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Exposure to Copper Nanoparticles Causes Gill Injury and Acute Lethality in Zebrafish (*Danio rerio*)

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Increasing use of metallic nanomaterials is likely to result in release of these particles into aqueous environments; however, it is unclear if these materials present a hazard to aquatic organisms. Because some dissolution of metal particles will occur, it is important to distinguish effects of nanoparticulates from dissolved metals. To address this issue, acute toxicity of soluble copper and 80 nm copper nanoparticle suspensions were examined in zebrafish. The results demonstrate that nanocopper is acutely toxic to zebrafish, with a 48 h LC_{50} concentration of 1.5 mg/L. Rapid aggregation of copper nanoparticles occurred after suspension in water, resulting in 50–60% of added mass leaving the water column. While dissolution of particulate copper occurred, it was insufficient to explain the mortality in nanocopper exposures. Histological and biochemical analysis revealed that the gill was the primary target organ for nanocopper. To further investigate the effects of nanocopper on the gill, zebrafish were exposed to 100 μg/L of nanocopper or to the concentration of soluble copper matching that present due to dissolution of the particles. Under these conditions, nanocopper produced different morphological effects and global gene expression patterns in the gill than soluble copper, clearly demonstrating that the effects of nanocopper on gill are not mediated solely by dissolution.

Introduction

Nanotechnology is one of the fastest growing sectors of the high tech economy. There are >200 separate consumer products alone using nanomaterials (*1*) with personal, commercial, medical, and military uses. As interest in the potential benefits of nanomaterials has increased, so too has concern about the potential toxic effects resulting from use or unintentional release into the environment (*2–4*). Much of the toxicological research to date has focused on atmospheric or inhalation exposures (*5, 6*); however, use of nanomaterials is likely to result in releases into aquatic systems and may pose a risk to aquatic ecosystems (*2*). Unfortunately, there is no accepted method for conducting aquatic toxicity tests with nanoparticles (*7*).

A growing number of nanotechnology applications utilize metallic components, many of which can be toxic to aquatic organisms. While regulations exist to protect aquatic life from soluble forms of toxic metals, it is crucial to determine if metallic nanomaterials are toxic and if so, whether the toxicity is quantitatively or mechanistically different than that of soluble metals. This information can then be used to develop appropriate guidelines for exposure to metallic nanomaterials. The situation is exemplified by nanosized copper particles, which are widely used as a bacteriocide (*8*), for air and liquid filtration, as coatings on integrated circuits and batteries, and to increase thermal and electrical conductivity in coatings and sealants. Soluble forms of copper are highly toxic to fish (*9*); however, little information exists on the toxicity of copper nanomaterials in aquatic species or on the behavior of copper nanoparticulates in aqueous environments, and it is unclear whether environmental release of nanocopper poses significant ecological risk.

The goal of this study was to determine if copper nanoparticles are toxic to zebrafish, and if so, determine if the observed toxicity is solely due to dissolution of particles. Therefore, the behavior of copper nanoparticles in natural water was examined and the acute toxicity of nanoparticulate copper was compared to that of soluble copper using adult zebrafish (*Danio rerio*).

Materials and Methods

Materials Used in Research. Copper nanoparticles with a nominal diameter of 80 nm used in this research were provided gratis from Quantum Sphere Incorporated (Santa Ana, CA). Optima grade nitric acid (70%, Fisher Scientific), Ultrex II 30% hydrogen peroxide (J.T. Baker), and copper sulfate pentahydrate were purchased from Fisher Scientific. Tricaine (MS-222) was purchased from Western Chemical inc. (Ferndale, WA). Ouabain, adenosine triphosphate, and paraformaldehyde were obtained from Sigma Chemical Company. All other chemicals were purchased through Fisher Scientific and were of the highest purity available.

Fish and Fish Care. Wild-type zebrafish were purchased from Ekk-Will (Gibsonton, FL), and maintained in the Aquatic Toxicology Laboratory at the University of Florida. Fish were fed pelleted feed (Ziegler Bros., Gardners, PA) at 1% of body weight daily. This facility uses dechlorinated municipal water and forced air aeration. Water quality parameters were as follows for flow-through tanks: DO was 8.5–8.9 mg/L; pH was 8.2 ± 1; hardness was 142 ± 12 mg $CaCO_3$/L; and total un-ionized ammonia was always less than 0.5 mg/L. Water temperature was 27 ± 2 °C. Fish were maintained on a 14:10 h light:dark cycle.

Particle Characterization. Prior to use in exposures, copper particles were characterized for surface area, size, and zeta potential. To determine the behavior of nanocopper in the water used for exposures, 270 mg/L of nanocopper was suspended in filtered aquatic facility water. Samples of this suspension were taken at 0, 24, and 48 h for analysis of particle size distribution, zeta potential, and particle dissolution. Samples were obtained following agitation to obtain size distribution for all particles, as well as without agitation to examine the particle size distribution for particles remaining in the water column. Particle size distributions were obtained using a Coulter LS 13 320 laser particle size analyzer and a CPS 100 Centrifugal Particle Sizer. Zeta potential of the particles was measured with a Zeta Reader Mk 21-II (Zeta

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TABLE 1. QPCR primer specifications for hypoxia-inducible factor 1 (HIF1), heat-shock protein 70 (HSP70), copper transport regulatory protein (CTR), metal transcription factor 1 (MTF1), sodium potassium ATPase (NKA), multidrug resistant protein (MDR1)

primer sequence (5′-3′)	gene (direction)	organ surveyed	T_A (C)	primer efficiency
aatgtggagctgctttttgc	CTR1 (F)	liver, gill	60	
acacagccaacaagaacacg	CTR1 (R)	liver, gill	60	91.1
gattgagcttctccgtcagg	MTF1 (F)	liver, gill	60	
tcctcgtcttcctcttctcg	MTF1 (R)	liver, gill	60	96.0
agacagggtgccattgtagc	NKA (F)	gill	60	
gcaatacccatagccacacc	NKA (R)	gill	60	97.3
gcagcagactgtgactgagc	HIF1A (F)	liver, gill	60	
gagcaattgaggctttctgg	HIF1A (R)	liver, gill	60	93.4
ggaaaagagggaagctttgg	HSP1 (F)	liver, gill	60	
acgttccatgtttccagacc	HSP1 (R)	liver, gill	60	93.5
aaagcacaaaggcaaaaagg	MDR1 (F)	liver	60	
agcatgaggaacacctctgg	MDR1 (R)	liver	60	96.1
cggctaccacatccaaggaa	18S (F)	liver, gill	60	
cctgtattgttatttttcgtcactacct	18S (R)	liver, gill	60	99.1

