Long-Term Effects of Copper Nanopesticides on Soil and Sediment Community Diversity in Two Outdoor Mesocosm Experiments

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ABSTRACT: The use of novel pesticides containing nanomaterials (nanopesticides) is growing and is considered a promising approach to reduce the impacts of agriculture on the environment and human health. However, the environmental effects of these novel agrochemicals are not fully characterized, and more research is needed to determine the benefits and risks they confer. Here, we assessed the impacts of repeated exposures to a $Cu(OH)_2$ nanopesticide on the soil and sediment biodiversity of target (terrestrial) and nontarget (wetland) ecosystems by performing long-term outdoor mesocosm experiments. As pesticides are often used concomitantly with other agrochemicals, we also tested for interactive effects between nanopesticide exposure and fertilization treatments in both ecosystems. We used high-throughput sequencing on three marker genes to characterize effects on bacterial, fungal, and total eukaryotic community structure and diversity. Interestingly, we found limited effects of nanopesticide exposure on the terrestrial soil communities. Conversely, we found significant shifts in the sediment communities of the wetland mesocosms, especially for eukaryotes (protists, fungi, and algae). In the absence of fertilization, fungal and total eukaryotic community compositions exposed to nanopesticides for long periods of time were distinct from unexposed communities. We identified 60 taxa that were significantly affected by nanopesticide exposure, most of which were microeukaryotes affiliated to cercozoans, Gastrotricha, or unicellular algal taxa. Our study suggests that this nanopesticide has limited effects on the soil biodiversity of a target terrestrial agroecosystem, while nontarget aquatic communities are more sensitive, particularly among protists which are not targeted by this bactericide/fungicide.
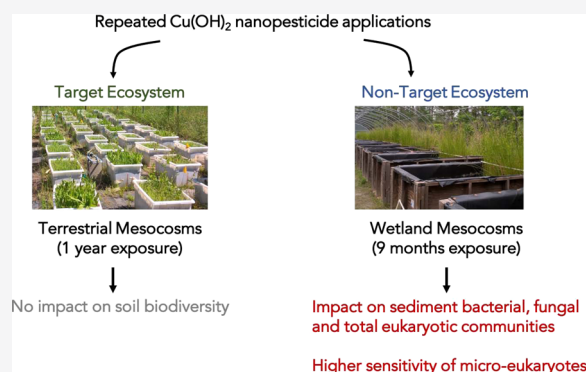
1. INTRODUCTION

A pre-eminent challenge of the 21st century is to increase global food production to keep pace with the growing human population. The complexity of this challenge is heightened by the need to balance agricultural production with ecological resilience and function in natural and managed systems, especially as these systems continue to be threatened by climate change. In this context, new solutions using nano-agrochemicals are emerging to reduce the impact of agriculture on the environment while maintaining or enhancing crop productivity.[1,2]

Agriculture can have strong impacts on nontarget species, communities, and ecosystems. For example, agricultural development can disrupt important interspecies interactions, leading to both short- and long-term decreases in species diversity.[3] Inputs of pesticides or fertilizers dramatically and directly alter nutrient inputs into aquatic ecosystems, with consequences on the global scale,[4−6] and pesticides can alter patterns of primary consumption by herbivores,[7,8] thus indirectly altering the flow of nutrients through systems.[9] Many of the studies documenting effects of agriculture and

associated contaminants on natural systems have focused on macroscopic species and communities, although responses of microbial communities are also gaining attention. These studies show that agricultural activities, especially agrochemical application, impact microbial community composition and function, altering soil fertility, water quality, and greenhouse gas emissions.[10−12] Microbial associations also have strong impacts on the health of both crop plants[13] and wild plant and animal species,[14] so microbial responses to agrochemical exposure have direct implications for the health and productivity of managed and natural systems.
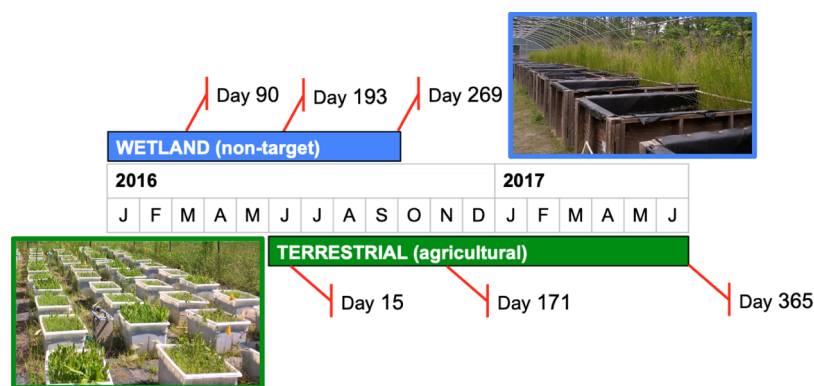
Identifying and describing the ecological effects of novel agrochemical formulations on ecosystems and their component species are critical to minimizing negative outcomes such as

Figure 1. Conceptual schematic of the experimental design and timeline. The width of each colored bar represents the duration of each mesocosm experiment, relative to the calendar months indicated. Red lines mark the timing of sampling for soil or sediment collections. The wetland (nontarget; blue shapes) mesocosms were implemented in January 2016 and were supplemented with both the nanocopper pesticides and fertilizer weekly for the duration of the 9 month experiment. The terrestrial (agricultural; green shapes) mesocosms were implemented in June 2016; they received a single application of slow-release fertilizer 8 months prior to day 0 and were treated with the nanocopper pesticide on days 0, 75, and 155. See Materials and Methods for additional details.

biodiversity loss and decreased ecosystem function. However, the implementation of novel technologies with limited up-front risk assessment makes this a significant challenge. Nano-agrochemicals such as nanopesticides are one such development. They are utilized in both conventional and organic agriculture,[15] as they promise improved fungicidal and bactericidal effects with lower inputs required compared to older pesticide formulations.[16] Some copper-based nano-pesticides have a general mechanism of antimicrobial action,[17] so it is likely that they influence nontarget microbial taxa in addition to crop pathogens. However, the nontarget effects of these compounds are still poorly described. Several metal nanomaterials have been found to impact soil microbial communities in terms of composition, structure, and function.[18,19] However, a very limited number of these nano-toxicology studies have been performed with commercial formulations of nano-agrochemicals and even fewer have simulated concentrations or repeated exposures that are representative of commercial agricultural uses.

The inherent challenge of predicting the impacts of new technologies early after they are adopted into agricultural practices is further complicated by potential interactions with other agrochemicals. For example, the use of conventional fertilizers in conjunction with novel nanomaterials can exacerbate the detrimental effects on wetland ecosystems.[20] There is growing evidence that these emerging technologies interact with nutrients or other contaminants in ways that can influence their environmental fate and uptake by organisms.[21−23] However, these interactive effects between nanomaterials and other environmental stressors have still rarely been tested.

