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ACS Sustainable Chemistry & Engineering

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Research Article

Dissolution Dynamics and Accumulation of Ag Nanoparticles in a Microcosm Consisting of a Soil−Lettuce−Rhizosphere Bacterial Community

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Cite This: *ACS Sustainable Chem. Eng.* 2021, 9, 16172−16181

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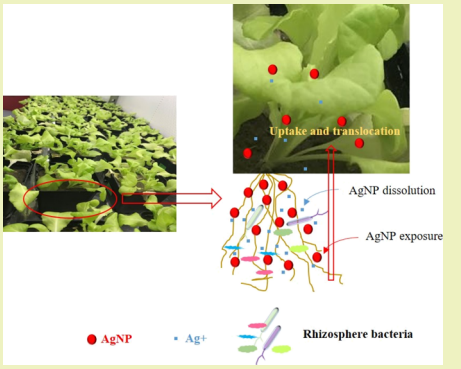
ABSTRACT: Assessment of chronic impact of metallic nanoparticles (NPs) in soil ecosystems is a necessity for ensuring safe and sustainable application. NPs affect plants and their associated microbial life, while the plants and their associated microbiota affect the NPs' fate. Here, we measured the available Ag pool (determined as diethylenetriaminepentaacetic acid-extractable Ag) in AgNP-amended sandy loam soil (1, 10, and 50 mg Ag per kg of soil) over a period of 63 d with and without lettuce. The associated impacts on soil pH, Ag accumulation in lettuce, and the responses of the rhizosphere bacterial community were determined. We found that the addition of AgNPs significantly increased the soil pH from 7.70 to 7.87 after a short-term (7 d) incubation. Noteworthily, the extractability of Ag in AgNP-amended soil was concentration-dependent and changed over time because of their continuous dissolution and uptake by plants. Ag uptake and upward translocation in lettuce positively correlated with the extractable Ag content in soil. Furthermore, a long-term (63 d) exposure to 50 mg/kg of AgNPs altered the structure and composition of the rhizosphere bacterial community potentially by regulation of bacterial groups associated with element (e.g., N and S) cycling and stress tolerance. In conclusion, our results demonstrated that the dynamic dissolution of AgNPs in sandy loam soil plays an important role in influencing the overall Ag bioavailability of the NPs in plants. The enhanced effects of AgNPs on the alterations in the rhizosphere bacterial community highlight that the long time-resolved dynamics of NP exposure should be taken into consideration for accurate ecological risk assessment of NPs in the soil ecosystem.



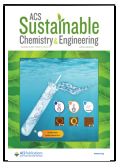
KEYWORDS: *silver nanoparticles, plant, bioavailability, agrochemical, rhizosphere soil bacteria*

INTRODUCTION

The rapid development of nanotechnology over the past two decades has inspired the production and application of nano-agrochemicals, and claims have been made that these nano-agrochemicals can improve the sustainability of agriculture.[1,2] As more and more nano-agrochemicals are introduced in agriculture as fertilizers or pesticides, agricultural soil is inevitably becoming an important sink for nanomaterials.[3] Silver nanoparticles (AgNPs) are one of the most extensively used commercialized nanomaterials worldwide, and the global production of AgNPs will reach a value of USD 2.45 billion by 2022.[4,5] Given their excellent antimicrobial properties, they have shown great potential in crop protection as insecticidal agrochemicals and against plant pathogens (phytopathogenic fungi, bacteria, and viruses).[1] This makes the impact assessment of AgNPs in soil ecosystems a necessity for the safe and sustainable usage of nanoscale products.

The impacts of metallic nanoparticles (NPs) on soil ecosystems have been reported to largely depend on their bioavailable fractions.[6] For instance, Pu et al.[6] reported that the toxicity of CuO NPs in maize plants and microbes was mainly modulated by the gradually released bioavailable Cu concentration. Soil properties are known to be a key factor affecting the bioavailability of metallic NPs in natural soil.[7,8] An important property is the soil pH, which modulates the bioavailability of metallic NPs by affecting the oxidation, aggregation, transformation, and dissolution processes of metallic NPs in the soil.[9,10] Importantly, plants, a key component of soil ecosystems,[11] can alter the soil properties directly by themselves or indirectly by the interaction with NPs. For instance, the amount of soil organic material in soil can be influenced by the presence of plants as nearly 5−40% of the photosynthetically fixed carbon is transported to the rhizosphere by plant root exudates.[12] Moreover, the interaction between plant roots and metallic NPs can alter the abundance and composition of root exudates and the soil pH.[12−14] Soil

Received: July 23, 2021
Revised: November 9, 2021
Published: November 18, 2021



organic matter and root-secreted chelators (such as phytosiderophores) can immobilize/sequester NPs and the released metal ions.[15,16] These changes in the soil environment may modify the available pool of Ag derived from AgNPs and in turn influence the plant responses.[13,17]

However, to our knowledge, the information regarding how plant roots influence the labile pool of Ag in a AgNP-amended rhizosphere and the consequent relationship with Ag accumulation in plants is scarce. Recently, Pradas del Real et al.[18] used diethylenetriaminepentaacetic acid (DTPA) and $CaCl_2$ extractions to assess the lability of Ag in soil mixed with AgNP-containing sludges (18−400 mg/kg) at a single-time point (4 weeks). The authors demonstrated that the low Ag content in wheat is consistent with the low lability of Ag in soil.[18] However, it should be noted that plant growth over time can dynamically change the soil environment and thus the dynamic particle dissolution, which may make the bioavailable Ag concentration time-dependent.[19,20] To capture these dynamics and their impacts on ecosystems, experiments of longer timescales need to be performed in which a series of time points are included at which the bioavailability of Ag in soil is assessed in a toxicity assay.

Similar to plants, soil bacteria also play an important role in soil ecosystems by promoting soil fertility and governing soil biological processes such as nutrient transformation and cycling and energy flow.[21,22] The impacts of AgNPs on bacterial communities of unplanted soil have been extensively reported with inconclusive findings.[21−25] It is suggested that the responses of the soil microbial community to AgNPs depend on the soil properties, exposure concentration, exposure duration, and the behavior of AgNPs in soil.[24,25] Therefore, the alterations in the soil environment induced by plants may modify the impacts of AgNPs on the behavior of rhizosphere soil bacteria,[26] which may result in either detrimental or beneficial impacts on the soil ecosystem.[15,17,27] To date, little information is available about how AgNPs alter rhizosphere soil bacterial communities.[14,28,29] This is surprising because it is known that soil rhizosphere bacteria play a crucial role in supporting the host plant growth by regulating nutrient uptake and to some extent by supporting against environmental stressors.[27,30] Thus, long-term impacts of AgNPs on the rhizosphere soil bacterial community deserve more investigation.

