Interactions shape aquatic microbiome responses to Cu and Au nanoparticle treatments in wetland manipulation experiments

Running title: Interactions mediate microbiome nanoparticle responses

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Abstract

In natural systems, organisms are embedded in complex networks where their physiology and community composition is shaped by both biotic and abiotic factors. Therefore, to assess the ecosystem-level effects of contaminants, we must pair complex, multi-trophic field studies with more targeted hypothesis-driven approaches to explore specific actors and mechanisms. Here, we examine aquatic microbiome responses to long-term additions of commercially- available metallic nanoparticles [copper-based (CuNPs) or gold (AuNPs)] and/or nutrients in complex, wetland mesocosms over 9 months, allowing for a full growth cycle of the aquatic plants. We found that both CuNPs and AuNPs (but not nutrient) treatments showed shifts in microbial communities and populations largely at the end of the experiment, as the aquatic plant community senesced. we examine aquatic microbiomes under **chronic** dosing of NPs and nutrients Simplified microbe-only or microbe + plant incubations revealed that direct effects of AuNPs on aquatic microbiomes can be buffered by plants (regardless of seasonal As mesocosms were dosed weekly, the absence of water column accumulation indicates the partitioning of both metals into other environmental compartments, mainly the floc and aquatic plants photosynthetically-derived organic matter. Overall, this study identifies the potential for NP environmental impacts to be either suppressed by or propagated across trophic levels via the presence of primary producers, highlighting the importance of organismal interactions in mediating emerging contaminants' ecosystem-wide impacts.

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Introduction

Among emerging contaminants, engineered metallic nanoparticles (NPs) have received increased attention as their consumer applications have expanded (Saravanan et al., 2021). NPs' small size (1-100 nm) and large surface area: volume ratio generally increase their reactivity relative to their bulk counterparts (Auffan et al., 2009). The biological impacts of inorganic nanoparticles are generally attributed to the release of dissolution products or nano-specific effects due to their physical properties. For example, metallic NPs can disrupt cell membranes and generate oxidative stress, resulting in lipid and protein peroxidation and DNA damage (Clar et al., 2016; Maurer-Jones et al., 2013). Concerns about NP ecotoxicity have grown, as rates of anthropogenic nanoparticle deposition now rival those of natural NPs in some areas (Hochella et al., 2019). While most nanomaterial studies initially focused on model NPs, there is a growing interest in expanding our understanding of the ecosystem-level impacts of commercially-available NPs, which primarily enter the environment through disposal or application (Carley et al., 2020; Mitrano et al., 2015; Ward et al., 2019). In these commercial applications, NPs' advantages include lower substrate requirements; for example, while copper has been used as a pesticide for over a hundred years, newer copper-NP based biocides like Kocide® 3000 (Dupont) both enhance antimicrobial properties and reduce Cu usage (Giannousi et al., 2013; Kah et al., 2018). As biocides are commonly used in conjunction with other agrochemicals like fertilizers, these co-occurring contaminants could alter ecosystems with impacts distinct from either the fertilizer or pesticide alone (Kah, 2015).

However, it may be difficult to *a priori* predict the ecosystem outcomes of these NP-containing agrochemical mixtures because of *(i)* NP metastability which means their (bio)transformation and fate are dynamic, and differ from bulk counterparts (Avellan et al., 2020), or *(ii)* interactions between multiple contaminants that can lead to unexpected biological impacts (Brennan and Collins, 2015; Hagenbuch and Pinckney, 2012). For example, nutrients can attenuate contaminant toxicity directly by binding contaminants, or indirectly by increasing the organism's biomass or energy investment in detoxification (Aristi et al., 2016; Leflaive et al., 2015; Pieters et al., 2005; Skei et al., 2000). Conversely, nutrients can increase toxicity through enhanced contaminant uptake (Hu et al., 2013). As we cannot robustly predict ecosystem outcomes from short-term, laboratory studies, recent research has focused on environmentally realistic conditions including contaminant mixtures or co-occurring stressors in complex multi-trophic systems.

Microbes are a critical component of all ecosystems: with high diversity, short generation times and as critical mediators of biogeochemical cycles, the microbiome can be a sensitive and ecologically-important indicator of disturbance (Aylagas et al., 2017; Hunt and Ward, 2015). Although early NP microbiome research focused on acute exposures in bacterial model systems (e.g. *Escherichia coli*), the field has shifted toward chronic exposures and whole community microbiome analyses. These community-level microbiome studies incorporate key, often uncultured organisms; account for different responses within microbiomes and incorporate modification of NP by other organisms or ecosystem components (Chae et al., 2014; Colman et al., 2014; Ward et al., 2019). Thus, there is a growing body of literature on how interactions with other organisms, and their associated biomacromolecules and ligands, can alter microbial

responses to toxicants through competition for resources, alteration of organic matter quantity and/or quality, or transformation and accumulation of contaminants (Bone et al., 2012; Ge et al., 2014).

