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Differential Sensitivity of Wetland-Derived Nitrogen Cycling Microorganisms to Copper Nanoparticles

Vincent C. Reyes,[†] Phillip B. Gedalanga,[†,‡] Nancy Merino,[†,§] Joy D. Van Nostrand,[⊥] Scott P. Keely,[∥] Susan K. De Long,[¶] Jizhong Zhou,[⊥,⊗,+] and Shaily Mahendra*[,†,∞,#]

[†]Department of Civil and Environmental Engineering, University of California, Los Angeles, Los Angeles, California 90095, United States

[‡]Department of Health Science, California State University, Fullerton, California 92834, United States

[§]Earth-Life Science Institute, Tokyo Institute of Technology, Ookayama, Meguro-ku, Tokyo 152-8550, Japan

[⊥]Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, Oklahoma 73072, United States

[∥]National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268, United States

[¶]Department of Civil and Environmental Engineering, Colorado State University, Fort Collins, Colorado 80523, United States

[⊗]Earth Science Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

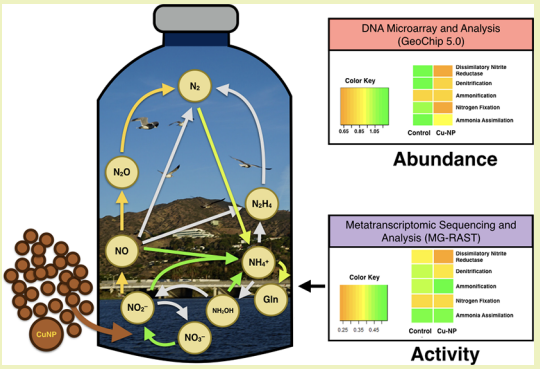
[+]State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China

[#]Institute of the Environment and Sustainability, University of California, Los Angeles, Los Angeles, California 900095, United States

[∞]California NanoSystems Institute, University of California, Los Angeles, Los Angeles, California 900095, United States

Supporting Information

ABSTRACT: Metallic nanoparticles (NPs), the most abundant nanomaterials in consumer and industrial products, are the most probable class to enter and potentially affect the environment. In this study, wetland-derived microcosms were incubated with copper nanoparticles (Cu-NP) and ionic $CuCl_2$ to investigate acute (10 days) and chronic (100 days) exposures to nitrogen cycling microorganisms. Gene abundance and expression changes were monitored using the GeoChip 5.0 high throughput functional gene microarray and metatranscriptomic sequencing (RNA-seq), respectively. After 10 days, the Cu-NP impacted microbial communities experienced structural shifts within microorganisms associated with dissimilatory nitrogen reduction accompanied by lower nitrate removal as compared to the unexposed controls. By day 100, these differences were largely resolved and nitrate removal was similar to the unexposed control. Furthermore, the Cu-NP exposed microcosms tolerated copper and were more resilient and adaptive than the unexposed controls based on the abundance of copper oxidase (*cueO*), copper efflux (*cusC*), and bacterial adaptive response (*opuE, soxS, desR, baeS*) genes. These findings suggest that sudden influxes of Cu-NPs into wetland systems may impair nitrogen removal initially, but long-term microbial shifts and functional redundancy would promote the net flux of total nitrogen out of the wetlands.



KEYWORDS: *Nanoscale, Lagoon, Sewage, Anaerobic, Microbiome, Stress response*

INTRODUCTION

Copper nanoparticles (Cu-NPs) and other metallic nanoparticles (NPs) are the most utilized engineered nanoparticles in consumer and industrial products.[1] Because of their growing applications in antimicrobial coatings, electronics, textiles, cosmetics, wood additives, and ceramics, NPs will enter the environment through their intended uses and subsequent waste disposal.[2] NPs influence the microbial composition in natural environments, implying that NPs have the potential to alter microbially driven processes like carbon, phosphorus, sulfur, and nitrogen cycling.[3−5]

Managing the nitrogen cycle is recognized by the National Academy of Engineering as one of the Grand Challenges of the 21st century due to the staggering nitrogen cycle imbalances

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caused by human activity.[6,7] Nitrogen transformations in wetlands and in wastewater treatment plants are shaped by nitrifying and denitrifying microbial communities, while fertility of agricultural soils is controlled by nitrogen-fixing bacteria. Cu-NPs have the potential to exacerbate existing nitrogen cycle imbalances by differentially impacting microbial populations involved in these processes. For example, greater inhibition of denitrifying microorganisms compared to nitrifying microorganisms could lead to elevated levels of nitrate in surface waters. Previous research has shown that denitrifying microorganisms in wastewater-derived sludge were more sensitive (IC_{50} = 0.95 mg/L) than nitrifying microorganisms (IC_{50} = 26.5 mg/L) to copper salts.[8] Cu-NPs may have greater impacts than copper salts due to inherently higher catalytic activity resulting from increased surface to volume ratios.[9,10] Denitrifying bacteria impacted by Cu-NP may contribute to climate change given that nitrous oxide, a potent greenhouse gas, is an intermediate of denitrification.[11] Thus, branches of the nitrogen cycle that are sensitive to NPs need to be identified to facilitate proactive management strategies.

Previous research into NP impacts on nitrogen cycling microorganisms has mainly focused on pure cultures, while NP impacts on environmental microbial communities have yet to be adequately explored.[12−16] Within mixed microbial communities, microorganisms function differently because of synergistic growth, functional redundancy, exchange of nutrients, and fortuitous novel gene variants.[17] Further, the few studies examining NP interactions with mixed microbial communities have focused on engineered wastewater systems and wastewater-derived microcosms.[18−20] Thus, limited information exists regarding the influence of NPs on the health of other important ecosystems. One study has assessed the effects of Ag- and Cu-NPs on freshwater wetland mesocosms, providing insight into the microbial community structure and composition over time.[21] However, NP impacts on the abundance and activity of specific microbial populations were not examined for wetlands.[21]

Wetlands play an essential role in balancing nitrogen. In rural and less developed areas, natural or constructed wetlands act as cost-effective alternatives to advanced wastewater treatment systems by removing excess nitrogen.[22,23] Biological nitrification and denitrification rates in wetland sediments have been recorded as high as 56.1 and 21.6 mg-N·kg^{-1} h^{-1}, respectively.[24] Further, wetlands play an important role in atmospheric nitrogen fixation. Estimates of nitrogen fixation rates for wetlands in the Florida Everglades are greater than 100 mg-N·m^{-2} d^{-1}.[25] Therefore, understanding how NPs may affect the growth and activity of specific wetland nitrogen cycling microorganisms is critical to understanding NP impacts on the local, as well as the global, nitrogen cycle.

