vs 6%) [4]. In addition, many microalgae are relevant for eutrophication of surface water bodies and the generation of renewable resources, such as biodiesel and hydrogen [5,6]. It is therefore important to understand and minimize potential negative Cu-NP interactions with algae to develop ecoresponsible applications of Cu-NPs.

Copper-containing NPs are reported to be toxic to a wide variety of organisms, including human cells, marine invertebrates, bacteria, and algae [7–11]. Previous research with copper oxide NPs (CuO-NPs) suggests that the release of copper ions was the primary source of toxicity. [9,12]. Free ions were implicated in producing reactive oxygen species (ROS)-mediated damage to DNA and mitochondria, protein oxidation, osmotic burst, and disruption of the cell membranes. Other studies with dissolved metals reflect similar toxic modes of action observed in studies with equivalent NPs [13–16]. Those reports, however, acknowledge that ions cannot completely explain toxicity and that metallic NPs might confer unique nanoscale-specific effects. For example, only 50% of the toxicity in *Saccharomyces cerevisiae* exposed to nano-sized, bulk-sized, and ionic forms of copper was explained by dissolved copper ions [17].

Alternative models have been proposed, such as a Trojan horse mechanism, by which metallic NPs enter cells and cause disruptions of cellular processes and structures directly and indirectly through dissolution into free ions [18]. For example, Wang et al. [19] found that internalization of bulk copper did not affect the cyanobacterium *Microcystis aeruginosa*. Instead, toxicity from CuO-NPs was defined by the amount of copper that had either aggregated within the cell or had dissolved into free ions once inside the cell. These differences in proposed toxicity mechanisms of metal-containing nanomaterials might be the result of the various exposure parameters used among studies.

Presently, the majority of research exploring various exposure parameters focuses on the role of chemical properties,

This article includes online-only Supplemental Data.

* Address correspondence to mahendra@seas.ucla.edu

Published online 21 September 2015 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3254

such as pH, particle aging, and media components in changing the amount of free ions in the media and altering toxicity [20–23]. Fewer studies have focused on how biological parameters such as nutritional status or the interactions with cell walls, membranes, or transport proteins can change metal NP toxicity toward the cells. For example, it was previously reported that the model alga, *Chlamydomonas reinhardtii*, was more resistant to silver NP (Ag-NP) toxicity in later growth stages because of increased extracellular dissolved organic carbon, which detoxified NPs [24].

Unlike other zerovalent metals produced as nanomaterials, such as silver or titanium, copper is an essential micronutrient important in many proteins involved in electron and oxygen transport [25]. In phototrophs, for example, the copper protein plastocyanin is required as an electron transport agent between the cytochrome *b6f* complex and photosystem I [26]. Previous studies have shown that the nutritional status with respect to metals prior to exposure influences susceptibility of cells. In studies with *C. reinhardtii*, researchers found that $Cd^{2+}$ toxicity increased when cells were supplanted with excess copper, but was reduced when zinc and cobalt individually or all 3 metals were present together [27]. In another study, $Ag^{+}$ ions were more toxic to Cu-deficient *C. reinhardtii* cells than to Cu-replete ones, because $Ag^{+}$ ions were able to enter more rapidly as a result of the increased abundance of $Cu^{+}$ transporters in the plasma membrane of the Cu-deficient cells [28]. It is therefore important to investigate how Cu-NP toxicity changes when algal cells are pregrown in the presence (replete) or absence (deplete) of copper.

To test the hypothesis that nutritional status, specifically copper status, of an organism determines sensitivity toward nutrients and toxicants, we investigated the interactions of Cu-NPs with the fresh water alga *C. reinhardtii* under copper-replete and copper-deplete conditions. Because little research has explored this topic, the present study tested the hypothesis that environmental factors that change copper homeostasis might alter the mode of action or interaction of cells with NPs that enter into the environment.

## MATERIALS AND METHODS

### *Cell growth conditions*

*Chlamydomonas reinhardtii* is an excellent test organism for environmental and toxicological studies because of its presence in multiple freshwater environments, extensive use in genomic and physiological studies, and relevance as a reference species for understanding photosynthesis [29]. *Chlamydomonas reinhardtii* (CC-4532) cells were grown in liquid media with one of the following types of algal trace elements: tris-acetate-phosphate medium with Hutner's trace metal solution, Kropat's trace metal solution, or copper-deplete Kropat's trace metal solution [30] (Supplemental Data, Table S1). The tris-acetate-phosphate medium was comprised of the following ingredients: $NH_4Cl$ ($7.5 \times 10^{-3}$ M), $CaCl_2$ ($3.4 \times 10^{-4}$ M), $MgSO_4$ ($4.0 \times 10^{-4}$ M), $K_2HPO_4$ ($6.8 \times 10^{-4}$ M), $KH_2PO_4$ ($4.4 \times 10^{-4}$ M), Trizma base ($2.0 \times 10^{-2}$ M), $C_2H_4O_2$ ($1.8 \times 10^{-2}$ M), and trace metals. Hutner's trace metal solution is a general formulation for microbial growth media, whereas Kropat's trace metal solution was specifically optimized for *C. reinhardtii* growth (Supplemental Data, Table S1). Hunter's trace metal solution was only used for initial stock incubations of *C. reinhardtii*, whereas Kropat's trace metal solution was used for all toxicity trials. The tris-acetate-phosphate medium was also supplemented with acetate allowing for both phototrophic and heterotrophic growth modes. *Copper-replete medium* refers to tris-acetate-phosphate medium using Kropat's trace metal solution, whereas *copper-deplete medium* refers to tris-acetate-phosphate medium using Kropat's trace metal solution without copper. Cells grown in these conditions will be referred to as either copper-replete or copper-deplete cells, respectively. Although a rich medium, tris-acetate-phosphate was used because *C. reinhardtii* exposed to Cu-NPs and $CuCl_2$ in simpler media did not show robust growth in toxicity assays. Furthermore, this was the only medium in which the growth was strong enough in the presence as well as absence of copper to understand how algal cells reacted to copper-replete and copper-deplete conditions in the environment.

To begin seed culture stocks, mature *C. reinhardtii* cells grown in copper-replete tris-acetate-phosphate medium or tris-acetate-phosphate with Hutner's solution were transferred (1% v/v) to the freshly sterilized media and incubated at 25 °C with a shaking rate of 180 rpm in the presence of continual light (2 cool white bulbs for each warm white fluorescent bulb; ~100 μmol/m$^2$/s photon flux density; New Brunswick Scientific). Mid-logarithmic phase cells (growth after 3–4 d) were inoculated in biological duplicates into the fresh medium, diluted to a final concentration of $10^4$ cells/mL, and incubated as previously described. For the experiments that employed both copper-replete and copper-deplete cells, 2 cell transfers were used to eliminate residual copper carried over from the initial seed culture, which has been previously shown to be efficient in removing residual copper [31].

### *NP preparation and characterization*

Commercially available Cu-NPs, with a nominal diameter of 20 nm to 50 nm were obtained from QuantumSphere. To assess the contribution of $Cu^{2+}$ ions to observed Cu-NP toxicity, $CuCl_2$ (>99% purity, Sigma Aldrich) was used in parallel experiments. All stock solutions were prepared by dispersing 1000 mg/L of Cu-NPs in 10 mL of sterile Milli-Q ultrapure water and sonicating for 30 min at maximum power (FS30H, Fisher Scientific, 100 W, 42 kHz). Stock solutions were stored on ice in the absence of light to minimize NP degradation and instability and were used within 3 h of preparation. Stock solutions were sonicated for 1 min prior to collecting aliquots for each experiment.

To assess particle characteristics under exposure conditions, aliquots of the particle dispersion were introduced to both copper-replete and copper-deplete tris-acetate-phosphate media, incubated under standard shaking and growth conditions used for *C. reinhardtii* cells, and assayed over 96 h. According to US Environmental Protection Agency (USEPA) standard method 3050B, inductively coupled plasma–optical emission spectrometry (ICP–OES) was used to analyze the extent of copper dissolved in the growth medium at 20 mg/L as $Cu^{2+}$ for Cu-NPs, which was near the median inhibitory concentration [IC50]) determined by the adenosine triphosphate (ATP) assay. Briefly, Cu-NPs and the control $CuCl_2$, were incubated at 37 °C for 96 h in tris-acetate-phosphate medium. Every 24 h, aliquots were centrifuged and filtered through 3-kDa ultrafiltration membranes (Amicon Ultra-4 3K, Millipore) for 30 min at 2800 *g*. The filtrate was digested in $HNO_3$ before total dissolved copper concentrations were measured by ICP-OES. Ionic $CuCl_2$ (>99% purity, Sigma Aldrich) was used as a standard with concentrations ranging from 50 mg/L to 0.2 mg/L. Furthermore, to assess the colloidal stability in the media, the hydrodynamic diameters of the particles were measured by dynamic light scattering (BI 90Plus, Brookhaven Instruments), and surface

zeta-potentials were computed from measured electrophoretic motilities (ZetaPALS, Brookhaven Instruments). These measurements were also taken at 20 mg/L as $Cu^{2+}$ for $CuCl_2$. All measurements were performed as experimental triplicates.