Here, to investigate how ecosystem complexity and co-occurring anthropogenic contaminants shape microbial responses to NPs, we examine aquatic microbiomes under chronic dosing of NPs and nutrients (N and P) in wetland mesocosms. We focus on two NPs: the commercially-available agricultural biocide Kocide® 3000 [containing $Cu(OH)_2$ NPs] and citrate-coated gold nanoparticles (AuNPs). While AuNPs were initially used primarily as a tracer of NP fate (Avellan et al., 2018), they have potential commercial applications in catalysts, sensors and medical treatments as well as potential ecotoxicity. Both these NP-based contaminants were chronically dosed into wetland mesocosms with either nanoparticles (CuNPs or AuNPs), nutrients, or both a single NP type and nutrients, over a 9-month period. Prior research on these mesocosms has revealed unexpected organismal responses (Perrotta et al., 2020), NP (bio)transformations (Avellan et al., 2020) and increased macroalgal blooms under chronic NP and nutrient dosing (Simonin et al., 2018a). However, it is still unclear how exposure to multiple stressors impacts aquatic microbial communities (Rillig et al., 2019). Further, as these mesocosms contain a complex food web including fish, snails, and plants, microbiome composition reflects both direct contaminant impacts and indirect effects mediated by interactions with other organisms (Hunt and Ward, 2015). To directly address the issue of ecosystem complexity, we employed a microcosm experiment (Bergemann et al., 2023) to address AuNP treatment effects in simplified communities composed of either only microbes or both microbes and the aquatic plant *Egeria densa*. Thus, this set of experiments focus on

identifying key environmental factors that mediate microbial responses to emerging pollutants in dynamic aquatic environments.

Methods

Wetland mesocosm experiments

Experiments were conducted at the Center of Environmental Implications of Nanotechnology (CEINT) mesocosm facility in the Duke University Forest (Durham, North Carolina, USA) from January 2016 - October 2016. Details about experimental set-up and monitoring were previously described (Lowry et al., 2012; Simonin et al., 2018a). Briefly, slantboard mesocosms (3.66 m long, 1.22 m wide and 0.8 m high) lined with a water-tight geotextile (0.45 mm reinforced polypropylene, Firestone Specialty Products, U.S.) were partially filled with sand, creating a permanently flooded zone (aquatic zone), a periodically flooded zone (transition zone), and a rarely flooded zone (upland zone). The mesocosms were filled with well water sourced at the site with an average starting water volume of 452 L; as the water level fluctuated over time with precipitation and evapotranspiration, therefore dosing is provided as weight rather than concentration. Organisms were introduced sequentially prior to starting the experiment in 2015, including the floating plant *Egeria densa*; aquatic snails *Physella acuta* and *Lymnaea* sp.; and the fish *Gambusia holbrookii* (eastern mosquitofish). An algal and zooplankton inoculum was added biweekly to reduce major divergences between mesocosms due to dispersal limitation and wetland plants were seeded in the transition zone.

Mesocosms were randomly assigned to one of six treatments (three replicates per treatment): control-ambient nutrient, control-nutrient enriched, AuNPs-ambient nutrient, AuNPs-nutrient enriched, Kocide (CuNPs)-ambient nutrient, and Kocide (CuNPs)-nutrient enriched. The synthesis and TEM characterization of citrate-stabilized AuNPs (average diameter: 11.9 $\pm$ 1.2 nm) and characterization of copper hydroxide NPs (average diameter: 38.7 $\pm$ 8.2 nm) (CuNPs; Kocide 3000; DuPont, Wilmington, Delaware, USA) were described previously (Simonin et al., 2018a). Mesocosms dosed with AuNPs received a weekly dose of 19 mg Au, resulting in a total dose of 750 mg Au after 9 months. As Kocide is 27% Cu, CuNP mesocosms received an initial pulse of 93.7 mg of Cu and then a weekly dose of 9.5 mg of Cu, resulting in a total dose of 450 mg Cu as Kocide per mesocosm after 9 months. Starting in September 2015, the nutrient-enriched treatments received 1 L of mesocosm water each week supplemented with 88 mg of N as KNO_3 and 35 mg of P as KH_2PO_4 to mimic agricultural run-off. This was a collaborative project and the nutrient amendment conditions were part of a complex experimental design that balanced the needs of many researchers, including preventing water column hypoxia.

Au and Cu concentrations in unfiltered surface water and other environmental metadata including temperature were collected as previously described (Avellan et al., 2020). To examine the microbial community, the aquatic zone was sampled immediately before dosing (D0), then 1 and 7 days (D1, D7) after dosing, as well as after the first (T1), second (T2) and third (T3) quarters. At each timepoint, ~250 mL of water was collected from the near-surface (~0.25 m depth) by submerging sterile polypropylene bottles, and microbial biomass was collected from ~100-250 mL of water on 0.22 μm Supor filters (Pall) via gentle vacuum filtration upon return to the lab. Samples were stored at −80 °C until DNA extraction.

194

195 **Jar microcosm experiments**

196

197 In this experimental follow up to the outdoor mesocosms, simplified microcosms in one-quart
198 acid-washed Ball® glass mason jars examined the impacts of ecosystem complexity and
199 seasonal conditions on AuNP-dosed microbiomes, as described previously (Bergemann et al.,
200 2023). Four treatments were chosen to compare with mesocosms mimicking both season (spring
201 and early fall) and ecosystem complexity (microbes only or microbes + *Egeria*). Environmental
202 Growth Chambers were set to match spring conditions (light: dark 12:12 hrs; irradiance 481.95
203 ± 4.14 lum ft^{-2} ; temperature 15 °C and 10°C in the light and dark periods, respectively) and
204 early fall (15:9 hrs light: dark cycle; irradiance 521.65 ± 3.08 lum ft^{-2}; temperature 30 °C and 20
205 °C in the light and dark periods, respectively). We note the "season" label differs between this
206 paper and a prior publication (Bergemann et al., 2023). Both spring and fall conditions were
207 assayed for two ecosystem complexities: microbes only and microbes + *Egeria densa*, with 6
208 replicate jars for each condition. Each microcosm was filled with 100 g of washed Quickrete
209 pool filter sand 700 mL of 0.25mm filtered water collected in July 2017 from a control
210 mesocosm (described above) and 1 mL of 0.25 mm filtered local wetland water; filtration
211 removed large organisms and debris to establish a microbiome. Macrophyte-containing
212 microcosms also included five rinsed shoots of *E. densa* with a total wet weight of 6g. Weekly
213 for 5 weeks, 143.3 µg of nitrogen (N) and 56.97 µg of phosphorus (P) were added to each
214 microcosm as KNO_3 and KH_2PO_4. Each jar was capped with Parafilm® to allow the exchange of
215 gases as well as light infiltration. After a week of acclimation, the AuNPs exposures began using

the same AuNP stock as the mesocosm experiment with 31.36 µg of Au added per week for a total of 125.44 µg over four weeks.