High-resolution analysis of microbial ecosystems, their community structures, and functional activities has been facilitated by advanced molecular techniques including functional gene arrays and next-generation sequencing. These platforms have significant advantages over traditional culture-based strategies because of their ability to genetically probe nonculturable microorganisms. The GeoChip functional gene microarray allows for simultaneous and repeatable analysis of environmentally relevant processes, such as carbon, phosphorus, sulfur, and nitrogen cycling.[26−28] Metatranscriptomic sequencing offers the advantage of identifying expressed genes, providing a more direct predictor of metabolic activity.[29] Applications of metatranscriptomic sequencing to environmentally relevant systems remain limited. However, this approach has been used to elucidate the metabolic and biogeochemical responses of marine microorganisms to day/night cycles[30] and to investigate adaptation mechanisms and microbial stress responses in acid mine drainage.[31]

This study describes the sensitivities to Cu-NPs among nitrogen cycling microorganisms derived from a wetland ecosystem. A primary objective included determining the most sensitive nitrogen cycling processes to Cu-NP stress. Further, changes in microbial community composition as well as functional activities of nitrogen cycling communities at the gene expression level were assessed after 10 and 100 days of exposure to differentiate acute and chronic effects because previous studies report that NP toxicity changes over time.[32,33]

EXPERIMENTAL SECTION

Nanoparticles. Copper nanoparticles (99.9% pure) of 50 nm nominal size were obtained from M. K. Impex Corp (Mississauga, ON, Canada). $CuCl_2$ (>99% purity) salt was used in parallel experiments to assess the contribution of Cu^{2+} ions to observed toxicity. Prior to microcosm amendments, fresh copper stock suspensions (1000 mg/L as Cu) were prepared by mixing Cu-NPs into deionized water, followed by sonication in an ultrasonic bath for 30 min at maximum power (FS30H, Fisher Scientific, 100 W, 42 kHz). All stocks were sonicated for 1 min before use and diluted to their final concentration within 3 h of preparation.

The stability of Cu-NPs and $CuCl_2$ was characterized in the exposure medium, which was a 1:1 blend of basal salt growth solution and environmental water derived from a slurry mix collected from the Malibu lagoon (see Supporting Information for details).[34] Hydrodynamic diameters of the particles were measured and surface zeta-potentials were computed to assess colloid stability in the medium (see Supporting Information).

Microcosm Conditions. Microcosm seed water and sediment were obtained from the Malibu Lagoon (34.0333°N, 118.6792°W; Malibu Lagoon State Beach in Malibu, CA). The Malibu Lagoon and the accompanying beach have a history of elevated nutrient levels and fecal indicator bacteria.[35] As a result, indigenous microbial populations may have had previous exposure and resistance to metals and other antimicrobials from anthropogenic inputs. Samples were collected in August 2013 prior to sunrise to reduce UV induced mortality of endemic organisms. Water and sediment samples were extracted from the surface water column down to 0.3 m into the sediment. Water and sediment samples were placed immediately on ice before transport to the laboratory, where they were stored at 4 °C, and used within 6 h. Samples contained less than 2 mg/L of nitrogen as ammonium, nitrate, or nitrite which was near the limit of detection for the ammonium assay (Hach, Loveland, CO, USA).

Microorganisms within the wetland slurry samples were enriched for a period of 10 days prior to the start of the microcosm study. This enrichment period was implemented to allow for growth of mesophilic microorganisms. For each microcosm, 25 mL of slurry was mixed with 25 mL of a basal salt solution (see Supporting Information for details) in butyl-rubber stopper sealed sterile 100 mL serum bottles.[36] Microcosms were flushed with N_2 gas to promote anaerobic conditions and incubations were carried out in a stationary incubator for 10 days at 30 °C in the dark to prevent photosynthesis from occurring in the microcosms prior to introduction of either Cu-NPs or $CuCl_2$. Incubation at 30 °C was chosen to maintain selection of mesophilic microorganisms because many of these bacteria are important contributors to nutrient removal processes in wetlands.[37−39] Samples were collected after the 10th day of enrichment and analyzed for ammonium, nitrite, and nitrate concentrations resulting from the basal salt solution and any potential microbial transformations occurring in this period.

Triplicate microcosms exposed to Cu-NPs or $CuCl_2$ (100 mg·L^{-1} as Cu) were established for 0-, 10-, and 100-day incubation periods to study acute (10 days) and chronic (100 days) effects of Cu-NPs and

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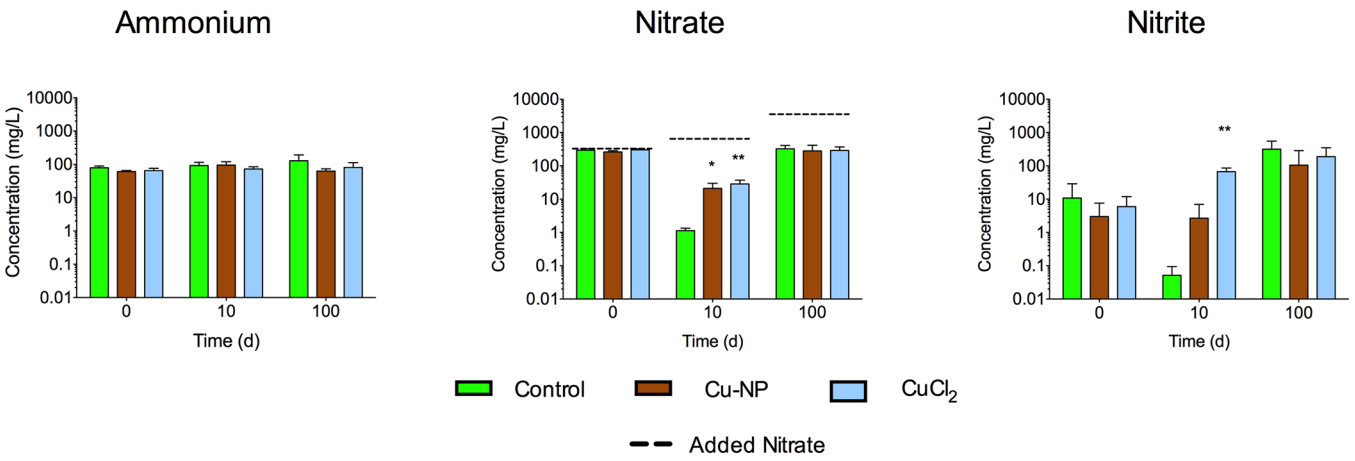

