Fate, accumulation and impact of metallic nanomaterials in the terrestrial environment

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Chapter 4

The dissolution dynamics and accumulation of AgNPs in a microcosm consisting of soil -lettuce - rhizosphere bacterial community

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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, whilst the plants and their associated microbiota affect nanoparticles fate. Here, we measured the available Ag pool (determined as the DTPA-extractable Ag) in AgNPs amended sandy-loam soil (1, 10 and 50 mg Ag per kg soil) over a period of 63d, 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 after short-term (7d) incubation. Noteworthy, the extractability of Ag in AgNPs amended soil was concentration-dependent and changed over time because of their continuous dissolution and uptake by plants. Ag uptake and upwards translocation in lettuce was positively correlated with the extractable Ag content in soil. Furthermore, long-term (63d) exposure to 50 mg/kg of AgNPs altered the structure and composition of the rhizosphere bacterial community potentially by regulation of bacterial groups associated to element (e.g., N and S) cycling and stress tolerance. In conclusion, our results demonstrated that the dynamic dissolution of AgNPs in sandy-loamy 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 NPs exposure should be taken into consideration for accurate ecological risk assessment of NPs in the soil ecosystem.

4.1. 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[21,238]. As more and more nano-agrochemicals are introduced in agriculture as fertilizers or pesticides, agricultural soil is inevitably becoming an important sink for nanomaterials[15]. 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[239,240]. 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)[21]. 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 on soil ecosystems have been reported to largely depend on their bioavailable fractions[241]. For instance, Pu et al.[241] reported that the toxicity of CuO nanoparticles 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 nanoparticles in nature soil[242,243]. An important property is soil pH, which modulates the bioavailability of metallic nanoparticles by affecting the oxidation, aggregation, transformation and dissolution processes of metallic nanoparticles in soil[24,244]. Importantly, plants, a key component of soil ecosystems[221], can alter the soil properties directly by themselves or indirectly by the interaction with nanoparticles. 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[142]. Moreover, the interaction between plant roots and metallic nanoparticles can alter the abundance and composition of root exudates as well as soil pH[142,245,246]. Soil organic matter and the root secreted chelators (such as phytosiderophores) can immobilize/sequester NPs and the released metal ions[247,158]. These changes in soil environment may modify the available pool of Ag derived from AgNPs and in turn influence the plant responses[146,245].

However, to our knowledge, the information regarding how plant roots influence the labile pool of Ag in a AgNPs amended rhizosphere and the consequent relationship with the Ag accumulation in plant is scarce. Recently, Del Real et al.[49] used DTPA and $CaCl_2$ extractions to assess the lability of Ag in soil mixed with AgNPs-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[49]. 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 concentration of Ag time-dependent[248,249]. To capture these dynamics and their impacts on ecosystems, experiments of longer time scales 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 plays important role in soil ecosystem by reflecting soil fertility and governing soil biological processes like nutrient transformation and cycling, and energy flow[137,250]. The impacts of AgNPs on bacterial communities of unplanted soil have been extensively reported with inconclusive findings[137,139,250,251,252]. It is suggested that the responses of soil microbial community to AgNPs depend on the soil properties, exposure concentration, exposure duration as well as the behaviours of AgNPs in soil[251,252]. Therefore, the alterations in the soil environment induced by plants may modify the AgNPs effects on the rhizosphere soil bacteria behaviours[253], which may result in either detrimental or beneficial impacts on the soil ecosystem[141,146,247]. To date, little information is available about how AgNPs alter rhizosphere soil bacterial communities[149,246,254]. 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[141,145]. Thus, long-term impact of AgNPs on rhizosphere soil bacterial community deserves 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 days. 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 AgNPs 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 nano-enabled agrichemicals.

4.2. Materials and Methods

4.2.1 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). Physico-chemical properties and information on the characterization of the AgNPs are summarized in JCR Reports[255]. AgNPs suspensions at 1, 10 and 50 mg/L were prepared by diluting the AgNPs stock in 1/4 Hoagland solution (pH 6.0 ± 0.1). The composition of the Hoagland solution is described in **Table S2.1** (Supplementary material). 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 is published in our previous publication[221] and provided in **Table S4.1**. The TEM picture of the AgNPs is also provided in **Figure S4.1** (Supplementary material).