Water samples were collected to measure Au concentration and dissolved organic carbon (DOC). To measure Au concentration in the microcosms, 5 mL of water was collected weekly, acidified with HNO_3 and HCl, then quantified using ICP-MS (Agilent 7700 and 7900). At the end of the experiment, 10 mL of GFF-filtered water was collected to measure dissolved organic carbon (DOC) using a TOC-VCPH Analyzer with a TNM-1 module (Shimadzu). At the end of the experiment, microbial biomass for community analysis was collected from 100 mL of water on 0.22 µm Supor filters (Pall) via gentle vacuum filtration. Samples were stored at −80 °C until DNA extraction.

Nucleic acid extraction, library preparation and sequence analysis

Genomic DNA for SSU rRNA gene libraries was extracted using the Gentra Puregene Yeast/Bacteria kit (QIAGEN) supplemented with bead beating (60 seconds; Biospec), cleaned using the Zymo *OneStep* PCR inhibitor removal kit and quantified using a Nanodrop ND-1000. 515F-926R (V4-V5) 16S rRNA gene libraries were constructed using a dual-barcode sequencing approach (Needham et al., 2019; Parada et al., 2016). PCR reactions were performed in triplicate with 20 µl reactions containing 20 ng template DNA, 1× Taq Buffer, 0.5 µM of each primer, 200 µM of dNTPs, and 0.4 U of non-proofreading Econo Taq (Lucigen). The thermal cycling conditions were 2 min at 95°C, followed by 25 cycles of 1 min at 95°C, 1 min at 50°C, 30 sec at 72°C, and a final extension at 72°C for 10 min. Triplicate PCR reactions were pooled and gel

purified (QIAquick, QIAGEN). Libraries were pooled at the same concentration, and the final pooled library concentration and purity verified by TapeStation (Agilent) and sequenced at the Duke Center for Genomic and Computational Biology using v2 2 x250 bp sequencing on the Illumina MiSeq.

Barcodes were removed and sequences were assigned to each sample using CASAVA (Illumina) and MacQIIME v1.9.1, sequences were then cleaned and clustered using USEARCH v.9.2 (Edgar, 2013). Low quality sequence ends were trimmed at a Phred quality score (Q) of 30 using a 10 bp running window. Paired-end reads were merged if the overlap was at least 10 bp with no mismatches. Sequences with expected errors >1 and/or a length <400 bp were removed. Potential chimeras were filtered with uchime2 in USEARCH v.9.2. MED v2.1 was then used to resolve amplicon sequence variants (ASVs) (Eren et al., 2015), with a minimum unique sequence abundance of 20. The remaining 10,374 ASVs represented 7,368,537 reads, representing 89% of all reads. The taxonomies of representative ASVs were classified using MacQIIME v1.9.1 using RDP classifier v2.2 (Wang et al., 2007). Mitochondrial and chloroplast sequences were removed and the libraries were then sub-sampled to 8,074 reads per library. SSU rRNA library sequences were deposited as Bioproject PRJNA613470.

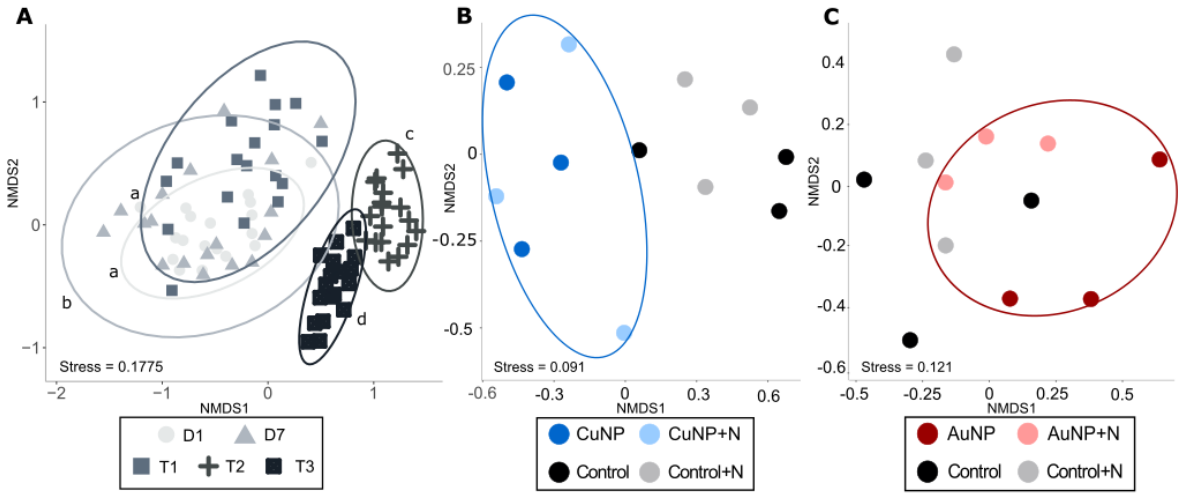
Bray-Curtis dissimilarities were visualized using non-metric multidimensional scaling (NMDS) ordination, and beta-diversity was analyzed by permutational multivariate ANOVA (PERMANOVA) using the adonis function in the *vegan* R package (Oksanen et al., 2015). ASV relative abundances >0.1%, with an added pseudo count of 1 to avoid excessive zeros inflating the model, were used to identify taxa with statistically significant effects of nanoparticles

(CuNPs or AuNPs), nutrients or interactions between factors using DESeq2 with a multifactor design (Love et al., 2014). Comparisons between environmental variables utilized the non-parametric Wilcoxon signed rank test, and significant differences were identified when $p < 0.05$ (Benjamini-Hochberg adjusted).