In this study, lettuce plants, a popular representative of the leafy vegetables worldwide, were exposed to 0, 1, 10, and 50 mg/kg of AgNPs over a period of 63 d. The objectives of this study are (a) to investigate if and to what extent the plants and AgNPs affect the soil pH and how this impacts the (potentially) available Ag concentrations shedding from AgNPs, (b) to quantify Ag accumulation and translocation in a soil−plant system, and (c) to determine the alterations of the rhizosphere soil bacterial community structure in response to exposure to AgNPs as a function of exposure concentration and exposure time. This study provides useful information to correlate the time-related changes of bioavailable Ag from AgNP-amended soil with the plant growth and soil microbial communities. Such information is important for risk assessment of nanomaterials in soil ecosystems and for safely and sustainably applying nanoenabled agrichemicals.

MATERIALS AND METHODS

Silver Nanoparticles. Stock suspensions of spherical AgNPs (NM-300K) with a nominal diameter of 15 nm and a concentration of 100 g/L were provided by RAS AG (Regensburg, Germany). Physicochemical properties and information on the characterization of the AgNPs are summarized in the JCR reports.[31] AgNP suspensions at 1, 10, and 50 mg/L were prepared by diluting the AgNP stock in a 1/4 Hoagland solution (pH 6.0 ± 0.1). The composition of the Hoagland solution is described in Table S1 (Supporting Information). The suspensions were sonicated for 5 min at 60 Hz (USC200T, VWR, Amsterdam, The Netherlands). The freshly prepared suspensions were used to determine the size distribution and zeta potential with a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., Royston, UK) at 1, 24, and 48 h of incubation. The data are published in our previous publication[11] and provided in Table S2. The transmission electron microscopy (TEM) picture of the AgNPs is also provided in Figure S1 (Supporting Information).

Soil Preparation. Surface agricultural soil (0−20 cm) was collected from a nonpolluted site (52°10′16.8″N 4°26′58.9″E, Leiden, The Netherlands), mixed thoroughly, sieved to 2 mm after being air-dried, and stored at 4 °C before use. The soil was sandy loam with a pH of 8.4 in water and 7.4 in the KCl solution, containing 2.2% of organic carbon, with a clay content of 18.4% and a cation exchange capacity of 0.39 $cmol^{(+)}\ kg^{-1}$. No Ag (< detection limit) was detected in the untreated soil. The exchangeable cations content and the content of various metals were determined, and these properties are reported in Table S3.

Plant Growth and Exposure Assay. *Lactuca sativa* seeds (Floveg GmbH, Kall, Germany) were first sterilized for 5 min in 0.5% (w/v) NaClO, followed by rinsing 3 times with tap water and immersing for 24 h in tap water. Afterward, the seeds were germinated in Petri dishes filled with a wet filter paper (15 seedlings/dish). After 3 d, the 1/4 Hoagland solution was added into the Petri dishes to supply nutrients for seedling growth. After pregrowing in Petri dishes for 1 week, the young seedlings were transferred to bottles (one seedling per bottle) with a height of 15 cm containing the Hoagland solution for further 2 weeks of growth. The suspensions in the Petri dishes and bottles were refreshed every 3 d.

The AgNP suspensions were prepared in the 1/4 Hoagland solution and sonicated at 60 Hz for 15 min before application to soil. Afterward, the AgNP suspensions were added to soil to achieve the nominal concentrations of 1, 10, and 50 mg Ag per kg of soil. The exposure concentrations of AgNPs were chosen based on the predicted and measured concentration of AgNPs in sludge/biosolids.[18] The soil was mechanically stirred with a mixer for 15 min to homogenize the AgNPs. Control treatment was the same as the AgNP treatments with the addition of the same volume of the 1/4 Hoagland solution. Next, two uniform pregrown seedlings were transferred into one plastic pot (9 cm long, 9 cm wide, and 9.5 cm high) containing 0.5 kg of AgNP-amended soil or clean soil. Treatments with 1, 10, or 50 mg Ag per kg of soil but without plants were also performed under the same conditions. In brief, this experiment consisted of three components: (a) AgNP application dose (0, 1, 10, and 50 mg/kg), (b) exposure time (3−63 d), and (c) the presence or absence of plants for a total of 17 treatments in triplicate, as described in Table S4. The pots were watered every 2 d, and all pots were placed in a climatic room under the conditions of day/night with a temperature of 20/16 °C and a light/dark cycle of 16/8 h with 60% relative humidity until harvest. After each exposure time point, the plants in each pot were harvested, and subsequently, the nonrhizosphere soil (further referred to as bulk soil) and soils with rhizospheres (further referred to as rhizosphere soil) were collected.

Plant Harvesting and Soil Sample Collection. At each selected sampling date, pots were picked up randomly and sacrificed for collecting plant samples and soil samples. The plants were carefully removed from the pots, and the soil which was left in the pots was defined as bulk soil in which the influence of plant roots was negligible.[13] The collected bulk soil was mixed thoroughly for further use. The soil that was loosely attached to the roots was first removed by shaking the plants (discarded), and then the soil that closely adhered to the roots was collected as the rhizosphere soil (<1 mm away from the root) by following the method reported by Guan et

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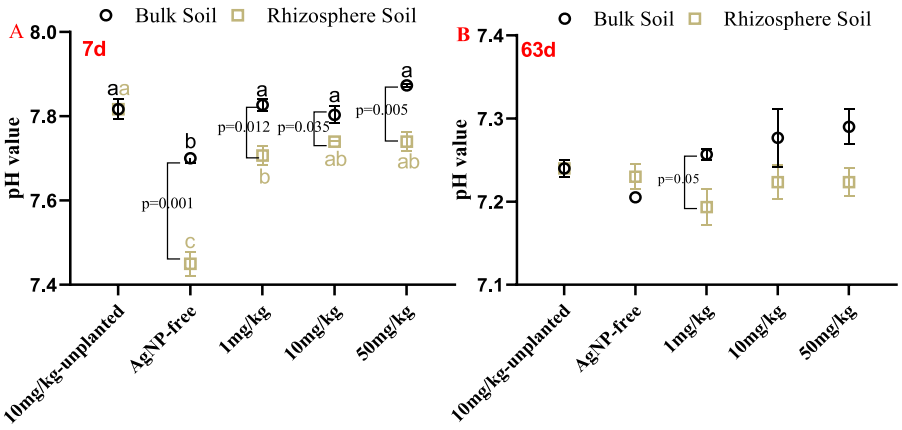

Figure 1. Soil pH in the rhizosphere soil and bulk soil exposed to AgNPs with or without plants for 7 d (A) and 63 d (B). Different letters indicate the significant difference among the treatments of the same soil ($p < 0.05$). The numbers indicate the significant differences between the bulk soil and rhizosphere soil under the same treatment ($p < 0.05$). Data are expressed as mean ± scanning electron microscopy (SEM) of triplicate samples.

al.[17] The collected rhizosphere soil was mixed thoroughly for further use. For Ag extraction and pH measurement, the bulk soil and rhizosphere soil samples were air-dried. The rhizosphere soil samples used for the soil DNA extraction were stored at 4 °C.