Soluble copper speciation in each exposure medium was predicted using the PHREEQC (Ver 3.0) platform in combination with the minteq.v4.dat database. No species were added to the original database. Speciation and batch-reaction modeling were based on the initial conditions to determine the equilibrium concentrations of each copper species. Speciation was modeled for 20 mg/L as $Cu^{2+}$ for Cu-NPs as well as for $CuCl_2$ introduced into tris-acetate-phosphate medium using Kropat's trace metal solution. Previous research with Cu-NPs suggests that copper oxide layers form on the surface of Cu-NPs exposed to oxygen, so both zerovalent copper and CuO were included in the model [32]. Speciation was identical for each copper source in tris-acetate-phosphate medium using Kropat's trace metal solution with or without copper.

*Optimization of ATP assay and IC50 determination*

As an indication of active metabolism, ATP content was used as a metric for enumerating living cells. A modified version of CellTiter-Glo® Luminescent Cell Viability Assay (Promega), was adapted for a high-throughput screening platform for use with *C. reinhardtii*. Although high-throughput screening methods have been used extensively for bacterial and yeast cells, they have only recently been applied to microalgae [33]. First, to assess the reagents' compatibility with *C. reinhardtii*, 2 standard curves were generated from a dilution series of purified ATP and live *C. reinhardtii* cells. After culturing, midexponential phase cells were diluted 11-fold into 96-well white microtiter plates (Thermo Scientific) to a final volume of 100 μL. At the time of assessment, 100 μL of CellTiter-Glo reagent was added to each well, and plates were incubated for 12 min before luminescence measurements were taken using the VICTOR 3V plate reader (PerkinElmer) with an integration time of 0.1 s. The purified ATP control was processed similarly.

To determine the IC50 concentrations of Cu-NPs and $Cu^{2+}$ ions supplied as $CuCl_2$, *C. reinhardtii* cells in midlogarithmic growth phase were exposed in 96-well white microtiter plates to a dilution series of concentrations of Cu-NPs (200–0.2 mg/L as $Cu^{2+}$). The IC50 concentrations were computed to find a range where Cu-NP stress could be measured for further experiments and to determine concentrations that may be hazardous for the environment. The control for estimating toxicity resulting solely from copper ions was $CuCl_2$. Because the concentration of Cu-NPs in the environment is unknown, a wide range of concentrations was used to determine a concentration at which biological responses could be enumerated [2]. Once the IC50 concentration was determined, all other toxicological tests were exposed to a slightly higher concentration to ensure that significant differences from the unexposed control would be observed. To control for Cu-NP interference, the assay was run in both the presence and absence of cells. After 12 h, luminescence was quantified as described in the previous paragraph. Each concentration was performed as an experimental triplicate. Luminescence data were fitted using a nonlinear regression dose–response model employing a 4-parameter logistic equation (Prism 4, GraphPad Software), which was used to calculate the IC50 values and 95% confidence intervals. The IC50 concentration was determined to define a sublethal concentration at which to perform the additional toxicity assessments of Cu-NPs to *C. reinhardtii* cells in copper-replete and copper-deplete media.

*Cell-associated copper*

The concentrations of extracellular, loosely bound, and tightly bound copper for copper-replete and copper-deplete cells exposed to 20 mg/L Cu-NPs or the control, $CuCl_2$ were measured using ICP–mass spectrometry (ICP–MS). Cell-associated copper (tightly bound) was measured to determine the potential for bioaccumulation in algal cells exposed to Cu-NPs. Nearly $10^8$ exposed cells were collected by centrifugation for 5 min at 5700 *g*. The supernatant was transferred, and any cell debris that might have been carried over was removed by centrifugation twice. The supernatant was designated as the extracellular copper fraction for the corresponding growth condition. The cell pellet was washed 3 times in ethylenediamine tetraacetic acid (EDTA; 1:2 EDTA:$H_2O$) and sedimented using centrifugation. The wash from the pellets was transferred, and debris was removed by centrifugation, which was designated as the loosely bound copper fraction. The resulting cell pellets were digested and designated as the tightly bound copper fraction. It was not clear whether this fraction was comprised of aggregates that penetrated cell membranes or dissolved copper species and ions that reprecipitated within cells. Previous studies, however, have shown that copper NPs aggregated to sizes above the nanoscale (> 100 nm) were capable of penetrating algal cell walls and becoming internalized by cells [34,35]. All fractions were acidified in 70% trace metal grade nitric acid for 48 h at 65 °C. Following the acidification, sterile Milli-Q ultrapure water was added to each pellet sample to a final concentration of nitric acid of 2.4%. The copper concentration in each sample was measured using the ICP-MS against copper standards prepared similarly to the experimental copper fractions. All values were measured as experimental triplicates.

*Transmission electron microscopy*

Potential morphological impacts to algal cells exposed to Cu-NPs were determined using transmission electron microscopy (TEM). The *C. reinhardtii* cells were prepared at the Electron Microscopy Services Center of the University of California, Los Angeles' Brain Research Institute for TEM imaging. Cells were exposed to 20 mg/L of Cu-NPs and $CuCl_2$ as $Cu^{2+}$ for 7 d. Under the growth conditions, control cells reached the stationary growth phase at 7 d but did not reach the death phase. The cells were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate-buffered saline. Samples were stained, dehydrated, and embedded using EPON 812 (Shell). After curing at 60 °C for 48 h, 60-nm sections were cut on an ultramicrotome (RMC MTX) with a diamond knife and deposited on single-hole carbon grids. The resulting sections were examined with a 100CX JEOL electron microscope. Differences between conditions were not quantified, but the results shown are representative of each condition examined.

*Chlorophyll concentration experiments*

Chlorophyll concentrations were measured for *C. reinhardtii* cells exposed to 20 mg/L Cu-NPs or $Cu^{2+}$ as $CuCl_2$ and unexposed cells grown in either copper-replete and copper-deplete tris-acetate-phosphate medium. Chlorophyll concentrations were measured to determine potential impacts on the photosynthetic abilities of algal cells exposed to sublethal concentrations of Cu-NPs. Following extractions in an 80% acetone:20% methanol solution (v/v), the absorbance of the chlorophyll was measured at 750 nm, 663.6 nm, and 646.6 nm (Thermo Scientific, NanoDrop 2000c Spectrophotometer).

Measurements were taken 3 times over 72 h (at 24 h, 48 h, and 72 h) for each experimental quadruplicate. Results were converted to mg/mL concentrations using the following formula:

$$\text{mg chlorophyll/mL} = 0.01776 \times E_{646.6} + 0.00734 \times E_{663.6}$$

where $E_{646.6}$ is the absorbance at 646.6 nm subtracted from the absorbance at 750 nm, and $E_{663.6}$ is the absorbance at 663.6 nm subtracted from the absorbance at 750 nm [36]. Each measurement was taken as experimental quadruplicates.

*Gene expression experiments*

Gene expression experiments were performed to determine the potential stresses algal cells might experience from Cu-NPs in the environment at sublethal concentrations. After cells were exposed to 20 mg/L of Cu-NPs or $CuCl_2$ for 6 h, total nucleic acids were extracted by a standard phenol–chloroform extraction protocol [37]. Following deoxyribonuclease (DNAse) treatment (Turbo DNAse; Ambion), complementary (c)DNA synthesis was performed using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was used to amplify the expression of 9 stress-induced genes (Supplemental Data, Table S2). Then SYBR Green reactions were prepared using Maxima SYBR Green Master Mix (Thermo Scientific), 300 nM forward and reverse primers, and 1:10 dilution of the cDNA template. The expression of *CYC6*, *MSD1*, and *FSD1* was assayed in comparison with the gene for the *Chlamydomonas* beta subunit-like polypeptide (*CβLP*), whose stability and use as a housekeeping gene has also been verified previously [30]. The primers were designed and optimized as previously described [26,37]. For all reactions, the following temperature program was used for the thermocycler (Applied Biosystems StepOnePlus Real-Time PCR System): 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 95 °C for 10 s, 65 °C for 15 s, 72 °C for 15 s, and 80 °C for 1 s, ending with 2 s at 70 °C and 15 s at 95 °C. Expression was normalized by the housekeeping gene, *CβLP*, and each respective gene's expression in the copper-replete unexposed control using the $\Delta\Delta C_T$ method [38]. Experimental triplicate measurements were taken for each data point.

