

# UCLA

## UCLA Previously Published Works

### Title

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

### Permalink

<https://escholarship.org/uc/item/0qf7d3x3>

### Journal

ACS Sustainable Chemistry & Engineering, 6(9)

### ISSN

2168-0485

### Authors

Reyes, Vincent C  
Gedalanga, Phillip B  
Merino, Nancy  
[et al.](#)

### Publication Date

2018-09-04

### DOI

10.1021/acssuschemeng.8b01868

Peer reviewed



# EPA Public Access

Author manuscript

*ACS Sustain Chem Eng.* Author manuscript; available in PMC 2020 December 21.

About author manuscripts

Submit a manuscript

Published in final edited form as:

*ACS Sustain Chem Eng.* 2018 September 04; 6(9): 11642–11652.

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

Vincent C. Reyes<sup>†</sup>, Nancy Merino<sup>†</sup>, Phillip B. Gedalanga<sup>†</sup>, Joy D. Van Nostrand<sup>‡</sup>, Scott P. Keely<sup>§, †</sup>, Susan K. De Long<sup>⊥</sup>, Jizhong Zhou<sup>†, †, +</sup>, Shaily Mahendra<sup>†, ?</sup>

<sup>†</sup>Department of Civil and Environmental Engineering, University of California, Los Angeles, CA 90095

<sup>‡</sup>Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73072

<sup>§</sup>National Exposure Research Laboratory, US Environmental Protection Agency, Cincinnati, OH 45268

<sup>⊥</sup>Department of Civil and Environmental Engineering, Colorado State University, Fort Collins, CO, 80523

<sup>†</sup>Earth Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

<sup>+</sup>State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China

<sup>?</sup>California NanoSystems Institute, University of California, Los Angeles, CA 900095

### Abstract

Metallic nanoparticles (NPs), the most abundant nanomaterials in consumer and industrial products, are the most probable class to enter the environment. In this study, wetland-derived microcosms were incubated with copper nanoparticles (Cu-NP) and ionic CuCl<sub>2</sub> to investigate acute (10 days) and chronic (100 days) exposure towards nitrogen cycling microorganisms. The microbial ecology of wetlands play a crucial role in balancing nitrogen in pristine environments as well as in areas impacted by high nutrient loads (e.g., at wastewater effluent discharges). Gene abundance and expression changes were monitored using the GeoChip 5.0 high throughput functional gene microarray and metatranscriptomic shotgun 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 and expression of other functions, including electron transfer,

Address correspondence to mahendra@seas.ucla.edu.

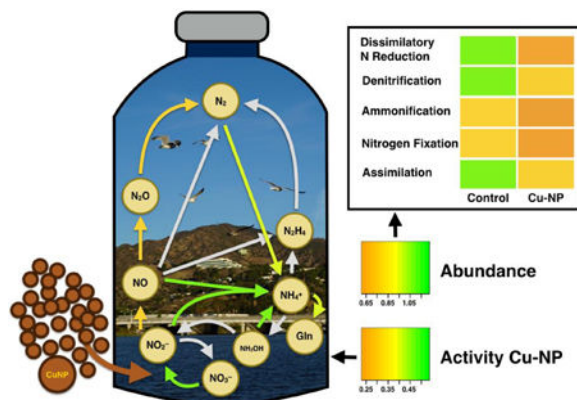
#### ASSOCIATED CONTENT

##### Supporting Information

Additional figures and tables describing the water quality characteristics, GeoChip 5.0 microarray data, and the RNA-seq data are in supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

metal homeostasis, and stress response. These findings suggest 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.

## Graphical abstract



## Keywords

nanomaterials; copper; lagoon; sewage; anaerobic; nitrogen transformation; toxic; stress

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 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<sup>-1</sup> h<sup>-1</sup>, 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<sup>-2</sup> d<sup>-1</sup>.(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).  $\text{CuCl}_2$  (>99% purity) salt was used in parallel experiments to assess the contribution of  $\text{Cu}^{2+}$  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  $\text{CuCl}_2$  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  $\text{N}_2$  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  $\text{CuCl}_2$ . 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  $\text{CuCl}_2$  (100  $\text{mg}\cdot\text{L}^{-1}$  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 CuCl<sub>2</sub>. Every 10 days, microcosms were flushed with N<sub>2</sub> to promote anaerobic conditions and amended with sucrose (100 mg·L<sup>-1</sup>) and nitrate (350 mg·L<sup>-1</sup>) 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<sub>2</sub>.