After collecting the soil samples, the plants were thoroughly washed with flowing tap water and rinsed in deionized water for 10 min, which was repeated 3 times. Subsequently, the plants were divided into root and shoot and after air drying, and the biomass of plant roots and shoots was recorded. To determine the Ag content in the plants, the plants were first washed with 10 mM HNO_3, 10 mM EDTA, and Milli-Q water to remove the attached AgNPs/Ag^+ ions, as described previously.[11] Next, the plants were oven-dried, weighed, and digested with HNO_3 (65%) and H_2O_2 (30%) at 120 °C.[11] Finally, the digests were diluted, and Ag concentrations were analyzed by means of graphite furnace atomic absorption spectrometry (AAS, PerkinElmer 1100 B, Waltham, MA, USA). The bioaccumulation factor (BAF) of Ag from soil to plant roots and the translocation factor (TF) of Ag from roots to shoots were calculated as follows[11]

$$\mathrm{BAF} = \frac{[\mathrm{Ag}]_{\mathrm{root}}}{[\mathrm{Ag}]_{\mathrm{soil}}} \quad (1)$$

$$\mathrm{TF} = \frac{[\mathrm{Ag}]_{\mathrm{shoots}}}{[\mathrm{Ag}]_{\mathrm{roots}}} \quad (2)$$

where $[Ag]_{root}$ represents the concentrations of Ag in the plants (mg/kg), $[Ag]_{soil}$ represents the exposure concentration of AgNPs in the soil (mg/kg), and $[Ag]_{shoots}$ represents the Ag concentration in plant shoot tissues (mg/kg).

Labile Ag Extraction from AgNP-Amended Soil and Soil pH Measurement. On each sampling day, ∼2.0 g of air-dried soil samples was extracted with 20 mL of the $CaCl_2$ extractant or 4 mL of the DTPA extractant.[13] $CaCl_2$ can extract the metals from the soil by making use of cation competition processes, which has been considered to be "readily available" to plants/soil organisms. DTPA extraction is used for extracting the "readily available" fraction and the "potentially available" fraction that is reversibly bound to the soil solid matrix, which has been suggested to be an indication for metallic NP dissolution in soil.[13] The $CaCl_2$ extractant was prepared by dissolving the $CaCl_2$ salt in Milli-Q water to reach a final concentration of 0.01 M. The DTPA extractant was a mixture of 0.005 M DTPA, 0.01 M $CaCl_2$, and 0.1 M triethanolamine. All extractions were conducted using a reciprocal shaker for 2 h at 180 rpm. After extraction, the samples were centrifuged at 4500 rpm for 30 min and the supernatants were filtered using a 0.22 μm filter. Afterward, the filter samples were acidified with concentrated HNO_3 (the final HNO_3 concentrations were less than 2%) and stored at 4 °C before performing inductively coupled plasma−mass spectrometry (ICP−MS) measurements. Standard Ag solutions of 0.5 mg/L (AAS) and 10 ng/L (for ICP−MS) were measured for every 20 samples to monitor the stability of the machines. Blanks and Ag standard solutions were included in the digestion procedure for the purposes of quality control. The average recovery of Ag for the digestion procedure was 91% with the standard deviation of 7%, and the recovery for the machines was between 99 and 101%. The detection limits for AAS and ICP−MS were 1 μg/L and 1 ng/L, respectively. The RSDs for all sample measurements were below 5%.

Within all sample treatments and times, the pH of the original supernatants (without centrifugation, filter, and acidification) was measured from the $CaCl_2$ extracts representing the soil pH of the soil-extractable available fraction.[19]

Rhizosphere Soil DNA Extraction and Illumina Miseq Sequencing. The DNA from the soil rhizosphere was extracted using a Qiagen DNeasy PowerSoil kit (Hilden, Germany). After quality control checking, a universal bacterial primer set (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 909R: 5′-CCCGTCAATTCMTTTRAGT-3′) was used for PCR amplification by targeting the variable V4−V5 regions of bacterial 16S rRNA genes. Paired-end sequencing was done using a 2 × 300 bp Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA) by BaseClear (Leiden, The Netherlands). The obtained sequences have been deposited into the National Center for Biotechnology Information (NCBI) database (project number: PRJNA732000). The quantitative insights into microbial ecology (QIIME2) pipeline was used to process the sequences. Sequence quality control was performed using the software package DADA2. Qualifying sequences were processed to construct the FeatureTable that was collapsed at the genus level (i.e., level 6 of the Greengenes taxonomy). The q2-phylogeny plugin was used to build the phylogenetic tree (Figure S2), and the q2-diversity plugin was used to compute alpha and beta diversity metrics. The sampling depth was rarefied to remove the heterogeneity (Figure S3). The q2-feature-classifier plugin was used for taxonomic assignment.