4.2.2 Soil preparation

Surface agricultural soil (0-20 cm) was collected from a non-polluted site (52°10'16.8"N 4°26'58.9"E, Leiden, Netherlands), mixed thoroughly, sieved to 2 mm after being air-dried, and stored at 4 °C before use. The soil is sandy-loam with pH of 8.4 in water and 7.4 in KCl solution, containing 2.2 % of organic carbon, with clay content of 18.4% and 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 S4.2**.

4.2.3 Plant growth and exposure assay

Lactuca sativa seeds (Floveg GmbH, Kall, Germany) were firstly sterilized for 5 min in 0.5% (w/v) NaClO, followed by rinsing three times with tap water and immersing for 24 h in tap water. Afterwards, the seeds were germinated in Petri dishes filled with a wet filter paper (15 seedlings/dish). After 3 d, 1/4 Hoagland solution was added into the Petri dishes to supply nutrients for seedling growth. After pre-growing in Petri dishes for one week, the young seedlings were transferred to bottles (one seedling per

bottle) with a height of 15 cm containing Hoagland solution for a further two weeks of growth. The suspensions in the Petri dishes and bottles were refreshed every 3d.

The AgNPs suspensions were prepared in 1/4 Hoagland solution and sonicated at 60 Hz for 15 min before application to soil. Afterwards, the AgNPs suspensions were added to soil to achieve the nominal concentration of 1, 10 and 50 mg Ag per kg soil. The exposure concentrations of AgNPs were chosen based on the predicted and measured concentration of AgNPs in sludge/biosolid[49]. The soil was mechanically stirred with a mixer for 15 min to homogenize the AgNPs. Control treatment was treated the same as the AgNPs treatments with the addition of same volume of 1/4 Hoagland solution. Next, two uniform pre-grown seedlings were transferred into one plastic pots (9 cm length, 9 cm wide, 9.5 cm high) containing 0.5 kg of AgNPs amended soil or clean soil. Treatments containing 1, 10 or 50 mg Ag per kg soil but without plants were also performed under the same conditions. In brief, this experiment consisted by 3 components: a) AgNPs applications 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 S4.3**. The pots were watered every two days and all pots were placed in a climate room under the conditions of day/night temperature at 20/16 °C and the 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 non-rhizosphere (further referred to as bulk soil) and soils with rhizospheres (further referred to as rhizosphere soil) were collected.

4.2.4 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. Plants were carefully removed from the pots, and the soil which was left in the pots was defined as bulk soil where the influence by plant roots is negligible[245]. The collected bulk soil was mixed thoroughly for further use. The soil that was loosely attached to the roots was firstly removed by shaking the plants (discarded) and then the soil that closely adhered to the roots was collected as rhizosphere soil (<1 mm away from the root) following the method reported by Guan et al.[146]. 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, plants were thoroughly washed with flowing tap water and rinsed in deionized water for 10 min, which was repeated three times. Subsequently, plants were divided into root and shoot and after air-drying, the biomass of plant roots and shoots was recorded. To determine the Ag content in plants, plants were firstly washed with 10mM HNO_3, 10mM EDTA and Milli-Q water to remove the attached AgNPs/Ag^+ ions as described previously[221]. Next, plants were oven-dried, weighed and digested with HNO_3 (65%) and H_2O_2 (30%) at 120 °C [221]. Finally, the digests were diluted, and Ag concentrations were analysed by means of a Graphite furnace atomic absorption spectrometer (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[221]:

$$BAF = \frac{[Ag]_{root}}{[Ag]_{soil}}; \mathrm{TF} = \frac{[Ag]_{shoots}}{[Ag]_{roots}}$$