To resolve some of these ambiguities, we conducted two long-term mesocosm experiments, testing the effects of a commercial copper-hydroxide nanopesticide (Kocide 3000, DuPont) and fertilization on the biodiversity of both target (terrestrial agricultural) and nontarget (natural wetland) ecosystems (Figure 1). Specifically, we asked: (1) Does repeated exposure to nanopesticides affect the community structure and diversity in soil and sediments? (2) Are the effects of nanopesticides modulated by nutrient addition? and (3) Which taxa are most sensitive to nanopesticide exposure? We used high-throughput sequencing of three taxonomic marker genes to assess how bacterial, fungal, and total eukaryotic communities responded

over time to repeated nanopesticide exposures under two fertilization regimes.

2. MATERIALS AND METHODS

2.1. Copper Nanopesticides. The nanopesticide used in the two mesocosm experiments was a commercial product called Kocide 3000 (DuPont) that is approved for conventional and organic farming. This nanopesticide contains $Cu(OH)_2$ nano-particles, micron-sized particles, and nanosheets, which constitute the active ingredients of this bactericide and fungicide.[24] The Kocide 3000 used in this experiment had a Cu content of $26.5 \pm 0.9\%$, and other elements (e.g., C, O, Na, Al, Si, P, S, and Zn) account for 73.5% of the dry mass of the product.[20] The Cu nanoparticles had an average primary particle size of 38.7 ± 8.2 nm and an average hydrodynamic diameter of 120 ± 30 nm when mixed with mesocosm water (measured via transmission electron microscopy and dynamic light scattering, respectively).[20] A secondary peak indicating particles greater than 700 nm was also detected using dynamic light scattering, suggesting some aggregation or the presence of larger particles in the Kocide formulation. This nanopesticide is sold as powder which is mixed with water and sprayed directly on plant foliage (see details below).

2.2. Mesocosm Experiments. The two outdoor mesocosm experiments were implemented in the Duke Forest (36°00′57.3″N 78°58′49.8″W, Durham, NC, United States) in 2016−2017. In both experiments, we performed repeated applications of the copper (Cu) nanopesticide over several months, but the application rates varied between the terrestrial (every 2.5 months for one year; three applications total) and wetland experiments (every week for 9 months; 38 applications total) because of the different exposure scenarios simulated in the two systems (Figure 1). In the terrestrial mesocosms, the three nanopesticide applications mimicked realistic agricultural practices for forage crops, where this nanopesticide is sprayed 15 days before each plant harvest. Here, we performed three harvests in the year, as it is recommended for a mixed forage culture by the North Carolina Cooperative Extension Service. In the wetland mesocosm experiment, we simulated a system exposed to weekly low pulses of nanopesticides to mimic wetlands exposed to chronic agricultural runoff. For both experiments, the nanopesticide treatments were crossed with a

nutrient addition treatment (ambient vs high) to investigate the interactive effects between the nanopesticide exposure and nutrient enrichment, which often occur concurrently in agroecosystems. The "ambient" treatment is not receiving any nutrient addition (i.e., control conditions), while the "high" treatment corresponds to the mesocosms receiving nutrient additions. The experimental designs of the two experiments are described briefly in Sections 2.2.1 and 2.2.2 below, and more details can be found in Simonin et al. (2018a)[20] and Simonin et al. (2018b).[25] In the two experiments, we used the same sandy-clay-loam soil (Sands and Soils, Durham, NC, United States) comprised of 57.7% sand, 20.5% clay, 21.9% silt, and 4% organic matter and a pH of 5.8.

2.2.1. Terrestrial Mesocosms. Each terrestrial mesocosm [51 cm (*l*) × 25 cm (*w*) × 51 cm (*h*)] was filled with 81 kg of soil. The mesocosms were seeded as a mixture of seven forage crop species commonly used in agricultural pasture regions of the surrounding North Carolina Piedmont: *Trifolium pratense* (legume, Fabaceae), *Chamaecrista fasciculata* (legume, Fabaceae), *Medicago sativa* (legume, Fabaceae), *Brassica napus* (annual forb, Brassicaceae), *Cichorium intybus* (invasive perennial forb, Asteraceae), *Sorghastrum nutans* (native perennial graminoid, Poaceae), and *Urochloa ramosa* (perennial graminoid, Poaceae). More information regarding the seeding of the mesocosms can be found in Table S1. A sprinkler system was installed in the summer to water each mesocosm every 3 days for 15 min unless a rain event occurred.

Two nutrient addition treatments (ambient and high) using the slow-release commercial Osmocote fertilizer (The Scotts Company, Marysville, OH, United States) were initiated on September 4, 2015, 8 months before the beginning of the nanopesticide applications. The ambient mesocosms received no fertilizer and the high mesocosms received 5.15 g of N, 2.32 g of P, and 3.86 g of K, simulating realistic farming practices.

On June 8, 2016, we initiated the nanopesticide additions. The Cu nanopesticide suspension was directly prepared in the field at a concentration of 6.68 mg/L, by adding 100 mg of Kocide 3000 powder in a carboy filled with 15 L of deionized water. The carboy was vigorously shaken for 30 s to ensure the homogenization of the suspension before its immediate application on the mesocosms. A handheld sprayer (Hudson, model 13581, Chicago, IL, United States) was used to treat the foliage in each mesocosm to ensure an aboveground plant biomass exposure of 30 mg of Kocide 3000/m² surface area, according to the manufacturer's instructions. Cu nanopesticide applications were performed on days 0, 75, and 155 of the experiment, each of which preceded plant and soil harvest 15 days later. The control mesocosms were sprayed with an equal volume of deionized water. The plant biomass Cu concentrations on day 170 (final plant harvest) in nanopesticide-treated mesocosms were double those of control mesocosms (14.3 ± 0.3 mg/kg vs 6.75 ± 0.18 mg/kg), confirming the good application of the treatments.[25] Over the duration of the experiment, the soil Cu concentrations were not significantly different between the control and Cu nanopesticide mesocosms ($P = 0.60$) because of the high natural Cu background of this soil (90.5 ± 4.4 mg Cu/kg) and the low Cu amount added with the nanopesticide treatment (a total of 5.43 mg of Cu per mesocosm).[25]

In summary, the terrestrial mesocosms were exposed to three consecutive nanopesticide exposures or to control exposures, under two different nutrient addition conditions, yielding 2 nanopesticide conditions × 2 nutrient addition treatments × 6

replicates per treatment = 24 terrestrial mesocosms. Soil samples were collected using small soil cores (2 cm diameter, 0−7 cm depth) 15 days following the first and last nanopesticide exposure (days 15 and 171) and finally on day 365, 6 months after the third exposure. The soil cores were homogenized and sieved to <2 mm mesh. From these soil samples, we stored samples at −20 °C for DNA extractions and at 4 °C for nutrient analysis (conducted within 2 days of collection). Soil moisture was determined by drying a 10 g subset of each soil core for 48 h at 105 °C and soil carbon content was determined by loss on ignition (5 h at 500 °C) on the same samples.[26] The soil NH_4^+ concentrations were determined after a KCl extraction (2 M) on a Lachat QuikChem 8000 (Lachat Instruments, Milwaukee WI, United States). Soil pH was measured according to ISO 10390 in pure water. We harvested the aboveground plant biomass using grass shears four times during the experiment: 15 days after each of the three Kocide exposures and at the end of the experiment (day 365). Different plant species were sorted and dried at 60 °C for 72 h to determine the dry biomass of each species.