Statistical Analysis. Statistically significant differences regarding the $CaCl_2$-extractable Ag, DTPA-extractable Ag, plant biomass, and Ag content in plants in treatment were analyzed by means of one-way ANOVA, followed by Duncan's honestly significant difference tests at $\alpha < 0.05$ using IBM SPSS Statistics 25 (no deviations in the data were found for normal distribution and homogeneity of variance with the Shapiro−Wilk test and Bartlett test prior to the ANOVA test). The *t*-test was performed to determine the differences of the tested end points between bulk soil and rhizosphere soil ($\alpha < 0.05$). The results are expressed as mean ± standard error of three replicates. The QIIME2 diversity alpha-group-significance plugin was used to test the significance of the Shannon index across the different treatments. The principal coordinates analysis (PCoA) based on the weighted UniFrac distance matrices was applied to compare community dissimilarities, and permutational multivariate analysis of variance (PERMANOVA) was used for the significance test. The featured taxa that are

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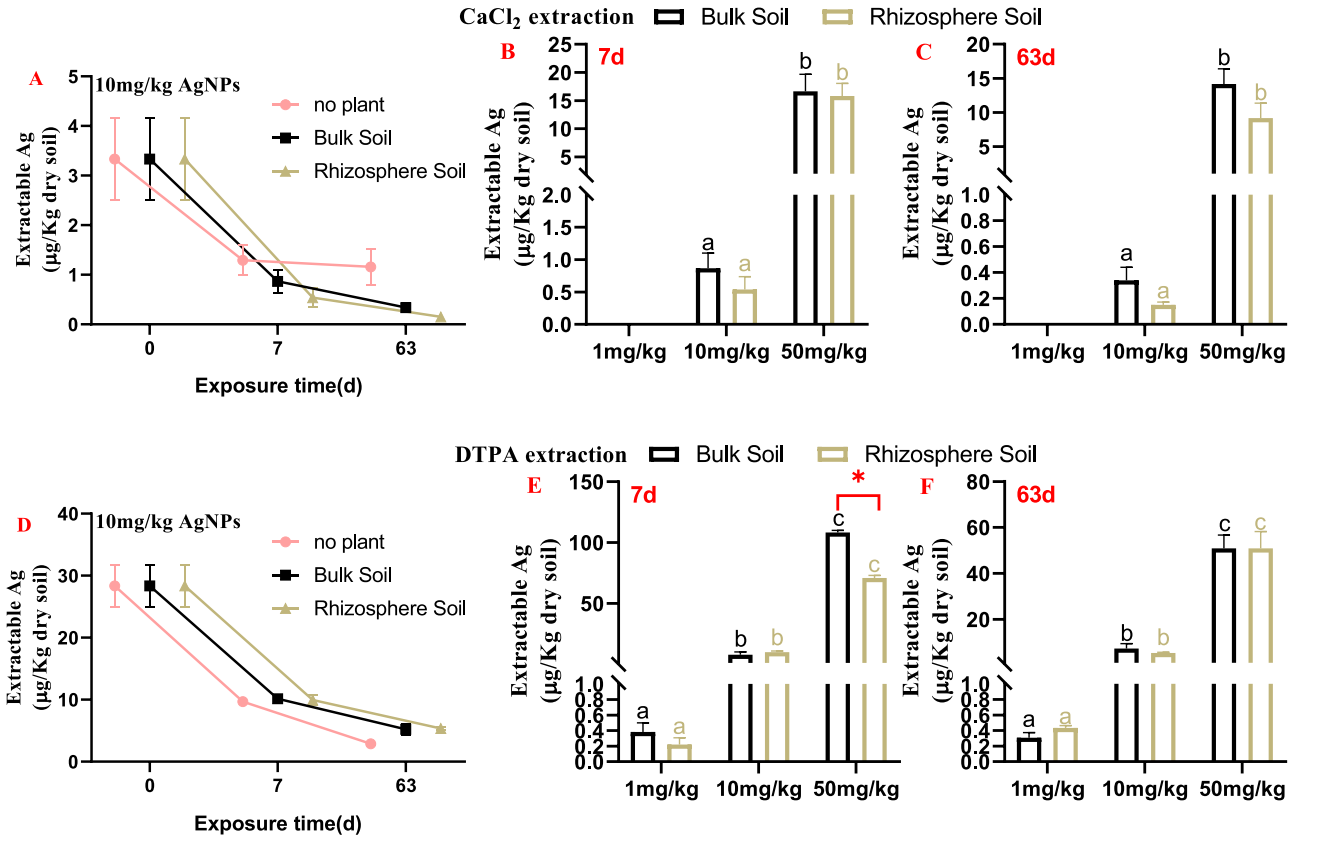

Figure 2. $CaCl_2$- and DTPA-extractable Ag in AgNP-amended soil. (A,D) Changes in the response to the presence of plants for 10 mg/kg AgNP treatment over time. (B,E) Changes in the bulk and rhizosphere soil with different concentrations of AgNPs after 7 d of exposure. (C,F) Changes in the bulk and rhizosphere soil with different concentrations of AgNPs after 63 d of exposure. Different letters indicate statistically significant differences among the treatments in the same soil ($p < 0.05$). The * indicates the significant differences between the bulk soil and the rhizosphere soil under the same treatment ($p < 0.05$). Data are expressed as mean ± SEM of triplicate samples.

differentially abundant in each treatment were identified using analysis of composition of microbiomes (ANCOM). The false discovery rate test was used to correct the *p*-values from false positives in the multicomparison tests. Spearman correlations between the tested end points were analyzed in R with the package of "ggcorrplot" and were considered significant when $p < 0.05$.

RESULTS

Soil pH Changes in Bulk and Rhizosphere Soil after Amendment. The results showed that the addition of AgNPs significantly increased the soil pH of both unplanted (from 7.70 to 7.82) and planted soil (from 7.70 to 7.87) after 7 d of incubation (Figure 1A). However, the significant differences between the control treatment and AgNP treatments disappeared after long-term exposure (63 d). Additionally, the soil pH of all treatments decreased after long-term exposure as compared to short-term exposure (7 d). Noteworthily, no significant difference in the soil pH was observed between 10 mg/kg AgNP planted and unplanted soil regardless of the exposure duration ($p = 0.184$ for 7 d and $p = 0.956$ for 63 d). In addition, the soil pH did not differ between the treatments amended with different concentrations of AgNPs regardless of the exposure duration (Figure 1).

Changes in Extractability of Ag in the Bulk and Rhizosphere Soil. For the freshly prepared 1 mg/kg AgNP-amended soil (day 0 refers to the transplantation date), the $CaCl_2$-extractable amounts of Ag were below the detection limit, while the corresponding DTPA-extractable amount of Ag was 7 ± 2 μg/kg. Similarly, the $CaCl_2$-extractable amounts of Ag were 3.3 ± 0.8 and 29 ± 5 μg/kg for the 10 and 50 mg/kg AgNP-amended soils, while the DTPA-extractable amounts of Ag were 28 ± 3 and 142 ± 4 μg/kg for the 10 and 50 mg/kg AgNP-amended soils, respectively. Upon increasing the incubation time, the extractable amount of Ag in both unplanted and planted soils decreased (Figure 2A). For example, for the 10 mg/kg AgNP unplanted soil, the DTPA-extractable amount of Ag decreased from 28 ± 3 to 9.7 ± 0.5 μg/kg (incubation for 7 d) to 2.9 ± 0.1 μg/kg (incubation for 63 d). Regarding the extractability of Ag in unplanted and planted soils for the same cultivation time, the DTPA-extractable amount of Ag in the bulk soil and rhizosphere soil was similar to 7 d or significantly higher than 63 d (ANOVA, $p = 0.01$) unplanted soil, while the $CaCl_2$-extractable amount of Ag in both cultivation time followed the order unplanted soil > bulk soil > rhizosphere soil.