Potential Instruments Inc.). Particle density and specific surface area was calculated using a Quantichrom Nova 1200 (Quantichrome Corp, Syosset, NY). Scanning electron micrographs were taken using a JEOL 6335F Field Emission SEM, with a working distance of 15 mm, at an accelerating voltage of 5.0 kV. Transmission electron micrographs were taken using a JEOL 2010 high resolution field emission TEM. Total copper was determined by acid digestion of water samples. Dissolved copper was defined as copper present in the supernatant of samples centrifuged at 100000*g* for 30 min Copper concentrations in both fractions were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) utilizing a Perkin-Elmer model 3200 monitoring 324.752 nm. Five repetitions were performed per sample and the average used to calculate copper concentration from a seven point standard curve.

Acute Toxicity Evaluation. Static 48 h toxicity tests were conducted to determine the dose–response curves for lethality in zebrafish exposed to soluble copper or nanocopper. All exposures were based on mass of copper added and were performed in triplicate in 10 L of 0.45 μm filtered facility water containing 10 female zebrafish per tank with continuous aeration. Dosing was accomplished by adding 10 mL of a 1000× stock prepared in ultrapure water (Milli-Q, 18.2 MΩ). Stock particle suspensions were prepared by adding required mass of particles to ultrapure water and sonicating for *six* seconds in 1 second bursts at 6 W output using a probe sonicator with a frequency of 22.5 kHz.

Based on the results of acute toxicity testing, exposures were performed to determine if toxicity of nanocopper differed from that caused by soluble copper. For this exposure four exposure groups were defined: Control (no treatment), soluble copper (0.25 mg/L as Cu^{2+} ion), low nanocopper (0.25 mg/L), and high nanocopper (1.5 mg/L). The soluble copper and high nanocopper concentrations represent 48 h LC_{50} concentrations for those forms of copper. The low nanocopper concentration was chosen to reflect an equivalent mass to the aqueous copper LC_{50}. After 48 h of exposure, surviving fish were euthanized with MS-222 and blood and tissue samples were obtained for assays described below. For each exposure, water samples were taken from the middle of the water column to quantify particle dissolution and sedimentation, and analyzed as described above.

Histopathology Analysis. Gills and carcasses were fixed separately by immersion in 10% buffered formalin for 24 h, washed twice in phosphate buffered saline and transferred to 75% ethanol until processing. After dehydration and embedding in paraffin, fish were step-sectioned to obtain longitudinal sections that include all major organ systems. Sections were stained with hematoxylin and eosin. Histopathological observations were made for each major organ system and ranked for severity. The analyst was blinded to sample identification. Five fish from each treatment were analyzed.

Biochemical Analysis of Toxicity. Plasma alanine aminotransferase (ALT) and urea nitrogen (BUN) levels were measured in surviving individuals to assess liver and kidney toxicity, respectively (*10*). For BUN analysis, 2 μL plasma was assayed using a QuantiChrom urea assay kit (BioAssay Systems, Hayward, CA) modified for use with reduced plasma volume. ALT activity was measured with a coupled enzyme assay as previously described (*11*) modified for use with 3 μL of plasma. Gill Sodium-Potassium ATPase (NKA) activity was measured in left gill arches to assess effects of soluble and nanoparticulate activity on gill function as described previously (*12*). For all assays, samples from five fish per treatment were analyzed, and each sample was analyzed in duplicate.

QPCR Analysis. At the termination of the exposure, gill and liver were dissected from five individuals and immediately homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated reverse transcribed into cDNA with random decamer primers using a Retroscript kit (Ambion, Austin, TX). Six genes responsive to environmental stress or metal exposure were analyzed in gill and/or liver tissues to investigate if soluble and nanoparticulate copper induced similar or divergent transcriptional responses in zebrafish (Table 1). Quantitative real-time PCR (QPCR) reactions were performed as duplicate 20 μL reactions on an iCycler (BioRAD, Hercules, CA) using 10 μL of iQ master mix, 0.25 μM each primer (final concentration) and 1 μL cDNA. Primers for the selected genes were designed using Primer3 software (*13*), and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primer sequences, annealing temperatures, organ surveyed, and amplification efficiencies are listed in Table 1.

Detailed Analysis of Gill Toxicity. Because the gills were determined to be the primary target of nanoparticulate copper, additional exposures were performed to assess zebrafish response to sublethal nanocopper exposure. Adult female zebrafish were exposed to either a sublethal concentration of nanocopper (100 μg/L) or to soluble copper at concentrations matching the release of soluble copper from nanocopper dissolution. Nanocopper exposures were conducted as described above. Soluble copper exposures were performed by incremental addition of the appropriate amounts of copper sulfate. Total and dissolved copper in these exposures was measured at 2, 24, and 48 h as described above using ICP-MS analysis (see below). This experimental design allowed

