

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

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1	Interactions shape aquatic microbiome responses to Cu and Au nanoparticle treatments in
2	wetland manipulation experiments
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4	Running title: Interactions mediate microbiome nanoparticle responses
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- 35 Abstract
- 36

In natural systems, organisms are embedded in complex networks where their physiology and 37 38 community composition is shaped by both biotic and abiotic factors. Therefore, to assess the 39 ecosystem-level effects of contaminants, we must pair complex, multi-trophic field studies with 40 more targeted hypothesis-driven approaches to explore specific actors and mechanisms. Here, we examine aquatic microbiome responses to long-term additions of commercially- available 41 metallic nanoparticles [copper-based (CuNPs) or gold (AuNPs)] and/or nutrients in complex, 42 43 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 44 45 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 46 nutrients Simplified microbe-only or microbe + plant incubations revealed that direct effects of 47 48 AuNPs on aquatic microbiomes can be buffered by plants (regardless of seasonal As mesocosms 49 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 50 51 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 52 53 presence of primary producers, highlighting the importance of organismal interactions in 54 mediating emerging contaminants' ecosystem-wide impacts.

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#### 81 Introduction

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Among emerging contaminants, engineered metallic nanoparticles (NPs) have received increased 83 attention as their consumer applications have expanded (Saravanan et al., 2021). NPs' small size 84 85 (1-100 nm) and large surface area: volume ratio generally increase their reactivity relative to 86 their bulk counterparts (Auffan et al., 2009). The biological impacts of inorganic nanoparticles 87 are generally attributed to the release of dissolution products or nano-specific effects due to their 88 physical properties. For example, metallic NPs can disrupt cell membranes and generate 89 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 90 91 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 92 expanding our understanding of the ecosystem-level impacts of commercially-available NPs, 93 94 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 95 include lower substrate requirements; for example, while copper has been used as a pesticide for 96 97 over a hundred years, newer copper-NP based biocides like Kocide® 3000 (Dupont) both 98 enhance antimicrobial properties and reduce Cu usage (Giannousi et al., 2013; Kah et al., 2018). 99 As biocides are commonly used in conjunction with other agrochemicals like fertilizers, these 100 co-occurring contaminants could alter ecosystems with impacts distinct from either the fertilizer 101 or pesticide alone (Kah, 2015).

103 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 104 105 and fate are dynamic, and differ from bulk counterparts (Avellan et al., 2020), or (ii) interactions 106 between multiple contaminants that can lead to unexpected biological impacts (Brennan and 107 Collins, 2015; Hagenbuch and Pinckney, 2012). For example, nutrients can attenuate 108 contaminant toxicity directly by binding contaminants, or indirectly by increasing the organism's 109 biomass or energy investment in detoxification (Aristi et al., 2016; Leflaive et al., 2015; Pieters 110 et al., 2005; Skei et al., 2000). Conversely, nutrients can increase toxicity through enhanced 111 contaminant uptake (Hu et al., 2013). As we cannot robustly predict ecosystem outcomes from 112 short-term, laboratory studies, recent research has focused on environmentally realistic 113 conditions including contaminant mixtures or co-occurring stressors in complex multi-trophic 114 systems.

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116 Microbes are a critical component of all ecosystems: with high diversity, short generation times 117 and as critical mediators of biogeochemical cycles, the microbiome can be a sensitive and 118 ecologically-important indicator of disturbance (Aylagas et al., 2017; Hunt and Ward, 2015). 119 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 120 121 microbiome analyses. These community-level microbiome studies incorporate key, often 122 uncultured organisms; account for different responses within microbiomes and incorporate 123 modification of NP by other organisms or ecosystem components (Chae et al., 2014; Colman et 124 al., 2014; Ward et al., 2019). Thus, there is a growing body of literature on how interactions with 125 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).