Figure 1. Concentrations of various nitrogen species during microcosm incubations. Only nitrate and nitrite levels were significantly higher at 10 days for Cu-NP and $CuCl_2$ exposed microcosms. After 100 days, however, nitrate and nitrite were not different from the control. Ammonium levels did not change over the assessment period. Experimental conditions were tested in triplicate, and asterisks indicate significance levels where (*) $P < 0.05$ and (**) $P < 0.01$. Error bars represent the standard deviation of biological triplicates samples.

$CuCl_2$. Every 10 days, microcosms were flushed with N_2 to promote anaerobic conditions and amended with sucrose (100 mg·L^{-1}) and nitrate (350 mg·L^{-1}) to replenish carbon and nitrogen, respectively. These amendments changed microcosm volumes by <10% throughout the experiment. Triplicate control microcosms were established following the above procedures without the addition of Cu-NPs or $CuCl_2$.

Sample Processing. At every sampling event, a replicate bottle was sacrificed for analysis. Each 50 mL replicate was centrifuged at 7,000*g* for 8 min to collect biomass and sediment. Supernatants were filtered (0.2 μm filter) and analyzed for water quality parameters (see Supporting Information) including ammonium, nitrite, and nitrate. Genomic DNA and RNA were extracted from pellets using the Powersoil DNA Isolation kit and Powersoil Total RNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's instructions. DNA and RNA were subjected to strict standards prior to downstream analyses (see Supporting Information).

DNA Microarray Hybridization, Scanning, and Data Processing. GeoChip 5.0 (Glomics, Norman, OK) was used for relative quantification of functional microbial populations. GeoChip 5.0 contains a total of 167 149 probes for genes involved in core biogeochemical cycles, xenobiotic degradation, metal homeostasis, antibiotic resistance, stress response as well as viral and protist genes and other categories. Additionally, GeoChip 5.0 has 6493 probes for genes involved in nitrogen cycling and phylogeny is assigned based on the probe sequence, as previously described.[26,27,40]

The specific nitrogen cycling gene targets analyzed in this study are listed in Table S5. Total DNA (800 ng) from each sample was labeled with CY3 and used for hybridization to the GeoChip 5.0, as previously described[27,28] (see Supporting Information for more details). Data normalization and quality filtering were performed as previously described.[41] Before statistical analysis, relative abundance was calculated for all spot signals. GeoChip sample processing was performed at the Institute for Environmental Genomics at The University of Oklahoma. Raw data are available at the institute's Web site (http://ieg.ou.edu/4download/).

Statistical Analysis. Shannon-Weiner's diversity index (H′) and alpha-diversity were calculated to evaluate the functional diversity of each treatment. H′ and alpha diversity were based on GeoChip data[26] and RNA-seq data, respectively. Principal component analysis (PCA) was used for comparing microbial communities among conditions to characterize overall structure differences between groups.[27] Both functional gene diversity analysis and PCA were performed using the vegan package in R 2.9.1 (www.R-project.org). Statistical differences were analyzed using analysis of variance (ANOVA) with a Bonferonni post-test. A significance level of $P < 0.05$ was adopted for all comparisons. Hypothesis testing was performed in GraphPad Prism 4.0 (La Jolla, CA).

Metatranscriptomic Sample Preparation, Sequencing, and Analysis. Total RNA was processed using the MicrobExpress kit (Ambion) to enrich mRNA. Metatranscriptomic libraries were prepared using the Ion RNA-Seq V2 Library (Life Technologies) for sequencing on a Life Technologies Ion Proton System using the Ion Seq 200 kit (Life Technologies) by the Colorado State University Next Generation Sequencing Core. The raw high-quality sequences were submitted to Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST)[42] for automated quality control, transcript identification, taxonomic assignment, comparative data analysis, and annotation using SEED Subsystems (level 3). Transcript abundance count values were normalized using MG-RAST to properly mitigate potential experimental and statistical sampling bias and allow comparisons among all gene responses.

RESULTS AND DISCUSSION

The impacts of Cu-NPs and ionic $CuCl_2$ on nitrogen cycling in wetland-derived microcosms were determined after acute and chronic exposures. Insights emerged from the collective analysis of water quality characteristics, including changes in nitrogen speciation (Figure 1), gene abundance (GeoChip 5.0), and gene expression (RNA-seq) (Figure 2). Quantitative and mechanistic data are provided to support eco-responsible design, applications, and disposal of metallic NPs.

Impact of Cu-NP on Water Quality and Nitrogen Species. To determine whether Cu-NPs or $CuCl_2$ altered the biogeochemistry of nitrogen cycling environments, water quality characteristics were measured (Figure 1 and Table S2). In general, pH and conductivity did not vary significantly among copper sources and the unexposed control throughout the 100 days. Interestingly, despite the introduction of 100 mg·L^{-1} of total copper as either Cu-NPs or $CuCl_2$, total dissolved copper did not significantly increase throughout the 100 days (Figure S1). Initially (day 0), dissolution of copper was minimal from Cu-NPs (2.8 ± 4.5 mg·L^{-1}) and $CuCl_2$ (8.3 ± 6.7 mg·L^{-1}), and the total dissolved copper in solution decreased with time. The overall low aqueous copper concentrations indicate that copper ions rapidly bound other dissolved species or that rapid aggregation did not allow for substantial release of copper ions from Cu-NPs or $CuCl_2$. Copper was likely chelated by natural organic matter, such as fulvic and humic acids, which are common in natural waters.[43]