### Sample Processing

At every sampling event, a replicate bottle was sacrificed for analysis. Each 50 mL replicate was centrifuged at 7,000g 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](http://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 MicroExpress 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<sub>2</sub> 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<sub>2</sub> 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<sup>-1</sup> of total copper as either Cu-NPs or CuCl<sub>2</sub>, total dissolved copper did not significantly increase throughout the 100 days (Figure S1). Initially (day 0), dissolution of copper was minimal from Cu-NPs ( $2.8 \pm 4.5$  mg·L<sup>-1</sup>) and CuCl<sub>2</sub> ( $8.3 \pm 6.7$  mg·L<sup>-1</sup>), 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<sub>2</sub>. Copper was likely chelated by natural organic matter, such as fulvic and humic acids, which are common in natural waters.(43) 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<sub>2</sub>) (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 ( $350 \text{ mg}\cdot\text{L}^{-1}$  every 10 days) for all conditions. At 10 days, microcosms exposed to Cu-NPs and salts contained significantly more nitrate ( $21.1 \pm 9.0$  and  $28.7 \pm 8.6 \text{ mg}\cdot\text{L}^{-1}$ , respectively) compared to the unexposed microcosms ( $1.1 \pm 0.2 \text{ mg}\cdot\text{L}^{-1}$ ), 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  $3175.3 \pm 85.0 \text{ mg}\cdot\text{L}^{-1}$ ,  $3219.3 \pm 134.8 \text{ mg}\cdot\text{L}^{-1}$ , and  $3210.7 \pm 80.7 \text{ mg}\cdot\text{L}^{-1}$   $\text{NO}_3\text{-N}$  were transformed in the control, Cu-NP, and  $\text{CuCl}_2$  microcosms incubated for 100 days, respectively. These findings verify that inhibition was not sustained after long-term exposure. Additionally, the nitrite levels in  $\text{CuCl}_2$  exposed microcosms were significantly higher after 10 days ( $67.0 \pm 19.2 \text{ mg}\cdot\text{L}^{-1}$ ) ( $P < 0.05$ ) 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  $\text{CuCl}_2$  toward nitrite transformation during short-term exposure. Thus, at the level of functional activity, nitrogen cycling microbial communities recovered from  $\text{CuCl}_2$  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  $\text{CuCl}_2$  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.<sup>(44)</sup> Microcosms exposed to Cu-NPs had significantly lower  $H'$  values than unexposed controls for all time points ( $p < 0.01$ ), while  $\text{CuCl}_2$  treated microcosms were only significantly lower than controls after 100 days ( $p < 0.001$ ) 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  $\text{CuCl}_2$  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  $\text{CuCl}_2$  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<sub>2</sub> 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<sub>2</sub> exposed communities, indicating Cu-NPs were less disruptive, potentially related to their lower initial dissolution than CuCl<sub>2</sub>. 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<sup>+</sup> (as CuO).<sup>(21)</sup> 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<sub>2</sub> exposed microcosms over time with acute exposures resulting in greater abundances compared to chronic Cu-NP and CuCl<sub>2</sub> 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<sub>2</sub> 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<sup>(45,46)</sup> and fullerenes (C<sub>60</sub>)<sup>(47)</sup> 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.<sup>(48)</sup> Furthermore, our results determined that microorganisms associated with nitrogen fixation, dissimilatory nitrogen reduction, and ammonification experienced phylogenetic changes after chronic Cu-NP or CuCl<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>, indicating a potential for Cu-NP or CuCl<sub>2</sub> 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<sub>2</sub>. 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<sub>2</sub> on the Relative Abundance and Activity of Nitrogen Cycling Genes

The impact of Cu-NP and CuCl<sub>2</sub> 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<sub>2</sub> exposures, whereas genes related to ammonification were significantly different depending on the copper source (Figure 2A). Specifically, 100-day incubation with CuCl<sub>2</sub> 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<sub>2</sub> exposed conditions relative to the control. In particular, short-term exposure to CuCl<sub>2</sub> 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<sub>2</sub> after 100 days (Figure 2A;  $P < 0.05$ ). This result verifies that ammonifying populations were stimulated after long-term CuCl<sub>2</sub> exposure but not by Cu-NP exposure and indicates a resilient group of microorganisms were responsible for ammonification after exposure to CuCl<sub>2</sub>. Indeed, a previous report determined increased ammonification in soil microbial communities receiving 100 mg-Cu-L<sup>-1</sup>.(49)

Interestingly, specific genes within these nitrogen cycling categories revealed increasing complexity associated with Cu-NP or CuCl<sub>2</sub> exposures. Genes encoding assimilatory nitrite reductases (*nirB* and *NiR*) showed higher relative abundances with Cu-NP and CuCl<sub>2</sub> exposures (Figure 2B). Specifically, short-term exposure to CuCl<sub>2</sub> resulted in significantly increased abundance of *nirB* compared to the unexposed control. While for denitrifying bacteria, microcosms incubated in the presence of CuCl<sub>2</sub> 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<sub>2</sub> exposures. Furthermore, the dissimilar sensitivities of microbial populations to short-term CuCl<sub>2</sub> 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.<sup>(50)</sup> 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 ( $C_{60}$ ).<sup>(21,45–47)</sup>

## Cu-NP and CuCl<sub>2</sub> 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<sub>2</sub> 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 *cdI* 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<sub>2</sub> 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 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<sub>2</sub> 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<sub>2</sub> 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 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<sub>2</sub> exposure: *opuE*, *soxS*, *pspB*, *desR*, and *baeS*. Excess copper from Cu-NPs may have affected cell osmolality more than equivalent concentrations from CuCl<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> impacted microbial community (Figure 3B). The resulting enrichment of *baeS* in CuCl<sub>2</sub>-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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation (NSF) Award 1134355 and performed in a renovated laboratory supported by the NSF award number 0963183 under the American Recovery and Reinvestment Act of 2009 (ARRA). V.C.R. received a one-year fellowship from the NSF Clean Energy for Green Industry IGERT Program 0903720. N.M. received the UCLA Dissertation Year Fellowship and the Earth-Life Science Institute Origin of Life (EON) Postdoctoral Fellowship, which is supported by a grant from the John Templeton Foundation. The opinions expressed in this publication are those of the author(s) and do not necessarily reflect the views of the John Templeton Foundation. The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names, products, or services does not convey, and should not be interpreted as conveying, official EPA approval, endorsement, or recommendation. US EPA had no role in the collection or analysis of data. Their role was limited to guidance of the analysis and interpretation of results.

