Amendment of Agricultural Soil with Metal Nanoparticles: Effects on Soil Enzyme Activity and Microbial Community Composition

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Abstract

Several types of engineered nanoparticles (ENPs) are being considered for direct application to soils to reduce the application and degradation of pesticides, provide micronutrients, control pathogens, and increase crop yields. This study examined the effects of different metal ENPs and their dissolved ions on the microbial community composition and enzyme activity of agricultural soil amended with biosolids. The activity of five extracellular nutrient-cycling enzymes was measured in biosolid-amended soils treated with different concentrations (1, 10, or 100 mg ENP/kg soil) of silver (nAg), zinc oxide (nZnO), copper oxide (nCuO), or titanium dioxide ($nTiO_2$) nanoparticles and their ions over a 30-day period. At 30 days, nZnO and nCuO either had no significant effect on soil enzyme activity or enhanced enzyme activity. In contrast, Ag inhibited selected enzymes when dosed in particulate or dissolved form (at 100 mg/kg). $nTiO_2$ either had no significant effect or slightly decreased enzyme activity. Next generation Illumina MiSeq sequencing of microbial communities indicated a shift in soil microbial community composition upon exposure to high doses of metal ions or nAg and negligible shift in the presence of $nTiO_2$. Some taxa responded differently to nAg and Ag^+. This work shows how metal ENPs can impact soil enzyme activity and microbial community composition upon introduction into soils amended with biosolids, depending on their type, concentration, and dissolution behavior, hence providing much needed information for the sustainable application of nanotechnology in agriculture.

Keywords: *nanopesticide, nanoparticle, agriculture, soil amendment, soil enzyme, sustainability*

Introduction

The UN Food and Agriculture Organization (FAO) and the World Bank are promoting the use of nanotechnology as a means to sustainably increase crop outputs to feed a growing population.[1-2] For example, the addition of polymeric nanocarriers,[3] silver nanoparticles (nAg), or TiO_2 nanoparticles ($nTiO_2$) can reduce required concentrations of the active ingredients of pesticides;[4] the addition of metal oxides such as nano-sized CuO (nCuO) has been shown to target soil pathogens (especially those in manure amendments);[5] and the addition of nano-sized ZnO (nZnO) can increase crop yields.[6]

The transformations of ENPs applied to agricultural soils and their trophic transfer and impacts in terrestrial environments have been critically reviewed by Gardea-Torresdey et al.[7] For instance, it has been shown that < 50 nm nCuO can improve the growth of maize by 51% when it is the source of Cu in a fertilizer solution and to a greater extent than $CuSO_4$.[8] Likewise, application of $nTiO_2$ resulted in a 76% increase in the dry weight of spinach when compared to the untreated control and a 47% increase when compared to bulk TiO_2.[9] Raliya and Tarafdar reported that nZnO improved the growth of cluster bean plants and increased the rhizospheric microbial population, while enhancing the activity of the soil enzymes acid phosphatase, alkaline phosphatase, and phytase.[10] Maize growth has also been improved by the addition of <100 nm nZnO at 0.28 ppm.[11] This latter study showed that nZnO at 0.05 and 0.5 ppm increased nitrate reductase activity which is important for plant growth. Nano-sized ZnO was also found to improve chickpea shoot dry weight more than bulk ZnO and $ZnSO_4$ when applied at 1.5 ppm.[12] Overall, nZnO also increased the total biomass of chickpea. Prasad

et al. added 400, 1000, or 2000 ppm nZnO to peanut plants and showed that it increased the root and stem dry weight more than $ZnSO_4$ at all concentrations.[13] Interestingly, the highest concentration tested (2000 ppm) resulted in the least growth, suggesting that excess nZnO can be wasteful and potentially detrimental to plant growth when concentrations are too high.

10-20% of crops grown in the U.S. are lost to plant pathogens.[14] Improving micronutrient bioavailability can help plants fight disease, thereby increasing crop yields.[14] Some ENPs show promise in suppressing plant disease by directly inhibiting pathogens (e.g., in the case of Ag) or improving delivery of essential micronutrients and stimulating plant defense mechanisms (e.g., in the case of Cu).[14] A recent literature survey by Servin et al. shows that metal/metal oxide exposure can have a significant positive impact on crop growth and/or plant disease suppression.[14] For example, nCuO was found to improve disease suppression in eggplants by 69%, while increasing Cu root content by 32% and plant fresh weights by 64%.[15] Clearly, a growing number of studies report on the impacts of ENP amendments on soil health and crop yields, however, few investigations have examined the influence of different ENPs in biosolid-amended soils.[16-20] Moreover, few studies have systematically examined the impact of ENP amendments on both soil enzyme activity and microbial community composition.[21-25]

Soil extracellular enzymes play a key role in biological soil processes such as the degradation of organic compounds, their mineralization, and recycling of nutrients including N, P, S, and C.[26] Furthermore, their response to environmental disturbances makes them a potential indicator of soil microbiological quality.[26-29] Thus, the determination of soil enzymatic activities is one approach of interest to study the impacts of ENPs, as an external

disturbance, on soil microbial processes.[30] Extracellular soil enzymes such as leucine amino peptidase and phosphatase had 52% and 27% lower activities, respectively, after exposure to nAg.[19] It has also been shown that soil protease, catalase, and peroxidase activities were inhibited in the presence of <100 nm nZnO and $nTiO_2$.[29] Single-walled carbon nanotubes (SWCNTs) at 300-1000 mg/kg soil significantly lowered microbial biomass and the activities of soil extracellular enzymes involved in P, N, and C cycling.[31] Peyrot et al. reported that 2-10 nm polyacrylate-coated nAg repressed the activity of hydrolases involved in hydrolysis of P, S, C, and N in the soil.[32] In contrast, a recent study showed that 50 nm citrate-coated gold nanoparticles (nAu) generally resulted in significant increases in the activity of five extracellular soil enzymes after 30 days of exposure, while exposure to 50 nm PVP-coated nAu resulted in an initial decrease in enzyme activity, but a recovery or increase after 30 days.[24]

The impact of ENP amendments on the soil microbial community is also important to consider. For instance, if changes in the composition of the soil microbial community alter the nutrient cycling capabilities of the soil or overall bacterial diversity, then such changes may have consequences on crop production. Studies on the effects of ENPs on soil microbial communities report different outcomes with respect to changes in relative abundance of different taxa, or diversity indices. Decrease in bacterial diversity upon addition of $nTiO_2$ and nZnO has been reported.[33-35] There are also indications that taxa involved in nitrogen cycling may be affected following addition of ENPs. For example, Ge et al. showed that the relative abundance of *Rhizobiales* decreased after exposure to nZnO and $nTiO_2$.[35] There are also studies reporting no or minimal effect of ENPs such as nZnO and nano-sized zerovalent Cu,[20]

$nTiO_2$,[36] and nano-sized Al_2O_3 and SiO_2 on the soil microbial community.[23] Weber et al. showed that 10 nm citrate-coated nAu enhanced the catabolic activity of microbial communities from loamy soil.[37] The observed impacts of applied ENPs varies depending on the composition, dose, and properties of ENPs such as coating, size,[24] and age,[38] or physicochemical properties of the receiving soils.[25]

In this study, we systematically investigated the impact of metal ENPs and their dissolved ions on soil enzyme activity and microbial community composition of a biosolid-amended agricultural soil. To this end, activities of five types of soil enzymes involved in nutrient cycling were measured in agricultural soil amended with biosolids and exposed to bare nZnO, nCuO, and $nTiO_2$, and citrate-coated nAg or their ions. Soil samples were exposed to different concentrations of ENPs or their corresponding metal ions and characterized after an exposure period of 30 days to determine enzyme activities and the microbial community composition. ENP dissolution was characterized in soil extract water using inductively coupled plasma mass spectrometry (ICP-MS). The physicochemical characteristics of the ENPs; namely, size and surface charge, were also monitored in soil extract water to support interpretation of the enzyme bioassays.