2.2.2. Wetland Mesocosms. Each wetland mesocosm was a large box built from weather-treated lumber (dimensions 3.66 × 1.22 × 0.81 m) that was partially filled with sand. This sand was graded to create a flat, deep bed of 0.8 m in length adjacent to a 2.8 m hillside rising at a 13° slope and then lined with a food-grade plastic liner. This setup allowed us to create three different hydrologic zones within each mesocosm: a permanently flooded zone (aquatic zone), a periodically flooded zone (transition zone), and a rarely flooded zone (upland zone). The mesocosms were initially filled with 250 L of groundwater from the experimental site in the Duke Forest but the water volume within the mesocosms fluctuated over the course of the experiment as a result of rainfall and evapotranspiration between 250 and 600 L, as expected in a wetland system. This study focuses only on the aquatic zone that was directly exposed to the chronic nanopesticide additions. The submerged portion of our mesocosms was dominated by the aquatic macrophyte *Egeria densa* (Hydrocharitaceae) and supported complex aquatic food webs including numerous macroinvertebrate taxa and large populations (hundreds of individuals) of *Gambusia holbrookii* (Poeciliidae) fish.

Two nutrient addition treatments (ambient and high) were initiated on 28 September 2015, three months before the beginning of the nanopesticide additions. Each week, the high-nutrient wetland mesocosms received 1 L of water collected from the mesocosm (i.e., mesocosm water) that had been supplemented with 88 mg of N as KNO_3 and 35 mg of P as KH_2PO_4, while the ambient mesocosms received 1 L of mesocosm water each week without any nutrients added to mimic the same disturbance in the water column. The mesocosm water had an average pH of 8.8 in early morning, a water conductivity of 111 ± 20 μS/cm, and a dissolved organic carbon concentration of 11.9 ± 4.4 mg/L over the course of the experiment (see detailed water chemistry in ref 27). On 18 January 2016, we initiated the weekly nanopesticide additions over the 270 d (9 month) experiment. The mesocosms were exposed to 35 mg of Kocide every week, except for the first week of the treatment when they received an initial pulse of 347 mg that resulted in a total dose of 1.664 g Kocide nanopesticide (450 mg of Cu) per mesocosm over the 9 months of the study. Over the course of the experiment, the Cu concentration in the water column in the mesocosm treated with the Cu nano-pesticide was 13.1 ± 10.9 μg/L, while the initial Cu

concentration in the mesocosm water before starting the nanopesticide dosing was 1.48 ± 0.93 μg/L. This dosing rate was chosen to simulate realistic runoff concentrations of pesticides in water bodies, resulting in concentrations in the low μg/L range.[28] The nanopesticide powder was mixed in 1 L of mesocosm water that was then dispensed back into the aquatic zone using a serological pipette, depositing the treatment homogeneously below the water surface and any algae blooms or other macroscopic organisms at the surface. Control treatments received the same volume of mesocosm water without any nanopesticides added. This long-term Cu nanopesticide dosing led to an accumulation of Cu in the surficial sediments (i.e., floc) that are the focus of this study. The average Cu concentration in the floc of control mesocosms was 22.9 ± 2 and 118 ± 26 mg/kg in the nanopesticide-treated mesocosms at the end of the experiment (day 269), indicating a 5-fold increase in the Cu concentration in this compartment.

In summary, the wetland mesocosms were exposed to 38 consecutive nanopesticide exposures or to control exposures during the experiment under two different nutrient addition conditions, yielding 2 nanopesticide conditions × 2 nutrient addition treatments × 3 replicates = 12 wetland mesocosms. Water chemistry was extensively monitored on a weekly or monthly basis for water dissolved organic carbon, *ortho*phosphate, total nitrogen, and Cu concentrations (see Simonin et al. 2018a[20] for detailed methods) throughout the experiment. Water volume in the mesocosms was also monitored every week. Quarterly (on days 90, 193, and 269), we used soil cores (5 cm diameter, 0−1 cm depth) to extract surficial sediments (i.e., floc) from the aquatic zone, from which samples were stored at −20 °C for DNA extraction and 4 °C for Cu and nutrient analysis (conducted within 2 days of collection). On these sampling dates, we also characterized the plant and animal communities (number of individuals, biomass, and species identification) present in the mesocosms. Carbon content in the floc was measured by loss on ignition and for Cu concentrations, the floc samples were oven-dried and ground for microwave-assisted acid digestion using 10:1 HNO_3/H_2O_2; following US EPA method 3052. Total Cu concentrations were then measured using ICP−MS (7500cx, Agilent Technologies, Santa Clara, CA, United States) following US EPA method 6020A.

2.3. DNA Extraction and Amplicon Sequencing. In both mesocosm experiments, high-throughput amplicon sequencing was performed to study the bacterial community with the 16S rRNA marker gene and the microeukaryotic community (fungi, protists, and algae) with the 18S rRNA marker gene. Given the interesting effects of the Cu nanopesticide observed in the wetland experiment, high-throughput amplicon sequencing of the ITS marker was also performed to obtain a more precise characterization of the response of the fungal community in this system. DNA was extracted from the frozen soil and sediment samples using a DNeasy PowerSoil kit (Qiagen) following the manufacturer's instructions and quantified yield using a Qubit Broad Range Kit (Invitrogen). This DNA was used as the template for PCR amplification of (i) the V4 region of the 16S rRNA gene using the updated Earth Microbiome Project primers (505F—806R, FWD: GTGYCAGCMGCCGCGG-TAA; REV: GGACTACNVGGGTWTCTAATREF); (ii) the V9 region of the 18S rRNA gene using the previously developed primers (1391F—1510R, FWD: GTACACACCGCCCGTC, REV: TGATCCTTCTGCAGGTTCACCTAC),[29] and (iii) the ITS1 region using the ITS1f and ITS2 primers (FWD: C T T G G T C A T T T A G A G G A A G T A A ; R E V :

GCTGCGTTCTTCATCGATGC).[30] Library preparation followed the standard Illumina protocol. Illumina MiSeq 150 paired-end sequencing with v2 chemistry was performed at the Duke Sequencing Core (Duke University, Durham, NC) for 16S rRNA gene amplicons. Amplicon sequencing for the 18S rRNA gene and ITS was completed at the Argonne National Lab using Illumina MiSeq at 250 and 150 paired-end sequencing for ITS and 18S rRNA genes, respectively.

2.4. Sequence Processing and Diversity Analyses. All paired-end sequence data were demultiplexed and checked for chimeras, denoised, and resolved into exact sequence variants[31] (ESVs) in Qiime 2.[32] For the bacterial community data (16S rRNA gene), we used Deblur[33] (p-trim-length 260 bp) to generate the ESVs, as this denoising pipeline was specifically designed for 16S rRNA-V4 region reads. Taxonomy assignment was performed using the database SILVA v128.[34] Reads were rarefied to the sampling depth threshold of 3387 sequences per sample (Figure S1), and singletons, chloroplasts, mitochondria, Archaea, and unassigned taxa were removed, leaving 38,267 ESVs in the final analysis. Archaea were excluded from the dataset because of their very low abundances in all samples (total number of sequences across all samples = 120,717, out of more than 11 million sequences). Several samples had very low 16S rRNA gene sequence counts after removal of mitochondrial and chloroplast sequences and had to be excluded from the analysis, including all the samples collected on day 90 in the wetland mesocosm experiment. Consequently, some treatments are missing replicates and the interpretation of the potential effects of these treatments has been performed with caution.