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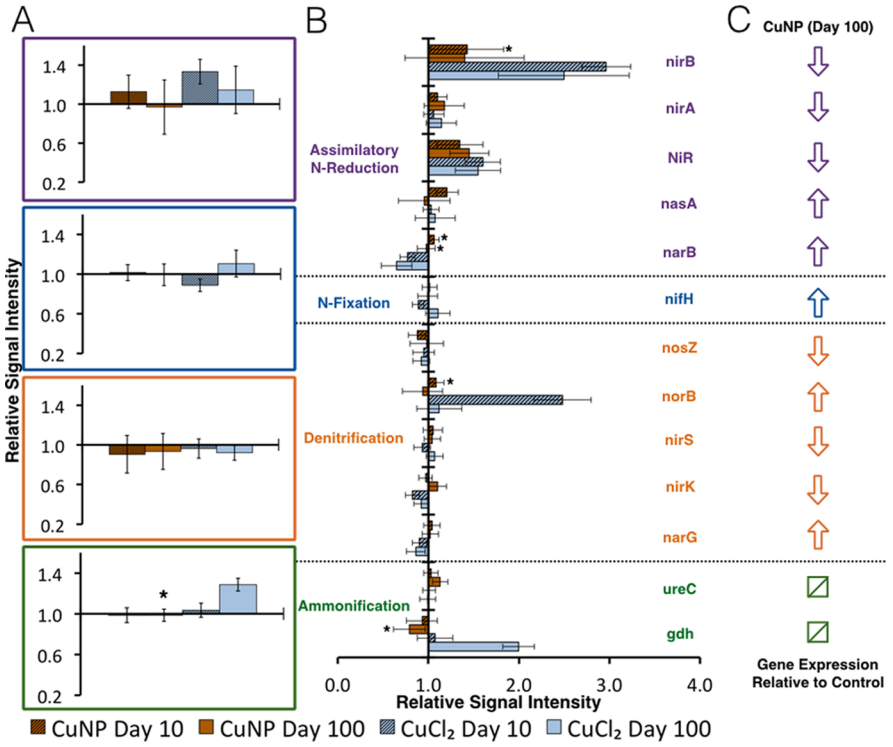

Figure 2. Relative abundances and expression for nitrogen cycling genes. Overall relative abundances (2A) and specific gene abundances (2B) for the Cu-NP and $CuCl_2$ treated microcosms on day 10 and 100 are represented as relative signal intensities from the GeoChip 5.0 microarray. Treatments were normalized against day 0, followed by the control at each time point. The top 5 gene functions for denitrification in panel B were selected based on the largest difference compared to the unexposed control. Gene expression (panel C) is represented as "up" (more normalized counts; more expression) and "down" (less normalized counts; less expression) arrows for the Cu-NP treated microcosms on day 100 compared to the control using the MG-RAST server (RNA-seq). Normalized counts are shown in Figures S8–S12. Squares represent no equivalent gene observed on the MG-RAST server. Error bars represent the standard deviation of triplicate samples, and asterisks indicate significant differences ($p < 0.05$) between the Cu-NP and $CuCl_2$ treated microcosms at the time point. Relative abundances and expression for all nitrogen cycling genes can be found in Table S5 and Figures S5–S9.

Similarly, others have observed low dissolution of copper ions from Cu-NPs in activated sludge.[3,18] Because copper is more stable in complexes with organic and inorganic ligands as well as in active sites of some enzymes, and can affect binding and free ion concentrations of other transition metals, the biologically essential metal iron (Fe) also was tracked. No significant changes were observed for total dissolved Fe over 100 days (Figure S1).

Ammonium, nitrate, and nitrite concentrations were measured to determine impacts on nitrogen cycling. For ammonium, no significant differences were observed between the unexposed control microcosms and the exposed microcosms for either copper source (Cu-NP or $CuCl_2$) (Figure 1). However, nitrate and nitrite levels differed among copper treatments. Nitrate reduction was evident as nitrate concentrations were lower than the cumulative amount of nitrate added (350 $mg{\cdot}L^{-1}$ every 10 days) for all conditions. At 10 days, microcosms exposed to Cu-NPs and salts contained significantly more nitrate (21.1 ± 9.0 and 28.7 ± 8.6 $mg{\cdot}L^{-1}$, respectively) compared to the unexposed microcosms (1.1 ± 0.2 $mg{\cdot}L^{-1}$), confirming that both copper sources inhibited nitrate transformation ($P < 0.05$). In contrast, at 100 days, levels of nitrate among unexposed and copper-exposed microcosms were similar even though nitrate amendments were performed every 10 days. Measured concentrations of nitrate indicate 3175.3 ± 85.0 $mg{\cdot}L^{-1}$, 3219.3 ± 134.8 $mg{\cdot}L^{-1}$, and 3210.7 ± 80.7 $mg{\cdot}L^{-1}$ NO_3–N were transformed in the control, Cu-NP, and $CuCl_2$ microcosms incubated for 100 days, respectively. These findings verify that inhibition was not sustained after long-term exposure. Additionally, the nitrite levels in $CuCl_2$ exposed microcosms were significantly higher after 10 days (67.0 ± 19.2 $mg{\cdot}L^{-1}$) ($P < 0.05$) compared to the unexposed controls. Cu-NP exposed microcosms also had higher nitrite levels, although differences were not statistically significant. These data imply that Cu-NPs were less inhibitory than $CuCl_2$ toward nitrite transformation during short-term exposure. Thus, at the level of functional activity, nitrogen cycling microbial communities recovered from $CuCl_2$ as well as Cu-NP exposure, which is supported by the concentration of nitrogen species in microcosms incubated for 100 days.