Materials and Methods

Characterization of Metal ENPs. 50 nm citrate-coated nAg (Nanocomposix), 40 nm bare nZnO (Nanostructured and Amorphous Materials Inc.), 40 nm bare nCuO and 5-15 nm bare $nTiO_2$ (US Research Nanomaterials Inc.) were used. ENPs were suspended in soil extract water

to simulate the solution chemistry of the soil slurries used in the enzyme assays described below. Soil extract water was obtained by mixing 1 g of soil with 50 mL of sterile deionized (DI) water at 100 rpm overnight. The mixture was then centrifuged at 580*g* for 10 min and the supernatant filtered at 0.1 μm (Millex, Millipore) was used as soil extract water for ENP suspension and characterization. Particle electrophoretic mobility was measured using ENPs suspended in soil extract water at 10 mg ENP/L water using laser Doppler electrophoresis (ZetaSizer Nano ZS, Malvern). Samples were taken after suspending ENPs in soil extract water for 2 hours or 30 days. In this study, 2 hour exposure time represents the initial time point for the characterization of the soil microbial community in the biosolid-amended soil and thus all parameters were characterized at this time-point and at 30 days. Measured particle electrophoretic mobility was converted to zeta potential using the Smoluchowski equation.[39] The change in hydrodynamic diameter of ENP suspensions over the 30-day period was measured by dynamic light scattering (ZetaSizer Nano ZS, Malvern) using the same samples.

Soil and Biosolids Sampling and Treatment. Surface soil (~35 cm depth) was collected in September 2014 from an agricultural site at the Macdonald campus of McGill University. After collection, soil samples were air-dried and passed through a 2 mm sieve. The pH was determined to be 6.7 (water) and 5.5 ($CaCl_2$).[40] 50 g of soil was mixed with 1 g of biosolids obtained at a municipal wastewater treatment plant (Saint-Hyacinthe, Quebec, Canada), and placed in a 250 mL Nalgene amber wide mouth HDPE bottle. ICP Optical Emission Spectrometry analyses of the biosolids after acid digestion showed negligible concentration of

total Ag (1.17±0.09 mg/kg dry biosolids), but high concentrations of total Zn (457 ± 30.8 mg/kg) and Cu (637 ± 24.4 mg/kg) (Table S1). Soils amended with biosolids were spiked with suspensions of ENP in 100 mL of DI water to achieve a target concentration of 1, 10, and 100 mg total ENP/kg soil. Standard metal ion solutions for Cu^{2+}, Zn^{2+}, and Ag^{+} in 4% HNO_3 (PlasmaCAL, SCP Science) at equivalent total metal concentrations were prepared for parallel biosolid-amended soils. Control samples were prepared by adding 100 mL of only DI water to biosolid-amended soil. The samples were kept static in the dark at 22±2°C for up to 30 days. Soil samples amended with biosolids and ENPs or standard ionic solutions hereafter are referred to as soil slurries.

Extracellular Enzyme Assay. The activities of five extracellular enzymes commonly found in soil, cellobiohydrolase (CBH), β-1,4-xylosidase (XYL), β-1,4-glucosidase (GLU), β-1,4-N-acetylglucosaminidase (AGA), and acid phosphatase (AP), were determined using 4-methylumbelliferyl-linked (MUB) substrates and MUB as a standard (Table S2). Details of the specific functions of each enzyme in soil are provided in Table S2. Substrates and standard were obtained from Sigma-Aldrich and used as received. Details regarding the enzyme activity assay are provided in an earlier study[24] and the Supporting Information.

5 mM and 30 mM stock solutions of MUB and MUB-linked substrates, respectively, were prepared in dimethylsulfoxide (DMSO) and stored in the dark at 4°C for up to two months. Stock solutions of 50 mM sodium acetate buffer were prepared in DI water and adjusted to pH 5.6 (~the soil pH of the study site in $CaCl_2$, i.e., pH 5.5).[41] Working solutions

of MUB substrates (200 μM) and MUB standard (10 μM) were prepared in buffer solution. Working solutions and buffer solutions were stored in the dark at 4°C for up to two weeks.

Extracellular enzyme activities were determined by fluorogenic substrate methods.[41-42] Briefly, 4 mL of soil slurries (containing 2 g soil) were added to 125 mL of sodium acetate buffer. These slurries were homogenized by magnetic stirring for 2 hours and added to a black polystyrene 96 well microplate (Corning). Substrates and the standard were added and baseline (initial) enzyme activities were determined after 2 hours incubation in the dark using a multi-well plate reader (Tecan Infinite m200) with 365 nm excitation and 450 nm emission settings. Each well was supplemented with 10 μL NaOH (1 M) within 1 min before measurement to amplify the fluorescence.[42] These assays were performed at 2 hours, 3 days, 20 days, and 30 days after treatment with ENP suspensions or ionic solutions. The enzyme activities are expressed as nmol MUB/(g h). All enzyme activity measurements are presented as a relative change from the untreated controls; thus, potential interference by natural colloids and organic matter (from the soil slurries) is accounted for.

Dissolution of ENPs in Soil Extract Water. A Perkin Elmer NexION 300X ICP-MS was used in standard mode to quantify the total metals (Ag, Cu, Zn) in soil extract water samples. Detailed parameters of the instrument can be found in Table S3. Calibration standards including one blank and 5 dissolved metal solutions containing Ag^{+}, Cu^{2+}, or Zn^{2+} at 0-50 ppb were prepared from a standard metal solution (Q.C. Standard 4, PlasmaCAL, SCP Science) in 1% HNO_3. 10 mg ENP/L water was added to soil extract water that did not contain biosolids

and aliquots were sampled for ICP-MS analysis at 2 hours and 30 days. 4 mL of each sample was centrifuged using 3 kDa Amicon ultra-centrifugal filtration units (Millipore) at 4000*g* for 30 min. Control experiments using nAg were used to confirm the efficiency of centrifugal filtration in separating ENPs from the dissolved metals in the soil extract water. Briefly, nAg suspensions at 10 mg ENP/L water were prepared in 4 mL of soil extract water. Single particle (SP)-ICP-MS analysis of the collected filtrates after centrifugation showed no nAg in the filtrate which indicates that the Amicon ultrafilters efficiently separated the nanoparticles from the dissolved ions. In addition, the ultra-filter retentate was also diluted adequately in DI water and analyzed using SP-ICP-MS. Nearly 99±1 wt% of nAg was found in diluted retentate. Moreover, to ensure that dissolved Ag was not lost by adsorption onto the filter membrane or was not complexed to any natural organic matter retained on the filter, control samples comprised of 10 mg Ag^{+}/L soil extract water were prepared. The filtrate was acid digested and analyzed for total Ag by ICP-MS. More than 95±4% of Ag^{+} in the control sample was found in the filtrate which showed that Ag^{+} was not retained on the filter during centrifugal ultrafiltration. It was assumed that centrifugal ultrafiltration would work similarly in retaining nCuO and nZnO as the ENPs were of similar size. Ti^{4+} was not examined due to the low solubility of $nTiO_2$.[43]

DNA Extraction, Sequencing, and Microbial Community Analysis.