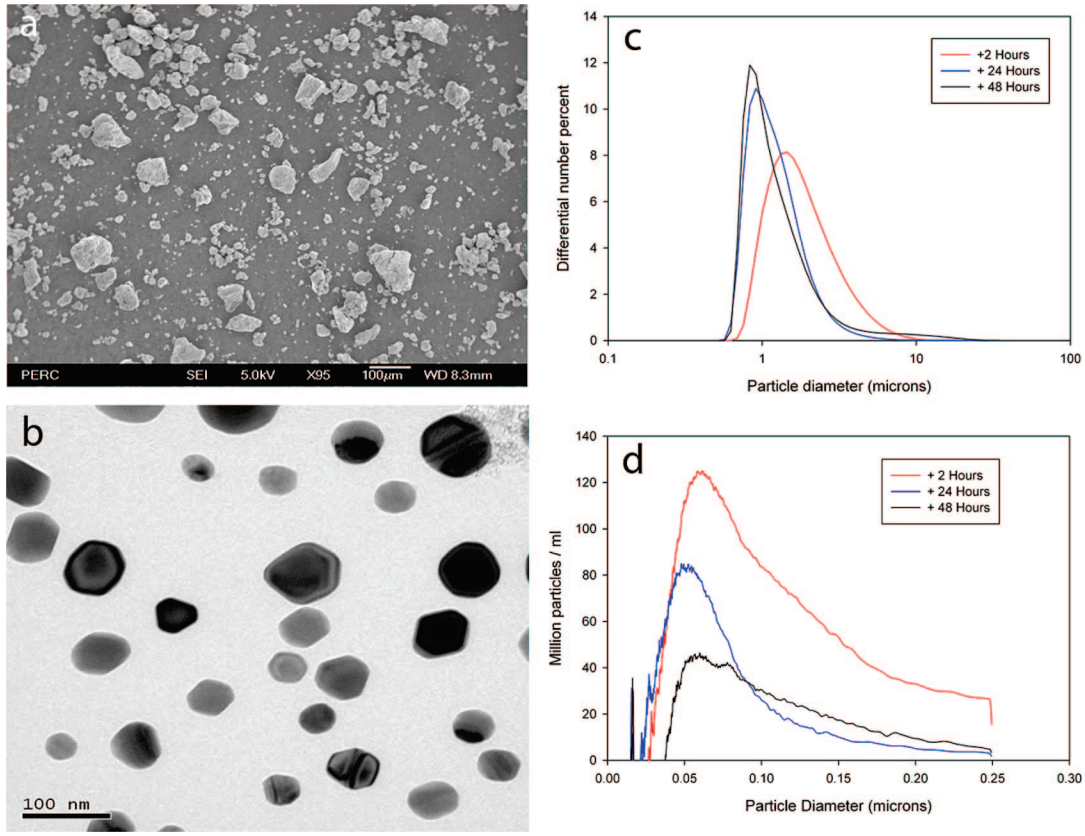

FIGURE 1. (a) SEM micrograph of nanocopper particles dispersed in water showing the wide disparity in particle aggregation states. (b) Higher magnification TEM micrographs of nanocopper particles dispersed in water. Note that particles are 50–60 nm in diameter. (c) Particle size distribution from 0.5 to 10 μm over 48 h as measured by dynamic light scattering. Nanoparticle suspensions were agitated prior to measurement to resuspend large particles and assess changes in large aggregate status. Dispersion curves represent the average of three separate tests. (d) Particle size distribution from 0.015 to 0.25 μm as measured by centrifugal particle sizing. Nanoparticle suspensions were not agitated prior to sampling to allow measurement of water column retention as well as particle size dispersion. Dispersion curves represent the average of three separate tests.

isolation of the effects due solely to nanoparticulate copper from the effects of soluble copper released by the particles.

Gill Metal Analysis. Following 48 h of exposure, gills from individual fish were weighed and digested with nitric acid-peroxide as described previously (*14*). Copper analysis was performed on a Thermo Electron X Series ICP-MS monitoring m/z 63 using iridium as an internal standard. The limit of quantitation of the method was 0.1 ppb in 2% nitric acid. Recovery was determined from samples spiked with 0.1–10 ppb of copper and determined to be between 94 and 111%.

Gill Histopathology. Gills were fixed in 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS) for 24 h at 4 °C. The tissues were rinsed twice in 10 mM PBS, dehydrated, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin wax. Tissue blocks were cut at 7 μm and the orientation of the tissue was lamellar cross sections and filamental sagittal sections. After mounting and rehydration, slides were processed through a series of histological stains following a modification of the Masson Trichrome (*15*). To determine filamental width (defined as the width of the epithelial cells in the interlamellar region from either the gill vasculature or cartilage), four fish in each treatment were analyzed. For each fish, a complete series of slides (approximately 20 slides per gill) were analyzed by randomly measuring five filaments/slide. On each filament, five randomly chosen filament widths were measured for a total of 500 measurements per fish.

Microarray Analysis. Transcriptome responses were measured using the Agilent 1 × 22 k Zebrafish microarray, using a two-color reference design. Total RNA was isolated using TRIzol (Invitrogen) following manufacturer protocol. Quality and concentration of the RNA samples was assessed using a Bioanalyzer and Nanodrop ND-1000. Only RNA Samples with RNA integrity number (RIN) greater than 8.0 and showing no visible evidence of RNA degradation were used. Equal amounts of total RNA from three separate fish were pooled on each microarray. A reference pool was constructed with equal amounts of RNA from all available samples. cRNA synthesis and in vitro transcription were performed according to manufacturers protocols. Due to low levels of Cy3 incorporation in the reference samples, the amount of sample loaded on each array was increased to 1000 ng for both sample and reference. Three arrays were analyzed for each treatment. The pooled samples were hybridized to the arrays for 17 h at 65 °C and scanned at the Microarray Core facility located at UF. Scanned microarrays were analyzed using JMP Genomics 3.0 (SAS, Cary NC). Individual spots flagged as absent or marginal were removed from the analysis, and any spot that was present in less than 75% of the arrays was removed. Missing values were calculated using the missing value imputation function in JMP Genomics as the median of each separate treatment. Using this data set, a ratio analysis was performed, normalizing the sample signal to the reference signal, and performing a within chip Loess normalization.