For the eukaryotic community data (18S rRNA gene) and the fungal community data (ITS gene), we used DADA2 to generate ESVs[35] (for both: -p-trim-left-f 13/-p-trim-left-r 13/-p-trunc-len-f 150/-p-trunc-len-r 150). For the eukaryotic community, the sequences were rarefied at 11,999 sequences per sample (Figure S1), and after removing singletons, the final number of ESVs included was 20,478. Taxonomy assignment was performed using the database SILVA v128 and Protist Ribosomal Reference database (PR2) databases.[36] For the fungal community, the reads were rarefied to the sampling depth threshold of 9602 sequences per sample (Figure S1), and singleton taxa were filtered out of the dataset, leaving 1951 ESVs. We assigned taxonomic affiliations to fungal ESVs, when available, using the UNITE v7 database.[37]

Alpha diversity (ESV richness) was assessed using the R package vegan[38] v 2.5. The effects of the treatments and their interactions (nanopesticide × fertilization × sampling day) were tested using generalized linear mixed models[39] (glmer function of the lme4 package) with the mesocosm treated as a random effect to account for serial correlation among observations from the same mesocosms over time. Post-hoc comparisons were performed using the lsmeans package in R.[40] Nonmetric mutidimensional scaling (NMDS) ordinations were prepared in the R package vegan based on a Bray−Curtis distance matrix to visualize the treatment effects on the community structure. Permutational multivariate analyses of variance on distance matrices using the "Adonis" function in vegan were performed to assess the effects of the sampling day, nanopesticide exposure, and fertilization treatment and their interactions. The "envfit" function in vegan was used to test for significant loading of environmental variables (e.g., soil moisture, carbon content, and water chemistry) onto NMDS axes, to assess which variables were correlated with the community structure.
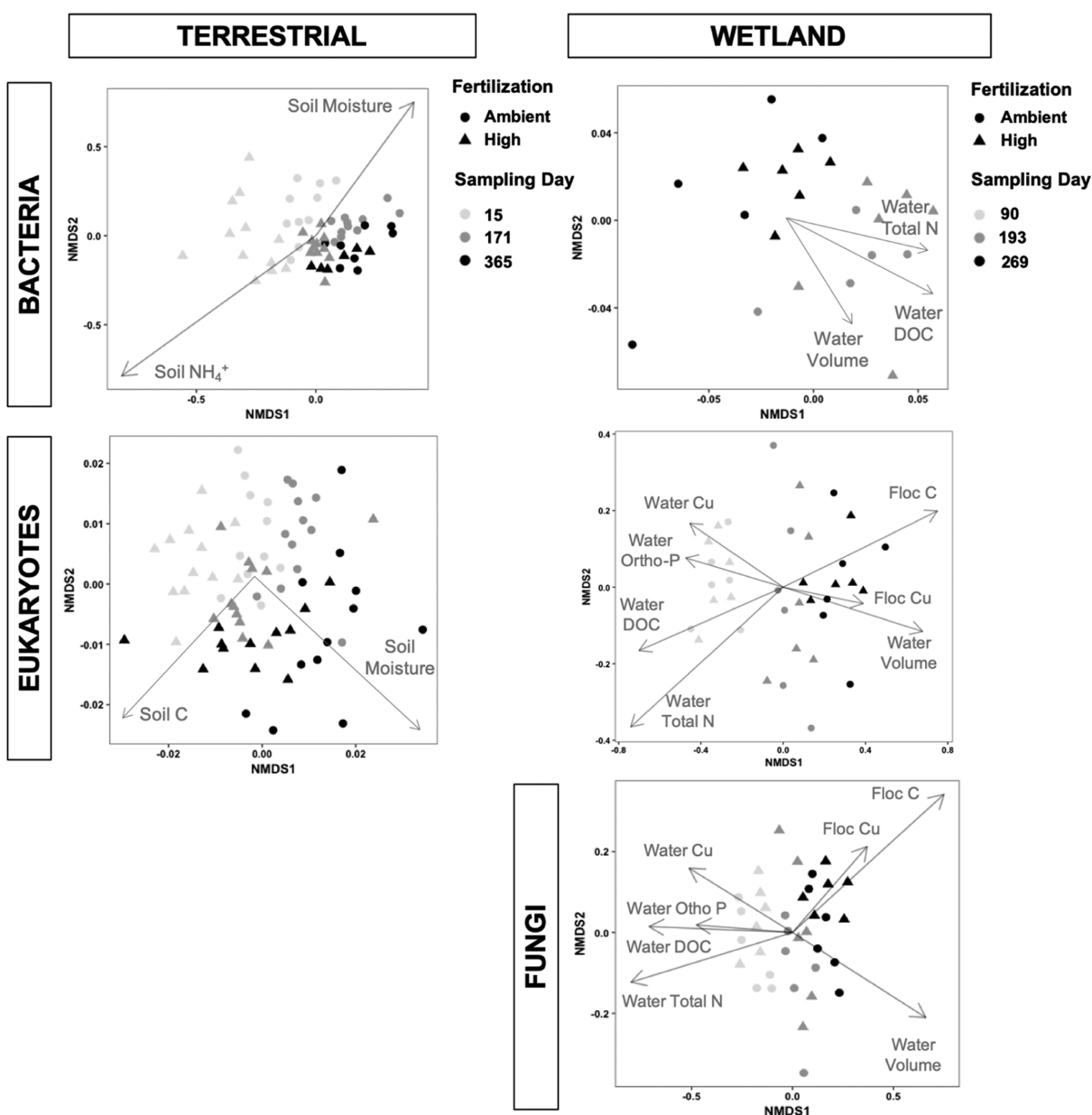
Figure 2. NMDS ordinations showing variation in microbial community composition in response to the fertilization treatment and sampling day: bacteria (top), eukaryotes (middle), and fungi (bottom) in the terrestrial (left) and wetland (right) mesocosms. Within each panel, environmental variables significantly correlated with the NMDS axes are plotted in ordination space and scaled according to the strength of correlation. Abbreviations: soil C = soil carbon content; water DOC = water dissolved organic carbon concentration; ortho P = orthophosphate concentration; floc Cu = floc copper concentration; water Cu = water copper concentration; and water total N = water total nitrogen concentration.

Using differential abundance testing in the R package DESeq 2 v 1.22.2,[41] we identified the taxa (ESVs) that significantly increased or decreased in relative abundance in the nanopesticide treatments in both experiments. To assess nanopesticide-induced shifts independently of fertilization effects, these analyses were conducted separately on subset data for each fertilization treatment.