Genomic DNA was extracted from the soil samples using the commercial DNA extraction kit (PowerLyzer PowerSoil DNA Extraction kit, MO-BIO Laboratories). The DNA concentration and purity were measured by optical density using a NanoDrop ND1000

spectrophotometer (NanoDrop Technologies, USA) to account for any treatment-related bias in extraction yield. The concentration of genomic DNA samples was adjusted to 10 ng/μL in a volume of 10 μL. Illumina MiSeq PE250 platform for 16S rRNA gene based amplicon sequencing was performed at McGill University and Genome Quebec Innovation Center (Montreal, Canada). PCR targeting the 16S rRNA gene was done using the 16S rRNA gene bacterial primers, 515F, GTGCCAGCMGCCGCGGTAA, and 907R, CCGTCAATTCMTTTRAGTTT. Two replicates were sequenced for the control and all of the treatments with ENPs at 1 and 100 mg ENP/kg soil for 2 h and 30 d. Sequence preparation, including trimming of tags and primers, quality assurance, and chimeric sequence removal for MiSeq data analysis were done using MOTHUR 1.36.1 software under the guidelines of the standard operation procedure.[44] A distance matrix was calculated and the sequences were clustered into operational taxonomic units (OTUs) on a 97% sequence similarity basis. OTU analysis included calculation of richness and between-group differences based on the weighted UniFrac pairwise distance matrix. The observed richness was calculated by the number of OTUs detected in a sample. The similarities between the bacterial communities were ordinated using non-metric multidimensional scaling plots (NMDS). These plots were based on a weighted UniFrac pairwise distance matrix[45] of all samples at two time points, 2 h and 30 d, and calculated from a neighbor joining tree of all sequences. Hypothesis testing statistical analysis of molecular variance (AMOVA)[46-47] was performed on the samples in the weighted UniFrac pairwise distance matrix (2 replicates). Phylogenetic

classification was done using the Silva taxonomy reference file available on the MOTHUR website. Sequences were then clustered to class level phylogeny.[48]

Statistical Analysis. Soil enzyme activities are expressed as mean ± one standard deviation of the mean with a sample size of n=4 and normalized against appropriate controls (soil + biosolids treatment at each respective timepoint). Experimental data was analyzed using the Student's *t*-test to determine significant differences ($p<0.05$) in enzyme activity between treated samples (ENP/ion) and the control (soil + biosolids alone).

Results and Discussion

Physicochemical Properties of the Metal ENPs. Surface charge and size are key factors determining ENP bioavailability and behavior in environmental systems.[49] ENP zeta potentials were determined for different ENPs suspended in soil extract water at 2 hours (to establish the initial condition) and 30 days (Table 1). ENP surface charges were negative for all types of ENPs suspended in soil extract water, and became less negative after 30 days. This may have been caused by sorption of natural organic matter or other ions, aggregation with other colloids and/or due to sulfidation of nAg, nCuO, and nZnO with soil constituents such as organics with thiol groups or inorganic sulfides.

ENP hydrodynamic diameters were considerably different from the reported nominal sizes, especially for nCuO and $nTiO_2$ (Table 1). Only the hydrodynamic size of $nTiO_2$ increased significantly ($p<0.05$) over the 30-day period suggesting aggregation occurred. In

general, there were no clear trends in particle aggregation behavior with respect to zeta potential.

It is not straightforward to determine ENP dissolution in the complex soil matrix. Thus, experiments to determine the dissolution of ENPs were carried out in soil extract water by quantifying the total metal content in filtrates using ICP-MS. The ENP content as a percentage of total metal in soil extract water over time is presented in Table 1. The results show that the mass of metals in nanoparticulate form significantly decreased after a short exposure time indicating the rapid dissolution of ENPs in soil extract water. For nAg, nCuO, and nZnO, dissolution occurred within the first 2 hours and remained stable up to 30 days, likely because of binding of soil dissolved organic matter to reactive (dissolution) sites on the ENP surface.[50] nCuO and nZnO exhibited greater initial dissolution with approximately 70% dissolution in a 2 hour period, compared to ~40% dissolution for nAg. At longer exposure time, ENPs exhibited different behaviors; while the particulate (undissolved) content remained constant for nAg and nZnO in soil extract water, it continued to decrease from 29% at 2 hours to 23% at 30 days for nCuO. Given that $nTiO_2$ is well known to be relatively insoluble, dissolution was not determined for this ENP.[43]

Effect of Exposure Time to ENPs and Ions on Soil Enzyme Activity. The enzymatic activities of CBH, XYL, AP, GLU, and AGA in soil-biosolids samples treated with four different ENPs (nAg, nZnO, nCuO, or $nTiO_2$) and three standard metal ion solutions (Ag^+, Zn^{2+}, or Cu^{2+}) at 1, 10, and 100 mg/kg soil were measured over 30 days of exposure and

normalized to their respective controls (soil + biosolids) (Figures 1, 2, S1-S3). Ti^{4+} was not examined due to the negligible solubility of $nTiO_2$.[43] The enzyme activities in soil alone were also normalized to those of the corresponding controls (soil + biosolids) at 2 hours (initial condition) and 30 days (final condition) to understand the effect of the biosolids (Table S4, Figure S4). Figures S1-S3 also include measurements taken at 3 and 20 days of exposure.

Inhibitory effect of nAg and Ag^+ on enzyme activity. At the start of the experiment (t=2 hours), nAg inhibited the activities of AP, GLU, and AGA at 100 mg/kg soil compared to the control ($p<0.05$) (Figure 1c-e). Exposure to Ag^+ solution at the highest concentration tested inhibited all enzyme activities significantly (up to 80% at 100 mg/kg soil, $p<0.05$); however, at low concentration, it did not have a significant effect on enzyme activities (Figure 1b). Ag^+ amended systems exhibited a "dose-response" effect on all enzyme activities whereby increasing the concentration of Ag^+ resulted in increased inhibition of enzyme activities. The moderate inhibitory effect of addition of nAg compared to Ag^+ could be because only 37% of the nAg was dissolved at 2 hours (Table 1).

Exposure to nAg for a 30-day period resulted in different enzyme activity profiles. Activities of CBH, XYL, AP, and AGA were significantly inhibited ($p<0.05$) by at least 40% after 30 days exposure to nAg at 100 mg/kg soil and exhibited a dose-response behavior for CBH, XYL, and AP (Figure 2a-c). Soil-biosolids slurries treated with Ag^+ solution exhibited no inhibitory effect on all tested enzyme activities after 30 days at 1 and 10 mg/kg soil (Figure 2); inhibitory effects at 30 days were observed only at 100 mg/kg soil for CBH, GLU, and AGA ($p<0.05$).