Statistical Analysis. Acute mortality indices (LC_{50} and 95% CI) were calculated using the Trimmed Spearman–Karber test. Alterations in metal body content and NKA, BUN, and ALT activity from control levels were assessed by Analysis of Variance followed by Dunnett's Test using the statistical package SPSS. In each case, 3–5 fish per treatment were measured with replication and the replicate average used in

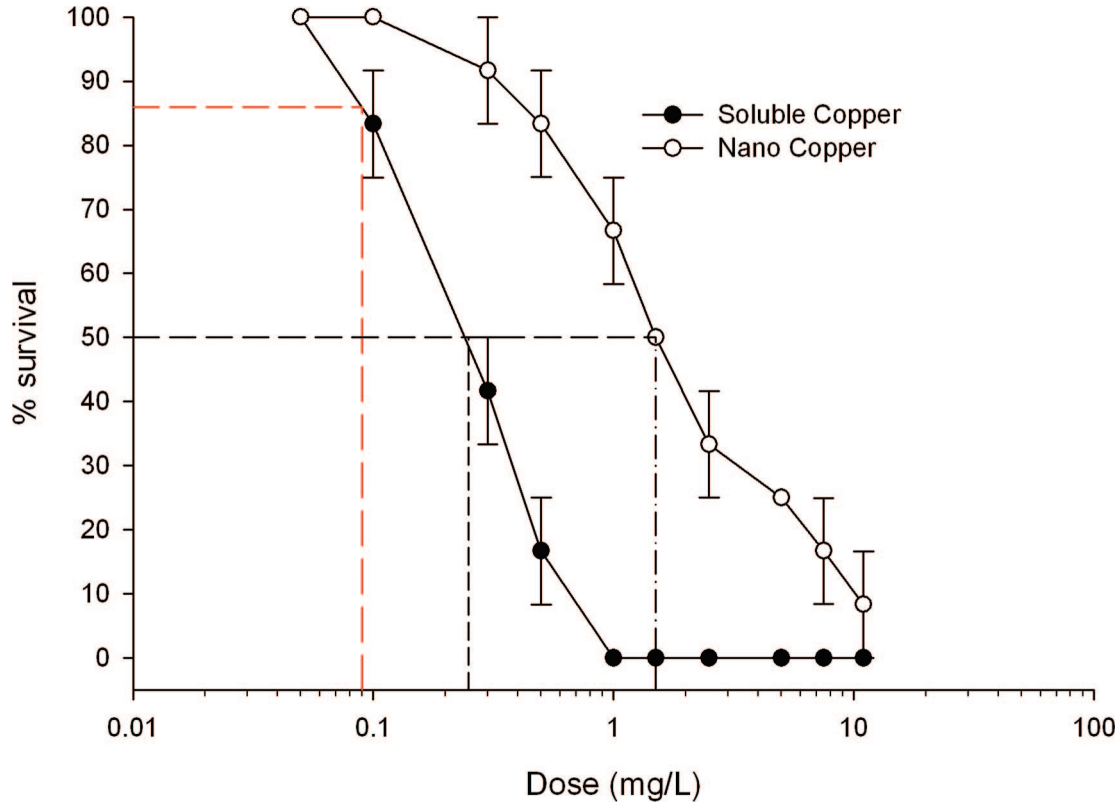

FIGURE 2. Toxicity of nanocopper (○) and soluble copper (•) to adult zebrafish. Data points indicate percent survival (mean ± SEM of three replicate experiments). Dashed black drop lines indicate LC_{50} concentrations for each exposure, as calculated by Trimmed Spearman Karber analysis. Red drop line indicates concentration and associated mortality of dissolved copper present in 1.5 mg/L nanocopper exposures, showing that the concentration of dissolved copper present in an LC_{50} nanocopper exposure is insufficient to explain the observed mortality.

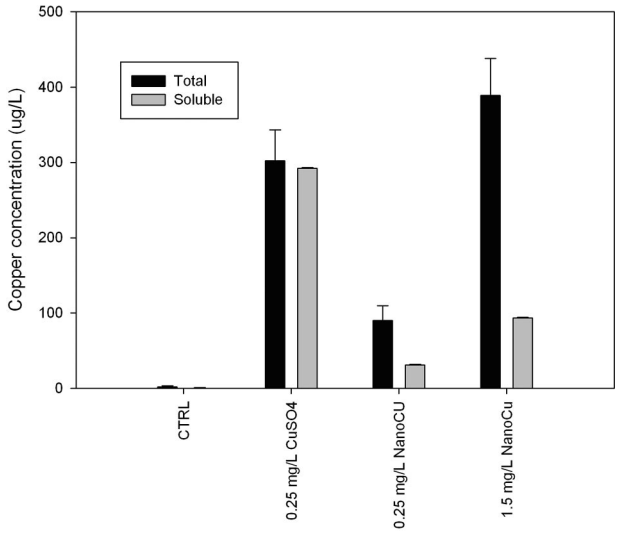

FIGURE 3. Dissolution and water column retention of copper nanoparticles after 48 h in zebrafish nanocopper exposures. Total and soluble copper concentrations were measured at 48 h in zebrafish exposures. Data are mean ± SEM ($n = 3$).

analysis. Results were considered statistically significant for $p < 0.05$. QPCR gene expression analysis was performed using the BioRAD iQ5 software. Means and standard deviations for fold change for each gene were calculated as the average of five individuals using the $\Delta\Delta C_T$ approximation corrected for primer efficiency and normalized to 18S expression values. Statistical significance was assessed by Student's *t* test, with a *p*-value <0.05 denoting significance. For microarray data, differentially expressed genes were identified by performing a one-way ANOVA with treatment as the single factor. Significance was assigned at a *p* value of 0.05, with false detection rate (FDR) multiple test correction. Genes identified as significant were clustered in JMP using the Fast Ward two-way clustering algorithm.