The differences in $CaCl_2$-extractable Ag and DTPA-extractable Ag for the AgNP-amended soil with different concentrations of AgNPs in the bulk soil and rhizosphere soil are shown in Figure 2. A clear concentration-dependent impact on the extractable amount of Ag was observed for both bulk soil and rhizosphere soil regardless of the $CaCl_2$ extractant or DTPA extractant. For the low AgNP concentration (1 mg/kg), the amount of Ag extracted by $CaCl_2$ extraction was below the detection limit, while the DTPA-extractable amount of Ag was less than 0.5 μg/kg soil. Compared to the concentration of the 10 mg/kg AgNP-amended soil, the extractable amount of Ag in the soil amended with 50 mg/kg AgNPs was significantly

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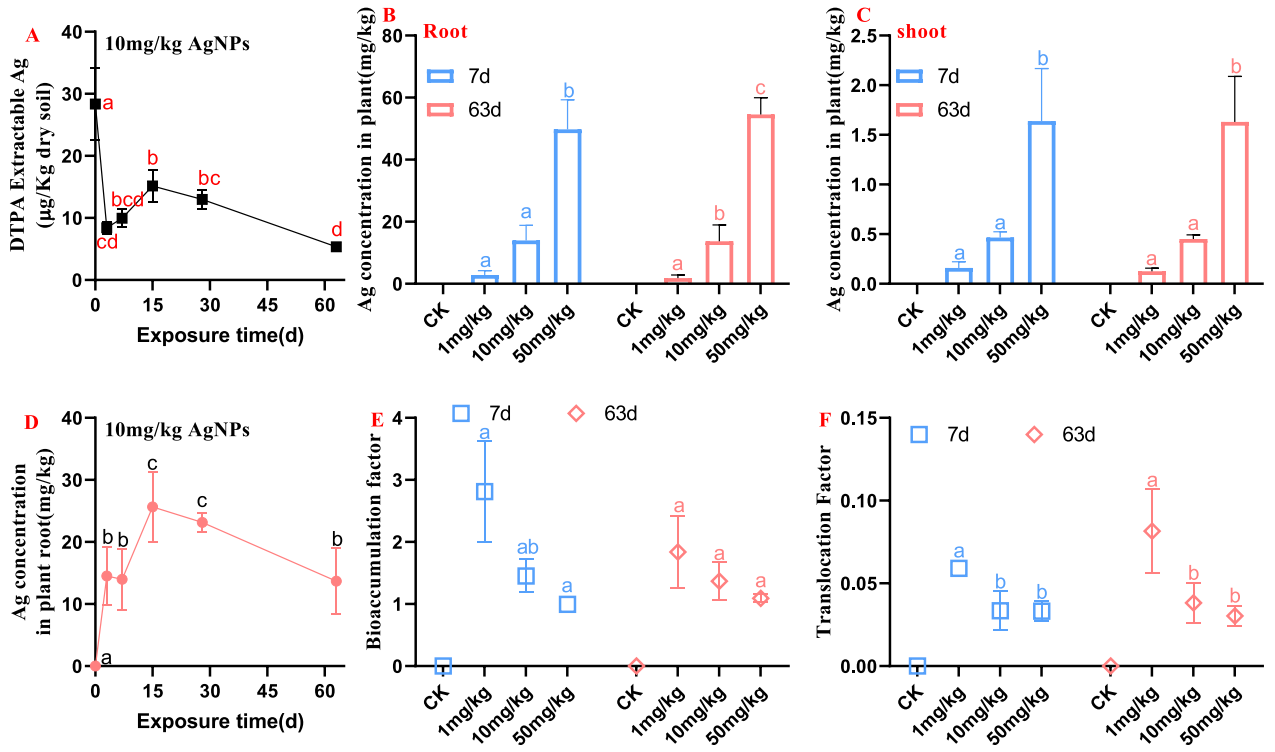

Figure 3. DTPA-extractable Ag in rhizosphere soil (A) and Ag accumulation in plant roots (D) in 10 mg/kg AgNP treatment over time. Ag accumulation in the plant root (B), plant shoot (C), and the BAFs (E) and TFs (F) at different AgNP concentrations after 7 and 63 d of exposure. The concentrations of Ag in plant roots and shoots for the control treatment were considered as 0 as they were below the detection limit. Different letters indicate a statistically significant difference among the treatments with the same exposure duration ($p < 0.05$). Data are expressed as mean ± SEM of triplicate samples. CK means the control treatment.

increased by a factor of 19−61 for $CaCl_2$ extraction and 7−14 for DTPA extraction under different conditions. Between the bulk soil and rhizosphere soil, no significant differences were observed for the $CaCl_2$-extractable Ag regardless of the exposure concentration or time. For DTPA-extractable Ag, significant differences between the bulk and rhizosphere soil were only observed for the soil to which 50 mg/kg AgNPs were added (*t*-test, $p < 0.005$).

We also investigated the changes in the extractability of AgNPs in the rhizosphere soil (10 mg/kg AgNPs) over time by DTPA extraction, as shown in Figure 3A. An interesting tendency was observed as the DTPA-extractable Ag in the rhizosphere soil decreased rapidly in the first 3 d of cultivation and then increased gradually after 7 and 15 d of cultivation but decreased again from 15 to 63 d of cultivation. In addition, the extractable amount of Ag in the planted soil after 7 d of cultivation was slightly higher when compared to the extractable amount of Ag after 63 d of cultivation in all experimental scenarios. However, statistically significant differences were only observed in 50 mg/kg AgNP-amended bulk soil for DTPA extraction ($p < 0.005$, *t*-test).

Ag Accumulation and Translocation in the Soil−Plant System. During the same exposure duration (7 or 63 d), no significant differences in the plant biomass were observed between the control treatment and AgNP treatments regardless of the exposure concentration ($p = 0.858$ for 7 d and $p = 0.541$ for 63 d, Figure S4).