Peyrot et al. showed that 2-10 nm polyacrylate-coated nAg and Ag^+ decreased phosphomonoesterase, β-D-glucosidase, arylsulfatase, and leucine-aminopeptidase activity in soil, after an exposure of 6 weeks.[32] Similarly, Colman et al. found that the addition of nAg to biosolid-amended soils decreased leucine amino-peptidase activity by 52%, and phosphatase activity by 27% after 50 days.[19] Feng et al. reported that Ag^+ can interact with thiol groups in proteins as well as inhibit DNA replication in *Escherichia coli* and *Staphylococcus aureus*.[51] In the present study, exposure to dissolved silver generally did not result in greater inhibition of enzyme activity than exposure to an equivalent dose of nAg after 30 days. However, measurements of enzyme activities taken at 3 and 20 days reveal that the patterns of enzymatic activity for nAg and Ag^+ are very different at the highest dose. For instance, activity of all five enzymes was significantly enhanced ($p<0.05$) upon exposure to nAg at 100 mg/kg soil (compared to Ag^+) at 20 days; but these differences were less important at 30 days (Figure S3a,b).

nZnO can enhance enzyme activity at 30 days exposure. At the start of the experiment (t=2 hours), all enzyme activities were inhibited or remained close to that of the control upon exposure to nZnO (Figure 1a-e). Particularly, the activities of AP and GLU were significantly inhibited ($p<0.05$) by 50% at 2 hours exposure (Figure 1c-d). Zn^{2+} was inhibitory to CBH, AP, GLU, AGA at 2 hours (Figure 1a,c-e). At this initial timepoint, the comparable inhibitory effects of the added nZnO and Zn^{2+} to most of the tested enzymes could be attributed to the high dissolution extent of nZnO (~70 weight %). nZnO has been reported to partially dissolve in environmental and biological media and exert toxicity to freshwater algae through the

liberated Zn^{2+}.[52] nZnO has also shown antibacterial activity by damaging the membrane of *Escherichia coli* via ROS mechanisms.[53]

After 30 days of exposure to 10 mg/kg nZnO, the enzymes CBH, XYL and GLU exhibited enhanced activities compared to the control (Figure 2a,b,d, $p<0.05$). Moreover, samples treated with 10 mg/kg Zn^{2+} exhibited greater enzymatic activity at 30 days compared to the initial condition, except for CBH (Figure 2b-e). Kim et al. showed that the activity of acid phosphatase and β-glucosidase was inhibited in soil samples treated with 2000 mg/kg soil of 50 nm nZnO and Zn^{2+} for 8 weeks.[54] They also showed that Zn^{2+} showed greater acid phosphatase inhibition than nZnO, while there was no significant difference between nZnO and Zn^{2+} on β-glucosidase inhibition. Although our work was done at a lower concentration and for a shorter period, there was either no significant effect of nZnO on enzyme activity at 30 days or enzyme activity was enhanced (e.g., AP and CBH at 100 mg/kg soil, $p<0.05$).

nCuO tends to stimulate enzyme activity at longer exposure time. Initially (t=2 hours), exposure to nCuO or Cu^{2+} had an inhibitory or no effect on all tested enzymes (Figure 1). At 30 days, the soil-biosolids slurry exposed to nCuO exhibited a similar response to that of nZnO in that no significant enzyme inhibition was observed (Figure 2). In fact, the activities of most of the enzymes were generally enhanced; by up to 85% in some cases (XYL) at the highest nCuO concentration (100 mg/kg soil). Soil-biosolids slurries treated with Cu^{2+} exhibited a moderate inhibitory effect ($p<0.05$) on AGA activity at the highest concentration at 30 days (Figure 2e).

Adhikari et al. also found that <50 nm nCuO at 0.01 and 0.02 ppm reduced guaiacol peroxidase, catalase, and succinate dehydrogenase activity, while enhancing superoxide dismutase, and glucose-6-phosphate dehydrogenase activity in maize plants after 21 days of exposure.[8] Likewise, Kim et al. showed that 50 nm nCuO and nZnO increased superoxide dismutase, catalase, and peroxidase activity in *Cucumis sativus* roots after only five days of exposure at concentrations ranging from 10-1000 mg/L.[55]

In general, nCuO and Cu^{2+} had similar effects on soil enzyme activities at the start of the experiment; however, the stimulatory effect was not observed with Cu^{2+} at 30 days, except for AP at 100 mg/kg Cu^{2+}. Over 70% of the nCuO was dissolved at 2 hours, and this dissolution increased at 30 days suggesting that most of the nCuO ended up as Cu^{2+} or Cu-organic complexes explaining their similar trends for some of the enzymes. The initial decrease in enzyme activity observed at 2 hours may be linked to the antimicrobial activity of Cu^{2+} and nCuO.[56-57] Nonetheless, our data shows that the activity of the five extracellular soil enzymes generally recovers after 30 days of exposure to nCuO (Figures S1e-S3e). Cu is a micronutrient required as a co-factor in several enzymes. Thus, it is not surprising that when added as nCuO, a higher enzyme activity was observed. XYL shows a "dose-response" trend in enzyme activity upon exposure to nCuO. Specifically, amendment with nCuO stimulates XYL activity with increasing dosage. This could have positive impacts on soil fertility as XYL facilitates the breakdown of cellulose. The Cu^{2+} ion also shows a "dose-response" trend with the enzyme AP at 30 days of exposure.

$nTiO_2$ has minimal impact on soil enzyme activity. At 2 hours, the soil enzyme activity of the slurries treated with different doses of $nTiO_2$ was the same ($p<0.05$) as that of the untreated control, except for AGA at 1 mg/kg $nTiO_2$ (Figure 1e). Thirty days of exposure to $nTiO_2$ did not result in any measurable difference in enzyme activity levels ($p<0.05$), with few exceptions (e.g., GLU and AGA are inhibited at 10 mg/kg $nTiO_2$, as well as AP at 1 mg/kg $nTiO_2$).

Ebrahimi et al. showed that $nTiO_2$ exposure could increase the activity of antioxidant enzymes and inhibit ROS in pinto bean leaves.[58] Servin et al. showed that inorganic soil exposed to 250-750 mg/kg $nTiO_2$ for 120 days can increase catalase activity, while inhibiting ascorbate peroxidase activity in cucumber plant leaves.[59] Laware and Raskar found that amylase, protease, catalase, and peroxidase activities increased when exposed to low concentrations of $nTiO_2$ (10-30 μg/mL) for 7 days, but decreased in the presence of higher concentrations of $nTiO_2$ (50 μg/mL).[60] Conversely, we did not observe much significant effects of $nTiO_2$ over the timescale of our study. Overall, $nTiO_2$ had a minimal effect on enzyme activity when compared to nAg, nZnO, and nCuO. This could be due to its low solubility. Ion release is commonly a primary mechanism for enzyme inhibition or antimicrobial activity.[51, 61] It is worth noting that only the nAg were coated with citrate while the other ENPs were bare. The increased aggregation of $nTiO_2$ (Table 1) also likely influences its interaction with soil microorganisms and enzymes. Finally, our study was conducted in the dark, thus $nTiO_2$ would not contribute to ROS production under these conditions unlike those studies where samples were exposed to light.[59]

Is the nano effect important? Student's t-test was used to compare levels of enzyme activity in the presence of metals added in nanoparticulate or ionic form. A clear picture of whether ENPs or ions are more inhibitory to enzyme activity is not evident from our data. For example, after 30 days, all systems that received Cu^{2+} and Zn^{2+} at 100 mg/kg had significantly lower ($p<0.05$) activities than the corresponding ENPs at an equivalent dose, with the exception of AP for Cu and GLU for Zn. At lower doses, and for 2 hours exposure, the results were variable for different enzymes. In the case of Ag, after 30 days, systems that received Ag^{+} at 100 mg/kg exhibited lower enzyme activity only in GLU ($p<0.05$) compared to systems that received 100 mg/kg nAg. However, at 2 hours exposure, systems that received Ag^{+} at 100 mg/kg had lower activity than nAg at the same dose, for CBH, XYL, GLU, and AGA. Thus, it appears that Cu^{2+} and Zn^{2+} were more inhibitory to enzyme activity over the 30 day period and Ag^{+} was more inhibitory over the 2 hour period than their corresponding ENPs.