Results

Particle Characterization. In the bulk powder, nanocopper particle density was measured as 8.96 g/cc, and specific surface area was measured to be 30.77 m^2/g. Zeta potential in deionized water was −0.69 mV. Initial particle size analysis indicated a biphasic size distribution, with broad peaks centered around 80 and 450 nm diameter.

Following suspension at high concentrations in moderately hard freshwater, copper nanoparticles aggregated rapidly and much of the mass of added copper was present as agglomerates greater than 1 μm in diameter (Figure 1a and c). However, many particles with a diameter less than 100 nm were present in the water column for at least 48 h (Figure 1b and d), indicating that potential for exposure to copper nanoparticles was present for the course of the 48 h exposure. Zeta potential of the particles did not change appreciably over time, staying between 0 and +6 mV (data not shown). Particle dissolution was minimal over 48 h as concentrations of dissolved copper never exceeded 0.19 ± 0.05 mg/L, representing <0.1% of the initial copper added as particulate (270 mg/L) in the absence of fish.

Acute Toxicity Evaluation. *Acute Mortality.* Copper sulfate was highly toxic to female zebrafish, with a 48 h static LC_{50} of 0.25 mg Cu/L (95% CI = 0.16 – 0.33 mg/L), while nanocopper was moderately toxic, with a 48 h LC_{50} of 1.56 mg nanocopper/L (95% CI = 0.79 – 3.08 mg/L) (Figure 2).

Exposure Characterization. Dissolution and settling of particulates occurred during zebrafish exposures. Soluble

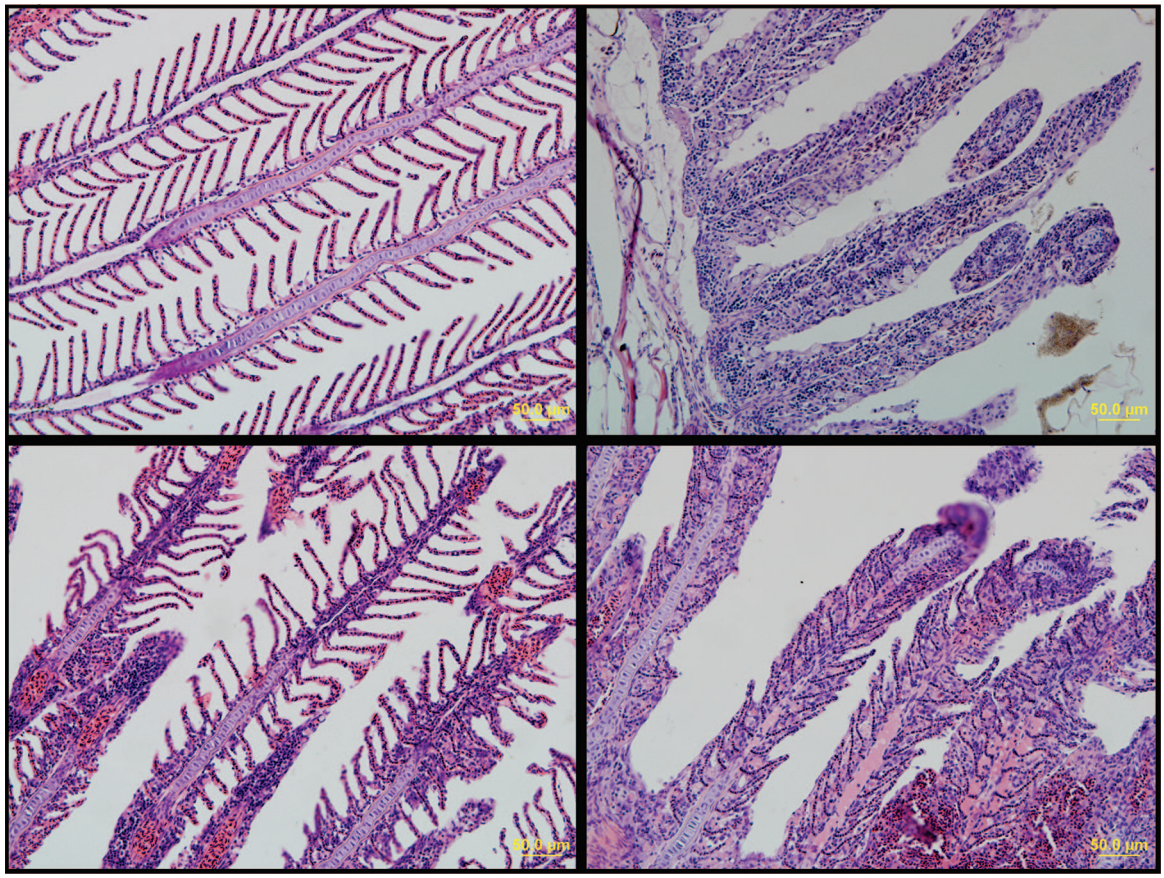

FIGURE 4. Micrographs showing gill injury induced by 48 h copper exposure. Soluble copper and nanocopper induced dramatic changes in gill morphology. Clockwise from top left: Control, 0.25 mg/L Soluble Cu^{2+}, 1.5 mg/L nanocopper, 0.25 mg/L nanocopper.

copper concentration after 48 h of 0.03 ± 0.01 mg/L in the low nanocopper exposures, and 0.09 ± 0.01 mg/L in the high nanocopper exposures. Based on this data, 12 and 6% of the initial mass of copper, respectively, dissolved over the course of the experiment. In both nanocopper exposures, only ~40% of the added mass of copper remained in the water column after 48 h (Figure 3).

Histological Analysis. Exposure to copper sulfate and nanocopper suspensions caused damage to gill lamellae characterized by proliferation of epithelial cells as well as edema of primary and secondary gill filaments. Effects of nanocopper suspensions were dose dependent, with significantly greater damage observed at higher concentrations (Figure 4). No significant histological evidence of injury was observed in major internal organs, though all fish had some degree of vacuolation of the liver. There was no hepatocellular necrosis and eosinophilic vacuolation was minimal and no significant difference between livers in the control and treated fish were detected (data not shown).