*Statistical analysis*

To evaluate the statistical significance of Cu-NP and $CuCl_2$ impacts on IC50 values, cell-associated copper, chlorophyll content, and gene expression, a 2-way analysis of variance (ANOVA) with a Bonferroni post test was used (Prism 4). All measurements were made in at least experimental triplicates. Significance was determined at $p < 0.05$.

## RESULTS AND DISCUSSION

Our results demonstrate that differences in toxic responses were observed between copper-replete and copper-deplete *C. reinhardtii* cells exposed to Cu-NPs. After identification of a toxic range using our high-throughput ATP-based fluorescence assay, Cu-NP impacts on copper accumulation, chlorophyll content, cell morphology, and expression of stress genes were assessed. At sublethal conditions, for example, copper-deplete cells showed reduced depletion and greater recovery of chlorophyll after exposure to Cu-NPs compared with copper-replete cells. Although our overall results were based on Cu-NP exposure to *C. reinhardtii*, the changing nutritional status of an organism should be a consideration for any environmental toxicity assessment.

*NP characterization*

Total copper dissolution was measured over 72 h using ICP-OES to compare copper availability when 20 mg/L of copper was introduced into tris-acetate-phosphate media as either NPs (Cu-NPs) or a salt ($CuCl_2$). The data indicate rapid initial dissolution (~80%) of the Cu-NPs in the growth medium within 30 min of introduction (Supplemental Data, Figure S1). After further incubation, the particles were mostly dissolved ($97.9 \pm 1.8\%$) after 36 h. As expected, the copper ions from the copper chloride solution were quickly dissolved after the introduction to the algal growth medium (96% at 0 h).

The Cu-NPs were further characterized in both copper-replete and copper-deplete tris-acetate-phosphate media to determine the size of suspended Cu-NPs. Although Cu-NPs were received as 20-nm to 50-nm nominal size powders, the data suggest that the Cu-NPs rapidly aggregated to the micron size range after being introduced into both formulations of tris-acetate-phosphate media (Supplemental Data, Figure S2). The presence of aggregates past 36 h suggests the formation of insoluble complexes of $Cu^{2+}$ ions with organic or inorganic anions in the media or continual rapid dissolution and reaggregation of particles. In contrast, the particles were not significantly aggregated in deionized water. The zeta potentials were consistent with the findings of particle instability established by the hydrodynamic diameters and ICP-OES measurements (Supplemental Data, Figure S2). The fact that zeta potential values were relatively close to 0 suggests that Cu-NPs' stability was unaffected by the presence or absence of copper in the media. Aggregation of metal-based NPs in microbial growth media has been reported previously, and large aggregates of particles have been related to reduced reactivity and toxicity to various organisms [22,39,40].

Although these particles did not remain nanoscale (<100 nm) under algal exposure conditions, these results are still relevant as these experiments were undertaken to determine the effect of their release into the environment. When released into water bodies such as wastewater streams and wetlands, NPs are likely to encounter conditions similar to those found in tris-acetate-phosphate media, which contain high ionic strength with various organic ligands [3]. Thus, it is important to understand Cu-NP toxicity under these representative conditions where aggregation occurs, to determine if the released Cu-NPs may be significantly different from the releases of comparable concentrations of ionic copper. Furthermore, studies have shown that aggregated Cu-NPs above the nanoscale (>100 nm) still have the potential to cause toxicity to algae [35].

Speciation and batch-reaction analysis using PREEQC (Ver 3) was performed to determine the soluble copper species for Cu-NPs and $CuCl_2$ in tris-acetate-phosphate medium with Kropat's trace metal solution (Supplemental Data, Table S3). Chemical equilibrium modeling shows that for Cu-NPs, zerovalent copper does not dissolve into the tris-acetate-phosphate medium, but when oxidized to CuO, the Cu-NPs can fully dissolve. As the particles were synthesized as a zerovalent-Cu core with a CuO shell, ICP-OES measurements verified that the oxidized CuO layer promoted Cu-NPs dissolution into the tris-acetate-phosphate media. Indeed previous research with Cu-NPs suggests that copper oxide layers form on the surface of Cu-NPs after exposure to oxygen and affect their reactivity in the medium [32]. Speciation also suggests that CuO from both Cu-NPs and $CuCl_2$ dissolved

into the same charged species—$Cu(Tris)_4^{2+}$, $Cu(Tris)_3^{2+}$, $CuOH(Tris)_2^{+}$, and $Cu(Tris)_2^{2+}$—but with slightly different proportions. The high affinity of Tris ligands to Cu also explains the high levels of copper dilution. After initial dissolution of CuO shells, Cu-NP cores needed to be oxidized to CuO. Thus, after rapid dissolution and reprecipitation of nearly 80% Cu-NPs, slower kinetics prevailed after 36 h of Cu-NPs' introduction into the media. These differences in dissolution rates likely influenced subsequent differences in reactivity and toxicity between $CuCl_2$ and Cu-NPs.

*Optimization of ATP assay and IC50 determination*

To rapidly assess the toxic range of Cu-NPs to *C. reinhardtii* under both copper-replete and copper-deplete conditions, an ATP luminescence-based algal viability assay was developed for more uniform parameters across tests to ensure better comparability [41]. The assay was optimized at bench scale as well as in microtiter plates to determine the concentrations at which cell densities and luminescent signals would be within the linear range. Mid-log *C. reinhardtii* cells and the control, purified ATP, were diluted 11-fold and exposed to the CellTiter-Glo reagent. The standard curves developed for the ATP assay show strong correlations between luminescence, ATP content, and the number of *C. reinhardtii* cells with $R^2$ values of 0.9998 and 0.9887, respectively (Supplemental Data, Figure S3). The linear range for purified ATP was below $1.5 \times 10^{-6}$ M and below $1.5 \times 10^5$ cells/well for *C. reinhardtii* cells. This linear and predictable relationship between luminescence and the number of cells enabled the viability assay to determine IC50 concentrations for Cu-NPs. Alternative assays were attempted to estimate the viable, dead, and total cells, specifically PrestoBlue (Invitrogen), SYTOX Green (Invitrogen), and 4′,6-diamidino-2-phenylindole. However, because of the natural fluorescence of chlorophyll, overlapping excitation and emission spectra, interactions with Cu-NPs, and interference from the tris-acetate-phosphate medium, these other fluorescence-based methods were not successful under the conditions tested (Supplemental Data, Figure S4).

Copper-replete and copper-deplete *C. reinhardtii* cells were exposed to a dilution series of Cu-NPs and the ionic control, $CuCl_2$ (200–0.2 mg/L as $Cu^{2+}$), to determine differences in toxicity using ATP concentrations as indicators of overall cellular metabolism after 12 h of exposure. No statistically significant differences were observed for IC50 concentrations for Cu-NPs at either growth condition (copper-deplete: $16.2 \pm 1.2$ mg/L and copper-replete: $14.0 \pm 2.9$ mg/L [$p > 0.05$]; Figure 1). The Cu-NPs, however, were statistically less toxic than equivalent nominal doses of $CuCl_2$, as the IC50 values for copper ions were 7 mg/L at either copper condition ($p < 0.05$). The measured values for Cu-NPs and *C. reinhardtii* are within the range of IC50 values reported from studies of other microalgae (*Pseudokirchneriella subcapitata* and *Chlorella* sp.) for CuO-NPs (0.7–5 mg/L) [8,11]. Discrepancies are not surprising, as the particles, algal species, and assays were somewhat different in the previous studies.