The change in the Ag concentration in plant roots in 10 mg/kg AgNP treatment over time is shown in Figure 3D. The Ag concentrations in the plant roots increased after 3, 7, and 15 d of cultivation and then decreased during the cultivation period of 15−63 d, which followed the same pattern of the DTPA-extractable Ag in the corresponding rhizosphere soil over time. Interestingly, when comparing the Ag concentrations in plants upon 7 d cultivation to 63 d cultivation at the same applied AgNPs dose, no significant difference was observed (Figure 3B, *t*-test, $p > 0.05$).

Figure 3 also shows the accumulation and translocation of Ag in the plant tissues after cultivation for 7 and 63 d in soil to which different amounts of AgNPs were added. The Ag concentrations in the plant roots were more than 10 times higher than the Ag concentration in the corresponding shoots upon exposure to the same concentration and time. Moreover, Ag was taken up by plant roots and translocated into plant shoots in all AgNP-amended treatments with a general concentration-dependent increase. For example, the Ag concentrations in lettuce shoots were around 1.6 mg Ag/kg plant for the treatment of 50 mg/kg AgNPs, which is 10−13 times higher than that found in the shoots of plants exposed to 1 mg/kg AgNP-amended soils (0.16 mg/kg for 7 d and 0.13 mg/kg for 63 d).

In addition, the BAFs of Ag in all exposure treatments were higher than 1. The high Ag concentrations in plant roots and the high BAFs of Ag indicated the potential biomagnification of Ag from soil to the plant. The presence of Ag in plant shoots shows the translocation ability of Ag from plant roots to shoots, even though the TFs of Ag in all treatments were lower than 0.1.

Response of Soil Microbial Communities to AgNPs in the Rhizosphere Soil. The alterations of the bacterial community in the rhizosphere in response to AgNP exposure was further investigated. The Shannon index, which reflects the

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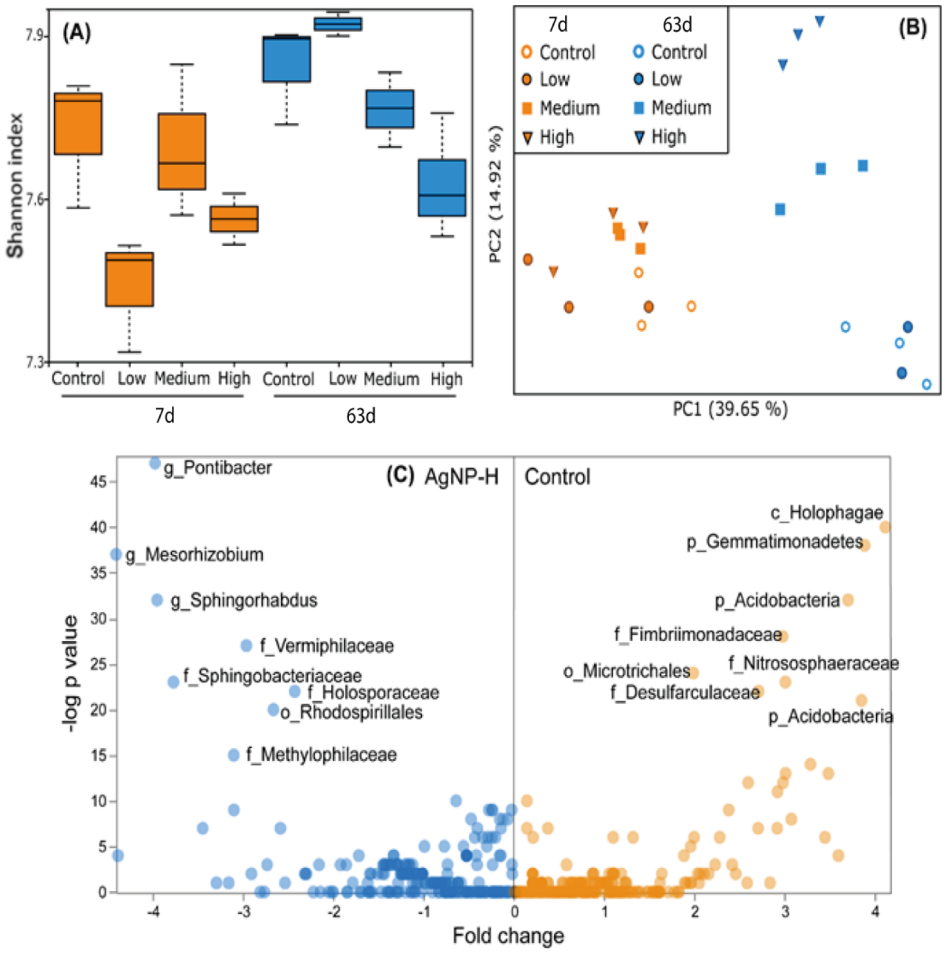

Figure 4. (A) Changes in the community (α) diversity shown as the Shannon index. (B) PCoA of the bacterial community structure. (C) Featured taxa identified between the control and soil amended with 50 mg/kg AgNPs upon 63 d of incubation.

species richness, was used to evaluate the alpha diversities of the rhizosphere bacteria in the control soil and in AgNP-amended soil. During 7 d of incubation, the changes in the Shannon indices among the control and the soil amended with different concentrations of AgNPs were irregular. However, after increasing the incubation duration to 63 d, a clear tendency was observed and the Shannon index decreased upon exposure to increasing concentration of AgNPs.

The shifts in the rhizosphere bacterial community structure induced by AgNP treatments over time were further analyzed by PCoA (Figure 4B). After incubation for 7 d, the bacterial communities in the control and different AgNP treatment samples clustered together. However, when the incubation time was increased to 63 d, the bacterial communities exposed to 10 mg/kg AgNPs and 50 mg/kg AgNPs were clearly separated from the control. Moreover, the bacterial communities in 10 and 50 mg/kg AgNP-amended soils separated from each other. This indicates that the impacts of AgNPs on the bacterial community structure are time-dependent.