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130 Here, to investigate how ecosystem complexity and co-occurring anthropogenic contaminants 131 shape microbial responses to NPs, we examine aquatic microbiomes under chronic dosing of 132 NPs and nutrients (N and P) in wetland mesocosms. We focus on two NPs: the commerciallyavailable agricultural biocide Kocide® 3000 [containing Cu(OH)2 NPs] and citrate-coated gold 133 134 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 135 136 medical treatments as well as potential ecotoxicity. Both these NP-based contaminants were 137 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 138 139 mesocosms has revealed unexpected organismal responses (Perrotta et al., 2020), NP 140 (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 141 142 stressors impacts aquatic microbial communities (Rillig et al., 2019). Further, as these 143 mesocosms contain a complex food web including fish, snails, and plants, microbiome 144 composition reflects both direct contaminant impacts and indirect effects mediated by 145 interactions with other organisms (Hunt and Ward, 2015). To directly address the issue of 146 ecosystem complexity, we employed a microcosm experiment (Bergemann et al., 2023) to 147 address AuNP treatment effects in simplified communities composed of either only microbes or 148 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 indynamic aquatic environments.

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152 Methods

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#### 154 Wetland mesocosm experiments

Experiments were conducted at the Center of Environmental Implications of Nanotechnology 155 156 (CEINT) mesocosm facility in the Duke University Forest (Durham, North Carolina, USA) from 157 January 2016 - October 2016. Details about experimental set-up and monitoring were previously 158 described (Lowry et al., 2012; Simonin et al., 2018a). Briefly, slantboard mesocosms (3.66 m 159 long, 1.22 m wide and 0.8 m high) lined with a water-tight geotextile (0.45 mm reinforced 160 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 161 rarely flooded zone (upland zone). The mesocosms were filled with well water sourced at the site 162 163 with an average starting water volume of 452 L; as the water level fluctuated over time with 164 precipitation and evapotranspiration, therefore dosing is provided as weight rather than 165 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 166 167 fish Gambusia holbrookii (eastern mosquitofish). An algal and zooplankton inoculum was added 168 biweekly to reduce major divergences between mesocosms due to dispersal limitation and 169 wetland plants were seeded in the transition zone.

171 Mesocosms were randomly assigned to one of six treatments (three replicates per treatment): control-ambient nutrient, control-nutrient enriched, AuNPs-ambient nutrient, AuNPs-nutrient 172 173 enriched, Kocide (CuNPs)-ambient nutrient, and Kocide (CuNPs)-nutrient enriched. The synthesis and TEM characterization of citrate-stabilized AuNPs (average diameter:  $11.9 \pm 1.2$ 174 175 nm) and characterization of copper hydroxide NPs (average diameter:  $38.7 \pm 8.2$  nm) (CuNPs; 176 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 177 178 dose of 750 mg Au after 9 months. As Kocide is 27% Cu, CuNP mesocosms received an initial 179 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-180 181 enriched treatments received 1 L of mesocosm water each week supplemented with 88 mg of N 182 as KNO<sub>3</sub> and 35 mg of P as KH<sub>2</sub>PO<sub>4</sub> to mimic agricultural run-off. This was a collaborative 183 project and the nutrient amendment conditions were part of a complex experimental design that 184 balanced the needs of many researchers, including preventing water column hypoxia. 185 186 Au and Cu concentrations in unfiltered surface water and other environmental metadata 187 including temperature were collected as previously described (Avellan et al., 2020). To examine 188 the microbial community, the aquatic zone was sampled immediately before dosing (D0), then 1 189 and 7 days (D1, D7) after dosing, as well as after the first (T1), second (T2) and third (T3) 190 quarters. At each timepoint, ~250 mL of water was collected from the near-surface (~0.25 m 191 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 192 193 the lab. Samples were stored at -80 °C until DNA extraction.

#### 195 Jar microcosm experiments

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  $\pm$  4.14 lum ft<sup>-2</sup>; temperature 15 °C and 10°C in the light and dark periods, respectively) and 203 early fall (15:9 hrs light: dark cycle; irradiance  $521.65 \pm 3.08$  lum ft<sup>-2</sup>; temperature 30 °C and 20 204 205 °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<sub>3</sub> and KH<sub>2</sub>PO<sub>4</sub>. 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 atotal of 125.44 µg over four weeks.