Overall Microbial Community Structure and Biodiversity. Given the observed negative short-term impacts of $CuCl_2$ and Cu-NP exposure, and subsequent functional recovery, we sought to determine impacts on microbial community structure and biodiversity over time. GeoChip detected between 35 000 and 58 000 gene variants within the microcosm conditions tested (Table S3) and over 99% of sequences generated from RNA-Seq passed quality control on MG-RAST. Biodiversity was determined using GeoChip DNA data to calculate the Shannon-Weiner's Diversity Index (H'). H' values ranged from 9.31 to 9.79 (Table S3) for microcosms, similar to previous diversity estimations for environmental and wastewater treatment samples.[44] Microcosms exposed to Cu-NPs had significantly lower H′ values than unexposed controls for all time points ($p < 0.01$), while $CuCl_2$ treated microcosms were only significantly lower than controls after 100 days ($p <$

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0.001) indicating a delayed influence of copper ions on species diversity. Additionally, Cu-NP impacts were more immediate and observed for the duration of the study. These results illustrate that exposure to Cu-NP and $CuCl_2$ can influence the total number of species and/or the proportions of these species within a microbial community. Interestingly, alpha diversity calculated using RNA sequencing results indicated minimal changes occurred at the species level for the Cu-NP exposed (69.24) and unexposed controls (74.00) after 100 days of incubation. The diversity based on RNA transcripts was similar to that of the unexposed controls supporting that Cu-NP exposure had a greater impact on abundance within species rather than impacting the total number of species. Taken together, these findings illustrate a lasting impact on the microbial community.

Shifts in the overall microbial community structure were observed for both acute and chronic exposure to Cu-NPs and $CuCl_2$ via principal component analysis (PCA) of GeoChip data (Figure S3). Acute exposure to copper did not strongly impact microbial community structures, as 10-day exposed microcosms grouped with 0-day exposed microcosms for all conditions. In contrast, chronic exposure led to more distinct community structures, as all 100-day microcosms were in different PCA quadrants than their respective 0- and 10-day microcosms. Interestingly, 100-day exposure to both Cu-NP and $CuCl_2$ led to community structures that grouped together and were both in a different quadrant than the control at 100 days, verifying that the microbial communities were similarly influenced by nanoparticulate and ionic forms of copper. Cu-NP exposed microbial communities showed less change over time than $CuCl_2$ exposed communities, indicating Cu-NPs were less disruptive, potentially related to their lower initial dissolution than $CuCl_2$. However, dissolution rates were not quantified because there was no significant difference over time (Table S2 and Figure S1). Similar results were observed in other studies of freshwater wetland mesocosms exposed to CuO-NPs, CuS-NPs, and Cu^+ (as CuO).[21] Drastic changes in microbial community structure due to chronic exposures may reflect the initial shock to the microbial community by metal exposure followed by the rebound of microorganisms over time. Indeed, similar trends in the relative abundance of microorganisms carrying copper detoxification genes were observed in both Cu-NP and $CuCl_2$ exposed microcosms over time with acute exposures resulting in greater abundances compared to chronic Cu-NP and $CuCl_2$ exposures (Table S7). This finding suggests acute exposures selected for copper resistant microorganisms and this selection was muted in chronic exposures possibly due to a rebound in other microorganisms that were not necessarily metal resistant. PCA analysis further supports this as chronic exposures to both Cu-NP and $CuCl_2$ conditions resulted in GeoChip probe distributions in relative close proximity (Figure S3). Previous studies have also observed the resiliency of microbial communities against Ag-NPs[45,46] and fullerenes (C_{60})[47] in complex environmental systems.

Phylogenetic Changes in Nitrogen Cycling Bacteria. Phylogenetic changes in nitrogen cycling bacteria were evaluated using the GeoChip functional gene array. PCA analysis indicated the microbial communities were most different after 100 days of exposure (Figure S3). Thus, details of 100-day phylogenetic changes are highlighted in Figure S5. Proteobacteria were the most dominant phyla identified within nitrogen cycling bacteria. Firmicutes, Bacteroidetes, and Actinobacteria were also represented throughout all of the nitrogen cycling categories examined. These findings are consistent with a meta-analysis of microorganisms, which determined Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes, and Actinobacteria as the predominant phyla in wetland soils.[48] Furthermore, our results determined that microorganisms associated with nitrogen fixation, dissimilatory nitrogen reduction, and ammonification experienced phylogenetic changes after chronic Cu-NP or $CuCl_2$ exposure (Figure S5). For example, nitrogen-fixing Proteobacteria were significantly lower ($p < 0.05$) in Cu-NP exposed microcosms while nitrogen-fixing Firmicutes were significantly higher ($p < 0.05$) in $CuCl_2$ exposed microcosms compared to the unexposed controls. In contrast, microorganisms associated with assimilatory nitrogen reduction, denitrification, and nitrification had similar phylogeny abundances for all conditions (Figure S5). These findings indicate nitrogen cycling microorganisms exhibited disparate sensitivities to Cu-NP and $CuCl_2$, indicating a potential for Cu-NP or $CuCl_2$ resistant microbial populations. However, ecosystem services related to ammonium, nitrite, and nitrate cycling were not significantly impacted after 100 days (Figure 1), indicating changes in phylogeny did not impact the functional activities of a resilient microbial community.

Of the nitrogen cycling microorganisms evaluated after 100 days of exposure, denitrifying microorganisms showed the most robust overall response to Cu-NP and $CuCl_2$. Specifically, denitrifiers were the most abundant (46.3% of total nitrogen cycling probes detected; Table S4), showed minimal declines in relative signal intensity (Table S5), and displayed fewer shifts in microbial phyla. Indeed, significant phylogenetic shifts in the denitrifying microbial community were not observed after 100 days, indicating a high resiliency to long-term copper toxicity for this group of nitrogen cycling microorganisms.

Influence of Cu-NPs and $CuCl_2$ on the Relative Abundance and Activity of Nitrogen Cycling Genes. The impact of Cu-NP and $CuCl_2$ exposures on the abundance and activity of nitrogen cycling genes independent of phylogenetic classification is illustrated in Figure 2. Nitrogen cycling genes associated with assimilatory nitrogen reduction, nitrogen fixation, and denitrification responded similarly to Cu-NP and $CuCl_2$ exposures, whereas genes related to ammonification were significantly different depending on the copper source (Figure 2A). Specifically, 100-day incubation with $CuCl_2$ resulted in significantly different relative signal intensity compared to the 100-day incubation with Cu-NPs (Figure 2A). While few differences were identified for nitrogen fixation and denitrification, differences were generally higher for assimilatory nitrogen reduction in Cu-NP and $CuCl_2$ exposed conditions relative to the control. In particular, short-term exposure to $CuCl_2$ resulted in a statistically significant difference in the relative abundance of microorganisms capable of assimilatory nitrogen reduction compared to the unexposed control (Figure 2A; $P < 0.05$). Microorganisms associated with ammonification showed significant differential sensitivity to Cu-NP compared to $CuCl_2$ after 100 days (Figure 2A; $P < 0.05$). This result verifies that ammonifying populations were stimulated after long-term $CuCl_2$ exposure but not by Cu-NP exposure and indicates a resilient group of microorganisms were responsible for ammonification after exposure to $CuCl_2$. Indeed, a previous report determined increased ammonification in soil microbial communities receiving 100 mg-Cu·L^{-1}.[49]