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

4.2.5 Labile Ag extraction from AgNPs amended soil and soil pH measurement

At each sampling date, ~2.0 g of air-dried soil samples were extracted with 20 mL of $CaCl_2$ extractant or 4 mL of DTPA extractant[245]. $CaCl_2$ can extract the metals from the soil 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 as well as the "potentially available" fraction that is reversibly bound to the soil solid matrix, which has been suggested to be an indication for metallic nanoparticle dissolution in soil[245]. The $CaCl_2$ extractant was prepared by dissolving $CaCl_2$ salt in Milli-Q water to reach the final concentration of

0.01M. The DTPA extractant was a mixture of 0.005 M DTPA, 0.01 M $CaCl_2$, and 0.1 M triethanolamine (TEA). 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 0.22 μm filter. Afterwards, 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 ICP-MS measurements. Standard Ag solutions of 0.5 mg/L (AAS) and 10 ng/L (for ICP-MS) was measured 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 from 99% to 101%. The detection limits for AAS and ICP-MS were 1 μg/L and 1 ng/L respectively. The RSDs for all samples measurement were below 5%.

Within all samples treatments and times, pH was measured within the original supernatants (without centrifugation, filter and acidifying) from the $CaCl_2$ extracts representing soil pH of soil extractable available fraction[248].

4.2.6 Rhizosphere soil DNA extraction and Illumina Miseq sequencing

The DNA from the soil rhizosphere was extracted using 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 2 × 300 bp Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA) by BaseClear (Leiden, the Netherlands). The obtained sequences are deposited into the National Center for Biotechnology Information (NCBI) database (Project number: PRJNA732000). Quantitative Insights Into Microbial Ecology (QIIME2) pipeline was used to process the sequences. Sequences quality control was performed using the software package DADA2. Qualified sequences were processed to construct the Feature Table 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 S4.2**), and the q2-diversity plugin was conducted to compute alpha and beta diversity

metrics. The sampling depth was rarefied to remove the heterogeneity (**Figure S4.3**). The q2-feature-classifier plugin was conducted for taxonomic assignment.

4.2.7 Statistical analysis

Statistically significant differences regarding the $CaCl_2$ extractable Ag, DTPA extractable Ag, plant biomass, Ag content in plants among treatments were analysed 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 of data were found for normal distribution and homogeneity of variance with Shapiro-Wilk test and Bartlett test prior to running the ANOVA). The t-test was performed to determine the differences of the tested endpoints between bulk soil and rhizosphere soil ($\alpha < 0.05$). Results are expressed as mean ± standard error of 3 replicates. The QIIME2 diversity alpha-group-significance plugin was used to test the significance of 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 the Permutational multivariate analysis of variance (PERMANOVA) was used for significance test. The featured taxa that are differentially abundant in each treatment was identified using analysis of composition of microbiomes (ANCOM). The FDR test was used to correct the p-values from false positives in the multi-comparison tests. Spearman correlations between the tested endpoints were carried out in R with the package of "ggcorrplot" and were considered significant when $p<0.05$.

4.3. Results

4.3.1 Soil pH changes in bulk and rhizosphere soil after AgNPs amendment

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 4.1**A). However, the significant differences between control treatment and AgNPs treatments disappeared after long-term exposure (63d). Additionally, the soil pH of all treatments deceased after long-term exposure as compared to short-term exposure (7d). Noteworthy, no significant difference in soil

pH was observed between 10 mg/kg AgNPs planted and unplanted soil regardless of exposure duration (p=0.184 for 7d and p=0.956 for 63d). In addition, the soil pH did not differ between the treatments amended with different concentrations of AgNPs regardless of exposure duration (**Figure 4.1**).