The variation in IC50 values between Cu-NPs and $CuCl_2$ for *C. reinhardtii* can be explained by the different behavior of the copper sources in the medium. Although both were able to release nearly all of their ions into the media, $CuCl_2$ was completely dissolved immediately, whereas Cu-NPs were only completely dissolved after 36 h (Supplemental Data, Figure S1). From speciation data and particle size data, Cu-NPs that remained unoxidized zerovalent copper formed insoluble complexes or aggregates (>1 μm), where less surface area of

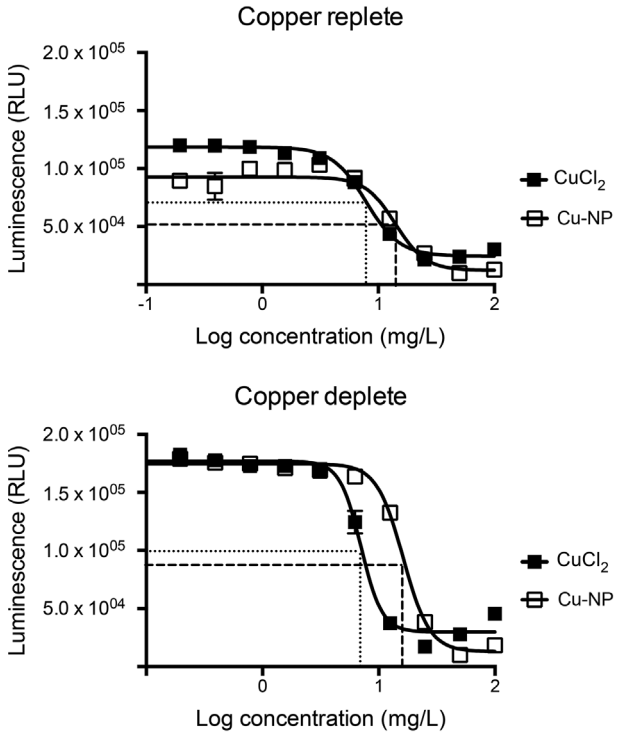


Figure 1. Median inhibitory concentration (IC50) values for (**A**) copper-replete and (**B**) copper-deplete cells exposed to copper nanoparticles (Cu-NPs) and $CuCl_2$ in tris-acetate-phosphate media. These data show the sensitivity of *Chlamydomonas reinhardtii* cells. Differences observed between copper-deplete and copper-replete IC50 values for either copper source were not statistically significant. For both conditions of algal growth, IC50 values were similar in that Cu-NPs (14–16 mg/L) were statistically less toxic than the ionic control, $CuCl_2$ (7 mg/L). RLU = relative light units.

the particle was likely available for direct interaction with algal cells or release of ions (Supplemental Data, Table S3 and Figure S2). This is consistent with previous studies revealing that increases in the size of NPs resulted in lower toxicity [42]. These data suggest that the speciation of Cu-NPs and copper ions present in the media is an important factor for establishing a predictive model for metal-containing NP toxicity [11,43]. They also suggest that particle aggregation and insoluble complexes formed by NPs might reduce the toxicity of Cu-NPs in the environmental exposure media.

*Disruption of cell membrane, reorganization of organelles, cellular swelling, and osmotic burst*

To observe the morphological effects resulting from Cu-NP exposure, cells were grown in media with 20 mg/L of Cu-NPs and $CuCl_2$ as $Cu^{2+}$ for 7 d and visualized by TEM (Figure 2). The micrographs presented are a representative sample of the majority of the cells assayed. At 7 d, control cells had reached the stationary growth phase, but had not yet reached the death phase. The images presented are reflective of the cells as a whole at each condition. In general, differences between copper-replete and copper-deplete cells under the same exposure conditions were not obvious. These results are not surprising because, under $CuCl_2$ or Cu-NP exposure, the algal cells were exposed primarily to dissolved copper species. After exposure to either Cu-NPs or $CuCl_2$, the cells clearly showed disrupted membranes and shrinking chloroplasts. Chloroplasts and other organelles appeared to be ruptured, and few cell constituents were distinguishable. The disruption of membranes has been documented in previous algal Cu-NP toxicity studies

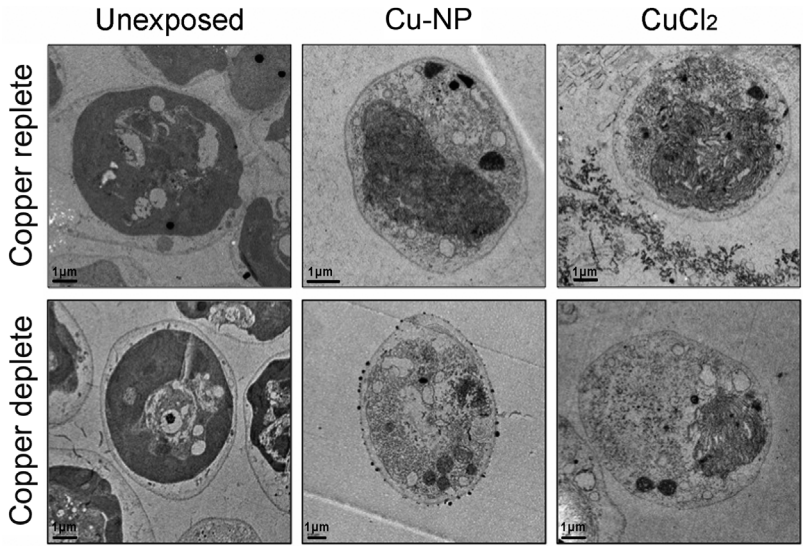


Figure 2. Transmission electron microscopy images of *Chlamydomonas reinhardtii* cells after copper nanoparticle (Cu-NP) and $CuCl_2$ exposure. Cells were exposed to 20 mg/L of Cu-NPs and $CuCl_2$ as $Cu^{2+}$ for 7 d. Both copper-replete and copper-deplete cells show disruption of membranes, cell wall thinning, reorganization of organelles, and swelling.

and was attributed to release of toxic ions and ROS generation rather than direct damage by the Cu-NPs [19]. This contrasted with the unexposed control cells, which had smooth intact membranes, and a clearly defined intracellular organization indicative of live healthy cells.

Furthermore, the TEM images also indicated swelling of the cells exposed to both Cu-NPs and $CuCl_2$. Cells increased to nearly twice the size of the unexposed control cells. Previous studies have shown that excess copper as low as 2 mg/L reduced the ability of the marine flagellate *Dunaliella marina* to properly regulate cell volume [44]. This osmotic swelling might be the result of an accumulation of copper inside the cell membranes that resulted from the formation of an ion gradient and resultant swelling with water. The space between the cell wall and the cell membrane widened, and the cell shape became more round, from oblong, particularly in the exposed copper-deplete cells, presumably to minimize surface to volume ratio. The swelling likely caused cell rupture and eventual cell mortality.

*Cell-associated copper*

To determine the amount of cell-associated copper after exposure to 20 mg/L of Cu-NPs, separate components as extracellular, loosely bound, and tightly bound fractions of copper were measured by ICP-MS (Figure 3). Copper-deplete cells exposed to Cu-NPs exhibited statistically greater copper accumulation in the tightly bound (replete: $0.7 \pm 0.1$ mg/L vs deplete: $4.6 \pm 0.4$ mg/L) and loosely bound (replete: $0.5 \pm 0.3$ mg/L vs deplete: $2.6 \pm 0.5$ mg/L; $p < 0.05$). The increased copper accumulation in Cu-NP exposed copper-replete cells is likely a reflection of its copper-deplete growth conditions. Previous studies with *C. reinhardtii* have found increased copper transport under copper-limited conditions. For example, Hill et al. [45] found that cupric reductase, a surface