219	Water samples were collected to measure Au concentration and dissolved organic carbon (DOC).
220	To measure Au concentration in the microcosms, 5 mL of water was collected weekly, acidified
221	with HNO3 and HCl, then quantified using ICP-MS (Agilent 7700 and 7900). At the end of the
222	experiment, 10 mL of GFF-filtered water was collected to measure dissolved organic carbon
223	(DOC) using a TOC-VCPH Analyzer with a TNM-1 module (Shimadzu). At the end of the
224	experiment, microbial biomass for community analysis was collected from 100 mL of water on
225	$0.22 \ \mu m$ Supor filters (Pall) via gentle vacuum filtration. Samples were stored at $-80 \ ^\circ C$ until
226	DNA extraction.
227	
228	Nucleic acid extraction, library preparation and sequence analysis
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230	Genomic DNA for SSU rRNA gene libraries was extracted using the Gentra Puregene
231	Yeast/Bacteria kit (QIAGEN) supplemented with bead beating (60 seconds; Biospec), cleaned
232	using the Zymo OneStep PCR inhibitor removal kit and quantified using a Nanodrop ND-1000.
233	515F-926R (V4-V5) 16S rRNA gene libraries were constructed using a dual-barcode sequencing
234	approach (Needham et al., 2019; Parada et al., 2016). PCR reactions were performed in triplicate
235	with 20 $\mu$ l reactions containing 20 ng template DNA, 1× Taq Buffer, 0.5 $\mu$ M of each primer, 200
236	$\mu M$ of dNTPs, and 0.4 U of non-proofreading Econo Taq (Lucigen). The thermal cycling
237	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
238	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.

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244 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.9.2 245 246 (Edgar, 2013). Low quality sequence ends were trimmed at a Phred quality score (Q) of 30 using 247 a 10 bp running window. Paired-end reads were merged if the overlap was at least 10 bp with no 248 mismatches. Sequences with expected errors >1 and/or a length <400 bp were removed. Potential 249 chimeras were filtered with uchime2 in USEARCH v.9.2. MED v2.1 was then used to resolve 250 amplicon sequence variants (ASVs) (Eren et al., 2015), with a minimum unique sequence 251 abundance of 20. The remaining 10,374 ASVs represented 7,368,537 reads, representing 89% of 252 all reads. The taxonomies of representative ASVs were classified using MacQIIME v1.9.1 using 253 RDP classifier v2.2 (Wang et al., 2007). Mitochondrial and chloroplast sequences were removed 254 and the libraries were then sub-sampled to 8,074 reads per library. SSU rRNA library sequences 255 were deposited as Bioproject PRJNA613470.

256

257 Bray-Curtis dissimilarities were visualized using non-metric multidimensional scaling (NMDS)

ordination, and beta-diversity was analyzed by permutational multivariate ANOVA

259 (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

262	(CuNPs or AuNPs), nutrients or interactions between factors using DESeq2 with a multifactor
263	design (Love et al., 2014). Comparisons between environmental variables utilized the non-
264	parametric Wilcoxon signed rank test, and significant differences were identified when $p < 0.05$
265	(Benjamini-Hochberg adjusted).
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268	Results and Discussion
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270	In order to characterize microbiome responses to NP-containing contaminant mixtures, here we
271	initially focus on the aquatic compartment of wetland mesocosms exposed to factorial NP and
272	nutrient treatments. In this experiment, CuNP treatments received a high initial dose (~94 mg of
273	Cu as Kocide) to mimic a high load due to storm-driven transport and then weekly doses of
274	CuNPs at concentrations approximating agricultural runoff (Simonin et al., 2018a). This
275	approach led to high initial Cu in the water column that gradually declined over time (Figure S1).
276	In contrast, AuNPs were applied at a steady rate and quickly sedimented out of the water
277	column, resulting in aquatic gold concentrations that were slightly elevated over controls
278	throughout the experiment (Figure S1). As mesocosms were dosed weekly, the absence of water
279	column accumulation indicates the partitioning of both metals into other environmental
280	compartments, mainly the floc and aquatic plants (Avellan et al., 2020). In order to understand
281	how NPs and nutrient additions might impact aquatic microbial communities, we examined
282	microbial community composition 1 day (D1) and 7 days (D7) after dosing initiation to identify
283	initial treatment effects and after 3, 6, and 9 months (T1, T2 and T3) to investigate potential
284	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 identify a significant effect of nutrient addition alone or of NP-nutrient interactions on the 286 287 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 macroalgal blooms, significantly altering competition between planktonic algae and floating 292 293 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 CuNP treatment microbial communities separated from controls on days 1 and 7 (Figure S2), 302 samples violated the assumption of equal dispersion (betadisper, p < 0.05), thus expected short-303 304 term CuNP treatment responses, potentially due to Cu toxicity, could not be evaluated 305 statistically (Table S2).