Additionally, the community composition at the phylum level in response to different treatments is provided in Figure S5. Proteobacteria (with an average relative abundance of 29−34%), Actinobacteria (27−31%), Bacteroidetes (9−14%), and Acidobacteria (9−11%) were the dominant bacterial phyla in both the control soil and AgNP-amended soil after 7 d of incubation. By increasing the incubation period from 7 to 63 d, the average relative abundance of Actinobacteria decreased from 27−31 to 16−20%, again indicating that the effect of AgNPs on the bacterial composition is time-dependent. The featured taxa that are differentially abundant in the different treatment samples were identified using ANCOM analysis (Figure 4C). In general, no featured taxa were found in the AgNP treatment samples after 7 d of incubation. After incubation for 63 d, a total of 16 featured taxa were observed, which greatly contributed to the observed differences between the 50 mg/kg Ag-amended soil and the control soil. From these featured taxa, eight taxa (including the phyla Acidobacteria and Gemmatimonadetes, the class Holophagae, the order Microtrichales, the families Fimbriimonadaceae, Nitrososphaeraceae, and Desulfarculaceae) were downregulated and eight taxa (including the order Rhodospirillales, the families Vermiphilaceae, Sphingobacteriaceae, Holosporaceae, and Methylophilaceae, and the genera Pontibacter, Mesorhizobium, and Sphingorhabdus) were upregulated in 50 mg/kg AgNPs when compared with the control. These results further confirmed a long-term impact of a high concentration of AgNPs on the rhizosphere bacterial community composition.

Correlation Analysis of Exposure Conditions, Soil pH, Extractable Ag in Soil, Plant Parameters, and Soil Bacterial Communities. As shown in the map of Spearman's correlations (Figure 5), the soil pH was negatively correlated with the exposure time but had no significant relationship with the exposure concentration. The amount of DTPA-extracted Ag in both bulk and rhizosphere soil was significantly and

https://doi.org/10.1021/acssuschemeng.1c04987
ACS Sustainable Chem. Eng. 2021, 9, 16172−16181

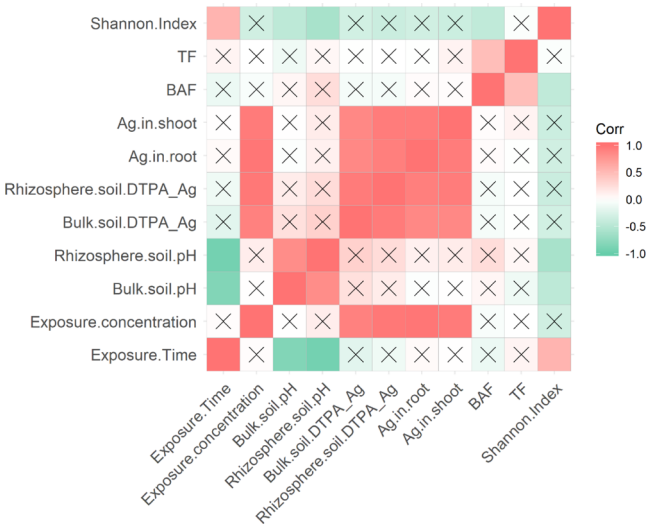

Figure 5. Spearman correlation map between the tested parameters including the exposure conditions, soil pH, extractable Ag in soil, plant-related parameters, and Shannon index of the soil bacterial community. Correlations with $p > 0.05$ fill with ×. Significant negative correlations (corr < 0) are given in gradations of green. Positive correlations (corr > 0) are given in gradations of red.

positively correlated with the exposure concentration of AgNPs since all correlation coefficients were higher than 0.9. In addition, the amount of Ag accumulated in the plant root and the shoots was correlated positively with DTPA-extracted Ag in the soil. Root and shoot concentrations were highly related because of the translocation of Ag after uptake via the roots. No relationships were observed between the exposure time or soil pH and extractable Ag in soil, as well as the Ag content in plants. On the contrary, the Shannon index of the soil bacterial community positively correlated with the exposure time but negatively correlated with the soil pH and the BAFs of Ag in plants.

DISCUSSION

Overall, this study enhanced the understanding of how the dynamic dissolution of AgNPs in soil affect their bioavailability in the rhizosphere−lettuce interface and of long-term impacts of AgNPs on the rhizosphere soil bacterial community. Our results revealed that the addition of AgNPs significantly increased the soil pH after 7 d of incubation regardless of the presence of lettuce plants (Figure 1A). This statistically significant increase is in line with the results of previous studies reporting an increase of the soil pH after amending soil with metallic NPs.[14,32] The alterations of the soil metabolite profiles and the abundance and composition of root exudates can change the soil pH.[13,14] For example, Zhang et al.[14] suggested that the increase of the soil pH might be attributed to the decrease of the concentrations of several fatty acids in metabolites of the soil induced by exposure to AgNPs. Additionally, the dissolution of AgNPs can also contribute to higher pH[19] as it can consume the H^+ in the system or release OH^- into soil following the stoichiometry below[33]

$$2Ag(s) + 1/2O_2 + 2H^+ \rightarrow 2Ag^+ + 2H_2O \quad (3)$$

or[34]

$$Ag_2O \text{ (oxide layer formed on the surface of AgNPs)} + H_2O \rightarrow 2Ag^+ + 2OH^- \quad (4)$$

However, the soil pH decreased after increasing the incubation time to 63 d and no significant differences were observed between treatments after 63 d of incubation. This pattern was similar to the findings of Das et al.,[35] who also reported that the soil pH increased during the initial exposure period but decreased after the long-term exposure. The findings suggest that the aging of AgNPs in the soil neutralized the pH changes between treatments.

The dissolution of metallic NPs is known to be the dominant process governing the availability of metals derived from metallic NPs.[19] Thus, the dissolution of AgNPs in soil over time was investigated using DTPA extraction. The DTPA-extractable Ag was less than 0.3% for all experimental scenarios, suggesting that the dissolution of AgNPs in soil was very limited, even in the rhizosphere. This was in line with previous studies,[18,36] which also revealed the very low lability/release of Ag from AgNP-amended soil. Interestingly, a gradual increase of DTPA-extractable Ag in the rhizosphere soil was observed during the cultivation period from 3 to 15 d (Figure 3A), indicating the gradual dissolution of AgNPs in the soil. However, the extractable Ag in the rhizosphere soil decreased as the cultivation period increased from 15 to 63 d. Also, the Ag concentration extracted from the AgNP-amended soil after 63 d of cultivation was slightly lower than the Ag concentration after 3 d and 7 d of cultivation. There are several possible explanations for this observation of declining Ag concentrations. First, the dissolution of AgNPs might have become slower after 15 d of cultivation, or the AgNPs dissolution reached saturation over after longer exposure duration. There is a plethora of information revealing the two-phase dissolution behavior of AgNPs, containing a short but rapid initial release phase and a longer but slower second release phase.[37,38] Second, the uptake of Ag by plant roots was much faster than the dissolution process of AgNPs in soil, as indicated by comparing the extractable Ag from AgNPs and the Ag uptake by plant roots over time (Figure 3A,D). This led to a relative decrease of Ag accumulation in the plants for the cultivation period from 15 to 63 d. Finally, this decline might be a result of the combination of Ag precipitation, irreversible binding of Ag^+/AgNPs to the solid soil matrix, and the transformation of AgNPs in soil.[10] After long-term exposure in soil, AgNPs have a large potential of being transformed to silver sulfide or other sulfur-bound Ag forms,[18,39] which will reduce the solubility and extractability of AgNPs. A previous study also found that the concentration of labile Ag in soil was significantly decreased by increasing the incubation time to 2 weeks and to 6 months and evidenced that the S group-bound Ag was the predominant form after the soil was amended with soluble Ag,[40] the so-called aging processes. The changes in the extractable concentration of Ag over time suggest that a single or fixed exposure duration cannot capture the actual dissolution and accumulation process of AgNPs in soil,[13] which may result in inaccurate assessment of the bioavailability or toxicity of AgNPs.