Biochemical Analysis of Toxicity. Gill NKA activity was monitored as a marker of gill function. Exposure to 0.25 mg Cu/L as copper sulfate decreased NKA activity by 88% (Figure 5a). Exposure to nanoparticulate copper decreased Na^+/K^+-ATPase activity by 29 and 58% in the low and high nanocopper exposures, respectively. Liver and kidney injury were assessed by monitoring plasma alanine aminotransferase (ALT) and blood urea nitrogen (BUN) levels, respectively. Copper sulfate and high nanocopper exposure elevated plasma BUN levels, though changes were not significant (Figure 5b). Plasma ALT levels were not significantly affected by any copper exposure (Figure 5c). This data suggests that gill function was impaired while there was little or no functional impairment of either liver or kidney by these copper exposures and is consistent with histological findings.

QPCR Results. Transcription of stress and metal responsive genes in the gill were markedly different following exposure to copper sulfate and nanocopper, and indicated that the transcriptional response elicited by stressful exposure to soluble and nanocopper were divergent. Expression levels for all genes surveyed were expressed to higher levels in nanocopper exposed fish than in copper sulfate exposed fish. HIF-1 (fold-induction 22.24 ± 4.8), HSP-70 (13.9 ± 3.2), and CTR (11.5 ± 3.2) were significantly upregulated by 1.5 mg/L nanocopper exposure ($p < 0.05$). In soluble copper exposures, those values were 0.67 ± 0.34 (HIF-1), 1.75 ± 0.95 (HSP-70), and 1.08 ± 0.54 (CTR). No significant gene expression differences were observed between the soluble and nanocopper treatments in the liver (data not shown).

Detailed Analysis of Gill Toxicity. *Exposure Characterization.* Total copper concentrations measured in the center of the water column were 57.2 ± 7.5, 49.5 ± 8.2, and 49.4 ± 3.0 ug/L at 2, 24, and 48 h, respectively. At these times, the concentration of soluble copper in the nanoparticulate exposures was 7.4 ± 0.9, 11.2 ± 1.1, and 13.3 ± 1.7 ug/L, respectively. Soluble copper exposures were well matched to the soluble contribution in nanocopper exposures. At 2, 24, and 48 h the concentration of soluble copper in the copper sulfate tanks was 7.6 ± 1.9, 9.9 ± 0.5, and 10.6 ± 2.8 ug/L, respectively.

Gill Metal Content. Gill copper levels were 5.1 ± 0.9 ng/mg in control fish, 9.1 ± 1.3 ng/mg in fish exposed to soluble copper, and 9.7 ± 2.1 ng/mg in zebrafish exposed to nanocopper. Both soluble and nanocopper exposures were

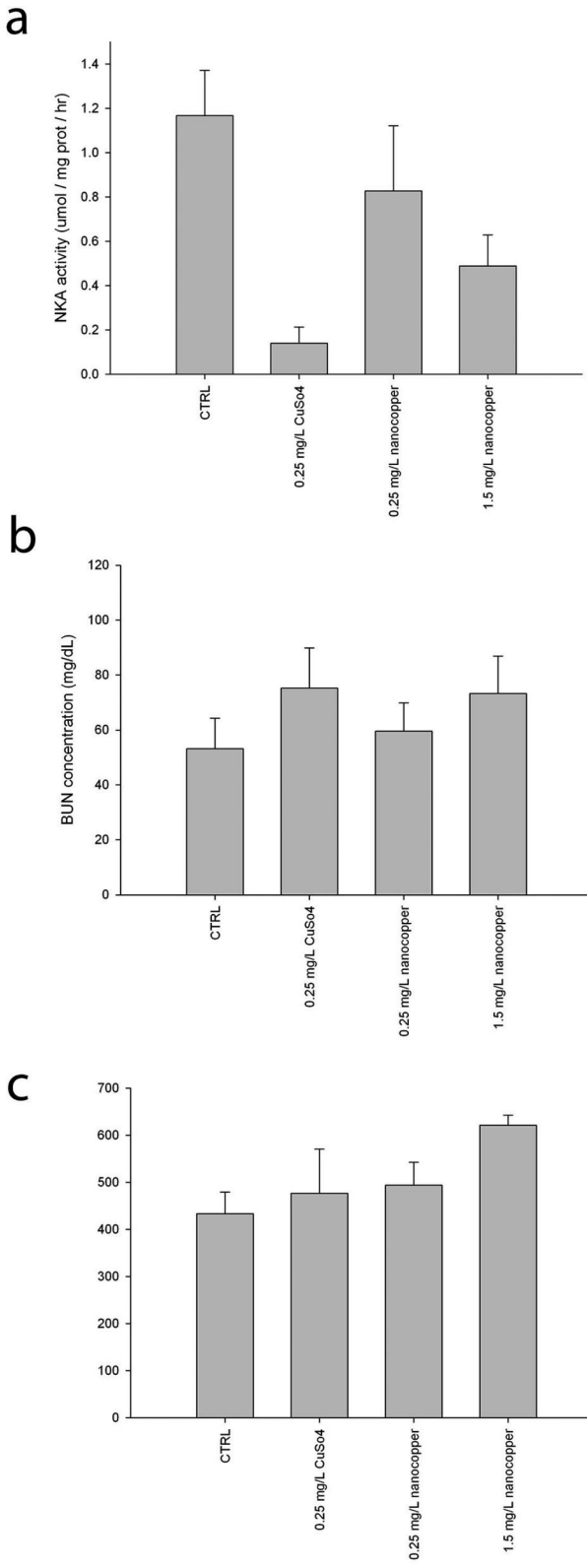

FIGURE 5. Biochemical indicators of toxicity following exposure to copper. (a) Gill Na^+/K^+-ATPase activity. (b) BUN activity (plasma). (c) plasma ALT activity. Data are mean ± SEM ($n = 4$).

significantly increased from control levels; there was no significant difference between soluble and nanocopper exposures.