## ABBREVIATIONS

<b>NPs</b>	nanoparticles
<b>Cu-NP</b>	copper nanoparticles
<b>COD</b>	chemical oxygen demand
<b>PCA</b>	principal component analysis
<b>Anammox</b>	ammonium oxidation
<b>TMAO</b>	trimethylamine N-oxide reductase

## REFERENCES

1. Consumer Product Inventory. The Project on Emerging Nanotechnologies. <http://www.nanotechproject.org/cpi>.
2. Nowack B; Ranville JF; Diamond S; Gallego-Urrea JA; Metcalfe C; Rose J; Horne N; Koelmans AA; Klaine SJ Potential Scenarios for Nanomaterial Release and Subsequent Alteration in the Environment. *Environ. Toxicol. Chem.* 2012, 31 (1), 50–59, DOI: 10.1002/etc.726 [PubMed: 22038832]
3. Chen YG; Wang DB; Zhu XY; Zheng X; Feng LY Long-Term Effects of Copper Nanoparticles on Wastewater Biological Nutrient Removal and N<sub>2</sub>O Generation in the Activated Sludge Process. *Environ. Sci. Technol.* 2012, 46 (22), 12452–12458, DOI: 10.1021/es302646q [PubMed: 23110389]
4. Collins D; Luxton T; Kumar N; Shah S; Walker VK; Shah V, Assessing the Impact of Copper and Zinc Oxide Nanoparticles on Soil: A Field Study. *PLoS One* 2012, 7 (8), DOI e42663 DOI: 10.1371/journal.pone.0042663.
5. Colman BP; Arnaout CL; Anciaux S; Gunsch CK; Hochella MF; Kim B; Lowry GV; McGill BM; Reinsch BC; Richardson CJ; Urnine JM; Wright JP; Yin LY; Bernhardt ES, Low Concentrations of Silver Nanoparticles in Biosolids Cause Adverse Ecosystem Responses under Realistic Field Scenario. *PLoS One* 2013, 8 (2), DOI e57189 DOI: 10.1371/journal.pone.0057189.
6. Sutton MA; Bleeker A Environmental Science: The Shape of Nitrogen to Come. *Nature* 2013, 494(7438), 435–437, DOI: 10.1038/nature11954 [PubMed: 23426258]

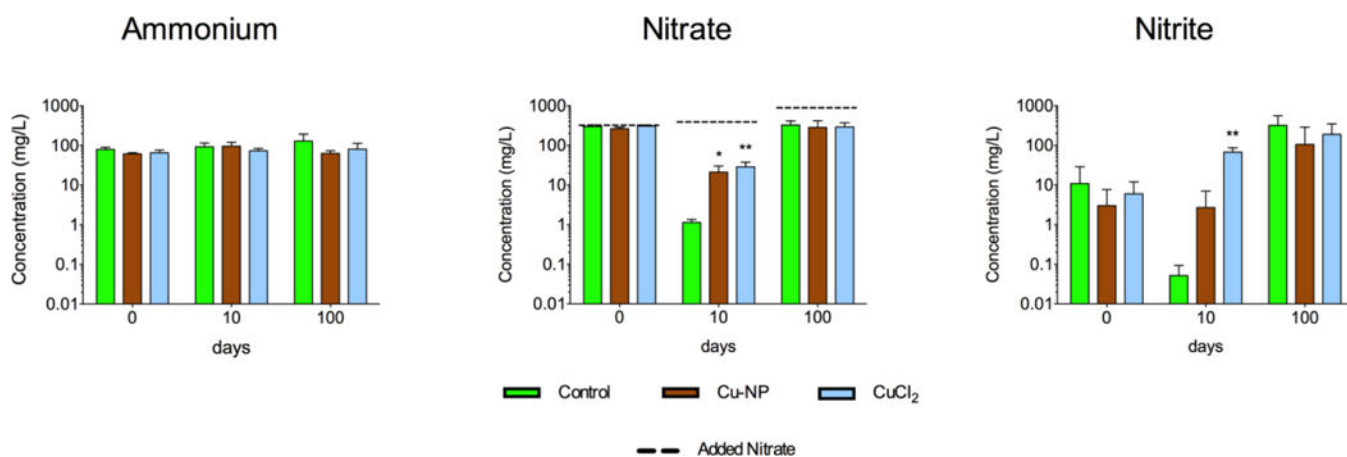
7. Grand Challenges of Engineering; National Academy of Engineering, 2008.
8. Ochoa-Herrera V; Leon G; Banihani Q; Field JA; Sierra-Alvarez R Toxicity of Copper(II) Ions to Microorganisms in Biological Wastewater Treatment Systems. *Sci. Total Environ.* 2011, 412, 380–385, DOI: 10.1016/j.scitotenv.2011.09.072 [PubMed: 22030247]
9. Reyes VC; Opot SO; Mahendra S Planktonic and Biofilm-Grown Nitrogen-Cycling Bacteria Exhibit Different Susceptibilities to Copper Nanoparticles. *Environ. Toxicol. Chem.* 2015, 34 (4), 887–897, DOI: 10.1002/etc.2867 [PubMed: 25556815]
10. Klaine SJ; Koelmans AA; Horne N; Carley S; Handy RD; Kapustka L; Nowack B; von der Kammer F Paradigms to Assess the Environmental Impact of Manufactured Nanomaterials. *Environ. Toxicol. Chem.* 2012, 31 (1), 3–14, DOI: 10.1002/etc.733 [PubMed: 22162122]
11. Thomson AJ; Giannopoulos G; Pretty J; Baggs EM; Richardson DJ Biological Sources and Sinks of Nitrous Oxide and Strategies to Mitigate Emissions. *Philos. Trans. R. Soc., B* 2012, 367 (1593), 1157–1168, DOI: 10.1098/rstb.2011.0415
12. Yang Y; Wang J; Zhu HG; Colvin VL; Alvarez PJ Relative Susceptibility and Transcriptional Response of Nitrogen Cycling Bacteria to Quantum Dots. *Environ. Sci. Technol.* 2012, 46 (6), 3433–3441, DOI: 10.1021/es203485f [PubMed: 22360857]
13. Yang Y; Wu L; Lin Q; Yuan M; Xu D; Yu H; Hu Y; Duan J; Li X; He Z; Xue K; van Nostrand J; Wang S; Zhou J Responses of the Functional Structure of Soil Microbial Community to Livestock Grazing in the Tibetan Alpine Grassland. *Glob Change Biol.* 2013, 19 (2), 637–648, DOI: 10.1111/gcb.12065
14. Radniecki TS; Stankus DP; Neigh A; Nason JA; Semprini L Influence of Liberated Silver from Silver Nanoparticles on Nitrification Inhibition of *Nitrosomonas Europaea*. *Chemosphere* 2011, 85 (1), 43–49, DOI: 10.1016/j.chemosphere.2011.06.039 [PubMed: 21757219]
15. Cherchi C; Gu AZ Impact of Titanium Dioxide Nanomaterials on Nitrogen Fixation Rate and Intracellular Nitrogen Storage in *Anabaena Variabilis*. *Environ. Sci. Technol.* 2010, 44 (21), 8302–8307, DOI: 10.1021/es101658p [PubMed: 20853867]
16. Mahendra S; Zhu HG; Colvin VL; Alvarez PJ Quantum Dot Weathering Results in Microbial Toxicity. *Environ. Sci. Technol.* 2008, 42 (24), 9424–9430, DOI: 10.1021/es8023385 [PubMed: 19174926]
17. Botton S; van Heusden M; Parsons JR; Smidt H; van Straalen N Resilience of Microbial Systems Towards Disturbances. *Crit. Rev. Microbiol.* 2006, 32 (2), 101–112, DOI: 10.1080/10408410600709933 [PubMed: 16850561]
18. Ganesh R; Smeraldi J; Hosseini T; Khatib L; Olson BH; Rosso D Evaluation of Nanocopper Removal and Toxicity in Municipal Wastewaters. *Environ. Sci. Technol.* 2010, 44 (20), 7808–7813, DOI: 10.1021/es101355k [PubMed: 20853883]
19. Choi OK; Hu ZQ Nitrification Inhibition by Silver Nanoparticles. *Water Sci. Technol.* 2009, 59 (9), 1699–1702, DOI: 10.2166/wst.2009.205 [PubMed: 19448303]
20. Alito CL; Gunsch CK Assessing the Effects of Silver Nanoparticles on Biological Nutrient Removal in Bench-Scale Activated Sludge Sequencing Batch Reactors. *Environ. Sci. Technol.* 2014, 48 (2), 970–976, DOI: 10.1021/es403640j [PubMed: 24364625]
21. Moore JD; Stegemeier JP; Bibby K; Marinakos SM; Lowry GV; Gregory KB Impacts of Pristine and Transformed Ag and Cu Engineered Nanomaterials on Surficial Sediment Microbial Communities Appear Short-Lived. *Environ. Sci. Technol.* 2016, 50, 2641, DOI: 10.1021/acs.est.5b05054 [PubMed: 26841726]
22. Truu M; Juhanson J; Truu J Microbial Biomass, Activity and Community Composition in Constructed Wetlands. *Sci. Total Environ.* 2009, 407 (13), 3958–3971, DOI: 10.1016/j.scitotenv.2008.11.036 [PubMed: 19157517]
23. Ladu JLC; Lu X; Osman AM Experimental Study on Anoxic/Oxic Bioreactor and Constructed Wetlands for Rural Domestic Wastewater Treatment. *Res. J. Appl. Sci., Eng. Technol.* 2014, 7, 354–363, DOI: 10.19026/rjaset.7.262
24. White JR; Reddy KR Nitrification and Denitrification Rates of Everglades Wetland Soils Along a Phosphorus-Impacted Gradient. *J. Environ. Qual.* 2003, 32 (6), 2436–2443, DOI: 10.2134/jeq2003.2436 [PubMed: 14674571]