307 Figure 1. Mesocosm microbial community compositional changes over time and in response to Cu and Au nanoparticles (NPs) and nutrient additions (+N). (A) Non-metric 308 multidimensional scaling (NMDS) ordination computed based on Bray-Curtis dissimilarity for 309 16S rRNA gene libraries of all samples over time. Ellipses show 95% confidence intervals 310 around the mean. Samples with different letters "a", "b", "c" and "d" indicate significant 311 312 differences over time (by combining all mesocosms at each time point regardless of treatment; pairwise PERMANOVA p<0.05). D1 and D7 indicate days relative to the initiation of NP 313 314 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 third quarter (T3; PERMANOVA, p < 0.05). (C) Shows the significant microbiome impacts of 320 AuNP-treatment in the third quarter (T3; PERMANOVA, p<0.05). "+N" indicates nutrient 321 322 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

As neither metal accumulated in the aquatic compartment (Figure S1), the chronic (T3) aquatic
microbiome effects in both NP treatments were likely mediated by changes in the abundance or

327 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 328 329 of the metals (Avellan et al., 2020). Other potential explanations, such as gradual changes in the 330 microbiome due to chronic exposure, were deemed unlikely due to rapid turnover in aquatic 331 microbial populations. However, as treatments altered the balance between the macrophyte 332 *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 333 pool available to microbial communities. We were specifically interested in explaining the 334 335 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 336 337 time points); as organic matter stabilizes and reduces the reactivity of NPs, lower organic matter 338 levels could increase the toxicity of metallic NPs or their dissolution products (Aristi et al., 2016; 339 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 340 341 coupling ecotoxicity with plant growth stage. In addition to complexation with the nanoparticles 342 directly, labile DOC produced by actively-growing primary producers could alleviate NP-343 toxicity by providing increased resources that allow microbial investment in detoxification etc. 344 In short, for this complex wetland experiment, we predict that the effect of NPs and their 345 transformation products on the water column microbial community are potentially predominately 346 indirect impacts mediated by complex ecosystem interactions.

347

348 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

350 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 351 352 effect of the CuNP treatment was identified by comparing all Kocide-treated mesocosms versus 353 the mesocosms without nanoparticles, regardless of nutrient addition (n = 6). As there were not 354 significant differences between ambient and nutrient-amended treatments (Figures S4, S5), we 355 again focus on nanoparticle treatments. At the population level, CuNP treatments showed 356 significant effects on day 7 as well as at the T2 and T3 time points (Figure 2; Table S4). At the 357 beginning of the experiment (D7), taxa comprising 5.9% of the community significantly declined 358 versus 4.9% which increased in CuNP treatments compared to controls (Figure 2), suggesting a 359 balance between toxicity effects and microbes which benefit from reduced competition or 360 additional resources released by dying cells. In contrast, at later time points, a larger percentage 361 of taxa significantly increased (12.9%, 15.6%) versus declined (1.9%, 7.0%) of the CuNPtreated community in T2 and T3, respectively, suggesting that responsive phylotypes do not 362 363 reflect environmental toxicity but also potentially include taxa which benefit from new niches or 364 altered physiology in the NP- treatments (Figure 2). We considered a number of potential 365 explanations for the observed Cu-treatment results including copper toxicity, Cu's role as a 366 micronutrient that could stimulate growth, and ecosystem-level impacts including shifts in 367 environmental resources. Treatment-responsive taxa were spread throughout the phylogenetic 368 tree (Figure 2); however, some trends emerged which provide insight into potential mechanisms. 369 Pertaining to the toxicity hypothesis, in T3 CuNP mesocosms, several cyanobacterial ASVs 370 decreased (Table S4); these declines in cyanobacteria are consistent with either Cu toxicity or 371 changes in the balance of primary producers, as observed previously (Simonin et al., 2018a), but 372 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 limiting concentrations even in non-CuNP treatments (Posacka et al., 2019), population increases 374 375 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 chronic AgNP treatment mesocosms (Ward et al., 2019), where similar responsive taxa were not 380 381 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 treatments both directly (e.g. toxicity) and indirectly (e.g. via interactions with CuNP-responsive 385 386 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, with AuNP treatment resulting in microbial community shifts relative to controls only at the end 393 394 of the experiment, through an unknown mechanism.