3. RESULTS

3.1. Nanopesticides Induce Compositional Shifts in Wetland but Not in Terrestrial Communities. We used NMDS ordinations (Figure 2) to visualize the effects of the different experimental conditions (nanopesticide, fertilization, and sampling date) on the community structure of bacteria, eukaryotes, and fungi. The statistical effects associated with

these experimental conditions were assessed using permutational multivariate analyses of variance with the adonis function (Table 1). Communities shifted over time in all datasets ($P < 0.001$, Table 1, Figure 2), and sample date explained the largest percentage of total variance in the community structure (R^2 up to 19.6% for fungi in the wetland experiment, Table 1). Fertilization explained 3−6% of the variance in community composition and caused significant compositional shifts in all communities ($P = 0.015$ to $P < 0.001$) except for eukaryotes in the wetland experiment ($P = 0.11$; Table 1).

As expected, these shifts in microbial community composition in response to time and fertilization treatment co-occurred with observed changes in environmental variables; axis loading on the NMDS ordinations showed that separation between ambient and high fertilizer treatments was correlated with variation in

Table 1. Statistical Summary of the Treatment Effects on the Community Structure of Bacteria, Eukaryotes, and Fungi in the Two Experiments (Adonis Test)[a]

wetland experiment	bacteria		eukaryotes		fungi	
	R^2	P-value	R^2	P-value	R^2	P-value
sampling day	0.085	**0.001**	0.141	**0.001**	0.196	**0.001**
nanopesticide	0.071	**0.003**	0.039	**0.031**	0.051	**0.013**
fertilization	0.061	**0.015**	0.031	0.113	0.055	**0.005**
sampling day × nanopesticide	0.034	0.987	0.034	*0.051*	0.017	0.656
sampling day × fertilization	0.032	0.999	0.021	0.556	0.011	0.976
nanopesticide × fertilization	0.054	*0.078*	0.044	**0.015**	0.054	**0.005**
sampling day × nanopesticide × fertilization	0.034	0.973	0.023	0.447	0.017	0.661

terrestrial experiment	bacteria		eukaryotes	
	R^2	P-value	R^2	P-value
sampling day	0.044	**0.001**	0.041	**0.001**
nanopesticide	0.017	0.347	0.015	0.221
fertilization	0.038	**0.001**	0.046	**0.001**
sampling day × nanopesticide	0.015	0.743	0.015	0.213
sampling day × fertilization	0.019	0.111	0.017	*0.082*
nanopesticide × fertilization	0.019	0.106	0.016	0.143
sampling day × nanopesticide × fertilization	0.016	0.511	0.013	0.393

[a]The R^2 values represent the proportion of the variance explained by each variable. Values in bold are significant ($P < 0.05$) and in italics are marginally significant ($P < 0.08$).

nutrient content (NH_4^+ and C) and soil moisture in the terrestrial mesocosms (Figure 2—Terrestrial). Conversely, in wetland mesocosms, shifts in community composition were most strongly correlated with the sampling date and concomitant seasonal variation in the water column nutrient concentration (dissolved organic carbon, total nitrogen, and *ortho*phosphate), water volume, and floc carbon content (Figure 2—Wetland). Interestingly, Cu concentration in the water column and in the floc was identified as significant environmental drivers of the eukaryotic and fungal community structure and composition in the wetland experiment.

Finally, Cu nanopesticide treatment also influenced microbial composition in wetland communities (Table 1; Figure 3); it caused significant compositional shifts in bacteria, fungi, and eukaryotes ($P = 0.01$ to $P = 0.003$) and explained 4–7% of the variance in community composition. This effect was modified by the fertilization treatment in two of the three communities (eukaryotes: $P_{nano×fert} = 0.02$; fungi: $P_{nano×fert} = 0.005$), such that the effect of the nanopesticide was greater in the absence of fertilization. For example, on day 269, the communities from the ambient-Kocide treatment were more clearly separated from the ambient control than were the communities from the high-Kocide treatment (Figure 3). In terrestrial mesocosms, nanopesticide exposures had no effect on microbial community composition, either through direct effects or through interactions with time or fertilization treatment (Table 1, Figure 3).

3.2. Taxonomic Richness was Unaffected by Nanopesticide Treatments in Both Terrestrial and Wetland Systems. The effects of repeated exposures to Cu nanopesticides on soil and sediment biodiversity were assessed by calculating ESV richness in each sample for the three marker genes. In the terrestrial experiment, the soil bacterial richness ranged between 1100 and 1600 ESVs and the soil eukaryotic richness (including fungi, oomycetes, protists, and some mesofauna) ranged between 200 and 500 ESVs (Figure 4). In the wetland experiment, the sediment bacterial richness ranged between 1000 and 1600 taxa and the total eukaryotic taxon

richness ranged between 250 and 700 taxa, while the fungal taxon richness ranged between 100 and 250 taxa. Richness varied significantly over time in terrestrial mesocosms for bacterial and eukaryotic communities (sampling day: $P < 0.001$ and $P = 0.004$, respectively), which both had the highest richness at intermediate time points (day 171). In the wetland experiment, only fungal richness varied over time (sampling day: $P < 0.001$), showing reduced richness at the intermediate time point (day 193). Richness of bacterial and eukaryotic wetland communities was stable over time (sampling day: $P = 0.52$ and $P = 0.09$, respectively).

Generally, nanopesticide exposure did not induce strong or consistent effects on microbial richness in either mesocosm type (Figure 4, nanopesticide: nonsignificant P values ranging between 0.18 and 0.94). However, there were sporadic effects of nanopesticide exposure on richness; bacterial and eukaryotic richness increased transiently in terrestrial mesocosms (on days 15 and 171, respectively), while eukaryotic richness decreased transiently in the wetland experiment (on day 193). These effects were only observed under ambient fertilization; richness did not differ in response to nanopesticide exposure under high fertilization. Fertilization also had direct effects on richness, reducing bacterial richness in both terrestrial and wetland communities (fertilization: $P = 0.02$ for terrestrial), especially at the earliest time points (significant sampling day × fertilization interactions, $P = 0.002$ for terrestrial and $P = 0.04$ for wetland).