25. Inglett PW; Rivera-Monroy VH; Wozniak JR Biogeochemistry of Nitrogen across the Everglades Landscape. *Crit. Rev. Environ. Sci. Technol.* 2011, 41, 187–216, DOI: 10.1080/10643389.2010.530933
26. He Z; Gentry TJ; Schadt CW; Wu L; Liebich J; Chong SC; Huang Z; Wu W; Gu B; Jardine P; Criddle C; Zhou J Geochip: A Comprehensive Microarray for Investigating Biogeochemical, Ecological and Environmental Processes. *ISME J.* 2007, 1 (1), 67–77, DOI: 10.1038/ismej.2007.2 [PubMed: 18043615]
27. He Z; Deng Y; Van Nostrand JD; Tu Q; Xu M; Hemme CL; Li X; Wu L; Gentry TJ; Yin Y; Liebich J; Hazen TC; Zhou J Geochip 3.0 as a High-Throughput Tool for Analyzing Microbial Community Composition, Structure and Functional Activity. *ISME J.* 2010, 4 (9), 1167–79, DOI: 10.1038/ismej.2010.46 [PubMed: 20428223]
28. Tu QC; Yu H; He ZL; Deng Y; Wu LY; Van Nostrand JD; Zhou AF; Voordeckers J; Lee YJ; Qin YJ; Hemme CL; Shi Z; Xue K; Yuan T; Wang AJ; Zhou JZ Geochip 4: A Functional Gene-Array-Based High-Throughput Environmental Technology for Microbial Community Analysis. *Mol. Ecol. Resour.* 2014, 14 (5), 914–928, DOI: 10.1111/1755-0998.12239 [PubMed: 24520909]
29. Poretsky RS; Bano N; Buchan A; LeClerc G; Kleikemper J; Pickering M; Pate WM; Moran MA; Hollibaugh JT Analysis of Microbial Gene Transcripts in Environmental Samples. *Appl. Environ. Microbiol.* 2005, 71 (7), 4121–4126, DOI: 10.1128/AEM.71.7.4121-4126.2005 [PubMed: 16000831]
30. Poretsky RS; Hewson I; Sun S; Allen AE; Zehr JP; Moran MA Comparative Day/Night Metatranscriptomic Analysis of Microbial Communities in the North Pacific Subtropical Gyre. *Environ. Microbiol.* 2009, 11 (6), 1358–75, DOI: 10.1111/j.1462-2920.2008.01863.x [PubMed: 19207571]
31. Chen LX; Hu M; Huang LN; Hua ZS; Kuang JL; Li SJ; Shu WS Comparative Metagenomic and Metatranscriptomic Analyses of Microbial Communities in Acid Mine Drainage. *ISME J.* 2015, 9 (7), 1579–1592, DOI: 10.1038/ismej.2014.245 [PubMed: 25535937]
32. Reyes VC; Li MH; Hoek EMV; Mahendra S; Damoiseaux R Genome-Wide Assessment in *Escherichia Coli* Reveals Time-Dependent Nanotoxicity Paradigms. *ACS Nano* 2012, 6 (11), 9402–9415, DOI: 10.1021/nn302815w [PubMed: 23039911]
33. Damoiseaux R; George S; Li M; Pokhrel S; Ji Z; France B; Xia T; Suarez E; Rallo R; Madler L; Cohen Y; Hoek EMV; Nel A No Time to Lose-High Throughput Screening to Assess Nanomaterial Safety. *Nanoscale* 2011, 3 (4), 1345–1360, DOI: 10.1039/c0nr00618a [PubMed: 21301704]
34. Lee CM; Lin TY; Lin CC; Kohbodi GA; Bhatt A; Lee R; Jay JA Persistence of Fecal Indicator Bacteria in Santa Monica Bay Beach Sediments. *Water Res.* 2006, 40 (14), 2593–602, DOI: 10.1016/j.watres.2006.04.032 [PubMed: 16793111]
35. Izbicki JA; Swarzenski PW; Burton CA; Van DeWerfhorst LC; Holden PA; Dubinsky EA Sources of Fecal Indicator Bacteria to Groundwater, Malibu California and the near Shore Ocean, Malibu, California, USA. *Annu. Environ. Sci.* 2012, 6, 36–86
36. Essen SA; Johnsson A; Bylund D; Pedersen K; Lundstrom US Siderophore Production by *Pseudomonas Stutzeri* under Aerobic and Anaerobic Conditions. *Appl. Environ. Microbiol.* 2007, 73 (18), 5857–5864, DOI: 10.1128/AEM.00072-07 [PubMed: 17675442]
37. Wang JX; Tai YP; Man Y; Wang R; Feng X; Yang YF; Fan JJ; Guo JJ; Tao R; Yang Y; Chen ZB; Vymazal J; Cai N Capacity of Various Single-Stage Constructed Wetlands to Treat Domestic Sewage under Optimal Temperature in Guangzhou City, South China. *Ecol Eng.* 2018, 115, 35–44, DOI: 10.1016/j.ecoleng.2018.02.008
38. Park JH; Kim SH; Delaune RD; Cho JS; Heo JS; Ok YS; Seo DC Enhancement of Nitrate Removal in Constructed Wetlands Utilizing a Combined Autotrophic and Heterotrophic Denitrification Technology for Treating Hydroponic Wastewater Containing High Nitrate and Low Organic Carbon Concentrations. *Agr Water Manage* 2015, 162, 1–14, DOI: 10.1016/j.agwat.2015.08.001
39. Mietto A; Politeo M; Breschigliaro S; Borin M Temperature Influence on Nitrogen Removal in a Hybrid Constructed Wetland System in Northern Italy. *Ecol Eng.* 2015, 75, 291–302, DOI: 10.1016/j.ecoleng.2014.11.027