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Interestingly, specific genes within these nitrogen cycling categories revealed increasing complexity associated with Cu-NP or $CuCl_2$ exposures. Genes encoding assimilatory nitrite reductases (*nirB* and NiR) showed higher relative abundances with Cu-NP and $CuCl_2$ exposures (Figure 2B). Specifically, short-term exposure to $CuCl_2$ resulted in significantly increased abundance of *nirB* compared to the unexposed control. While for denitrifying bacteria, microcosms incubated in the presence of $CuCl_2$ for 10 days resulted in significant enrichment ($p < 0.05$) of nitric oxide reductase genes (*norB*) highlighting the differential sensitivities of denitrifying microbial populations to acute Cu-NP and $CuCl_2$ exposures. Furthermore, the dissimilar sensitivities of microbial populations to short-term $CuCl_2$ exposure may explain the reduced capacity to remove nitrite compared to the unexposed controls (Figure 2).

The RNA-seq results showed impacts to gene expression after long-term exposure to Cu-NP compared to the control (Figure 2C). Within assimilatory nitrogen reduction genes, all of the detected nitrate reductases (*nasA* and *narB*) were upregulated while all nitrite reductases (*nirB*, *nirA*, and *NiR*) were down regulated. Additionally, the expression of specific genes, such as *norB* and *narG* were higher for the Cu-NP exposed microcosms compared to the controls (Figure 2C). Collectively, these genes are all involved in the dissimilatory and assimilatory conversion of nitrate to nitrite, respectively, and their relatively increased expression demonstrates the resiliency of the microbial community to resume nitrogen cycling functions after long-term Cu-NP exposure.

GeoChip microarray data imply exposure to Cu-NP did not significantly influence the number of nitrogen cycling microorganisms, as evidenced by relative gene abundances near 1.0 after 10 and 100 days (Figure 2A and Table S5), and RNA-seq analysis also corroborates minimal impacts at the level of expression (Figure 2C, Figures S8–S12). The SEED database includes subsystems, which are a collection of proteins grouped by a relationship in function.[50] The following level 3 SEED Subsystem nitrogen metabolism pathways were detected in both the control and Cu-NP exposed samples on day 100: allantoin utilization, ammonium assimilation, ammonification cyanate hydrolysis, denitrification, dissimilatory nitrite reductase, nitric oxide synthase, nitrogen fixation, and nitrosative stress. The control and Cu-NP exposed metatranscriptomes, respectively, contained a similar number of detected gene functions for allantoin utilization (control = 1, Cu-NP = 3), ammonium assimilation (control = 13, Cu-NP = 13), ammonification (control = 12, Cu-NP = 15), cyanate hydrolysis (control = 1, Cu-NP = 1), denitrification (control = 10, Cu-NP = 13), dissimilatory nitrite reductase (control = 6, Cu-NP = 4), nitric oxide synthase (control = 1, Cu-NP = 2), nitrogen fixation (control = 6, Cu-NP = 5), and nitrosative stress (control = 5, Cu-NP = 8). There were no significant differences between the control and Cu-NP for Subsystems containing 5 or more genes: ammonium assimilation ($p = 0.24$), ammonification ($p = 0.11$), denitrification ($p = 0.34$), dissimilatory nitrite reductase ($p = 0.17$) and nitrogen fixation ($p = 0.83$). However, statistical significance was observed for nitrosative stress ($p < 0.05$). These results indicate the microbial community experienced increased stress likely due to the presence of reactive nitrogen species, such as nitric oxide. Relative gene responses for ammonification, ammonium assimilation, denitrification, dissimilatory nitrite reductase, and nitrogen fixation confirm that wetland microbial communities exposed to Cu-NP primarily metabolized nitrogen by denitrification, ammonium assimilation and ammonification (Figures S8, S10, and S11). The genes for nitrogen fixation and dissimilatory nitrite reductase also were active in Cu-NP exposed samples, but there was no evidence for anammox or nitrification. However, the lack of detectable gene transcripts for anammox and nitrification was not surprising because the microcosms were maintained at conditions suitable for denitrification.

By day 100, no clear and significant stimulatory or inhibitory trends toward nitrogen cycling populations were observed for Cu-NPs relative to the unexposed controls. However, relative signal intensities were similar to unexposed controls (Figure 2A) and only subtle changes were observed in the expression of certain genes involved in nitrogen cycling processes in Cu-NP exposed microcosms (Figure 2C). These results imply that microorganisms were impacted by Cu-NPs, but the surviving microbial community after 100 days had generally recovered from Cu-NP exposure. Similar results have been shown previously with other engineered nanomaterials, such as Ag-NPs and fullerenes (C_{60}).[21,45−47]

Cu-NP and $CuCl_2$ Impacts on Other Functions in Wetland Microbial Communities. To elucidate the effects of Cu-NPs on other microbial functions, genes related to electron transfer, metal homeostasis, and stress response from the GeoChip microarray and RNA-seq data were also analyzed (Figures 3 and S13−S16). For RNA-seq data (Figure 3C), electron transfer functions were analyzed in the "Respiration" category on the MG-RAST server, and metal homeostasis functions were analyzed in the "Resistance to Antibiotics and Toxic Compounds" category in which metal resistance functions comprised 12 out of 18 total functions.