Gill Histopathology. When exposed to equivalent concentrations of dissolved copper, nanocopper produces 40% greater proliferation or hypertrophy of epithelial cells in the interlamellar region of the gill. After a 48 h exposure to copper nanoparticles or soluble copper, filament widths increased 3- and 1.9-fold, respectively, compared to control (Figure 6).

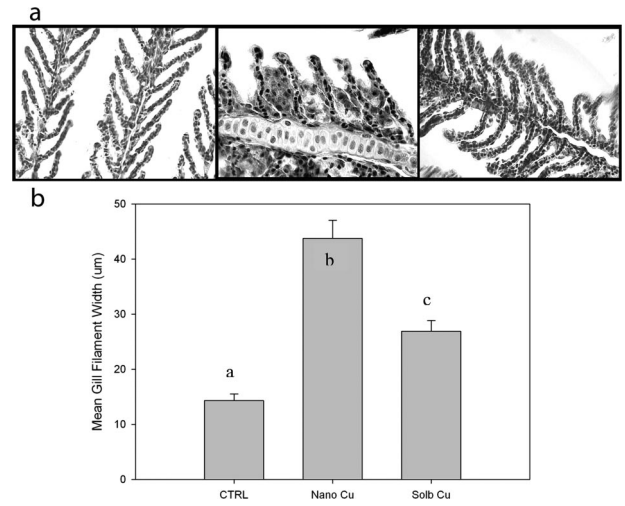

FIGURE 6. Gill injury induced by 48 h exposure to sublethal nanocopper exposures. (a) left to right: Control, nanocopper, soluble copper. (b) Gill filament widths following exposure to sublethal concentrations of soluble or nanocopper. Values are mean ± SEM ($n = 5$). Bars sharing the same letter are not significantly different from each other.

Microarray Results. 48 h exposure to sublethal concentrations of copper resulted in 82 genes identified as significantly differentially expressed. Cluster analysis of these genes demonstrated that the transcriptional response induced by nanocopper was highly divergent from that generated by only the soluble fraction of nanocopper exposures (Figure 7).

This indicates that the response was largely driven by nanoparticulate copper rather than by dissolution releasing soluble copper ions.

Discussion

The results of this study demonstrate that copper nanoparticles are acutely lethal to zebrafish. The observed 48 h LC_{50} of nanocopper would be classified as moderately toxic by the U.S. Environmental Protection Agency and is much lower than those reported for titanium dioxide particles in daphnia (*3, 16*), single walled nanotubes (SWNT) in copepods (*17*), or water-stirred fullerenes in fathead minnows (*18*). This is consistent with a previous report demonstrating that oral exposure to copper nanoparticles causes toxicity in rodents (*19*) and suggests that release of copper nanoparticles into the environment may pose a risk.

The gill was the primary target of acute toxicity caused by copper nanoparticles. Exposure to copper nanoparticles caused inhibition of Na^+/K^+ ATPase activity and caused proliferation of interlamellar cells (Figures 4 and 5), while no histological or biochemical evidence for damage to other organs was observed. Exposures that caused severe gill damage were associated with somewhat elevated plasma BUN levels (Figure 5). This is likely due to the role of gills in excretion of nitrogenous compounds from the blood, which would be impaired by the observed damage and not indicative of renal damage (*20*). However, the gill is also the primary target of dissolved copper which is known to inhibit Na^+/K^+ ATPase activity resulting in ionoregulatory toxicity (*21, 22*). Previous work has also demonstrated that exposure to high levels of soluble copper (>100 μg/L) are associated with proliferation of chloride cells (*22*). Therefore, it is critical to ascertain if toxicity is due to dissolution or to the particles themselves.