#### 396 Figure 2. Mesocosm amplicon sequence variants (ASVs) that significantly respond to CuNP

**397** or AuNP treatments. Log<sub>2</sub> fold change of each ASV was calculated as

398  $\log_2(\frac{avg \ abundance \ in \ NP \ treated \ mesocosms}{avg \ abundance \ in \ control \ mesocosms})$ . NP treated mesocosms include NP only and NP-

nutrient enriched mesocosms (n=6), and control mesocosms include both ambient control and

400 control + Nutrient addition mesocosms (n=6), as nutrients did not show any significant

401 individual or interactive effect with NPs on microbial community composition. ASVs are shown

402 in the plot if: (1) they are identified as significantly responding to CuNPs or AuNPs at any time

- 403 point using DESeq2 (Asterisks indicate the taxa relative abundance was significantly different
- 404 that controls p < 0.05); and (2) ASV relative abundance exceeds the threshold of 0.2% at the
- 405 corresponding time point. Gray shading indicates that the ASV does not exceed the 0.2%
- 406 abundance threshold at that time point. ASVs are organized by a maximum likelihood
- 407 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 (%).

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## 411 Microcosm experiments to explore ecosystem complexity in microbiome responses to NP 412

413 The question remains why T3 (fall) samples exhibited microbiome responses in AuNP and 414 CuNP treatments, with the prediction that environmental factors rather than accumulation drives 415 this response. We specifically focus on AuNP treatments, as elemental gold was historically 416 taken as an inert tracer not toxic to microbes (Ahmad et al., 2013; Zhang et al., 2015), although 417 recent examples of microbial toxicity have been noted in the literature (Sathiyaraj et al., 2021)... 418 While the mechanisms of AuNP microbiome responses are unclear, they may include AuNP 419 antimicrobial activity of either the NPs (Sathiyaraj et al., 2021) or their environmental 420 transformation products including potentially-toxic gold ions or gold-containing compounds 421 (Avellan et al., 2018). Moreover, the fact that AuNPs mesocosms exhibited a microbiome 422 response at a single time point suggests a role for ecosystem interactions (Gräf et al., 2023), 423 which we sought to test here through guided experimentation. In addition to direct toxicity, 424 microbial community shifts could be explained by multi-stressor effects (e.g. warmer water 425 temperatures in fall samples) or indirect effects through interactions with other AuNP-treatment 426 sensitive organisms (Hunt and Ward, 2015; Wang et al., 2021). To differentiate among the 427 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; 428 429 temperature and light incubations) and ecosystem complexity (presence of primary producers) 430 using simplified, month-long jar microcosms. Compared to the mesocosms, microcosms had 431 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 longterm 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.

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440 In the microcosm experiment, we observed a strong season-treatment effect (Figure S7),

441 consistent with the known impact of temperature on aquatic microbial communities (Wang et al., 442 2021; Ward et al., 2017) (Figure 3). However, contrary to our initial hypothesis of season-related 443 interactions with AuNPs, for both spring and fall regimes, AuNP treatment influenced 444 microbiome composition in the microbe-only but not in the microbiome + Egeria microcosms 445 (for a given ecosystem complexity and season, microbiomes were compared with or without 446 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 447 448 impacts of AgNPs on microbially-mediated biogeochemical cycles (He et al., 2022) and toxicity 449 effects on juvenile fish (Bone et al., 2012), suggesting a more general role for primary producers 450 in mediating NP toxicity. Consistent with these community-level results, more AuNP treatment-451 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., 452 453 2015), researchers have observed AuNP toxicity in microbial cultures (Ahmad et al., 2013; 454 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.