The resiliency and adaptability of microorganisms to excess copper over long-term exposure is further supported by the abundance and activity of electron transfer genes. While the abundance of overall electron transfer genes was generally lower than the control over time for both copper exposures (Figure 3A), these reductions were not statistically significant. Further, specific electron transfer genes were expressed more in the Cu-NP impacted microbial community, such as cytochrome and trimethylamine N-oxide (TMAO) reductase, on day 100 (Figures 3C and S12). These are either heme-copper or heme-iron containing oxidoreductases and may have benefited from the addition of Cu-NP.[3,18] Other genes encoding electron transfer enzymes, such as NiFe hydrogenase and various cytochromes (Figure 3B), were significantly more abundant in $CuCl_2$ treated microcosms by day 100 ($p < 0.05$). These genes previously have been shown to play a role in the nitrogen cycle, especially denitrification and ammonification.[51] For example, *c* type cytochromes are found in hydroxyl amine oxidoreductase and cytochrome *cd*1 nitrite reductase.[51] These electron transfer functions have been identified in other biogeochemical cycles, including the sulfur cycle,[52] and the coupling of the nitrogen and sulfur cycles has been documented previously.[53−55] The significantly higher relative signal intensities observed in $CuCl_2$ treated microcosms further demonstrates that select microorganisms, likely involved in ammonification (Figure 2A,B) and the sulfur cycle (Figure S13), withstood high copper concentrations and increased in abundance over 100 days.

Insights toward copper tolerance were revealed when analyzing genes related to metal homeostasis. These genes are related to metal uptake and efflux, and copper exposed

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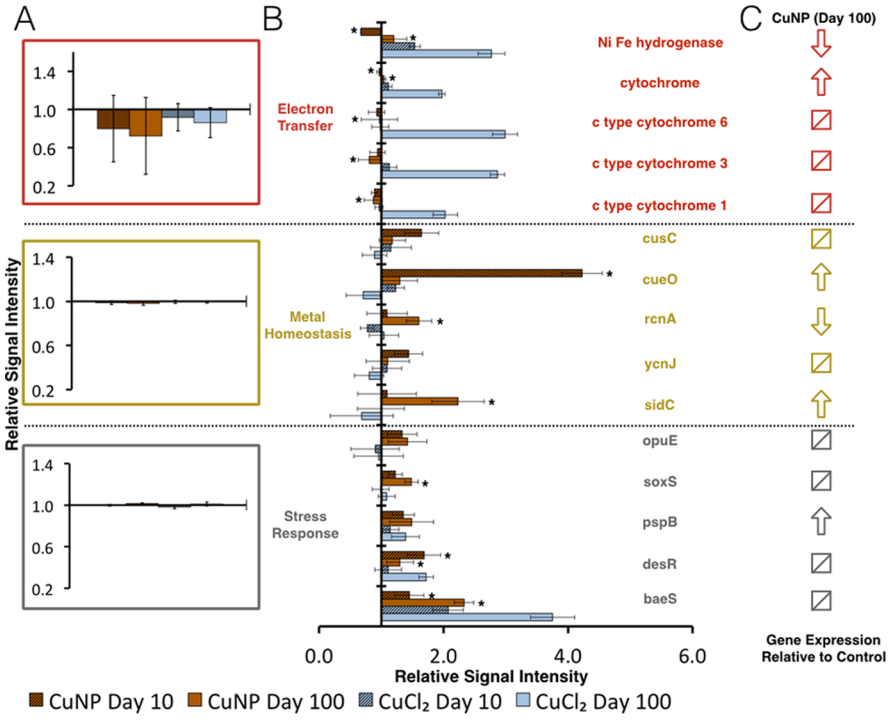

Figure 3. Relative abundances and expression of other functions. Overall relative abundances (panel A) and specific gene abundances (panel B) for the Cu-NP and $CuCl_2$ treated microcosms on day 10 and 100 are represented as relative signal intensities from the GeoChip 5.0 microarray. Treatments were normalized against day 0, followed by the control at each time point. Values for metal homeostasis ranged from ~0.98−0.99 and stress response values ranged from ~0.98−1.01. The top 5 gene functions for panel B were selected based on the largest difference compared to the unexposed control. Gene expression (panel C) is represented as "up" (more normalized counts; more expression) and "down" (less normalized counts; less expression) arrows for the Cu-NP treated microcosms on day 100 compared to the control using the MG-RAST server (RNA-seq). Normalized counts are shown in Figures S14−S16. Squares represent no equivalent gene observed on the MG-RAST server. Error bars represent the standard deviation of triplicate samples, and asterisks indicate significant differences ($p < 0.05$) between the Cu-NP and $CuCl_2$ treated microcosms at the time point. Relative abundances and expression for all electron transfer, metal homeostasis, and stress response functions can be found in Tables S6−S8 and Figures S14−S16.

microcosm incubations may have simultaneously selected for decreased metal importing genes and increased metal exporting genes. Such counterbalancing changes would make overall shifts in metal homeostasis appear neutral (Figure 3A). However, while the overall metal homeostasis category did not change in abundance compared to the control or over time, specific metal homeostasis-related genes showed changes in both abundance and expression (Figure 3B,C). Three metal homeostasis categories were selected to demonstrate the specific changes in abundance and expression over time: copper, cobalt, and nickel (Figure 3B). Copper-sensing and efflux genes, such as *cusC*, *cueO*, and *ycnJ*, (Figure 3B), were more abundant with Cu-NP exposure at day 10 relative to the $CuCl_2$ treated microcosms and the control. By day 100, the abundance of *cusC*, *cueO*, and *ycnJ* decreased. However, *cueO* was expressed higher in the Cu-NP treated microcosms relative to the control at day 100 (Figure 3C). These findings prove that Cu-NP impacted microbial communities maintained homeostasis and tolerated elevated copper concentrations (Figure S1), especially since *cueO* is the primary copper efflux system that is upregulated when excess copper is present.[56] The *ycnJ* gene was also upregulated when copper was present and is known to maintain essential levels of copper inside the cells.[57] However, *ycnJ* was not found on the MG-RAST server so discerning changes in *ycnJ* expression was not possible. Other metal homeostasis gene abundances were affected by the presence of copper, including *rcnA* and *sidC*. The *rcnA* gene maintains nickel and cobalt homeostasis while *sidC* maintains iron homeostasis and is closely linked to copper metabolism.[58,59] After 100 days, the Cu-NP treated microcosms had significantly ($P < 0.05$) higher abundances of both *rcnA* and *sidC* compared to the $CuCl_2$ treated microcosms and the control, and *sidC* expression was higher in the Cu-NP treated microcosms at day 100. The abundance and expression of these genes further demonstrate microbial community resiliency to Cu-NPs.