Effect of Exposure to Metallic ENPs and Ions on Soil Microbial Community

To study the impact of ENPs on soil microbial community, 32 samples were analyzed at phyla level (Figure 3a). We analyzed biosolid-amended soil slurries treated with nAg, nZnO, nCuO, and $nTiO_2$ and their ions (except for Ti^{4+}) at two concentrations (1 and 100 mg/kg soil) as well as control samples of biosolid-amended soil at 2 hours (initial condition) and 30 days (final condition). Seven major phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Proteobacteria*, *Firmicutes*) were present at relative abundances of more than 1% in all samples analyzed. *Proteobacteria* was the most abundant phylum (29%

average of all samples) and was mostly comprised of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. *Actinobacteria* was the second most abundant phylum with ~11% relative abundance averaged from all samples.

The microbial community composition at the start of the experiment (2 hours of exposure) was relatively uniform in the biosolid-amended systems (Figure 3a). Upon addition of biosolids to soils, a significant change in relative abundance of *Bacteroidetes* was observed, which significantly increased from 3.2% in the soil without biosolids to 7.5±2.1% in the biosolid-amended soil with and without metal ion and ENP treatment, at 2 hours (Figure 3a). This indicates that this phylum was abundant in the biosolids. However, at day 30, the relative abundance of this phylum decreased to 5.2±1.2% in biosolid-amended systems, whereas it remained nearly unchanged at 3.4% in unamended soil. Figure 3a shows that the relative abundance of different phyla were very similar in the soil and biosolid-amended soil after 30 days, and this suggests that the biosolids microbiome did not dominate the indigenous microbial community of the soil.

After 30 days of exposure, we observed shifts in the relative abundance of different phyla for different treatments. The relative abundance of the *Gammaproteobacteria* class was significantly higher in the 100 mg/kg soil levels for the three metal ions (Ag-100, Cu-100, and Zn-100) and nAg (nAg-100) at 30 days than the other treatments at 2 hours or 30 days ($p<0.05$) (Figure 3a). Yang et al. reported enrichment of *Gammaproteobacteria* upon exposure to nAg and attributed this to the presence of metal resistance integrons in members of this class.[62] *Alphaproteobacteria* was the most diverse and abundant class in the *Proteobacteria*

phylum. In general, treatments with Cu-100, Zn-100, nAg-100, nCuO-100, and nZnO-100 showed higher relative abundances for *Alphaproteobacteria* members such as nitrogen fixing *Bradyrhizobiaceae* family, or *Rhizomicrobium* genus. In contrast, the relative abundance of *Alphaproteobacteria* members was significantly lower in Ag-100. Therefore, the *Alphaproteobacteria* community responded differently to dissolved Ag and nAg. Generally, at 30 days, a significant decrease in the relative abundance of *Firmicutes* phylum ($p<0.001$) was observed.

The Shannon diversity index calculated at the OTU level, generally decreased over time based on the various 2 h and 30-day samples (Figure 3b), and a discussion is included in the Supporting Information. It is however noteworthy that diversity was not decreased for some 30-day samples, particularly those amended with Ag-100, nAg-100 and Cu-100, suggesting that the microbial community was rendered less dynamic by these amendments.

The non-metric multidimensional scaling (NMDS, Figure 4) at OTU level (97% similarity) showed two distinct clouds representing microbial community composition at 2 hours (light symbols) and 30 days (dark symbols) ($p<0.001$, AMOVA analysis). For the 30-day cloud, Cu-100, Zn-100, Ag-100, and the nAg-100 system were significantly distant from the center of the cloud, indicating a significant change in the community structure compared to the other treatments. Overall, Figure 4 indicates that at 30 days, there was a shift in the microbial community composition of the systems amended with the highest dose of metal ions or nAg, and metal ions had a greater impact on microbial community composition than ENPs.

Interestingly, even at 100 mg/kg, $nTiO_2$ did not significantly affect the microbial community composition.

Generally, *Acidobacteria* and *Betaproteobacteria* were more vulnerable, and *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes* were more resistant to addition of metal ions and ENPs. Where a change in relative abundance of major classes in 100 mg/kg nZnO and nCuO amended systems was observed, a qualitatively similar change was observed in 100 mg/kg Zn^{2+} and Cu^{2+} amended systems after 30 days (Figure 3a). In contrast, some microbial community classes responded differently to the addition of Ag^{+} and nAg at 100 mg/kg, indicating the nAg effect was driven by both Ag^{+} and nAg properties.

Environmental Implications

Soil enzyme activity measurements are useful for assessing the status or the condition of the soil environment. Enzymes are essential to the cycling of materials in soil and are thus critical to the availability of nutrients to both microbiota and plants;[63] yet, few studies have examined the potential effects of ENPs on soil enzyme activity.[29, 31-32]

Some heavy metals are essential for the proper function of enzymes; for instance, Zn is present in over 300 enzymes where it plays a catalytic, structural, or regulatory role.[64] However, these functions can be impaired when the metal concentration is too high. Metals can affect soil enzymes directly or indirectly by affecting their production by soil microorganisms, the composition of the soil microbial community, or root activity.[64] Du et al. demonstrated that the activity of the soil enzymes protease, catalase, and peroxidase is inhibited in the presence

of nZnO (50 mg/kg soil) and $nTiO_2$ (100 mg/kg soil) after 9 months of exposure.[29] In our study, we observed inhibition of AP and GLU activities immediately after exposure to nZnO, but generally enhanced activities of soil enzymes after 30 days of exposure. Exposure to $nTiO_2$ generally had little to no effect on soil enzyme activity. Peyrot et al. observed significant inhibition (~30%) in β-D-glucosidase activity after a month of exposure to 2 nm polyacrylate-coated nAg at 1.25 mg/kg soil.[32] In this study, we observed over 40% reduction in CBH, XYL, AP, and AGA activity after 30 days of exposure to 50 nm citrate-coated nAg at 100 mg/kg soil. In our previous work, we demonstrated that changing the particle coating of nAu from PVP to citrate generally resulted in significant increases in soil enzyme activity after 30 days of exposure.[24] More research is needed to better understand the potential impacts of ENP coatings and particle size on soil enzyme activities in biosolid-amended soils.