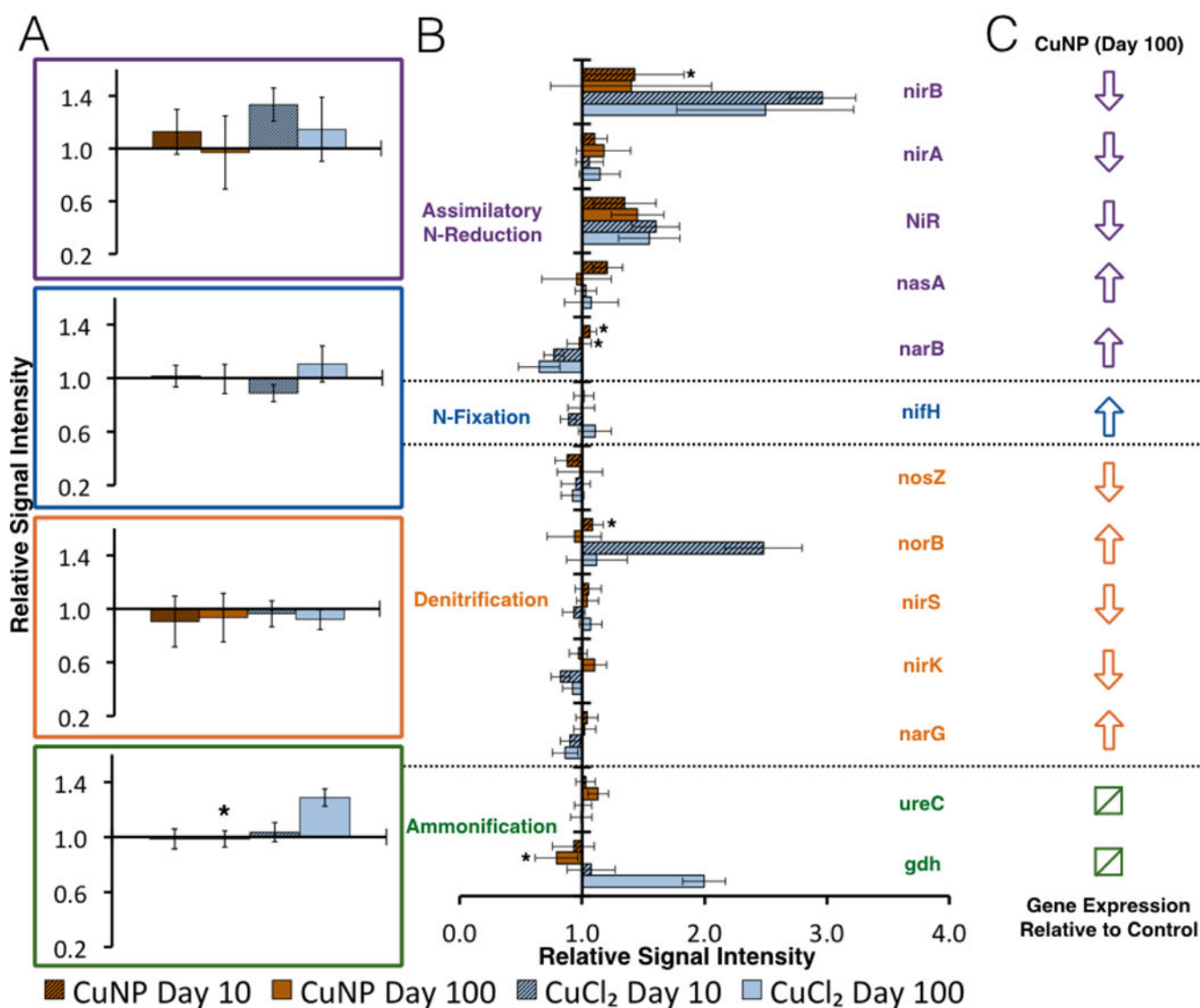
40. Bai SJ; Li JW; He ZL; Van Nostrand JD; Tian Y; Lin GH; Zhou JZ; Zheng TL Geochip-Based Analysis of the Functional Gene Diversity and Metabolic Potential of Soil Microbial Communities of Mangroves. *Appl. Microbiol. Biotechnol.* 2013, 97 (15), 7035–7048, DOI: 10.1007/s00253-012-4496-z [PubMed: 23135227]
41. Liang YT; He ZL; Wu LY; Deng Y; Li GH; Zhou JZ Development of a Common Oligonucleotide Reference Standard for Microarray Data Normalization and Comparison across Different Microbial Communities. *Appl. Environ. Microbiol.* 2010, 76 (4), 1088–1094, DOI: 10.1128/AEM.02749-09 [PubMed: 20038701]
42. Meyer F; Paarmann D; D'Souza M; Olson R; Glass EM; Kubal M; Paczian T; Rodriguez A; Stevens R; Wilke A; Wilkening J; Edwards RA The Metagenomics Rast Server -a Public Resource for the Automatic Phylogenetic and Functional Analysis of Metagenomes. *BMC Bioinf.* 2008, 9, 386, DOI: 10.1186/1471-2105-9-386
43. Brady NC; Weil RR *The Nature and Properties of Soil*, 14 ed.; Prentice Hall, 1996.
44. Liang YT; Van Nostrand JD; Deng Y; He ZL; Wu LY; Zhang X; Li GH; Zhou JZ Functional Gene Diversity of Soil Microbial Communities from Five Oil-Contaminated Fields in China. *ISME J.* 2011, 5(3), 403–413, DOI: 10.1038/ismej.2010.142 [PubMed: 20861922]
45. Bradford A; Handy RD; Readman JW; Atfield A; Muhling M Impact of Silver Nanoparticle Contamination on the Genetic Diversity of Natural Bacterial Assemblages in Estuarine Sediments. *Environ. Sci. Technol.* 2009, 43 (12), 4530–6, DOI: 10.1021/es9001949 [PubMed: 19603673]
46. Colman BP; Wang SY; Auffan M; Wiesner MR; Bernhardt ES Antimicrobial Effects of Commercial Silver Nanoparticles Are Attenuated in Natural Streamwater and Sediment. *Ecotoxicology* 2012, 21 (7), 1867–77, DOI: 10.1007/s10646-012-0920-5 [PubMed: 22569948]
47. Tong Z; Bischoff M; Nies L; Applegate B; Turco RF Impact of Fullerene (C60) on a Soil Microbial Community. *Environ. Sci. Technol.* 2007, 41 (8), 2985–91, DOI: 10.1021/es0619531 [PubMed: 17533868]
48. Lv X; Yu J; Fu Y; Ma B; Qu F; Ning K; Wu H A Meta-Analysis of the Bacterial and Archaeal Diversity Observed in Wetland Soils. *Sci. World J.* 2014, 2014, 437684, DOI: 10.1155/2014/437684
49. Premi PR; Cornfield, Ah Effects of Addition of Copper, Manganese, Zinc and Chromium Compounds on Ammonification and Nitrification During Incubation of Soil. *Plant Soil* 1969, 31 (2), 345, DOI: 10.1007/BF01373578
50. Overbeek R; Begley T; Butler RM; Choudhuri JV; Chuang HY; Cohoon M; de Crecy-Lagard V; Diaz N; Disz T; Edwards R; Fonstein M; Frank ED; Gerdes S; Glass EM; Goesmann A; Hanson A; Iwata-Reuyl D; Jensen R; Jamshidi N; Krause L; Kubal M; Larsen N; Linke B; McHardy AC; Meyer F; Neuweger H; Olsen G; Olson R; Osterman A; Portnoy V; Pusch GD; Rodionov DA; Ruckert C; Steiner J; Stevens R; Thiele I; Vassieva O; Ye Y; Zagnitko O; Vonstein V The Subsystems Approach to Genome Annotation and Its Use in the Project to Annotate 1000 Genomes. *Nucleic Acids Res.* 2005, 33 (17), 5691–5702, DOI: 10.1093/nar/gki866 [PubMed: 16214803]
51. Moir JWB *Nitrogen Cycling in Bacteria: Molecular Analysis*; Horizon Scientific Press, 2011; p 262.
52. Seitz HJ; Cypionka H Chemolithotrophic Growth of *Desulfovibrio-Desulfuricans* with Hydrogen Coupled to Ammonification of Nitrate or Nitrite. *Arch. Microbiol.* 1986, 146 (1), 63–67, DOI: 10.1007/BF00690160
53. Gu CH; Laverman AM; Pallud CE, Environmental Controls on Nitrogen and Sulfur Cycles in Surficial Aquatic Sediments. *Front. Microbiol.* 2012, 3, DOI DOI: 10.3389/fmicb.2012.00045.
54. Brettar I; Rheinheimer G Denitrification in the Central Baltic -Evidence for H<sub>2</sub>S-Oxidation as Motor of Denitrification at the Oxidic-Anoxic Interface. *Mar. Ecol.: Prog. Ser.* 1991, 77, 157–169, DOI: 10.3354/meps077157
55. Garciagil LJ; Golterman HL Kinetics of Fe<sup>2+</sup>-Mediated Denitrification in Sediments from the Camargue (Rhône Delta, Southern France). *FEMS Microbiol. Ecol.* 1993, 13 (2), 85–91, DOI: 10.1111/j.1574-6941.1993.tb00054.x