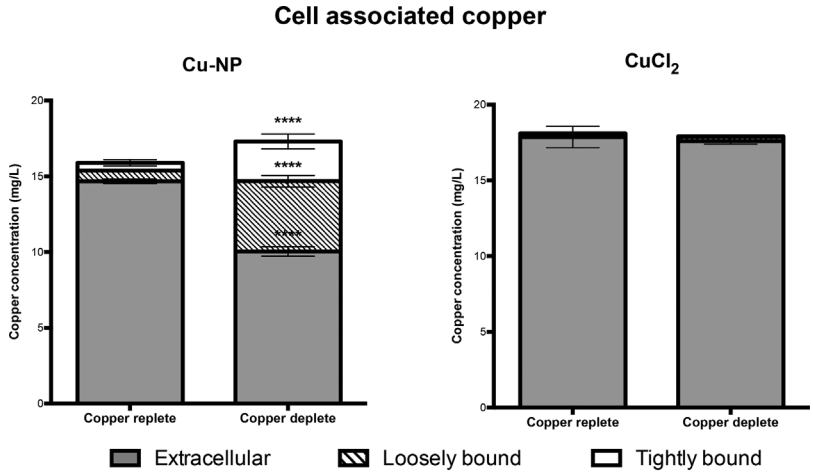


Figure 3. Cell association of copper in *Chlamydomonas reinhardtii*. After 6 h of exposure to 20 mg/L of copper nanoparticles as $Cu^{2+}$, copper-replete and copper-deplete cells exhibit different levels of extracellular, loosely bound, and tightly bound copper concentrations. (**A**) Copper-deplete cells showed statistically greater copper accumulation in the loosely bound (replete: $0.7 \pm 0.1$ mg/L vs deplete: $4.6 \pm 0.4$ mg/L) and tightly bound fractions (replete: $0.5 \pm 0.3$ mg/L vs deplete: $2.6 \pm 0.5$ mg/L; **** $p < 0.0001$). (**B**) In contrast, various cell-associated fractions were not statistically different for copper-replete and copper-deplete cells incubated with $CuCl_2$ ($p > 0.05$). Cu-NP = copper nanoparticle.

protein necessary for copper uptake, was more active during copper-deplete conditions. In addition, the CTR family genes, *CTR1* and *CTR2*, which are components of the Cu(I) assimilation pathway, are also more active during copper-deplete growth [31]. Therefore, the copper-deplete conditions promoted increased uptake of copper by *C. reinhardtii* cells during toxicity tests. These observations support ICP membrane fraction data, which showed marked accumulation of loosely bound and tightly bound copper.

In contrast to Cu-NPs, copper accumulations in copper-replete and copper-deplete cells were not statistically different after their exposure to $CuCl_2$ (tightly bound: ~0.22 mg/L and loosely bound: ~0.03 mg/L; $p > 0.05$). This difference, which hints at a potential effect of Cu-NPs, may be explained by differences in copper dissolution between Cu-NPs and $CuCl_2$. Dissolution data suggested that $CuCl_2$ was present in the media as free ions, whereas a small percentage of undissolved Cu-NPs was likely present as aggregates, as shown in the particle size data (Supplemental Data, Figures S1 and S3). Previous studies have also shown the capacity for metal-containing NPs and their aggregates to readily adsorb to algal cells [40,46]. For example, researchers studying $TiO_2$-NPs effects on the alga *P. subcapitata* found that after exposure, some cells had as much as 2.3× their own weight in $TiO_2$-NPs adsorbed [40].

These membrane-bound metal NPs and aggregates have the potential to be internalized by the cell. Maio et al. [47] found that Ag-NPs could be readily internalized by a Trojan horse mechanism in the alga *Ochromonas danica* but were not necessarily toxic unless they released a substantial amount of ions. Furthermore, Leclerc et al. [48] showed that for short-term exposure, Ag-NP had a greater capacity to bioaccumulate in *C. reinhardtii* than comparable concentrations of ionic silver. As copper export transporters act on ionic copper ions, Cu-NP and their aggregates may be less efficiently removed by these transporters, which could explain the increased loosely and tightly bound fractions [25]. This effect may be exaggerated under copper-deplete conditions in which copper importers were more active. The difference may also be the result of Cu-NPs adsorbing to the cells, whereas $Cu^{+/2+}$ ions, which exchange rapidly with ligands, likely do not [3].

*Chlorophyll content*

Chlorophyll content was measured after 24 h, 48 h, and 72 h of Cu-NP exposure to assess their affect on the cellular photosynthetic apparatus (Figure 4). Because chlorophyll concentrations remained consistent in the unexposed controls ($0.03 \pm 0.003$ mg/mL), all measured values were normalized to unexposed cells and reported as % chlorophyll content relative to the control. Over time, all cells exposed to copper as either Cu-NPs or $CuCl_2$ displayed reduced chlorophyll content. Specifically, all except copper-deplete cells exposed to Cu-NPs showed a statistically significant decrease after 48 h, and all cells showed a statistically significant decrease in chlorophyll content after 72 h ($p < 0.05$). This is consistent with previous research suggesting that excessive copper can drastically reduce chlorophyll concentration and negatively impact photosynthesis by inhibiting the primary photochemical reactions in photosystem II [49,50]. Furthermore, Saison et al. [51] found that core shell copper oxide NPs had a deteriorative effect on chlorophyll by inducing the inhibition of photosystem II. Interestingly, cells exposed to Cu-NPs retained more chlorophyll (45–82% relative to untreated cells) than did cells exposed to $CuCl_2$ (25–54%), suggestive of

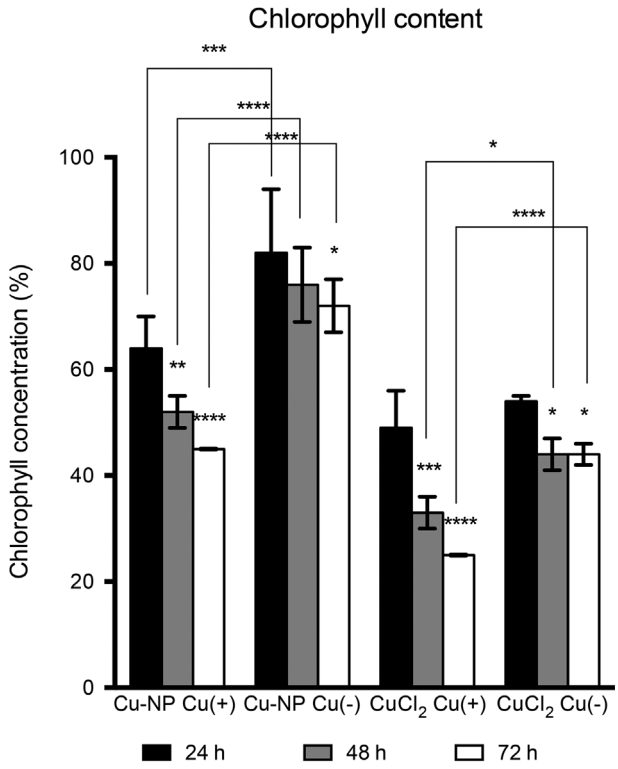


Figure 4. Chlorophyll concentration of cells after copper nanoparticle (Cu-NP) exposure in copper-replete Cu(+) and copper-deplete Cu(–) *Chlamydomonas reinhardtii* cells. Chlorophyll concentrations were measured after exposure to 20 mg/L of Cu-NPs as $Cu^{2+}$ for 24 h, 48 h, and 72 h. Concentrations were normalized to the unexposed controls, which remained consistent at $0.03 \pm 0.003$ mg/mL, and error bars represent standard deviation ($n = 4$). Consistent with the sensitivity indicated by median inhibitory concentration values, cells exposed to Cu-NPs resulted in statistically smaller decreases in chlorophyll content than $CuCl_2$ exposed cells. Over 72 h, copper-replete cells Cu(+) appeared more sensitive to toxicity from Cu-NPs as well as $CuCl_2$, with respect to chlorophyll content. Asterisks above bar represent statistical differences between times, whereas asterisks above connecting line represent statistical differences between copper-replete and copper deplete counter parts ($p < 0.05$(*), 0.01(**), 0.001(***), and 0.0001(****)).

reduced toxicity of Cu-NPs. This is consistent with IC50 values, which showed that $CuCl_2$ was somewhat more toxic than Cu-NPs.

Although chlorophyll content decreased after 72 h under all exposure conditions, copper-replete cells experienced a greater reduction in chlorophyll content than did copper-deplete cells for both Cu-NPs and $CuCl_2$. Specifically, for all exposure times except $CuCl_2$-exposed cells at 24 h, the copper-replete cells had statistically greater reduction in chlorophyll than each respective copper-deplete condition. After 72 h, the net reduction in chlorophyll concentration for copper-replete cells was 19% for Cu-NPs and 24% for $CuCl_2$, compared with only 11% net decrease for copper-deplete cells after exposure to either Cu-NPs or $CuCl_2$. Thus, copper-deplete cells exhibited greater resistance to chlorophyll loss than did copper-replete cells when exposed to either copper source. This may be because copper-deplete cells have a reservoir to handle copper equivalent to their usual copper quota.