Results and Discussion

In order to characterize microbiome responses to NP-containing contaminant mixtures, here we initially focus on the aquatic compartment of wetland mesocosms exposed to factorial NP and nutrient treatments. In this experiment, CuNP treatments received a high initial dose (~94 mg of Cu as Kocide) to mimic a high load due to storm-driven transport and then weekly doses of CuNPs at concentrations approximating agricultural runoff (Simonin et al., 2018a). This approach led to high initial Cu in the water column that gradually declined over time (Figure S1). In contrast, AuNPs were applied at a steady rate and quickly sedimented out of the water column, resulting in aquatic gold concentrations that were slightly elevated over controls throughout the experiment (Figure S1). As mesocosms were dosed weekly, the absence of water column accumulation indicates the partitioning of both metals into other environmental compartments, mainly the floc and aquatic plants (Avellan et al., 2020). In order to understand how NPs and nutrient additions might impact aquatic microbial communities, we examined microbial community composition 1 day (D1) and 7 days (D7) after dosing initiation to identify initial treatment effects and after 3, 6, and 9 months (T1, T2 and T3) to investigate potential chronic or accumulation-driven microbiome impacts. While there were strong seasonal changes

285 in the microbial community (PERMANOVA, $p < 0.05$; Figure 1A and Table S1), we did not
286 identify a significant effect of nutrient addition alone or of NP-nutrient interactions on the
287 microbiome (Figures 1, S2, S3 and Tables S2, S3). While nutrient addition previously alleviated
288 Kocide inhibition of soil microbes (Simonin et al., 2018b), here we posit that most of the
289 heterotrophic microbes are not nutrient-limited, thus low levels of added nitrogen and
290 phosphorous did not significantly alter microbial community composition. However, prior
291 research on these mesocosms found that nutrient-amended NP treatments intensified episodic
292 macroalgal blooms, significantly altering competition between planktonic algae and floating
293 plants and other environmental parameters (Simonin et al., 2018a). Somewhat surprisingly, the
294 impact of nutrients on primary producers did not propagate to the non- eukaryotic, planktonic
295 microbiome composition examined here; but higher nutrient concentrations or an increased
296 number of mesocosm replicates might have revealed statistically-significant effects on the
297 microbiome. As nutrient additions did not significantly alter the aquatic microbiome, we focused
298 on the NP treatments by grouping the mesocosms with and without nutrient additions in
299 subsequent analyses (n=6). To identify potential nanoparticle treatment effects, we compared all
300 NP-samples versus non-NP amended controls; significant community NP treatment effects were
301 observed only in T3 for both Cu and Au NPs (Figures 1, S2, S3 and Tables S2, S3). Although the
302 CuNP treatment microbial communities separated from controls on days 1 and 7 (Figure S2),
303 samples violated the assumption of equal dispersion (betadisper, $p < 0.05$), thus expected short-
304 term CuNP treatment responses, potentially due to Cu toxicity, could not be evaluated
305 statistically (Table S2).



306

307 **Figure 1. Mesocosm microbial community compositional changes over time and in response**
308 **to Cu and Au nanoparticles (NPs) and nutrient additions (+N).** (A) Non-metric
309 multidimensional scaling (NMDS) ordination computed based on Bray-Curtis dissimilarity for
310 16S rRNA gene libraries of all samples over time. Ellipses show 95% confidence intervals
311 around the mean. Samples with different letters "a", "b", "c" and "d" indicate significant
312 differences over time (by combining all mesocosms at each time point regardless of treatment;
313 pairwise PERMANOVA $p<0.05$). D1 and D7 indicate days relative to the initiation of NP
314 dosing. T1 (3-month), T2 (6-month) and T3 (9-month) represent quarterly samples. Panels (B)
315 and (C) show NMDS ordination based on Bray-Curtis dissimilarity for 16S rRNA gene libraries
316 at time points where the NP-treated mesocosms (combining NP and NP+N treatments)
317 statistically differed from the non-NP amended treatments (combining Control and Control +N).
318 The ellipses in (B) and (C) were manually drawn to highlight the effect of NPs on microbial
319 community composition. (B) Shows the significant microbiome impacts CuNP-treatment in the
320 third quarter (T3; PERMANOVA, $p<0.05$). (C) Shows the significant microbiome impacts of
321 AuNP-treatment in the third quarter (T3; PERMANOVA, $p<0.05$). "+N" indicates nutrient
322 additions, which did not significantly affect microbial community composition, nor interact with
323 CuNP or AuNP at any time point (PERMANOVA, $p>0.05$).