3.3. Nontarget Organisms, Especially Nonfungal Eukaryotes, Are Sensitive to Nanopesticide Exposure. Among the thousands of ESV taxa identified in these ecosystems, differential abundance testing revealed significant shifts in the proportional abundance of just 60 ESVs in response to nanopesticide treatment (Figure 5, Supporting Information 1 detailing taxonomic affiliations and sequences). Consistent with the community structure results, most of the taxa showing individual responses to nanopesticides were identified in the wetland experiment ($n = 54$ taxa, Figure 5), while just a few taxa in terrestrial communities responded significantly to nanopesticide exposure ($n = 6$ taxa). The majority of the taxa that
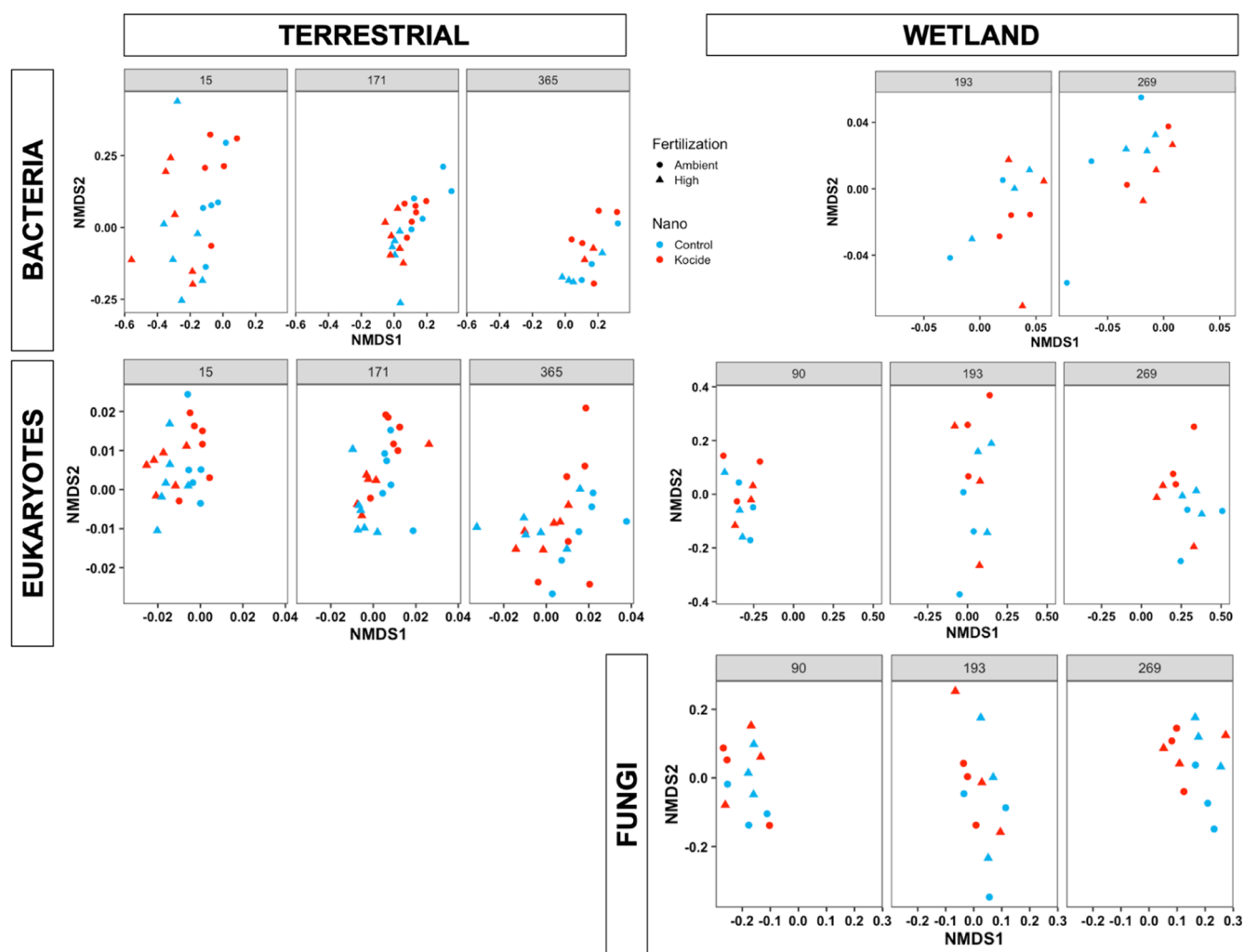
Figure 3. Effects of the nanopesticide exposures over time on the structure of the bacterial, eukaryotic, and fungal communities in the terrestrial and wetland mesocosm experiments. Each panel represents a different sampling day.

responded to nanopesticide treatment ($n = 45$) were eukaryotes identified with the 18S rRNA gene, while only five bacterial taxa and 10 fungal taxa (identified by ITS) were significantly affected. Cu nanopesticide exposure affected taxa from a great diversity of taxonomic groups including Proteobacteria, algae (Chlorophyta, Ochrophyta), fungi, Gastrotricha, Amoebozoa, or cercozoans (Figure 5). Approximately half of these taxa responded by increasing abundance in the nanopesticide treatment ($n = 32$ taxa), while the abundance of the other half of taxa decreased ($n = 28$ taxa). In the wetland experiment, the taxa that showed the largest positive responses to nanopesticide additions (Log2 fold change > 20) were uncultured taxa of unicellular algae affiliated to the Chrysophyceae, Chlorophyceae, and Cryptomonadaceae taxonomic groups and the species *Scenedesmus pupukensis* (Chlorophyta: Chlorophyceae: Scenedesmaceae). The taxa exhibiting the largest declines (Log2 fold change <−20) were affiliated to diverse eukaryotic taxa, including microscopic worms (Gastrotricha, e.g., genus *Chaetonus* and *Halichaetonotus schromi*), cercozoans (all uncultured), and the amoeba *Darbyshirella*. Analysis of wetland ESV responses over time shows that shifts in abundance in response to nanopesticide exposure were not localized to a single time point (Figure S2).

Overall, most microbial taxa in the terrestrial ecosystems did not respond strongly to nanopesticide treatment. The five taxa

that were identified as significant responders to nanopesticide exposure in terrestrial soils were eukaryotes affiliated to cercozoans, fungi, and Streptophyta. The taxa that exhibited the largest response to the treatments were three cercozoans, with two positive responders (*Platyreta germanica* (Rhizaria: Cercozoa: Vampyrellida: Leptophyridae), and an uncultured Trinematidae) and one negative responder (another uncultured Trinematidae).

4. DISCUSSION

The use of nanomaterials as agricultural pesticides can help to protect crops against yield loss to pathogens at doses lower than are required by conventional pesticides,[15] suggesting that they may produce fewer negative effects on nontarget species and ecosystems. However, the effects of nanopesticides on ecological communities are still poorly described. Here, we show that while impacts on target terrestrial microbial communities may indeed be minimal, copper nanopesticides significantly alter nontarget aquatic communities.