56. Outten FW; Huffman DL; Hale JA; O'Halloran TV The Independent Cue and Cus Systems Confer Copper Tolerance During Aerobic and Anaerobic Growth in *Escherichia Coli*. *J. Biol. Chem.* 2001, 276 (33),30670–30677, DOI: 10.1074/jbc.M104122200 [PubMed: 11399769]
57. Chillappagari S; Miethke M; Trip H; Kuipers OP; Marahiel MA Copper Acquisition Is Mediated by Ycnj and Regulated by Ycnk and Cso in *Bacillus Subtilis*. *J. Bacteriol.* 2009, 191 (7), 2362–2370, DOI: 10.1128/JB.01616-08 [PubMed: 19168619]
58. Arredondo M; Nunez MT Iron and Copper Metabolism. *Mol. Aspects Med.* 2005, 26 (4–5), 313–27, DOI: 10.1016/j.mam.2005.07.010 [PubMed: 16112186]
59. Blatzer M; Binder U; Haas H The Metalloreductase Freb Is Involved in Adaptation of *Aspergillus Fumigatus* to Iron Starvation. *Fungal Genet. Biol.* 2011, 48 (11), 1027–1033, DOI: 10.1016/j.fgb.2011.07.009 [PubMed: 21840411]
60. Baker-Austin C; Wright MS; Stepanauskas R; McArthur JV Co-Selection of Antibiotic and Metal Resistance. *Trends Microbiol.* 2006, 14 (4), 176–182, DOI: 10.1016/j.tim.2006.02.006 [PubMed: 16537105]
61. Kempf B; Bremer E Uptake and Synthesis of Compatible Solutes as Microbial Stress Responses to High-Osmolality Environments. *Arch. Microbiol.* 1998, 170 (5), 319–330, DOI: 10.1007/s002030050649 [PubMed: 9818351]
62. Watmough NJ; Butland G; Cheesman MR; Moir JWB; Richardson DJ; Spiro S Nitric Oxide in Bacteria: Synthesis and Consumption. *Biochim. Biophys. Acta, Bioenerg.* 1999, 1411 (2–3), 456–474, DOI: 10.1016/S0005-2728(99)00032-8
63. Li Y; Zhang W; Niu J; Chen Y Mechanism of Photogenerated Reactive Oxygen Species and Correlation with the Antibacterial Properties of Engineered Metal-Oxide Nanoparticles. *ACS Nano* 2012, 6 (6), 5164–5173, DOI: 10.1021/nl300934k [PubMed: 22587225]
64. Yin H; Casey PS; McCall MJ; Fenech M Effects of Surface Chemistry on Cytotoxicity, Genotoxicity, and the Generation of Reactive Oxygen Species Induced by Zn Nanoparticles. *Langmuir* 2010, 26 (19),15399–15408, DOI: 10.1021/la101033n [PubMed: 20809599]
65. Choi O; Hu Z Size Dependent and Reactive Oxygen Species Related Nanosilver Toxicity to Nitrifying Bacteria. *Environ. Sci. Technol.* 2008, 42 (12), 4583–4588, DOI: 10.1021/es703238h [PubMed: 18605590]
66. Ivask A; Bondarenko O; Jephthina N; Kahru A Profiling of the Reactive Oxygen Species-Related Ecotoxicity of Cu, Zn, TiO<sub>2</sub>, Silver and Fullerene Nanoparticles Using a Set of Recombinant Luminescent *Escherichia Coli* Strains: Differentiating the Impact of Particles and Solubilised Metals. *Anal. Bioanal. Chem.* 2010, 398 (2), 701–716, DOI: 10.1007/s00216-010-3962-7 [PubMed: 20623373]
67. Song W; Zhang J; Guo J; Zhang J; Ding F; Li L; Sun Z Role of the Dissolved Zinc Ion and Reactive Oxygen Species in Cytotoxicity of Zn Nanoparticles. *Toxicol. Lett.* 2010, 199 (3), 389–397, DOI: 10.1016/j.toxlet.2010.10.003 [PubMed: 20934491]
68. Horn G; Hofweber R; Kremer W; Kalbitzer HR Structure and Function of Bacterial Cold Shock Proteins. *Cell. Mol. Life Sci.* 2007, 64 (12), 1457–1470, DOI: 10.1007/s00018-007-6388-4 [PubMed: 17437059]
69. Leblanc SKD; Oates CW; Raivio TL Characterization of the Induction and Cellular Role of the Baer Two-Component Envelope Stress Response of *Escherichia Coli*. *J. Bacteriol.* 2011, 193 (13), 3367–3375, DOI: 10.1128/JB.01534-10 [PubMed: 21515766]
70. Sims A; Hu ZQ Simulated Storm-Water Runoff Treatment by Duckweed and Algae Ponds. *J. Environ. Eng.* 2013, 139 (4), 509–515, DOI: 10.1061/(ASCE)EE.1943-7870.0000593
71. Constructed Wetland and Aquatic Plant Systems for Municipal Wastewater Treatment; United States Environmental Protection Agency, 1988; pp 1–92.
72. 2nd Nature California. Malibu Lagoon Restoration Monitoring Plan Baseline Conditions Report; 2010; pp 1–73.
73. Frost JW; Schleicher T; Craft C Effects of Nitrogen and Phosphorus Additions on Primary Production and Invertebrate Densities in a Georgia (USA) Tidal Freshwater Marsh. *Wetlands* 2009, 29 (1), 196–203, DOI: 10.1672/07-79.1