Our results demonstrated that the DTPA-extractable Ag concentration in unplanted soil, bulk soil, and rhizosphere soil was almost equal (Figure 2D), suggesting that the effect of lettuce on the dissolution of AgNPs was limited. The $CaCl_2$-extractable amount of Ag followed the order unplanted soil >

https://doi.org/10.1021/acssuschemeng.1c04987
ACS Sustainable Chem. Eng. 2021, 9, 16172−16181

bulk soil > rhizosphere soil (Figure 2A). This indicates that $CaCl_2$-extractable Ag is a better predictor of Ag uptake by plants as it better represents the more "readily available" Ag form. Gao et al.[13] also suggested that DTPA extraction is a better indication for metallic NP dissolution in soil, while $CaCl_2$ extraction provides a more accurate prediction of the uptake of NPs by plants. The observed Ag in plant shoot tissues shows the translocation of Ag from roots to shoots. In addition, the Ag accumulation and translocation positively correlated with the extractable amount of Ag in soil (Figure 5), and the trend of Ag accumulation in the plants over time was similar to the dissolution of AgNPs in soil (Figure 3). These results indicate that the dissolution of AgNPs is the predominant process related to the Ag uptake by plant roots.

For the rhizosphere bacterial community, we did not observe any significant impact of AgNPs during a short-term exposure (7 d) regardless of the exposure concentration. However, after long-term exposure (63 d), the Shannon index decreased, and the bacterial communities separated from the control with increasing exposure concentration. This suggests that the effects of AgNP concentration on the diversity and composition of the rhizosphere bacterial community varied over time. Moreover, eight upregulated bacterial taxa and eight downregulated featured taxa were only observed in the treatment with 50 mg/kg AgNPs after 63 d of exposure, which contributed the most in inducing the differences between AgNP treatment and the control. Previous research stated that the soil microbiome can shift its composition by increasing the Ag-tolerant taxa in response to AgNP stress.[21,41] Similarly, the abundance of Ag-resistant and -sensitive genus *Mesorhizobium*[42] was found to be increased in the soil amended with high concentration of AgNPs in our study. In addition, several bacterial groups associated with the removal/degradation of a number of contaminants were stimulated in response to AgNPs, including the genus *Pontibacter* that is able to remove metals[42,43] and *Sphingorhabdus* and *Sphingobacteriaceae* that are related to the degradation of a variety of recalcitrant organic compounds.[14,15,44] In addition, *Pontibacter* (strongly associated with the N fixation gene nifH),[17] *Mesorhizobium*,[39] and *Rhodospirillales* (containing free-living N_2-fixing bacteria)[15,41] were also promoted, indicating that the long-term exposure of AgNPs stimulates the bacterial taxa related to nitrogen cycling. Besides these upregulated bacterial taxa, several bacteria such as *Acidobacteria* and *Desulfarculaceae* were significantly inhibited upon long-term exposure to high concentrations of AgNPs.[14,45,46] These bacteria are involved in carbon usage, sulfur reduction, and iron reduction. The alterations of the identified featured taxa highlight the potential disruption of agricultural systems because of AgNP exposure by affecting the functional bacterial groups associated with nutrient acquisition, stress tolerance, and biogeochemical element cycling (such as C, N, and S). Follow-up research, to determine the relationship among the content of the elements in soil (such as C, N, P, and S), the nutrients in plants (such as proteins and phospholipids), and the abundance of genes involved in the biogeochemical element cycling in the rhizosphere soil amended with NPs, would be very interesting and valuable for understanding the interaction of NPs−plants−soil bacteria. Furthermore, the changes in the diversity and structure of the rhizosphere soil bacterial community over time also emphasize the importance of investigating the dynamic impacts of NPs on the rhizosphere bacterial communities.

CONCLUSIONS

Overall, the presence of lettuce played a limited role in affecting AgNP dissolution in soil as the extractability of Ag in unplanted and planted soil was similar under the same exposure conditions. We found that the dissolution of AgNPs in soil is the dominant process influencing Ag uptake via the roots and translocation to the shoots. The Ag extractability from AgNP-amended soil and accumulation of Ag in the plants changed over time. The diversity and composition of the rhizosphere soil bacterial community were altered after long-term exposure to high concentrations of AgNPs. These results highlight the importance of taking time-resolved dynamics of the soil−plant system in consideration in response to NP exposure. The slow but continuous dissolution of AgNPs in soil can provide a sustained antimicrobial effect against plant pathogens (phytopathogenic fungi, bacteria, and viruses). This implies that repetitive applications of AgNPs are not needed, which likely diminishes the total Ag concentration applied. This is an important potential benefit of using AgNPs containing agrochemicals compared to applying ionic Ag solutions. However, attention should still be paid to control the potential negative effects of AgNPs in soil−plant systems as high amounts of Ag in plant roots and the long-term alterations of the composition of the rhizosphere bacterial community were observed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c04987.

TEM pictures of AgNPs, phylogenetic tree of the rhizosphere bacterial community, rarefaction curves of the rhizosphere bacterial communities, biomass of lettuce exposed to different treatments and the composition of the rhizosphere soil bacterial communities at the phylum/class levels, Hoagland's solution composition, hydrodynamic diameter and zeta potential of AgNP suspensions, and physicochemical properties of the soil and experimental design (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Guiyin Wang (Leiden University) for the help with soil collection and preparation and Dr. Fazel Abdolahpur Monikh (Leiden University) for help with the ICP−MS measurements. The China Scholarship Council (CSC) is gratefully acknowledged for its financial support to J.W. (201706750014).

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