Additionally, selection for metal-tolerant strains may have occurred as several functions related to antibiotic resistance showed increased transcript abundance for Cu-NP exposed compared to the control at day 100 (Figure S14; Multidrug efflux system, methicillin resistance, and fluoroquinolones resistance). Exposure to heavy metals has been well documented to select for metal- and antibiotic-resistant microorganisms.[60] This potential for NPs to increase environmental reservoirs of antibiotic resistance warrants further investigation given the negative public health implications.

The copper-impacted microbial communities could tolerate high copper concentrations likely due, in part, to several stress response functions that help to maintain homeostasis. The abundance (Figure 3B) and expression (Figures 3C and S11) of stress response genes were generally higher or similar compared to the unexposed control, although no net changes in abundance over time were observed (Figure 3A). The overall stress response category on GeoChip contains 19 specific categories, including envelope stress, osmotic stress, cold shock, oxidative stress, and more. Five stress response

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genes with relatively different abundances than the control are shown in Figure 3B to demonstrate changes in abundance and expression due to Cu-NP and $CuCl_2$ exposure: *opuE*, *soxS*, *pspB*, *desR*, and *baeS*. Excess copper from Cu-NPs may have affected cell osmolality more than equivalent concentrations from $CuCl_2$ because *opuE* (osmoprotectant) was generally more abundant in Cu-NP treated microcosms. OpuE has previously been proportionally linked to environmental osmolality and is continuously produced if an osmotic stimulus exists.[61] However, by day 100, the expression of overall osmotic stress functions in the Cu-NP impacted microbial communities were similar to the unexposed control (Figure S15), suggesting that Cu-NPs did not have a sustained impact on cell osmolality. In contrast, *soxS*, an oxidative stress gene sensitive to superoxide-generating compounds,[62] was significantly more abundant in Cu-NP treated microcosms compared to the $CuCl_2$ treated and unexposed microcosms on day 100 (Table S8). Furthermore, overall oxidative stress functions were more expressed in the Cu-NP exposed microcosms compared to the control on day 100 (Figure S15). The higher abundance and expression observed in this study is similar to previous studies analyzing oxidative stress functions in relation to metal nanoparticle exposure.[63−67] In addition to osmotic and oxidative stress, envelope stress functions were observed to have higher abundance over time for both Cu-NP and $CuCl_2$ treated microcosms than for the unexposed control (Figure 3B). The *pspB* and *baeS* genes are envelope stress genes while *desR* is categorized as a cold shock gene and encodes a protein that modifies cell membrane composition.[68] Although *pspB* did not significantly change over time for either treatment, *baeS* was impacted by excess copper. The main role of *baeS* is to maintain envelope homeostasis by communicating with the efflux pump MdtABC to remove harmful compounds, and *baeS* can be induced by indole, flavonoids, and zinc.[69] However, the Cu-NP impacted microbial community had significantly less abundance of *baeS* compared to the $CuCl_2$ impacted microbial community (Figure 3B). The resulting enrichment of *baeS* in $CuCl_2$ exposed microcosms may signify reduced biodiversity, which is also observed in Table S3, further indicating that copper ions represent more chronic threats than Cu-NPs to the diversity of microbial communities.

This study demonstrated that acute exposure to Cu-NPs may negatively impact the microbial community of wetlands, but over time, the microorganisms are able to adapt and recover from exposure to Cu-NPs. After acute exposure of wetland microcosms to Cu-NPs, nitrate and nitrite concentrations were elevated (Figure 1), indicating that sudden Cu-NP influxes may be problematic for wetlands in the short term and could also affect other systems with low hydraulic retention times. Indeed, wetlands often serve as important low cost and low energy-consuming stormwater management solutions[70] and hydraulic retention times as well as microbial residence times may be shorter than 10 days for constructed wetlands.[71] For example, residence times in the Malibu lagoon have been recorded to be as little as a few hours during rainfall events.[72] Thus, during storms, Cu-NPs reaching wetlands through urban runoff may significantly decrease the ability of wetlands to remove nitrogen resulting in excess nitrogen entering surrounding surface waters.

Furthermore, this study showed that Cu-NPs have the potential to shape long-term nitrogen transformations in wetlands by selecting for more resilient and metal-tolerant nitrogen cycling microorganisms that lead to the recovery of wetland microcosm activities. Utilizing a combination of sequence-based techniques like the GeoChip functional gene array and RNA-seq revealed important changes occurring within the microbial community. However, this approach remains limited because it indirectly measures microbial activity without directly quantifying the activity of the enzymes encoded by the functional genes. Regardless, this strategy is advantageous because we gain an understanding of changes occurring at both the DNA- and transcript-level within the microbial community as a result of elevated metal exposures. Denitrifying communities were able to adapt to the presence of Cu-NPs, with minimal declines in relative signal intensity and fewer shifts in microbial phyla. Ammonifying and nitrogen fixing bacteria experienced the most shifts in phyla and appeared to be sensitive to long-term Cu-NP exposure. However, imbalances in nitrogen cycling microorganisms, could be problematic for environmental health. First, nitrous oxide is an intermediate of denitrification and a potent greenhouse gas that has nearly 300-fold greater global warming potential than carbon dioxide.[11] Increased wetland denitrification may increase nitrous oxide emissions. Second, decreased nitrogen fixation, combined with increased denitrification, may result in net nitrogen losses in wetlands. Previous estimates of nitrogen cycling have found that inadequate nitrogen concentrations limit wetlands' primary and secondary productivity.[73] Therefore, Cu-NPs entering the environment may reduce the productivity of the wetlands through enhanced nitrogen losses leading to their overall degradation. Another potential impact from Cu-NP introduction is the selection of metal- and antibiotic-resistant microbes and mobilomes. Further research is needed to understand the influence of Cu-NPs on antibiotic-resistant and potentially pathogenic microorganisms and their implications for human and ecological health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b01868.

Additional figures and tables describing the water quality characteristics, GeoChip 5.0 microarray data, and the RNA-seq data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*S. Mahendra. E-mail: mahendra@seas.ucla.edu.

ORCID

Phillip B. Gedalanga: 0000-0003-0846-4486

Shaily Mahendra: 0000-0003-3298-9602

Notes

The authors declare no competing financial interest.

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