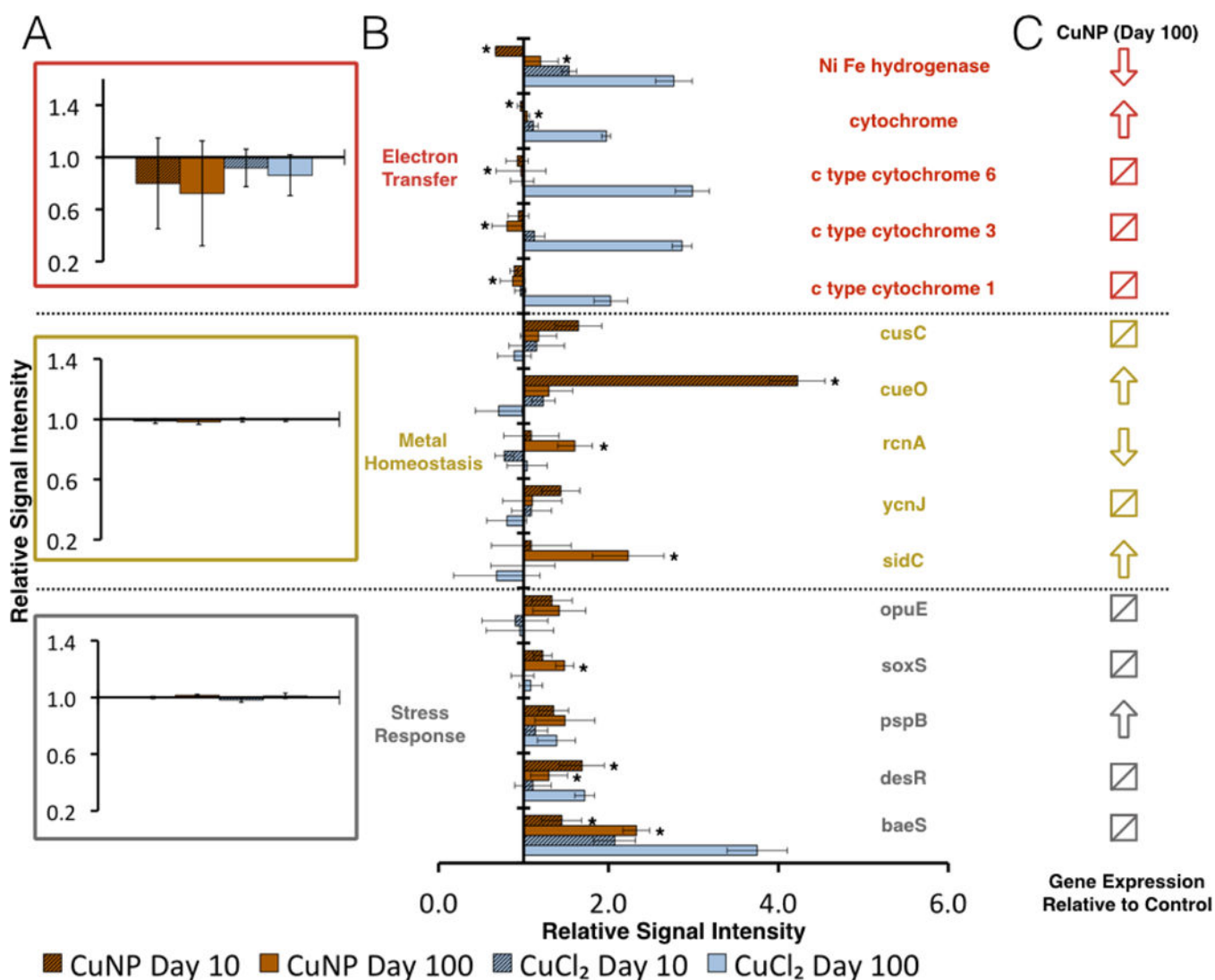
**Figure 1. Concentrations of Various Nitrogen Species during Microcosm Incubations.**