After 30 days of exposure, nCuO and nZnO enhanced the activity of certain soil enzymes. The similarity in the effects of nCuO and nZnO could be due to them both releasing divalent ions. The biosolids contained significant amounts of Zn and Cu (457 and 637 mg/kg, respectively) which, if labile and bioavailable, would have been comparable to the 1 and 10 mg/kg doses. It is noteworthy, that given these high background concentrations of Zn and Cu, the amendments of those metals as ENPs or ions produced inhibitory or stimulatory effects. This phenomenon is likely because the amendments were more bioavailable and labile than the metals contained in the biosolids, which would have been strongly complexed to organic matter. nAg was the most inhibitory among the tested ENPs. We also observed that the introduction of ENPs and metal ions at a high concentration into agricultural soil could shift

soil microbial community composition, which potentially influence N and P cycling and soil fertility. There are still uncertainties about the benefits and risks associated with the use of ENPs in agriculture and there are no nanomaterial-specific regulations in effect yet. Our results show that unlike other tested metal ENPs, long exposure to high concentrations of nAg at 100 mg/kg soil can have a negative influence on soil ecosystems. Silver in the form of Ag^+ showed the greatest inhibition of enzyme activity of the metals tested, with a consistent dose-response at 2 hours. After 30 days, most of the enzyme activity recovered for soils exposed to 1 and 10 mg/kg Ag^+. It is possible that over time, ionic silver is being complexed by organic matter, or forming new nanoparticles that are less toxic than the dissolved silver (see for example, Azodi et al.[50] for evidence of nanoparticle formation). The adaptation of soil microbes to the silver exposure could be another contributing factor for this observed phenomenon. However, soils exposed to 100 mg/kg Ag^+ still showed enzyme inhibition for certain enzymes after 30 days. Our results show that both ENP type and dose may influence soil fertility factors such as enzyme activity, but the changes may be positive or negative depending on the ENP type and dose. ENP type and dose also had a range of influence on microbial community composition. At high doses, ENPs showed no effect (TiO_2) or mild impact (nAg, nZnO, nCuO) on microbial community composition. The results of this study are also relevant to better understanding the impacts of ENP-contaminated wastewater biosolids that may be added to agricultural soils. A full assessment of the incremental toxicity of ENPs to soil microbiota in biosolids-amended agricultural soils requires additional studies assessing the role of soil types, environmental conditions (aqueous and soil surface chemistry, temperature and moisture

levels) and the role of other agricultural amendments (fertilizers, pesticides) and potentially toxic agents such as pharmaceuticals present in biosolids.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Additional microbial community analysis; Data at additional time points (Figure S1-S3). Enzyme activity of soil without biosolids (Figure S4); Metal content of biosolids (Table S1); Role of enzymes assayed in this study (Table S2); ICP-MS parameters (Table S3); Enzyme activity of soil and soil + biosolids (Table S4); Comparison of enzyme activity when exposed to ions vs ENPs (Table S5).

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Table 1. Size (number average of the hydrodynamic diameter) from dynamic light scattering, zeta potential (mV), and ENP content (percentage of total metal) for 10 mg ENP/L soil extract water after 2 hours and 30 days. The values are presented as the mean ± one standard deviation of the mean (n=6).

Exposure Time	nAg	nZnO	nCuO	$nTiO_2$	Soil Extract Water
	Size (nm)				
2 h	37.2 ± 1.0	53.6 ± 7.1	122.9 ± 7.5	222.4 ± 12.9	110.3 ± 4.9
30 d	37.8 ± 1.3	71.8 ± 9.3	98.8 ± 22.6	406.6 ± 8.3	114.3 ± 3.0
	Zeta Potential (mV)				
2 h	-23.8 ± 0.5	-18.7 ± 0.3	-13.0 ± 0.7	-18.9 ± 0.8	-17.0 ± 0.6
30 d	-21.6 ± 0.4	-10.7 ± 0.8	-8.4 ± 0.6	-16.5 ± 1.0	-17.8 ± 0.7
	ENP Content (% total metal)				
2 h	62.7 ± 2.1	27.5 ± 0.7	29.1 ± 1.6	n.d.	N/A
30 d	61.3 ± 0.8	31.2 ± 1.2	23.1 ± 1.1		

n.d.: not determined.
N/A: not applicable.

Figure Captions

Figure 1. Enzyme activities for (a) cellobiohydrolase (CBH); (b) β-1,4-xylosidase (XYL); (c) acid phosphatase (AP); (d) β-1,4-glucosidase (GLU); and (e) β-1,4-N-acetylglucosaminidase (AGA) in soils treated with nAg, nZnO, nCuO, and $nTiO_2$ and their ionic counterparts at different concentrations (1, 10, and 100 mg/kg soil) determined at 2 hours of exposure. The enzyme activities of treated samples were normalized to those of the control (soil + biosolids alone) (dashed line) at 2 hours and reported as a fold change in enzyme activity. Error bars indicate one standard deviation of the mean (n=4). Significant differences from the control ($p<0.05$) are indicated by an asterisk*.

Figure 2. Enzyme activities for (a) cellobiohydrolase (CBH); (b) β-1,4-xylosidase (XYL); (c) acid phosphatase (AP); (d) β-1,4-glucosidase (GLU); and (e) β-1,4-N-acetylglucosaminidase (AGA) in soils treated with nAg, nZnO, nCuO, and $nTiO_2$ and their ionic counterparts at different concentrations (1, 10, and 100 mg/kg soil) determined at 30 days of exposure. The enzyme activities of treated samples were normalized to those of the control (soil + biosolids alone) (dashed line) at 30 days and reported as a fold change in enzyme activity. Error bars indicate one standard deviation of the mean (n=4). Significant differences from the control ($p<0.05$) are indicated by an asterisk*.

Figure 3. (a) Overall phylogenetic diversity of bacteria in 2 hours and 30 days soil samples treated with nAg, nZnO, nCuO, and $nTiO_2$ and their ionic counterparts at two concentrations (1 and 100 mg/kg soil), based on 16S rRNA gene sequences. Soil+BS-2h and Soil+BS-30d

refer to the control biosolid-amended soil samples at 2 hours and 30 days, respectively. Phyla and *Proteobacteria* classes with more than 1% abundance in all samples are presented in the figure, and the rest are grouped as 'Other'. (b) OTU level Shannon diversity indices for soil samples treated with nAg, nZnO, nCuO, and $nTiO_2$ and their ionic counterparts at 1 and 100 mg/kg soil.

Figure 4. Non-metric multidimensional scaling plot showing the effect of soil samples treated with nAg, nZnO, nCuO, and $nTiO_2$ and their ionic counterparts at 1 and 100 mg/kg soil on bacterial community structure based on 16S rRNA gene results (stress=0.19, r^2=0.87). BS-2h and BS-30d refer to the control biosolid-amended soil samples at 2 hours and 30 days, respectively. The arrows indicate classified abundant OTUs labeled with phylum/class name and lowest taxa level in parentheses if available.