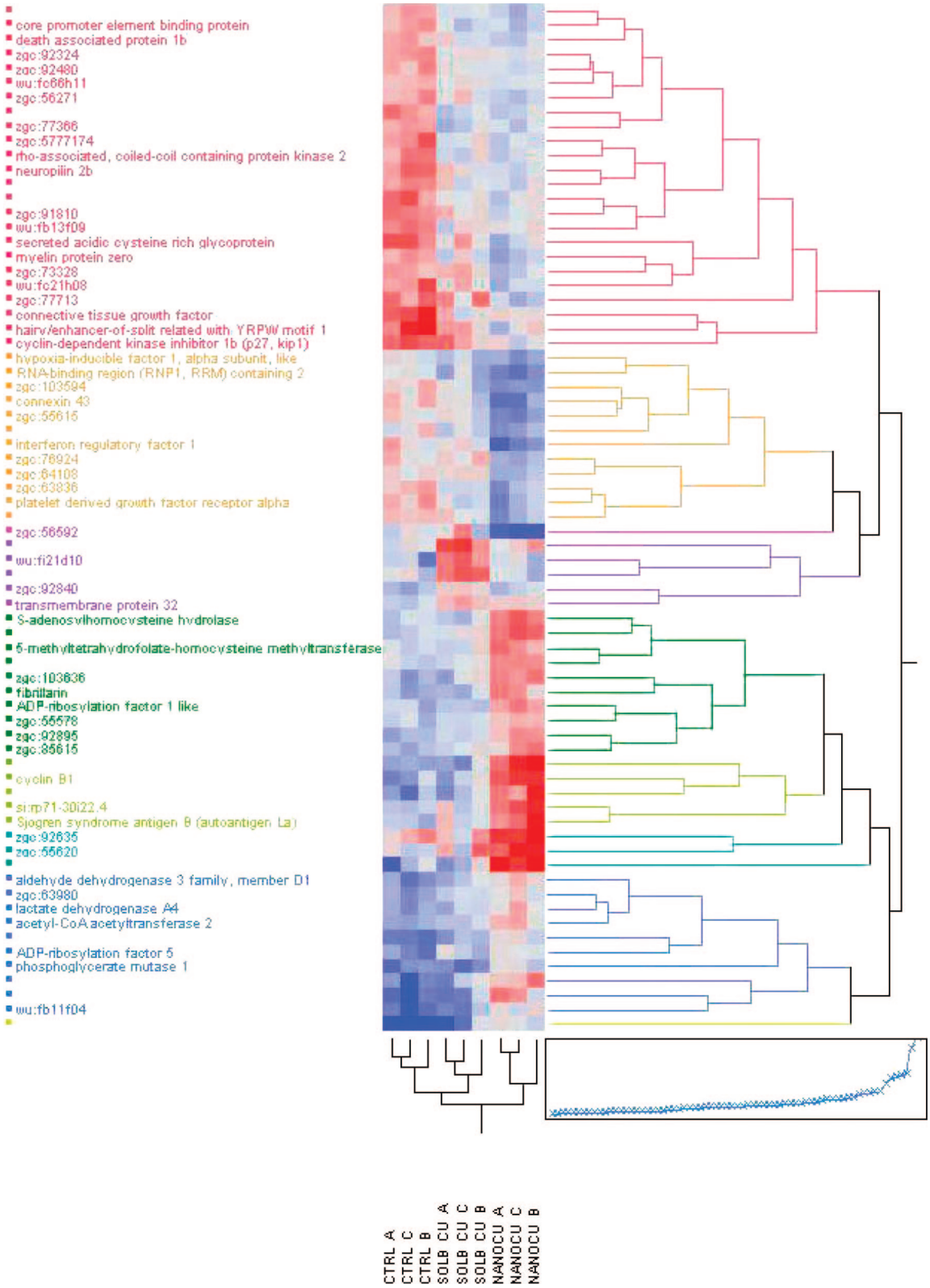

FIGURE 7. Hierarchical cluster analysis of significantly differentially expressed genes following exposure to sublethal concentrations of soluble or nanocopper. Gene expression values were clustered using Fast Ward hierarchical two-way clustering in JMP Genomics 3.0. Red indicates gene expression in the sample is greater than the pooled reference; blue indicates expression in the sample is less than the pooled reference.

Our data demonstrates that dissolution of copper nanoparticles does occur, but during exposure to the LC_{50} concentration of copper nanoparticles (1.5 mg/L) only 0.09 mg/L of dissolved copper was present (Figure 3). Based on the toxicity of soluble copper, dissolution accounts for only 16% mortality (Figure 1), leaving the majority of the observed mortality unexplained and suggesting that particles are causing toxicity by a route other than dissolution. The degree of Na^+/K^+ ATPase inhibition caused by the LC_{50} concentration of nanocopper was significantly

lower than that caused by an equally toxic concentration of soluble copper (Figure 5a), also suggesting that dissolution of particles is not solely responsible for toxicity. Further evidence that nanocopper particles have an effect separate from dissolution is provided by the preliminary transcriptional data, where soluble copper produced little effect on the expression of selected genes in the gill while exposure to 1.5 mg/L nanocopper produced significant induction of HIF-1, HSP-70, and CTR.

By comparing the responses of fish exposed to nanocopper, which contains both particulate and soluble copper, with those exposed to soluble copper concentrations that matched the dissolution profile of nanocopper, it is possible to isolate the effects of nanocopper. Under these conditions, it is clear that nanocopper produces morphological (figure 6) and transcriptional effects (figure 7) that are not solely due to dissolution.

The mechanism responsible for the effects of nanocopper are unclear, though they do not appear to be mediated by increased gill uptake of nanocopper as gill copper levels were similar following exposure to nanocopper and its soluble fraction. While total copper levels in the gill were similar, it is possible that nanocopper exposure results in uptake of particulate copper as gills have been shown to take up silica nanoparticles in medaka (*23*). It is also possible that copper particles induce oxidative stress gills as both HIF-1 and HSP70 are induced by oxidative stress in fish (*24*) and copper particles catalyze production of reactive species at their surface (*25–27*).

This study highlights the need to characterize nanoparticle suspensions under conditions that mimic exposure, which should closely mimic the conditions an organism is likely to see in the environment (*28, 29*). The presence of fish in tanks markedly increased the dissolution of copper, perhaps due to ingestion. When suspended in natural water, much of the mass of copper nanoparticles aggregated into larger, micron-sized particles that sedimented rapidly such that only 40–50% of the mass of added particles was present in suspension between 2 and 48 h after addition to tanks. The aggregation is likely driven by the low zeta potential of copper particles and the presence of divalent ions in natural water (*30, 31*). Despite aggregation, large numbers of monodispersed particles or small aggregates with a mean diameter of 50.2 $\pm$ 31.4 nm remained in suspension for at least 48 h. As our data indicates that there is a significant effect of nanoparticulate copper, it becomes important to determine appropriate dosimetry parameters (e.g., mass vs particle number vs particle surface area). Because these parameters change throughout the exposures and toxicity occurs at particle concentrations too low to be accurately characterized by existing instrumentation, appropriately expressing nanoparticulate exposure is problematic and must be addressed.

In conclusion, we have characterized the toxicity and behavior of nanoparticulate copper in an environmentally relevant aqueous exposure setting. We have shown (1) that nanocopper is acutely toxic to zebrafish, (2) that the toxicity appears to occur primarily at the gills, and (3) that this toxicity is not adequately explained by dissolution of the particles alone. We have also demonstrated that while significant aggregation occurs, a large number of particles <100 nm remain in suspension providing ongoing exposure to nano-sized particles. We conclude that copper nanoparticles exert a toxic effect on zebrafish gills separate from the well-understood effects of soluble copper. This research highlights the need for integrated toxicological assessment and suggests that existing regulations for soluble copper may not adequately address the safety concerns associated with metallic nanoparticles.

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Supporting Information Available

Additional experimental details and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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