324

325 As neither metal accumulated in the aquatic compartment (Figure S1), the chronic (T3) aquatic

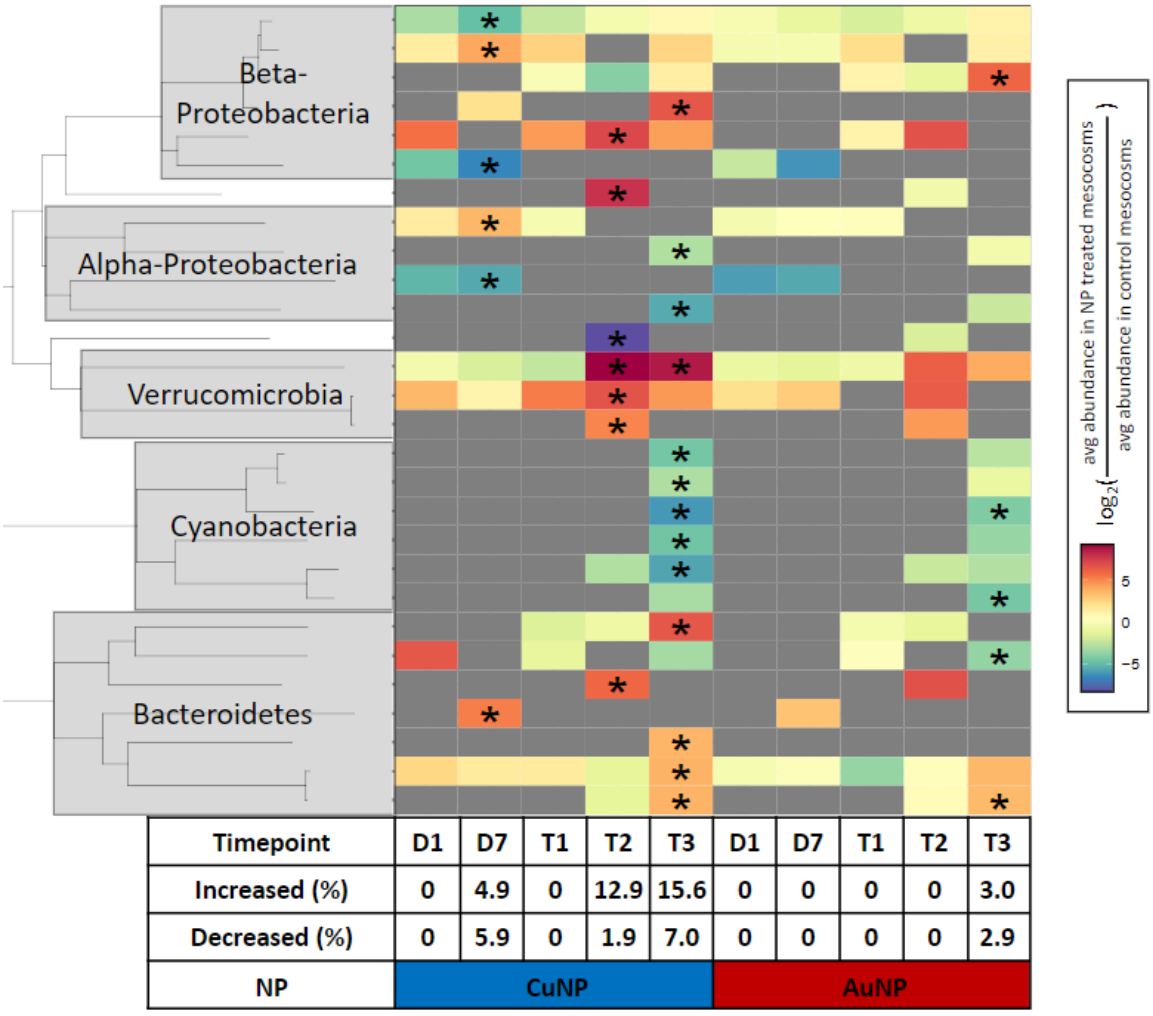
326 microbiome effects in both NP treatments were likely mediated by changes in the abundance or

physiology of other organisms (i.e. biological interactions), impacts of seasonality (e.g. effect of temperature, prevalence of sensitive organisms), or changes in the speciation and bioavailability of the metals (Avellan et al., 2020). Other potential explanations, such as gradual changes in the microbiome due to chronic exposure, were deemed unlikely due to rapid turnover in aquatic microbial populations. However, as treatments altered the balance between the macrophyte *Egeria* and planktonic algae in the aquatic zone (Simonin et al., 2018a), changes in the primary producer composition or metal-induced physiology could potentially alter the organic matter pool available to microbial communities. We were specifically interested in explaining the timing of the nanoparticle effect on microbiomes. While not a treatment effect, dissolved organic carbon (DOC) declined at the end of the experiment (~ 5 mg L^{-1} at T3 versus >10 mg L^{-1} at other time points); as organic matter stabilizes and reduces the reactivity of NPs, lower organic matter levels could increase the toxicity of metallic NPs or their dissolution products (Aristi et al., 2016; Bone et al., 2012; Diegoli et al., 2008; Miao et al., 2009). These lower DOC levels in T3 are likely due to *Egeria* senescence (Avellan et al., 2020; Simonin et al., 2018a), potentially coupling ecotoxicity with plant growth stage. In addition to complexation with the nanoparticles directly, labile DOC produced by actively-growing primary producers could alleviate NP-toxicity by providing increased resources that allow microbial investment in detoxification etc. In short, for this complex wetland experiment, we predict that the effect of NPs and their transformation products on the water column microbial community are potentially predominately indirect impacts mediated by complex ecosystem interactions.