4.1. Community-Level Responses. By assessing bacterial and eukaryotic community composition using a metabarcoding approach, we found that community richness was relatively stable across experimental treatments and shifted only occa-
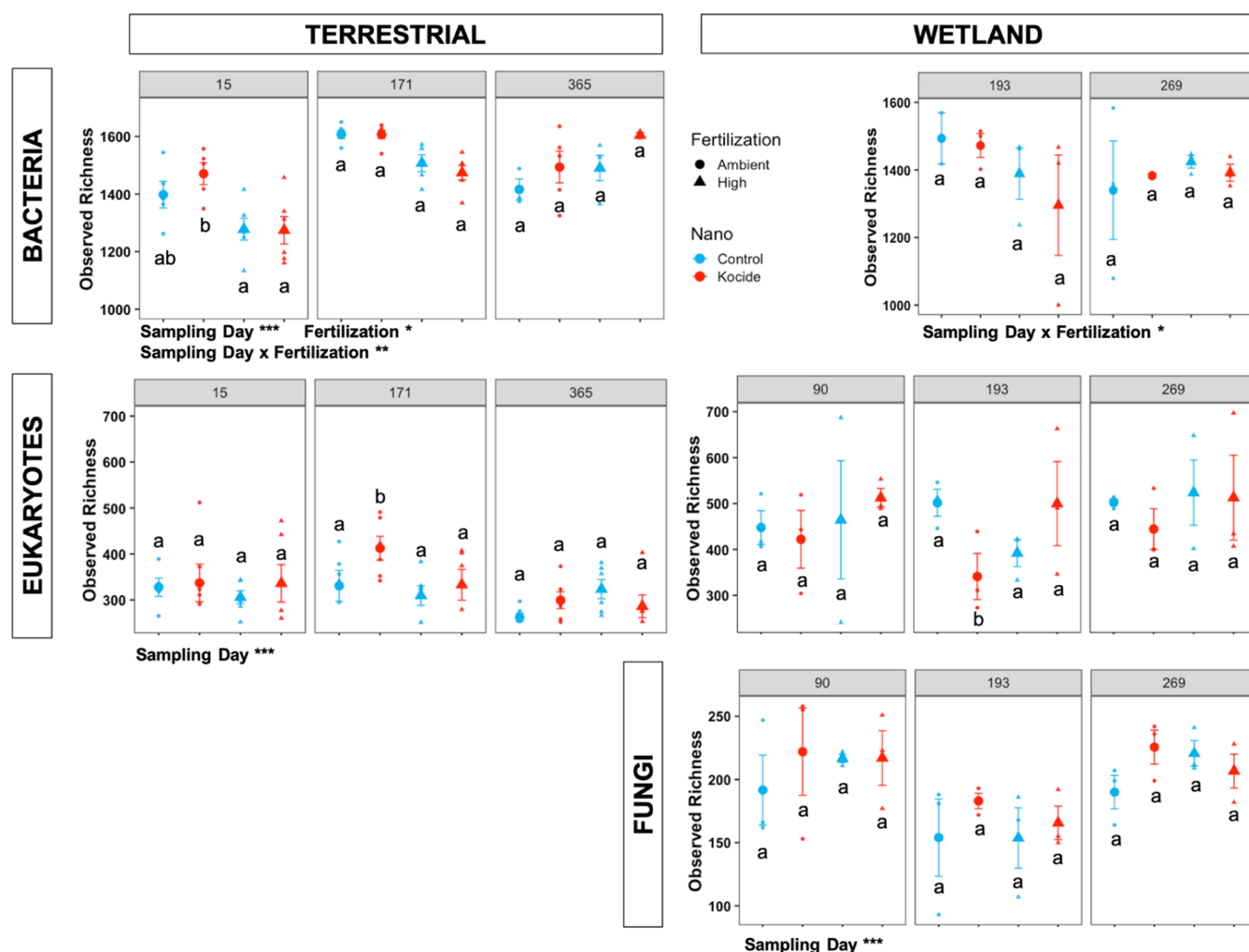
Figure 4. Effects of the nanopesticide and fertilizer treatments over time on the observed taxon richness (ESV number) of bacterial, eukaryotic, and fungal communities in the terrestrial and wetland mesocosm experiments. The results at different sampling dates are displayed in different panels: 15 days, 171 days, and 365 days for the terrestrial experiment; and 90 days, 193 days, and 269 days for the wetland experiment. Beneath each panel, significant effects and interactions identified using glms are presented with asterisks corresponding to the P value observed: *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Within each panel, categories not linked by the same letter differed significantly in richness, as determined by a Tukey HSD post-hoc test.

sionally over time (Figure 4). This finding is consistent with previous studies in wetland or soil systems that reported limited or no effects of metal nanomaterials on microbial diversity.[42,43] However, while richness showed only subtle changes, the composition of both wetland and terrestrial communities shifted significantly over time and in response to the agrochemical treatments (Figure 3; Table 1). Because compositional changes generally occurred without accompanying shifts in richness, this suggests that taxon turnover rather than community simplification (or diversification) is driving these patterns.

In terrestrial communities, compositional shifts only occurred in response to sampling time and fertilization treatment, with nanopesticides eliciting little effect. In this experiment, the effects of fertilization on the community structure were mainly driven by changes in soil NH_4^+ concentration (for bacterial community) and carbon content (for eukaryotic community) and also soil moisture, which was likely driven by higher water uptake of plants in fertilized mesocosms.

In wetland communities, sampling time had a large impact on the community structure that was mainly associated with temporal changes in water column nutrient concentrations and

volumes and also with changes in floc C and Cu concentrations for eukaryotic and fungal communities. In contrast to terrestrial communities, wetland community composition shifted significantly in response to nanopesticide exposures (Table 1). Thus, we found that nontarget wetland ecosystems are sensitive to repeated applications of fertilizers and nanopesticides and that these two agrochemicals elicit changes of similar magnitudes, each explaining 3−7% of the overall community composition for different taxonomic groups (Table 1). These results are consistent with previous studies that reported significant shifts in the bacterial community structure, following single exposure to copper oxide nanoparticles in wetland, paddy soils, or salt marsh ecosystems.[42,44,45] However, it is worth noting that our study is one of the first to report effects on eukaryotic communities (fungi, algae, and protists), particularly in the context of chronic exposure to a commercial nanopesticide.

Furthermore, in the wetland mesocosms, we observed significant interactive effects of fertilization and nanopesticides on fungal and eukaryotic communities (Table 1); in this case, the effect of nanopesticides on community composition was greater under ambient conditions than that under fertilized
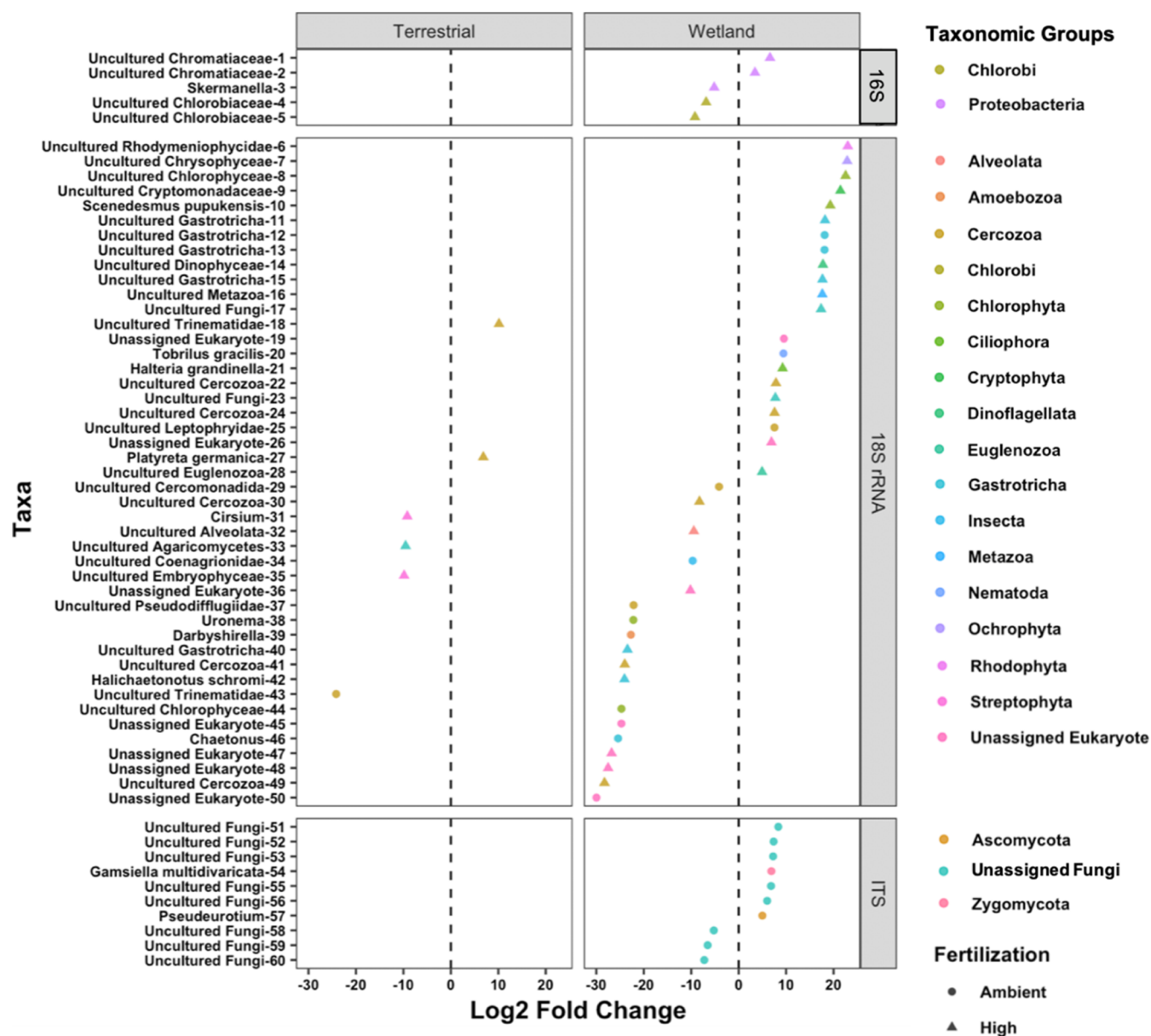
Figure 5. Bacterial, eukaryotic, and fungal taxa significantly affected by nanopesticide exposures in the two mesocosm experiments. The direction (positive or negative responses) and the magnitude of the responses are indicated on the x-axis, which shows the Log2 fold change of each taxon's abundance in the nanopesticide treatment relative to the control. The number to the right of each taxon name indicates the ESV identification number; see the Supporting Information 1 for detailed taxonomic information.