Because copper-replete cells also displayed increased copper accumulation in the loosely bound and tightly bound fraction of copper-replete cells, these combined results suggest a potential protective effect as copper-deplete cells may scavenge copper from Cu-NPs to drive overall metabolism that is dependent on

copper. Under various conditions, Cu-NPs have been shown to enhance growth of various photosynthetic organisms [52]. The Cu-NPs were found to increase lettuce root to shoot ratio in lettuce plants [53]. In the case of the copper-deplete *C. reinhardtii*, it may rapidly utilize copper from any source, even Cu-NPs, as copper-dependent enzymes are often preferable to alternative copper-independent enzymes [31]. Copper-replete cells, in contrast, had adequate amounts of copper, so any excess copper likely had an exaggerated toxic effect because they did not contribute to copper metabolism.

Alternatively, studies with yeast have shown that yeast deprived of zinc prepare themselves for a sudden influx of zinc and can control ion uptake in such a zinc shock situation [54]. A similar mechanism might be present in algae, and the copper-deprived algae might be better prepared for the sudden influx of copper. The phenomenon may be unique to copper and other biologically relevant metals. For example, *C. reinhardtii* exposed to $TiO_2$ at sublethal concentrations exhibited substantial photosynthesis inhibition with no measured positive effects [15]. In any case, the increased chlorophyll reduction across both conditions suggests that cells were unable to respond to excess copper over time. Taken together, these results highlight the need for predictive toxicity models to factor in not only the amount of bioavailable ions of a metal-containing NPs but also the essential trace nutrient status of the exposed organisms.

Although the copper-replete and copper-deplete cells show different resistances to chlorophyll loss, they share nearly equivalent IC50 values. This is likely the result of the different toxic metrics used. The IC50 values were based on ATP abundance, which represents overall metabolic activity, whereas chlorophyll measurements are specific to photosynthesis. Under the growth conditions tested, *C. reinhardtii* had the ability to grow mixotrophically because the tris-acetate-phosphate media contained acetate. Acetate metabolism may have supplemented losses from photosynthetic activity and masked the differences between copper-deplete and copper-replete growth conditions in the ATP assessment. Furthermore, acetate-supplemented growth also has a protective effect on photosynthesis, which suggests that under substrate-limited conditions such as those found in oligotrophic natural environments, the differential responses of copper-deplete and copper-replete cells may be intensified in reduction of both chlorophyll and ATP losses [55].

In addition, these results highlight the importance of using and understanding different assays with varying toxic endpoints, as different assays can yield different results. There is no ideal or most appropriate assay because each single mechanism assay has its own set of biases [41]. The choice of assay is dependent on the toxic endpoint under study, and it is important to ensure that similar assays and toxic endpoints are being compared when making comparisons across organisms and studies.

*Gene expression experiments*

The expression of copper homeostasis and stress genes was tracked following exposure to Cu-NPs and $CuCl_2$ to assay the impact on *C. reinhardtii* gene expression. The gene *CYC6* is expressed in many green algae and also cyanobacteria only under conditions of copper deficiency. The gene product, Cyt $c_6$, is a replacement for the copper-dependent photosynthetic electron transport protein, plastocyanin [56]. Therefore, the *CYC6* gene can be used as a biomarker for intracellular copper status. In the present study, expression of the Cu deficiency marker *CYC6* was quantified after 6 h of exposure to 20 mg/L of Cu-NPs or $CuCl_2$ to determine if the Cu-NPs were recognized by the cell as a copper source. The average $C_T$ and $\Delta\Delta C_T$ values for *CYC6* and the housekeeping gene *CβLP* are compared in Table 1. Although the expression of *CYC6* was high in the unexposed copper-deplete cells (low $C_T$ value), it was expressed at low levels throughout both Cu-NP and $CuCl_2$ exposure conditions (high $C_T$ value). The $\Delta\Delta C_T$ values of 13.2 and 12 show significant down-regulation of *CyC6* when the cells were supplied with Cu-NP or $CuCl_2$ (Table 1; $p < 0.0001$) [38]. These data suggest that although the severity of toxicity may be different for either copper source, ions originating from both $CuCl_2$ and Cu-NP can be utilized by the cell, and thus result in a down-regulation of *CYC6* expression. The high expression of *CYC6* in the unexposed copper-deplete control group is consistent with its role in copper-deficient cells and confirms that copper-deplete cells were indeed grown in the absence of copper.

In addition to *CYC6*, expression of the ROS stress genes *MSD1* and *FSD1* was tracked in response to *C. reinhardtii* exposure to Cu-NPs and $CuCl_2$ (Table 2). Although *MSD1*, a gene specific to oxidative stress, was significantly up-regulated in cells exposed to Cu-NP and $CuCl_2$-exposed cells relative to the unexposed control ($p < 0.0001$), the expression of *FSD1*, the gene for the iron superoxide dismutase did not significantly change between treatments ($p > 0.05$; Supplemental Data, Table S2). The gene *MSD1*, encoding mitochondrially targeted manganese superoxide dismutase, is responsive to oxidative stress, which is consistent with previous studies reporting ROS generation from Cu-NPs as well as $Cu^{2+}$ ions [12]. The finding that the *FSD1* gene does not respond suggests that ROS generation and/or $Cu^{2+}$-associated stress was compartmentalized. Previous studies have shown that under metal ion stress, $Cu^{2+}$ ions were compartmentalized in *C. reinhardtii* cells to reduce the stress [56]. A similar response mechanism may be responsible. The response of the *MSD1* gene was not dramatic, but, as previously suggested, that might be because the added copper could initially serve as a nutrient in copper-deplete

Table 1. Expression of *CyC6* following exposure to copper nanoparticles (Cu-NPs) and $CuCl_2$[a]

| | Cu-NPs | | $CuCl_2$ | | No added Cu |
|---|---|---|---|---|---|
| Gene | $C_T$ | $\Delta\Delta C_T$ | $C_T$ | $\Delta\Delta C_T$ | $C_T$ |
| *CYC6* | $33.3 \pm 0.6$ | $13.2 \pm 0.7$**** | $32.5 \pm 0.3$ | $12 \pm 0.2$**** | $21.1 \pm 0.1$ |
| *CβLP* | $16.8 \pm 0.1$ | | $17.1 \pm 0.1$ | | $17.7 \pm 0.0$ |

[a]Although the expression of *CYC6* was high in the unexposed copper-deplete cells (low threshold cycle [$C_T$] value), it was expressed at low levels throughout all other conditions (high $C_T$ value). The $\Delta\Delta C_T$ values of 13.2 and 12 show statistically significant down-regulation of *CyC6* when the cells are supplied with Cu-NP or $CuCl_2$. Higher $\Delta\Delta C_T$ values correspond to lower relative gene expression [38]. Means and standard deviations are presented for all values.
**** Statistical significance at $p < 0.0001$.

Table 2. Expression of *FSD1* and *MSD1* following exposure to copper-nanoparticles (Cu-NPs) and $CuCl_2$[a]

| | Copper-replete | | | | | Copper-deplete | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cu-NPs | | $CuCl_2$ | | No added Cu | Cu-NPs | | Cu-$Cl_2$ | | No added Cu |
| Gene | $C_T$ | $\Delta\Delta C_T$ | $C_T$ | $\Delta\Delta C_T$ | $C_T$ | $C_T$ | $\Delta\Delta C_T$ | $C_T$ | $\Delta\Delta C_T$ | $C_T$ |
| *FSD1* | 26.4 ± 0.1 | −0.5 ± 0.1 | 26.5 ± 0.3 | −0.4 ± 0.3 | 25.5 ± 0.0 | 25.3 ± 0.0 | −0.3 ± 0.1 | 25.7 ± 0.2 | −0.4 ± 0.1 | 26.5 ± 0.1 |
| *CβLP* | 19.2 ± 0.1 | | 19.2 ± 0.1 | | 17.8 ± 0.1 | 17.6 ± 0.1 | | 18.1 ± 0.1 | | 18.5 ± 0.1 |
| *MSD1* | 22.8 ± 0.3 | −3.3 ± 0.4**** | 22.6 ± 0.0 | −3.5 ± 0.1**** | 24.7 ± 0.0 | 21.3 ± 0.1 | −3.0 ± 0.1**** | 21.8 ± 0.0 | −3.0 ± 0.0**** | 25.2 ± 0.0 |
| *CβLP* | 19.3 ± 0.0 | | 19.3 ± 0.1 | | 17.9 ± 0.1 | 17.8 ± 0.1 | | 18.3 ± 0.1 | | 18.7 ± 0.1 |

[a]*MSD1*, a gene specific to oxidative stress, was significantly up-regulated in both Cu-NP-exposed and $CuCl_2$-exposed cells relative to the unexposed control, but the expression of *FSD1*, the gene for the iron superoxide dismutase, did not change significantly between treatments. Furthermore, expression of *MSD1* was lower for copper-deplete cells. Lower $\Delta\Delta C_T$ values correspond to higher relative gene expression [38]. Means and standard deviations are presented for all values. $C_T$ = threshold cycle.
**** Statistical significance at $p < 0.0001$.

situations. For all genes examined, expression was similar between copper-deplete and copper-replete cells exposed to either copper source, as no statistically significant differences in expression were observed between conditions.