In order to gain greater insight into potential NP and nutrient effects on specific taxa, we examined population-level treatment responses using 16S rRNA gene amplicon sequence

variants (ASVs). Again, a two-factor design was applied to examine the impacts of nanoparticles, nutrient additions and interactions between these two factors. For example, the effect of the CuNP treatment was identified by comparing all Kocide-treated mesocosms versus the mesocosms without nanoparticles, regardless of nutrient addition (n = 6). As there were not significant differences between ambient and nutrient-amended treatments (Figures S4, S5), we again focus on nanoparticle treatments. At the population level, CuNP treatments showed significant effects on day 7 as well as at the T2 and T3 time points (Figure 2; Table S4). At the beginning of the experiment (D7), taxa comprising 5.9% of the community significantly declined versus 4.9% which increased in CuNP treatments compared to controls (Figure 2), suggesting a balance between toxicity effects and microbes which benefit from reduced competition or additional resources released by dying cells. In contrast, at later time points, a larger percentage of taxa significantly increased (12.9%, 15.6%) versus declined (1.9%, 7.0 %) of the CuNP-treated community in T2 and T3, respectively, suggesting that responsive phylotypes do not reflect environmental toxicity but also potentially include taxa which benefit from new niches or altered physiology in the NP- treatments (Figure 2). We considered a number of potential explanations for the observed Cu-treatment results including copper toxicity, Cu's role as a micronutrient that could stimulate growth, and ecosystem-level impacts including shifts in environmental resources. Treatment-responsive taxa were spread throughout the phylogenetic tree (Figure 2); however, some trends emerged which provide insight into potential mechanisms. Pertaining to the toxicity hypothesis, in T3 CuNP mesocosms, several cyanobacterial ASVs decreased (Table S4); these declines in cyanobacteria are consistent with either Cu toxicity or changes in the balance of primary producers, as observed previously (Simonin et al., 2018a), but by themselves are not conclusive. Second, we examined the potential for copper to act as a key

373 micronutrient (Clar et al., 2016; Jamers et al., 2013). As aquatic Cu concentrations are >10x
374 limiting concentrations even in non-CuNP treatments (Posacka et al., 2019), population increases
375 in CuNP-treatments are unlikely to reflect alleviation of Cu limitation. Finally, we examined the
376 evidence for ecosystem-level changes in the system; in addition to declines in cyanobacterial
377 relative abundance, in T2 CuNP treatments a number of Verrucomicrobia ASVs increased;
378 Verrucomicrobia are known polysaccharide degraders and may reflect increased environmental
379 availability of these compounds (He et al., 2017). Notably, these results contrast with previous
380 chronic AgNP treatment mesocosms (Ward et al., 2019), where similar responsive taxa were not
381 observed in both initial and long term exposure time points; the results in this study suggest
382 either strong microbiome seasonality (Figure 1) or different factors governing microbial
383 responses across the time course of the experiment. Thus, we conclude that chronic dosing of
384 CuNPs yields a complicated response, with microbial populations potentially affected by CuNP
385 treatments both directly (e.g. toxicity) and indirectly (e.g. via interactions with CuNP-responsive
386 ecosystem components), as evidenced by microbial populations that increased as well as
387 decreased in abundance. Compared to CuNP treatments, fewer taxa significantly increased or
388 decreased at any time point in AuNP treatment mesocosms (Figure 2). All 5 AuNP treatment-
389 responsive taxa were in T3; yet there was not an apparent phylogenetic signal (i.e. no clustering
390 of responsive taxa in the phylogenetic tree) and responsive taxa both increased and decreased in
391 relative abundance (Figure 2). In summary, population-level analysis shows that compared to
392 AuNPs, CuNP treatment caused more widespread impacts across both time and microbial taxa,
393 with AuNP treatment resulting in microbial community shifts relative to controls only at the end
394 of the experiment, through an unknown mechanism.



Timepoint	D1	D7	T1	T2	T3	D1	D7	T1	T2	T3
Increased (%)	0	4.9	0	12.9	15.6	0	0	0	0	3.0
Decreased (%)	0	5.9	0	1.9	7.0	0	0	0	0	2.9
NP	CuNP					AuNP				

Figure 2. Mesocosm amplicon sequence variants (ASVs) that significantly respond to CuNP or AuNP treatments. Log_2 fold change of each ASV was calculated as $\log_2(\frac{avg\ abundance\ in\ NP\ treated\ mesocosms}{avg\ abundance\ in\ control\ mesocosms})$. NP treated mesocosms include NP only and NP-nutrient enriched mesocosms (n=6), and control mesocosms include both ambient control and control + Nutrient addition mesocosms (n=6), as nutrients did not show any significant individual or interactive effect with NPs on microbial community composition. ASVs are shown in the plot if: (1) they are identified as significantly responding to CuNPs or AuNPs at any time point using DESeq2 (Asterisks indicate the taxa relative abundance was significantly different that controls $p<0.05$); and (2) ASV relative abundance exceeds the threshold of 0.2% at the corresponding time point. Gray shading indicates that the ASV does not exceed the 0.2% abundance threshold at that time point. ASVs are organized by a maximum likelihood phylogenetic tree with major phyla labelled. Underneath the heatmap, the total relative

abundance of ASVs that significantly increased in the NP- treated mesocosms or declined in the control mesocosms are labeled with Increased (%) and Decreased (%).