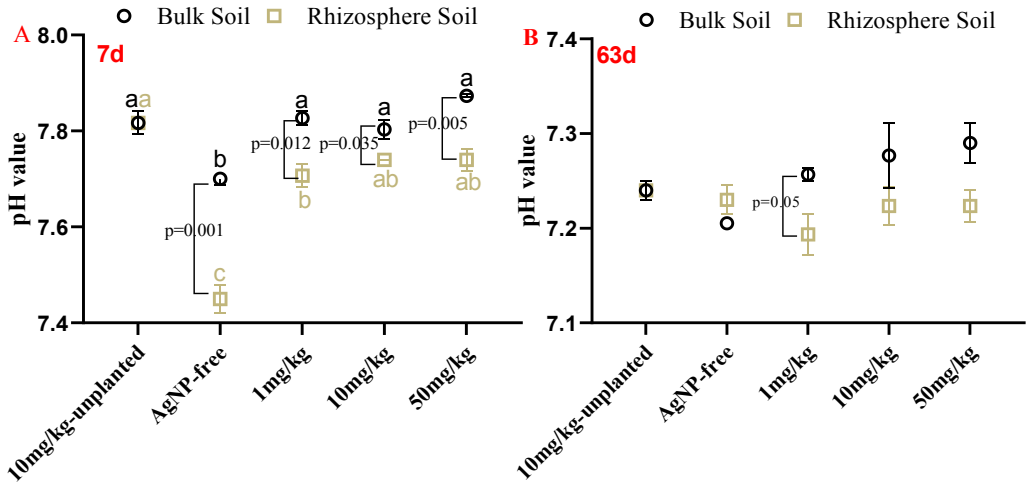

Figure 4.1. Soil pH in rhizosphere soil and bulk soil exposed to AgNPs with or without plant for 7 d (A) and 63 d (B). Different letters indicate the significant difference among the treatments in same soil ($p<0.05$). The numbers indicate the significant differences between the bulk soil and rhizosphere soil under same treatment($p<0.05$). Data expressed as mean ± SEM of triplicate samples.

4.3.2 Changes of extractability of Ag in bulk and rhizosphere soil

For the freshly prepared 1 mg/kg AgNPs amended soil (day 0-referred to transplanting date), the $CaCl_2$-extractable amount of Ag was 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 µg/kg and 29 ± 5 µg/kg for 10 mg/kg and 50mg/kg AgNPs amended soil; while the DTPA-extractable amounts of Ag were 28 ± 3 µg/kg, 142 ± 4 µg/kg for 10 mg/kg and 50mg/kg AgNPs amended soil, respectively. Upon increasing incubation time, the extractable amount of Ag in both unplanted and planted soil decreased (**Figure 4.2**A). For example, for the 10 mg/kg AgNPs unplanted soil, the DTPA- extractable amount of Ag decreased from 28 ± 3 to 9.7 ± 0.5 µg/kg (incubation for 7 d) and to 2.9 ± 0.1 µg/kg (incubation for 63 d). Regarding the extractability of Ag in unplanted and planted soil at the same

cultivation time, the DTPA-extractable amount of Ag in 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 of unplanted soil > bulk soil > rhizosphere soil.

The differences of $CaCl_2$ extractable Ag and DTPA extractable Ag for AgNPs amended soil with different concentrations of AgNPs in bulk soil and rhizosphere soil are shown in **Figure 4.2**. A clear concentration-dependent impact on the extractable amount of Ag was observed for both bulk soil and rhizosphere soil regardless of $CaCl_2$ extractant or DTPA extractant. For the low AgNPs concentration (1 mg/kg), the amount of Ag extracted by the $CaCl_2$ 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 10 mg/kg AgNPs amended soil, the extractable amount of Ag in soil amended with 50 mg/kg AgNPs was significantly increased by a factor of 19 ~ 61 for $CaCl_2$ extraction and 7 ~ 14 for DTPA extraction under different conditions, respectively. Between bulk soil and rhizosphere soil, no significant differences were observed for the $CaCl_2$ extractable Ag regardless of exposure concentration or time. For DTPA extractable Ag, significant difference between bulk and rhizosphere soil was only observed for the soil to which 50 mg/kg AgNPs were added (t-test, $p<0.005$).

We also investigated the changes of the extractability of AgNPs in rhizosphere soil (10 mg/kg AgNPs) over time by DTPA extraction, as shown in **Figure 4.3**A. 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 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 AgNPs amended bulk soil for DTPA extraction ($p<0.005$, t-test).