## CONCLUSIONS

Under copper-deplete and copper-replete conditions, Cu-NPs were less toxic than the control $CuCl_2$ toward *C. reinhardtii*. This effect was observed in terms of higher IC50 values and lower reduction in chlorophyll content. These differences in severity may have resulted because of the differences in dissolution behavior after introduction into the medium. When introduced as $CuCl_2$, copper dissolved rapidly (30 min), whereas copper from Cu-NPs required longer incubation (36 h) for complete dissolution. The Cu-NPs formed complexes or aggregates with a reduced capacity to release toxic ions and interact with cells. Despite this difference in particle fate and stability, both copper sources displayed photosystem II damage as measured by chlorophyll content, and caused membrane and organelle damage and cellular swelling, and comparable up- and down-regulation of genes related to copper ion stress and ROS generation. The occurrence of these shared biological responses following Cu-NP and $CuCl_2$ exposure suggests that ion release was the primary toxicity mechanism. Indeed, Cu-NPs showed eventual full dilution in the media. Differences in severity likely resulted because Cu-NP aggregation hindered but did not stop the dissolution of ions in the medium, which resulted in oxidative stress and membrane and organelle damage.

Copper dosage alone, however, could not explain all of the observed differences in toxic responses of copper-replete and copper-deplete *C. reinhardtii* cells to Cu-NPs. Relative to copper-replete cells, copper-deplete cells displayed greater accumulation of copper, resistance to chlorophyll reduction, and expression of genes related to ROS damage. These results suggest that copper-deplete cells had increased transport and resistance to Cu-NPs and $CuCl_2$, which may result from copper-deplete cells using excess copper for metabolically favored copper-dependent enzymes or being better equipped for a sudden influx of copper [31,54]. In aquatic environments, nutrient-limited conditions often occur after algal blooms, resulting in anaerobiosis and the rapid loss of essential micronutrients [57].

The present study's results suggest that antialgal applications using Cu-NPs would be less effective in these situations, as copper-limited algae may be more resistant to Cu-NPs, and algal blooms could be amplified. These results endorse toxicity assessments that account for the characteristics of impacted organisms such as nutritional status and use toxicity models based on estimations of the bioavailable fraction.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3254.

*Acknowledgment*—The present study was supported in part by the National Science Foundation (NSF) award 1134355. V.C. Reyes received a 1-yr fellowship from the NSF UCLA Science and Engineering of the Environment of Los Angeles (SEE-LA) GK-12 program. M.R. Spitzmiller was funded by UCLA's Eugene V. Cota-Robles Fellowship and Women's Faculty Club Fellowship. M. Wang graciously performed all PHREEQC analyses. S.S. Merchant, A. Hong-Hermesdorf, and J. Kropat were supported by the National Institutes of Health (NIH) award GM42143. Support for the Molecular Screening Shared Resource from the National Cancer Center Support grant P3016042-35 is acknowledged.

*Disclaimer*—Any opinions, finding, conclusions, or recommendations expressed in the present study are those of the authors and do not necessarily reflect the views of any of the aforementioned agencies. The present study has not been subjected to NIH or NSF review, and no official endorsement should be inferred.

*Data availability*—Please contact the corresponding author for raw data (mahendra@seas.ucla.edu).

## REFERENCES

1. Consumer Product Inventory. 2013. *The Project on Emerging Nanotechnologies*. [cited 2014 July 8]. Available from: http://www.nanotechproject.org/cpi/
2. Lee J, Mahendra S, Alvarez PJJ. 2010. Nanomaterials in the construction industry: A review of their applications and environmental health and safety considerations. *ACS Nano*. 4:3580–3590.
3. Klaine SJ, Koelmans AA, Horne N, Carley S, Handy RD, Kapustka L, Nowack B, Kammer von der F. 2011. Paradigms to assess the environmental impact of manufactured nanomaterials. *Environ Toxicol Chem* 31:3–14.
4. Vasumathi KK, Premalatha M, Subramanian P. 2012. Parameters influencing the design of photobioreactor for the growth of microalgae. *Renew Sust Energ Rev* 16:5443–5450.
5. Georgianna DR, Mayfield SP. 2012. Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature* 488:329–335.
6. Thilakaratne R, Wright MM, Brown RC. 2014. A techno-economic analysis of microalgae remnant catalytic pyrolysis and upgrading to fuels. *Fuel* 128:104–112.
7. Minocha S, Mumper RJ. 2012. Effect of carbon coating on the physicochemical properties and toxicity of copper and nickel nanoparticles. *Small* 8:3289–3299.
8. Heinlaan M, Ivask A, Blinova I, Dubourguier H-C, Kahru A. 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere* 71:1308–1316.