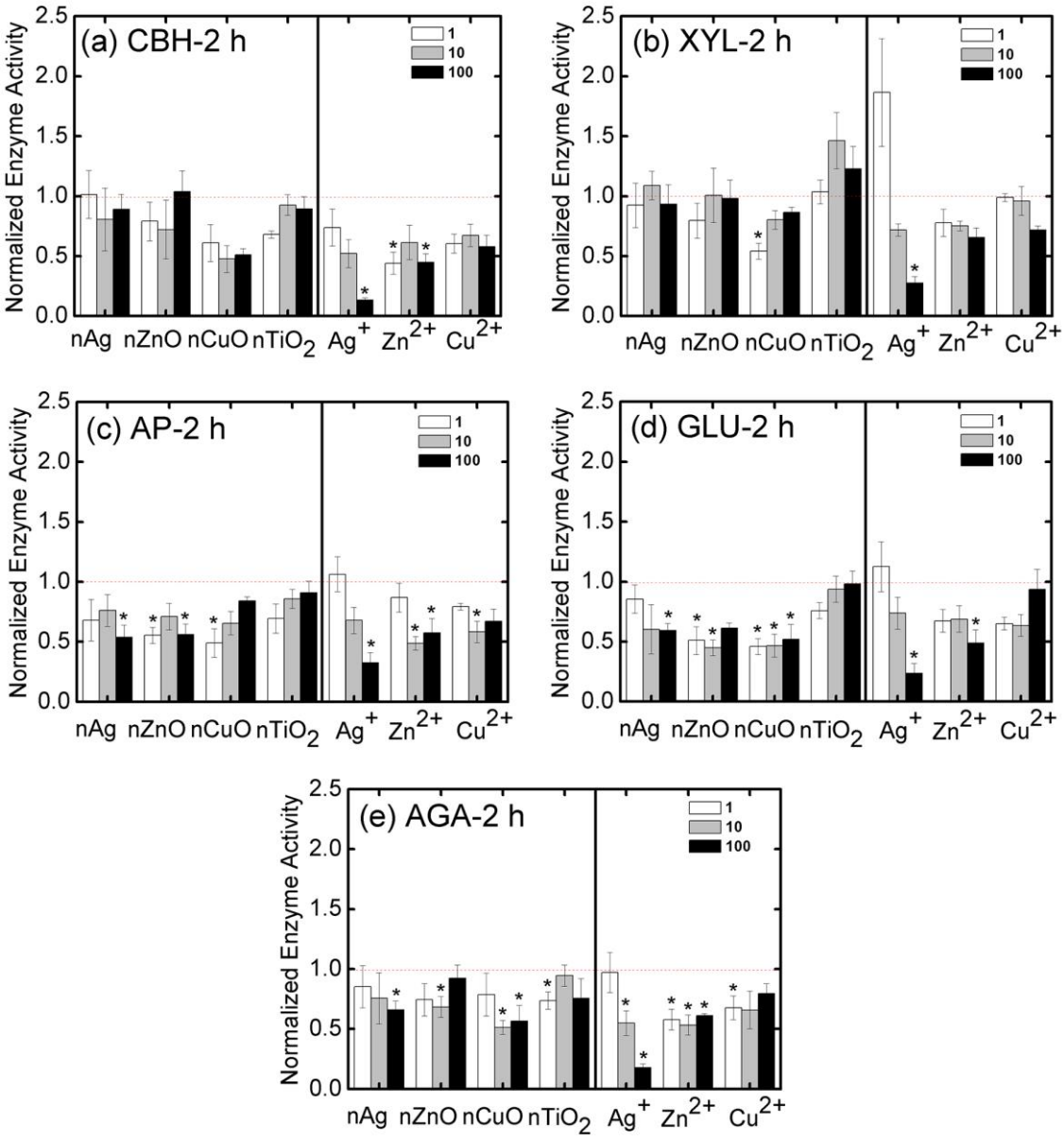

Figure 1.

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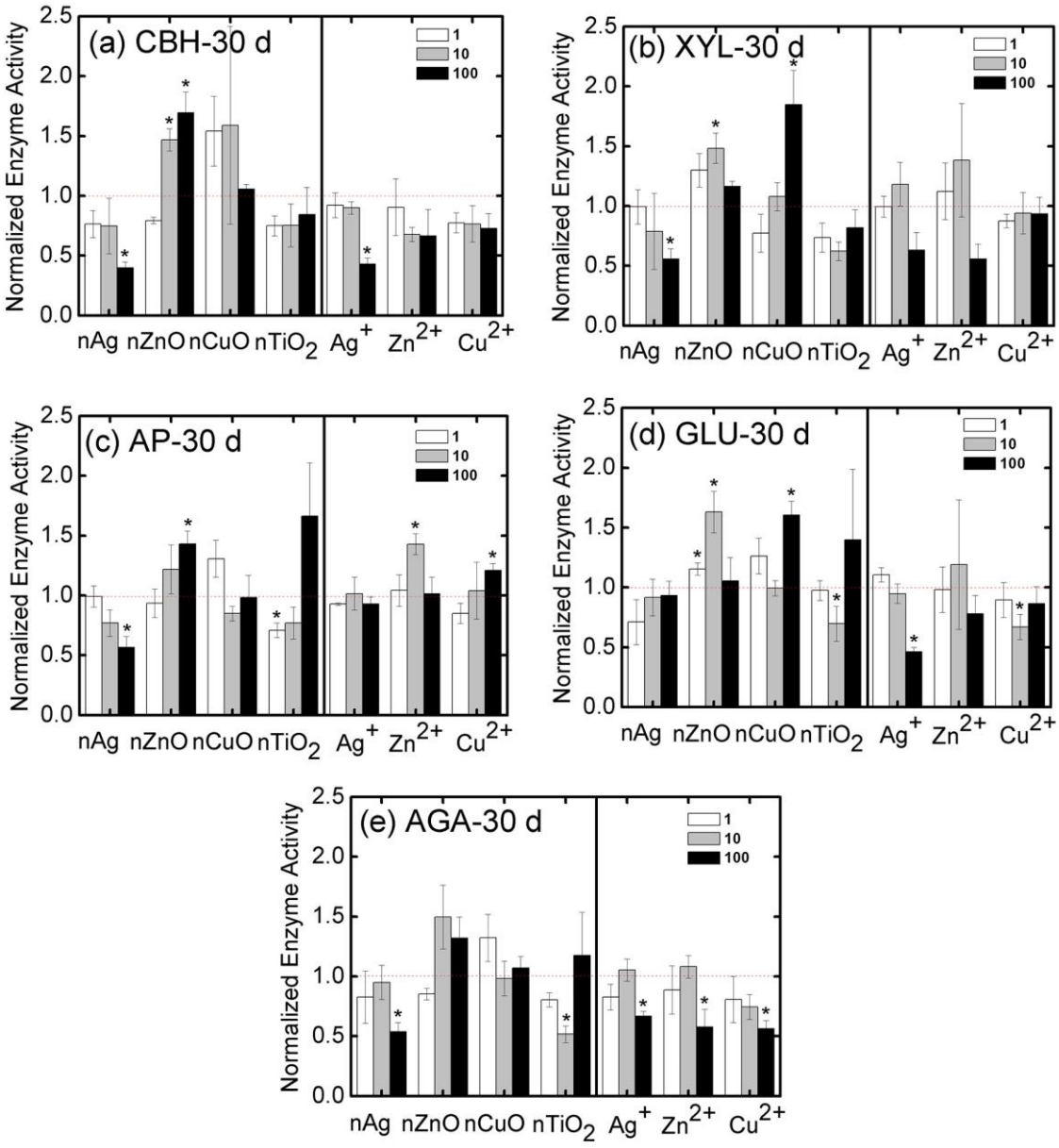

Figure 2.