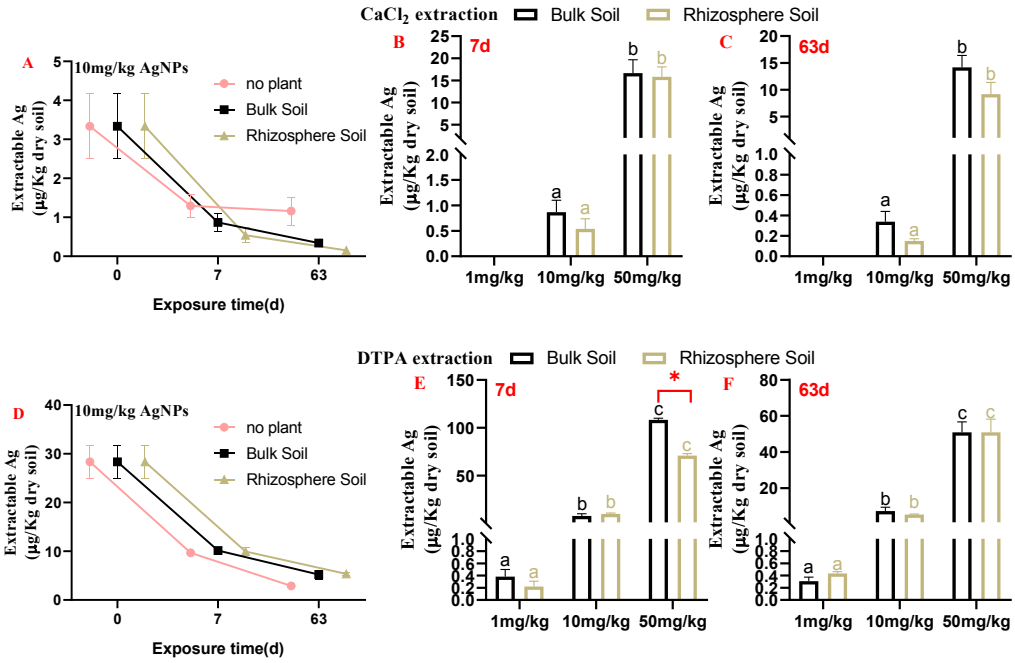

Figure 4.2. $CaCl_2$ and DTPA extractable Ag in AgNPs amended soil. A and D: Changes in response to the presence of plants for 10 mg/kg AgNPs treatment over time. B and E: Changes in bulk and rhizosphere soil with different concentration of AgNPs after 7 d of exposure. C and F: Changes in bulk and rhizosphere 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.

4.3.3 Ag accumulation and translocation in the soil-plant system

At the same exposure duration (7d or 63d), no significant differences in plant biomass were observed between control treatment and AgNPs treatments regardless of the exposure concentration ($p = 0.858$ for 7d and $p=0.541$ for 63d, **Figure S4.4**).

The change of Ag concentration in plant roots in 10 mg/kg AgNPs treatment over time is shown in **Figure 4.3**D. The Ag concentrations in the plant roots increased after 3, 7, and 15d of cultivation and then decreased in the cultivation period of 15 d to 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 4.3**B, t-test, $p>0.05$). **Figure 4.3** also shows the accumulation and translocation of Ag in plant tissues after cultivation for 7 and 63 d in soil to which different amounts of AgNPs were added. The Ag concentrations in plant roots were more than 10 times higher than the Ag concentration in the corresponding shoots upon the same exposure concentration and time. Moreover, Ag was taken up by plant roots and translocated into plant shoots in all AgNPs 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 found in the shoots of plants exposed to 1 mg/kg AgNPs 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 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.