9. Gunawan C, Teoh WY, Marquis CP, Amal R. 2011. Cytotoxic origin of copper(II) oxide nanoparticles: Comparative studies with micron-sized particles, leachate, and metal salts. *ACS Nano* 5:7214–7225.
10. Reyes VC, Opot SO, and Mahendra S. 2015. Planktonic and biofilm grown nitrogen cycling bacteria exhibit different susceptibilities to copper nanoparticles. *Environ Toxicol Chem* 34:887–897.
11. Aruoja V, Dubourguier H-C, Kasemets K, Kahru A. 2009. Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae *Pseudokirchneriella subcapitata*. *Sci Total Environ* 407:1461–1468.
12. Bondarenko O, Ivask A, Kakinen A, Kahru A. 2012. Sub-toxic effects of CuO nanoparticles on bacteria: Kinetics, role of Cu ions and possible mechanisms of action. *Environ Pollut* 169:81–89.
13. Domingos RF, Simon DF, Hauser C, Wilkinson KJ. 2011. Bioaccumulation and effects of CdTe/CdS quantum dots on *Chlamydomonas reinhardtii*—Nanoparticles or the free ions? *Environ Sci Technol* 45:7664–7669.
14. Chen Z, Zhu L, Wilkinson KJ. 2010. Validation of the Biotic Ligand Model in Metal Mixtures: Bioaccumulation of Lead and Copper. *Environ Sci Technol* 44:3580–3586.
15. Simon DF, Domingos RF, Hauser C, Hutchins CM, Zerges W, Wilkinson KJ. 2013. Transcriptome sequencing (RNA-seq) analysis of the effects of metal nanoparticle exposure on the transcriptome of *Chlamydomonas reinhardtii*. *Appl Environ Microbiol* 79:4774–4785.
16. Zhou H, Wang X, Zhou Y, Yao H, Ahmad F. 2014. Evaluation of the toxicity of ZnO nanoparticles to *Chlorella vulgaris* by use of the chiral perturbation approach. *Anal Bioanal Chem* 406:3689–3695.
17. Kasemets K, Ivask A, Dubourguier H-C, Kahru A. 2009. Toxicity of nanoparticles of ZnO, CuO and TiO2 to yeast *Saccharomyces cerevisiae*. *Toxicol In Vitro* 23:1116–1122.
18. Navarro E, Piccapietra F, Wagner B, Marconi F, Kaegi R, Odzak N, Sigg L, Behra R. 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environ Sci Technol* 42:8959–8964.
19. Wang Z, Li J, Zhao J, Xing B. 2011. Toxicity and internalization of CuO nanoparticles to prokaryotic alga *Microcystis aeruginosa* as affected by dissolved organic matter. *Environ Sci Technol* 45:6032–6040.
20. Kennedy AJ, Chappell MA, Bednar AJ, Ryan AC, Laird JG, Stanley JK, Steevens JA. 2012. Impact of organic carbon on the stability and toxicity of fresh and stored silver nanoparticles. *Environ Sci Technol* 46:10772–10780.
21. Li M, Pokhrel S, Jin X, Mädler L, Damoiseaux R, Hoek EMV. 2011. Stability, bioavailability, and bacterial toxicity of ZnO and iron-doped ZnO nanoparticles in aquatic media. *Environ Sci Technol* 45:755–761.
22. Jin X, Li M, Wang J, Marambio-Jones C, Peng F, Huang X, Damoiseaux R, Hoek EMV. 2010. High-throughput screening of silver nanoparticle stability and bacterial inactivation in aquatic media: Influence of specific ions. *Environ Sci Technol* 44:7321–7328.
23. Mahendra S, Zhu H, Colvin VL, Alvarez PJ. 2008. Quantum dot weathering results in microbial toxicity. *Environ Sci Technol* 42:9424–9430.
24. Stevenson LM, Dickson H, Klanjscek T, Keller AA, McCauley E, Nisbet RM. 2013. Environmental feedbacks and engineered nanoparticles: Mitigation of silver nanoparticle toxicity to *Chlamydomonas reinhardtii* by algal-produced organic compounds. *PLos One* 8:e74456.
25. Merchant SS, Allen MD, Kropat J, Moseley JL, Long JC, Tottey S, Terauchi AM. 2006. Between a rock and a hard place: Trace element nutrition in *Chlamydomonas*. *Biochim Biophys Acta* 1763:578–594.
26. Quinn JM, Merchant S. 1995. Two copper-responsive elements associated with the *Chlamydomonas* Cyc6 gene function as targets for transcriptional activators. *Plant Cell* 7:623–638.
27. Lavoie M, Fortin C, Campbell PGC. 2012. Influence of essential elements on cadmium uptake and toxicity in a unicellular green alga: The protective effect of trace zinc and cobalt concentrations. *Environ Toxicol Chem* 31:1445–1452.
28. Howe G, Merchant S. 1992. The biosynthesis of membrane and soluble plastidic c-type cytochromes of *Chlamydomonas reinhardtii* is dependent on multiple common gene products. *EMBO J* 8:2789–2801.
29. Nishimura Y, Stern DB. 2010. Differential replication of two chloroplast genome forms in heteroplasmic *Chlamydomonas reinhardtii* gametes contributes to alternative inheritance patterns. *Genetics* 185:1167–1181.
30. Kropat J, Hong-Hermesdorf A, Casero D, Ent P, Castruita M, Pellegrini M, Merchant SS, Malasarn D. 2011. A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. *Plant J* 66:770–780.
31. Page MD, Kropat J, Hamel PP, Merchant SS. 2009. Two *Chlamydomonas* CTR copper transporters with a novel Cys-Met motif are localized to the plasma membrane and function in copper assimilation. *Plant Cell* 21:928–943.
32. Boyd RD, Pilch I, Garbrecht M, Halvarsson M, Helmersson U. 2014. Double oxide shell layer formed on a metal nanoparticle as revealed by aberration corrected (scanning) transmission electron microscopy. *Mater Res Express* 1:1–11.
33. Nestler H, Groh KJ, Schönenberger R, Behra R, Schirmer K, Eggen RIL, Suter MJF. 2012. Multiple-endpoint assay provides a detailed mechanistic view of responses to herbicide exposure in *Chlamydomonas reinhardtii*. *Aquat Toxicol* 110–111:214–224.
34. Perreault F, Oukarroum A, Melegari SP, Matias WG, Popovic R. 2012. Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga *Chlamydomonas reinhardtii*. *Chemosphere* 87:1388–1394.
35. Melegari SP, Perreault F, Costa RHR, Popovic R, Matias WG. 2013. Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*. *Aquat Toxicol* 142–143:431–440.
36. Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* 975:384–394.
37. Allen MD, Kropat J, Tottey S, Del Campo JA, Merchant SS. 2006. Manganese deficiency in *Chlamydomonas* results in loss of photosystem II and MnSOD function, sensitivity to peroxides, and secondary phosphorus and iron deficiency. *Plant Physiol* 143:263–277.
38. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3:1101–1108.
39. Reyes VC, Li M, Hoek EMV, Mahendra S, Damoiseaux R. 2012. Genome-wide assessment in *Escherichia coli* reveals time-dependent nanotoxicity paradigms. *ACS Nano* 6:9402–9415.
40. Sharma VK. 2009. Aggregation and toxicity of titanium dioxide nanoparticles in aquatic environment—A review. *J Environ Sci Health A* 44:1485–1495.
41. Damoiseaux R, George S, Li M, Pokhrel S, Ji Z, France B, Xia T, Suarez E, Rallo R, Mädler L, Cohen Y, Hoek EMV, Nel A. 2011. No time to lose—High throughput screening to assess nanomaterial safety. *Nanoscale* 3:1345.
42. Burchardt AD, Carvalho RN, Valente A, Nativo P, Gilliland D, Garcìa CP, Passarella R, Pedroni V, Rossi F, Lettieri T. 2012. Effects of silver nanoparticles in diatom *Thalassiosira pseudonana* and *Cyanobacterium synechococcus* sp. *Environ Sci Toxicol* 46:11336–11344.
43. Franklin NM, Rogers NJ, Apte SC, Batley GE, Gadd GE, Casey PS. 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility. *Environ Sci Technol* 41:8484–8490.
44. Rilsgard HU. 1979. Effect of copper on volume regulation in the marine flagellate *Dunaliella marina*. *Mar Biol* 50:189–193.
45. Hill KL, Hassett R, Kosman D, Merchant S. 1996. Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability. *Plant Physiol* 112:697–704.
46. Hajipour MJ, Fromm KM, Ashkarran AA, de Aberasturi DJ, de Larramendi IR, Rojo T, Serpooshan V, Parak WJ, Mahmoudi M. 2012. Antibacterial properties of nanoparticles. *Trends Biotechnol* 30:499–511.
47. Miao A-J, Luo Z, Chen C-S, Chin W-C, Santschi PH, Quigg A. 2010. Intracellular uptake: A possible mechanism for silver engineered nanoparticle toxicity to a freshwater alga *Ochromonas danica*. *PLos One* 5:e15196.
48. Leclerc S, Wilkinson KJ. 2014. Bioaccumulation of nanosilver by *Chlamydomonas reinhardtii*—Nanoparticle or the free ion? *Environ Sci Technol* 48:358–364.
49. Hsu B-D, Lee J-Y. 1988. Toxic effects of copper on photosystem II of spinach chloroplasts. *Plant Physiol* 87:116–119.
50. Shakya K, Chettri MK, Sawidis T. 2007. Impact of heavy metals (copper, zinc, and lead) on the chlorophyll content of some mosses. *Arch Environ Contam Toxicol* 54:412–421.
51. Saison C, Perreault F, Daigle J-C, Fortin C, Claverie J, Morin M, Popovic R. 2010. Effect of core–shell copper oxide nanoparticles on cell culture morphology and photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas reinhardtii*. *Aquat Toxicol* 96:109–114.
52. Masarovičová E, KráĽová K. 2013. Metal nanoparticles and plants. *Ecol Chem Eng Sci* 20:9–22.

53. Shah V, Belozerova I. 2008. Influence of metal nanoparticles on the soil microbial community and germination of lettuce seeds. *Water Air Soil Pollut* 197:143–148.
54. MacDiarmid CW, Milanick MA, Eide DJ. 2003. Induction of the ZRC1 metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. *J Biol Chem* 278:15065–15072.
55. Roach T, Sedoud A, Krieger-Liszkay A. 2013. Acetate in mixotrophic growth medium affects photosystem II in *Chlamydomonas reinhardtii* and protects against photoinhibition. *BBA-Bioenergetics* 1827:1183–1190.
56. Hsieh SI, Castruita M, Malasarn D, Urzica E, Erde J, Page MD, Yamasaki H, Casero D, Pellegrini M, Merchant SS, Loo JA. 2013. The proteome of copper, iron, zinc, and manganese micronutrient deficiency in *Chlamydomonas reinhardtii*. *Mol Cell Proteomics* 12:65–86.
57. Castruita M, Casero D, Karpowicz SJ, Kropat J, Vieler A, Hsieh SI, Yan W, Cokus S, Loo JA, Benning C, Pellegrini M, Merchant SS. 2011. Systems biology approach in *Chlamydomonas* reveals connections between copper nutrition and multiple metabolic steps. *Plant Cell* 23:1273–1292.