Microcosm experiments to explore ecosystem complexity in microbiome responses to NP

The question remains why T3 (fall) samples exhibited microbiome responses in AuNP and CuNP treatments, with the prediction that environmental factors rather than accumulation drives this response. We specifically focus on AuNP treatments, as elemental gold was historically taken as an inert tracer not toxic to microbes (Ahmad et al., 2013; Zhang et al., 2015), although recent examples of microbial toxicity have been noted in the literature (Sathiyaraj et al., 2021).. While the mechanisms of AuNP microbiome responses are unclear, they may include AuNP antimicrobial activity of either the NPs (Sathiyaraj et al., 2021) or their environmental transformation products including potentially-toxic gold ions or gold-containing compounds (Avellan et al., 2018). Moreover, the fact that AuNPs mesocosms exhibited a microbiome response at a single time point suggests a role for ecosystem interactions (Gräf et al., 2023), which we sought to test here through guided experimentation. In addition to direct toxicity, microbial community shifts could be explained by multi-stressor effects (e.g. warmer water temperatures in fall samples) or indirect effects through interactions with other AuNP-treatment sensitive organisms (Hunt and Ward, 2015; Wang et al., 2021). To differentiate among the mechanisms behind AuNP aquatic microbiome responses and to remove co-occurring changes with season (e.g. plant growth stage), we specifically tested the impact of season (spring or fall; temperature and light incubations) and ecosystem complexity (presence of primary producers) using simplified, month-long jar microcosms. Compared to the mesocosms, microcosms had reduced organismal complexity: microbes alone or microbes incubated with the aquatic plant

Egeria, the biomass-dominant primary producer in the aquatic compartment of the mesocosms, which provides heterotrophic bacteria with carbon, competes with microbial primary producers and alters water quality parameters (Figure S6). The month-long duration enables observation of microbiome shifts with seasonal incubation conditions and/or AuNP treatment, without the long-term accumulation effects that occurred over 9 months in the mesocosms. Microcosm conditions were set to match either the beginning (spring: D1, D7) or end of the experiment (fall: T3), when a significant AuNP microbiome treatment effect was observed.

In the microcosm experiment, we observed a strong season-treatment effect (Figure S7), consistent with the known impact of temperature on aquatic microbial communities (Wang et al., 2021; Ward et al., 2017) (Figure 3). However, contrary to our initial hypothesis of season-related interactions with AuNPs, for both spring and fall regimes, AuNP treatment influenced microbiome composition in the microbe-only but not in the microbiome + *Egeria* microcosms (for a given ecosystem complexity and season, microbiomes were compared with or without AuNP treatment: PERMANOVA, $p<0.05$; Figure 3). Thus, the presence of *Egeria* buffers the AuNP-treatment effect on microbes. Similarly, wetland plants were shown to mitigate the impacts of AgNPs on microbially-mediated biogeochemical cycles (He et al., 2022) and toxicity effects on juvenile fish (Bone et al., 2012), suggesting a more general role for primary producers in mediating NP toxicity. Consistent with these community-level results, more AuNP treatment-responsive taxa were identified in microbe-only (37) vs. microbes with *Egeria* treatments (1), (Figure S8). While AuNPs were initially predicted to exhibit minimal toxicity (Zhang et al., 2015), researchers have observed AuNP toxicity in microbial cultures (Ahmad et al., 2013; Hernández-Sierra et al., 2008) and Au bound to ligands (e.g. cyanide, hydroxyl and thiol) has

unknown microbial effects (Avellan et al., 2020; Avellan et al., 2018). Responsive taxa in the microcosm experiments included a number of shifts (both positive and negative) in the relative abundance of Bacteroidetes, potentially reflecting replacement of AuNP-treatment-sensitive taxa with resistant taxa that filled similar ecological niches (Figure S8). Thus, we examined other microcosm parameters to identify potential mechanisms for *Egeria*'s mediation of AuNP's microbiome impacts.

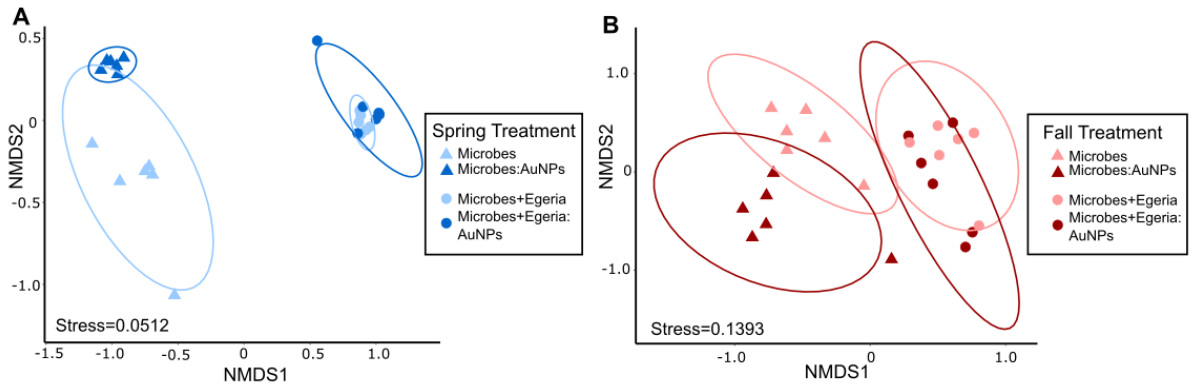


Figure 3. Microcosm microbial community changes (16S rRNA gene libraries) with gold nanoparticle conditions for different seasonal conditions and ecosystem complexity. Jar microcosm microbiomes at the end of the experiment are shown as non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity. (A) Spring treatment conditions: average 12.5 ^{o}C, light:dark: 12:12 hours (B) Fall conditions: average 26.25 ^{o}C, light:dark: 15:9 hours Ellipses (95% confidence intervals around the mean) show significant effects of AuNP treatment for a given seasonal treatment in the Microbe-only microcosms (PERMANOVA, $p<$ 0.05). Triangles indicate microcosms with microbes only and circles those containing both microbes and the plant *Egeria densa*.