Asadishad et al.

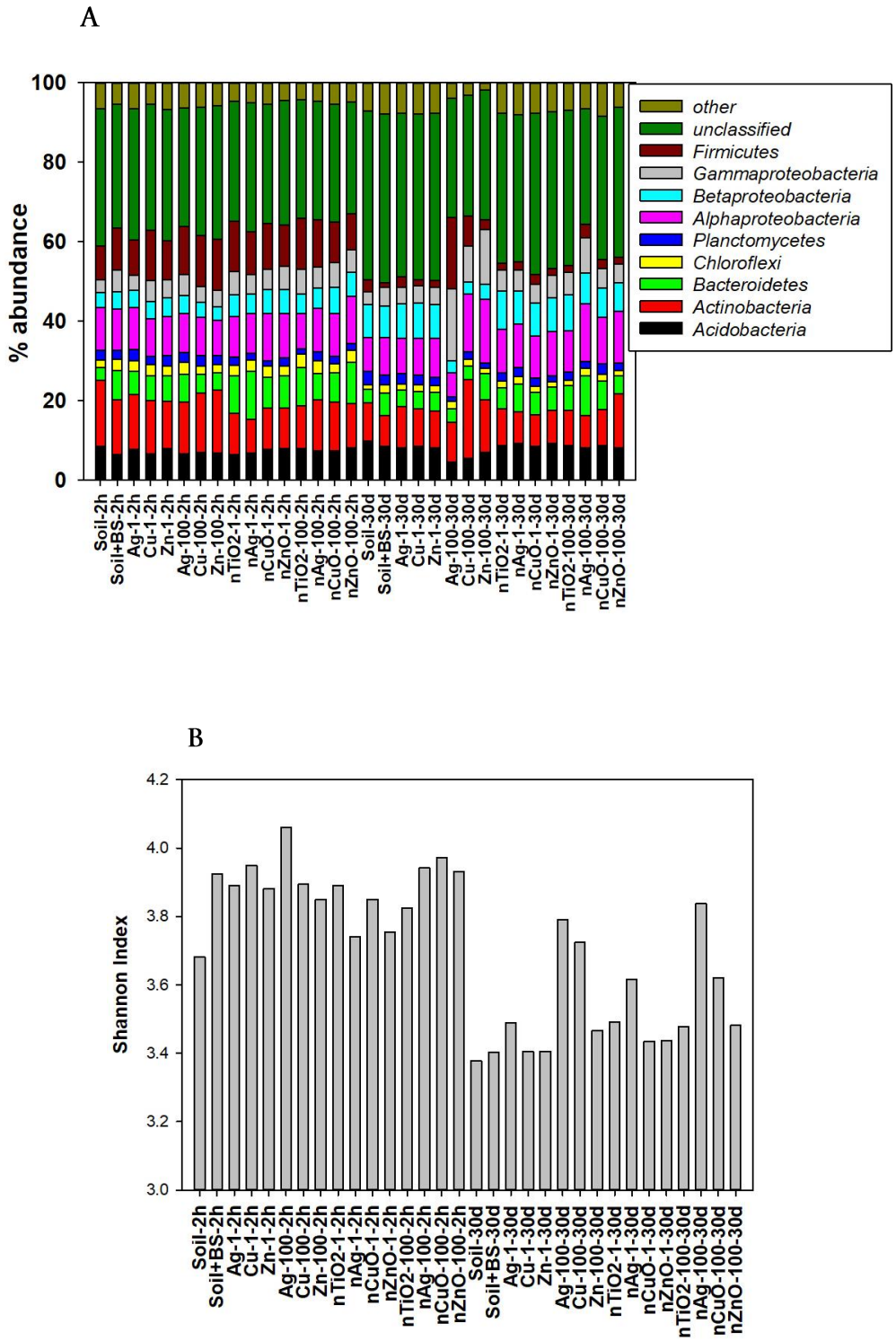

Figure 3.

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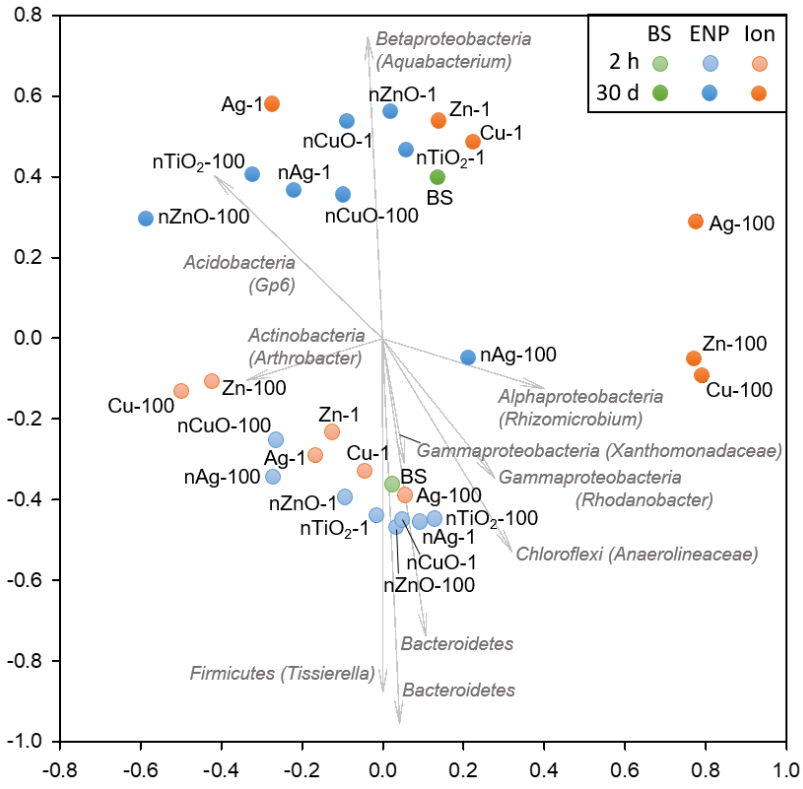

Figure 4.

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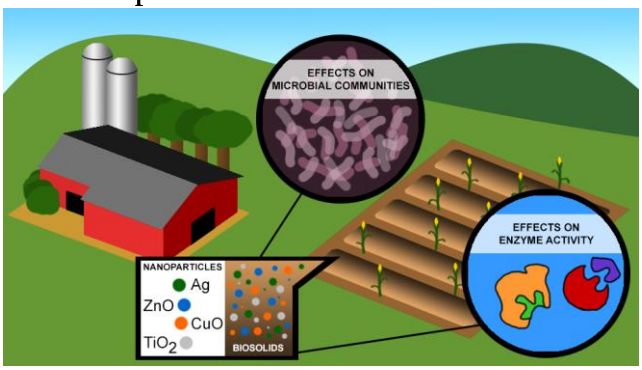
TOC Graphic


EFFECTS ON
MICROBIAL COMMUNITIES
EFFECTS ON
ENZYME ACTIVITY
NANOPARTICLES
Ag
ZnO
CuO
TiO_2
BIOSOLIDS