conditions (Figure 3). These results are consistent with another study documenting an attenuated impact of silver nanoparticles on lake phytoplankton communities with phosphorus addition.[22] These findings suggest that aquatic communities already stressed by nutrient limitation might be more sensitive to an additional stressor such as a nanopesticide.[46] It is also possible that fertilization modifies the speciation and fate of Cu nanopesticides in this wetland system, but our extensive characterization of Kocide 3000 transformation and accumulation in all compartments in this experiment does not support this hypothesis.[27]

The two mesocosm experiments that we conducted are not directly comparable as the application scenarios were different (weekly vs 75 day interval) based on the ecosystem to simulate realistic exposures. Hence, the larger effects in the community structure in the wetland experiment are likely associated to the

higher cumulative dose of Cu nanopesticides that ended up in the sediments than in the soils of the terrestrial experiment. Still, it is important to note that the shifts in sediment community structures were clearly observed only at the end of experiment after nine months of chronic exposures. These results align with other studies that detected impacts on microbial communities only after chronic and long-term exposures.[47,48] This observation encourages additional studies to monitor the long-term effects of Cu nanopesticides in agroecosystems, as unwanted consequences on soil biodiversity and functions might become detectable only after several years of applications.

4.2. Taxon-Level Responses. Our differential abundance analyses identified a suite of ~60 taxa, which occurred at significantly higher or lower abundance in the nanopesticide-treated mesocosms relative to the control mesocosms. These taxa make up a very small proportion of all taxa identified in

these experiments and are largely uncharacterized or identified only to coarse taxonomic levels; however, they do reveal interesting patterns about sensitivity to Cu nanopesticides. First, consistent with the community-level analyses, we found that overall, the majority of responsive taxa were found in the wetland mesocosms, whereas relatively few responsive taxa were found in the terrestrial mesocosms. Furthermore, while these pesticides are intended to target bacterial and fungal phytopathogens, we found that the majority of responsive taxa were nonfungal eukaryotes, especially protists (Figure 5). Sensitive taxa were affiliated to diverse microeukaryotic groups (Gastrotricha, cercozoans, and amoeba), with many lacking good taxonomic affiliations and/or putative functions. This highlights an important gap in protist systematics and ecology, despite the important role these species play in nutrient cycling and in controlling bacterial communities through predation.[49] It is worth noting that diverse unicellular algae of the Chrysophyceae, Chlorophyceae, and Cryptomonadaceae taxonomic groups were found to be highly stimulated by Cu nanopesticide additions in the high fertilization treatments. These results are consistent with observations of more frequent and intense algal blooms observed in these conditions (nanopesticide + fertilizer addition) during this experiment.[20] Stimulation of algal growth at low concentrations of nanoparticles is frequently observed (i.e., hormesis), and it is likely that in our experiment, weekly addition of low concentrations of the Cu nanopesticide favored the growth of unicellular algae in the water column.[50] This finding reinforces the idea that emerging contaminants such as Cu nanopesticides may play an underappreciated role in driving eutrophication of aquatic ecosystems by stimulating algal growth.

In terrestrial mesocosms, among the few taxa that responded to nanopesticide treatments, most of them were Cercozoan protists and no bacterial taxa responded significantly. These findings are consistent with the recent work, demonstrating that soil protist communities are more sensitive to nitrogen fertilization than other microbial groups in agricultural soils.[51] Altogether, our results indicate that nontarget organisms such as protists are more strongly impacted by repeated nanopesticide treatments than are bacteria or fungi. We encourage further research to better characterize the ecology of these microeukaryotes and their roles in sediment and soil function.

Beyond controlling agricultural pathogens, Cu nanopesticides have the capacity to elicit changes in natural communities. Here, we found evidence that microbial community compositions shift in response to both Cu nanopesticide, fertilization, and their interaction. Strikingly, we find that both nontarget organisms (predominantly protists) and nontarget ecosystems (nonagricultural wetlands) appear to be more sensitive to repeated Cu nanopesticide exposures, while target organisms (bacteria and fungi) in target ecosystems (terrestrial agricultural systems) showed minimal responses. Furthermore, this study demonstrates the importance of taking an integrative approach to assess the impact of emerging contaminants on biodiversity. Had this study been conducted only in terrestrial systems, or only targeting DNA from bacteria and fungi, we would have failed to detect the significant effects of Cu nanopesticides on eukaryotic biodiversity. Thus, we recommend explicit testing of both nontarget organisms and nontarget ecosystems that are susceptible to exposure (e.g., wetlands) when undertaking environmental risk assessments associated to novel nano-technologies used in agriculture.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c00510.

Table of all responsive ESVs to the treatments along with their taxonomic affiliations (xlsx)

Detailed information on the seven plant species seeded in the terrestrial mesocosms. Rarefaction curves of the three datasets (16S rRNA gene, 18S rRNA gene, and ITS) for all samples. Heatmap representing the abundance of taxa significantly affected by the nanopesticide exposures in the wetland mesocosm experiment (PDF)

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Notes

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