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463 Figure 3. Microcosm microbial community changes (16S rRNA gene libraries) with gold 464 nanoparticle conditions for different seasonal conditions and ecosystem complexity. Jar 465 microcosm microbiomes at the end of the experiment are shown as non-metric multidimensional 466 scaling (NMDS) ordination based on Bray-Curtis dissimilarity. (A) Spring treatment conditions: 467 average 12.5 °C, light:dark: 12:12 hours (B) Fall conditions: average 26.25 °C, light:dark: 15:9 hours Ellipses (95% confidence intervals around the mean) show significant effects of AuNP 468 469 treatment for a given seasonal treatment in the Microbe-only microcosms (PERMANOVA, p< 470 0.05). Triangles indicate microcosms with microbes only and circles those containing both 471 microbes and the plant Egeria densa.

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473 Although AuNP treatments with *Egeria* did not exhibit shifts in microbiome composition in

474 either season, water column parameters suggest *Egeria* has different effects on the AuNPs: in

475	spring, removal of gold from the water column and in fall stabilization and inactivation of water
476	column gold through enhanced DOC concentrations (Glenn and Klaine, 2013). In the spring
477	AuNP treatment microcosms, aquatic gold concentrations are significantly higher in the microbe-
478	only condition (Figure 4A, p<0.05 Wilcoxon signed- rank test), and more gold accumulated in
479	the Egeria (Microbe+ Egeria treatment) (Bergemann et al., 2023). In contrast, under fall
480	conditions, water column gold was significantly higher (mean ~ 40 $\mu$ g L <sup>-1</sup> ) in the microbe +
481	<i>Egeria</i> AuNP treatment than in the microbe-only treatment (~ 10 $\mu$ g L <sup>-1</sup> , Figure 4A, p<0.05
482	Wilcoxon signed rank test). This higher aquatic gold concentrations in the fall microbe + Egeria
483	AuNP treatment could be explained by high <i>Egeria</i> -produced dissolved organic carbon (DOC)
484	concentrations which stabilized aquatic Au and potentially reduced its toxicity (Figure 4B;
485	p<0.05 Wilcoxon signed rank test) (Aristi et al., 2016; Diegoli et al., 2008; Glenn and Klaine,
486	2013; Miao et al., 2009). As the DOC concentration is elevated in <i>Egeria</i> -containing AuNP and
487	Control treatments (Figure 4B), DOC levels are due to "fall" conditions rather than the AuNP
488	treatment. Although we cannot definitively assign a mechanism, these results complement the
489	field mesocosm's conclusions that microbe-only studies may not readily translate to complex
490	ecosystems, where interactions with other organisms (and environmental factors) mediate
491	contaminant microbiome responses in complex and unpredicted ways. Overall, these combined
492	experiments suggest that growing aquatic plants attenuates NP-toxicity; however, this protective
493	effect is lost during <i>Egeria</i> senescence with the accompanying decline in aquatic DOC (as
494	observed in the mesocosm experiment).



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497 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 498 499 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). 500 501 Error bars show one standard deviation. (B) Microcosm DOC concentrations on day 28 labeled 502 with the same letter are not significantly different (Wilcoxon ranked sum test, p < 0.05). Error 503 bars show one standard deviation. Seasonal comparisons between spring (avg. 12.5 °C, light:dark; 12:12 hours) and fall (avg. 26.25 °C, light:dark; 15:9 hours) treatments. 504 505

#### 507 Conclusions

509 In these set of two linked experiments, we found that CuNPs and AuNPs treatments can exert 510 significant effects on aquatic microbial communities, but that microbiome responses are likely a 511 combination of direct effects as well as interactions with other ecosystem components. While 512 NPs can generate broad ecosystem-level effects either as synthesized or as transformation 513 products, as well as indirect effects mediated by interactions with other organisms (Hunt and

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