Only nitrate and nitrite levels were significantly higher at 10 days for Cu-NP and CuCl<sub>2</sub> 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 performed in triplicate and asterisks indicate significance levels where (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ , and (\*\*\*)  $P < 0.001$ . Error bars represent the standard deviation of triplicate samples.



**Figure 2. Relative abundances and expression for nitrogen cycling genes.**

Overall relative abundances (3A) and specific gene abundances (3B) for the Cu-NP and CuCl<sub>2</sub> treated microcosms on day 10 and 100 are represented as relative signal intensities from the GeoChip 5.0 microarray. Treatments were compared against day 0, followed by the control at each time point. The top 5 gene functions for denitrification in Figure 3B were selected based on the largest difference compared to the unexposed control. Gene expression (3C) is represented as “up” (more expression) and “down” (less expression) arrows for the Cu-NP treated microcosms on day 100 compared to the control using the MG-RAST server (RNA-seq). Squares represent no equivalent gene observed on the MG-RAST server. Error bars represent the standard deviation of triplicate samples, and asterisks indicate significantly different ( $p < 0.05$ ) between the Cu-NP and CuCl<sub>2</sub> treated microcosms at the time point. Relative abundances and expression for all nitrogen cycling genes can be found in Table S5 and Figure S5–S9.



**Figure 3. Relative abundances and expression for other functions.**

Overall relative abundances (panel A) and specific gene abundances (panel B) for the Cu-NP and CuCl<sub>2</sub> 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<sub>2</sub> 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.