Although AuNP treatments with *Egeria* did not exhibit shifts in microbiome composition in either season, water column parameters suggest *Egeria* has different effects on the AuNPs: in

spring, removal of gold from the water column and in fall stabilization and inactivation of water column gold through enhanced DOC concentrations (Glenn and Klaine, 2013). In the spring AuNP treatment microcosms, aquatic gold concentrations are significantly higher in the microbe-only condition (Figure 4A, $p<0.05$ Wilcoxon signed- rank test), and more gold accumulated in the *Egeria* (Microbe+ *Egeria* treatment) (Bergemann et al., 2023). In contrast, under fall conditions, water column gold was significantly higher (mean ~ 40 $\mu g\ L^{-1}$) in the microbe + *Egeria* AuNP treatment than in the microbe-only treatment (~ 10 $\mu g\ L^{-1}$, Figure 4A, $p<0.05$ Wilcoxon signed rank test). This higher aquatic gold concentrations in the fall microbe + *Egeria* AuNP treatment could be explained by high *Egeria*-produced dissolved organic carbon (DOC) concentrations which stabilized aquatic Au and potentially reduced its toxicity (Figure 4B; $p<0.05$ Wilcoxon signed rank test) (Aristi et al., 2016; Diegoli et al., 2008; Glenn and Klaine, 2013; Miao et al., 2009). As the DOC concentration is elevated in *Egeria*-containing AuNP and Control treatments (Figure 4B), DOC levels are due to "fall" conditions rather than the AuNP treatment. Although we cannot definitively assign a mechanism, these results complement the field mesocosm's conclusions that microbe-only studies may not readily translate to complex ecosystems, where interactions with other organisms (and environmental factors) mediate contaminant microbiome responses in complex and unpredicted ways. Overall, these combined experiments suggest that growing aquatic plants attenuates NP-toxicity; however, this protective effect is lost during *Egeria* senescence with the accompanying decline in aquatic DOC (as observed in the mesocosm experiment).

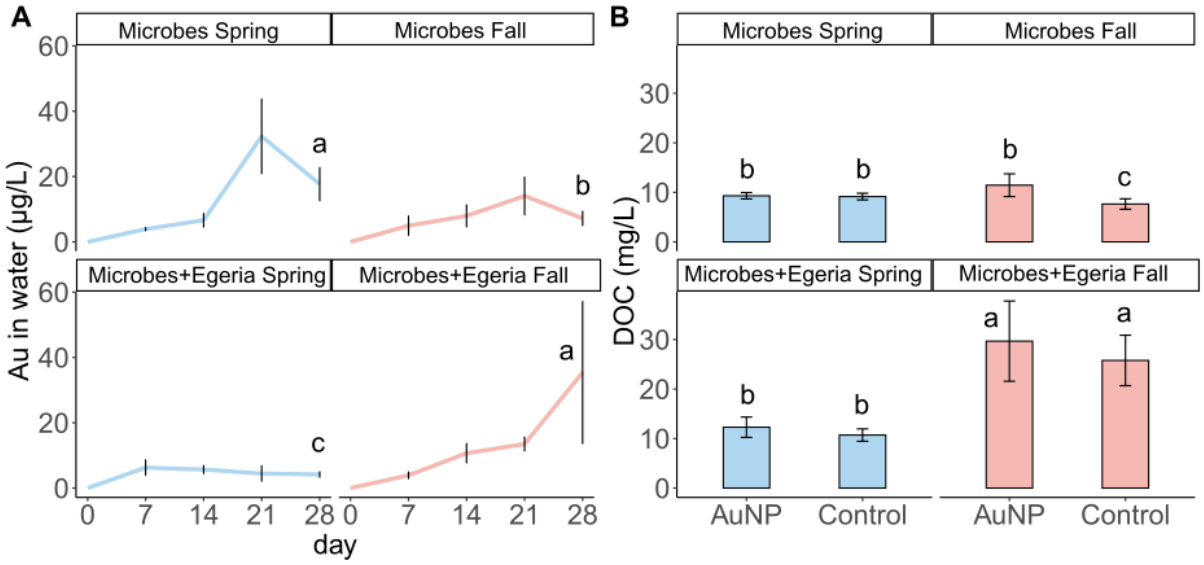


Figure 4. Microcosm gold and dissolved organic carbon (DOC) colonized by either microbes or microbes + *Egeria* incubated under spring and fall conditions. (A) Total gold in the water column over the 28-day incubation for AuNP treatment microcosms. Means on day 28 labeled with the same letter are not significantly different (Wilcoxon ranked sum test, $p<0.05$). Error bars show one standard deviation. (B) Microcosm DOC concentrations on day 28 labeled with the same letter are not significantly different (Wilcoxon ranked sum test, $p<0.05$). Error bars show one standard deviation. Seasonal comparisons between spring (avg. 12.5 ºC, light:dark; 12:12 hours) and fall (avg. 26.25 ºC, light:dark; 15:9 hours) treatments.

Conclusions

In these set of two linked experiments, we found that CuNPs and AuNPs treatments can exert significant effects on aquatic microbial communities, but that microbiome responses are likely a combination of direct effects as well as interactions with other ecosystem components. While NPs can generate broad ecosystem-level effects either as synthesized or as transformation products, as well as indirect effects mediated by interactions with other organisms (Hunt and

Ward, 2015), the impacts of nanoparticles are strongly mediated by environmental complexity. Here, we speculate that, compared to other taxa, primary producers have the potential to either suppress or propagate the effects of contaminants to other trophic levels due to their position at the base of the food web and biomass dominance in many ecosystems (Ge et al., 2014; Slaveykova, 2022). This research suggests that rather than requiring full-ecosystem complexity, simplified microcosms containing primary producers may allow greater insights into the impacts of nanoparticles and other contaminants on microbiomes. Thus, by focusing on critical ecosystem components, we can better understand the processes by which contaminants transform and are transformed by ecosystems.

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