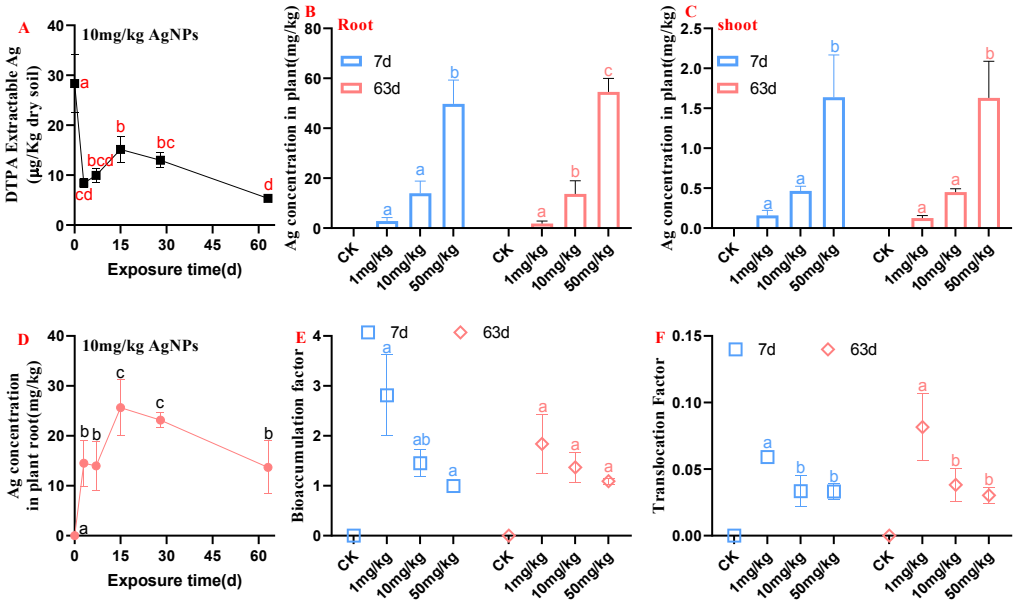

Figure 4.3. DTPA extractable Ag in rhizosphere soil (A) and Ag accumulation in plant root (D) in 10 mg/kg AgNPs treatment over time. Ag accumulation in plant root (B), plant shoot (C), and the BAFs (E) and TFs (F) among different AgNPs concentrations after 7 and 63 d of exposure. The concentration of 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 at the same exposure duration ($p<0.05$). Data are expressed as mean ± SEM of triplicate samples. CK means the control treatment.

4.3.4 Response of Soil Microbial Communities to AgNPs in rhizosphere soil

The alterations of the bacterial community in the rhizosphere in response to AgNPs exposure were further investigated. The Shannon index, which reflects the species richness, was used to evaluate the Alpha diversities of the rhizosphere bacteria in control soil and in AgNPs-amended soil. For 7 d of incubation, the changes of Shannon indices among the control and the soil amended with different concentrations of AgNPs were irregular (**Figure 4.4**A). However, after increasing the incubation duration to 63 d, a clear tendency we observed was that the Shannon index decreased with the increasing exposure concentration of AgNPs.

The shifts in the rhizosphere bacterial community structure induced by AgNPs

treatments over time were further analysed by principal coordinate analysis (PCoA) (**Figure 4.4**B). After an incubation of 7 d, the bacterial communities in control and different AgNPs treatments clustered together with each other. However, when the incubation time 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 AgNPs amended soil 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 S4.3**. Proteobacteria (with the average relative abundance of 29%-34%), Actinobacteria (27%-31%), Bacteroidetes (9%-14%) and Acidobacteria (9%-11%) were the dominant bacterial phyla in both control soil and AgNPs-amended soil after 7d of incubation. By increasing the incubation period from 7 d 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 between the different treatments, were identified using ANCOM analysis (**Figure 4.4**C). In general, no featured taxa were found in the AgNPs treatments after 7d incubation. After incubation for 63d, a total of 16 featured taxa were observed, which greatly contributed to the observed differences between 50 mg/kg Ag amended soil and the control soil. From those featured taxa, 8 taxa (including the phyla of Acidobacteria and Gemmatimonadetes, the class of Holophagae, the order of Microtrichales, the family of Fimbriimonadaceae, Nitrososphaeraceae and Desulfarculaceae) were down-regulated and 8 taxa (including the order of Rhodospirillales, the family of vermiphilaceae, sphingobacteriaceae, Holosporaceae and Methylophilaceae, and the genus of *Pontibacter*, *Mesorhizobium* and *Sphingorhabdus*) were up-regulated 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.

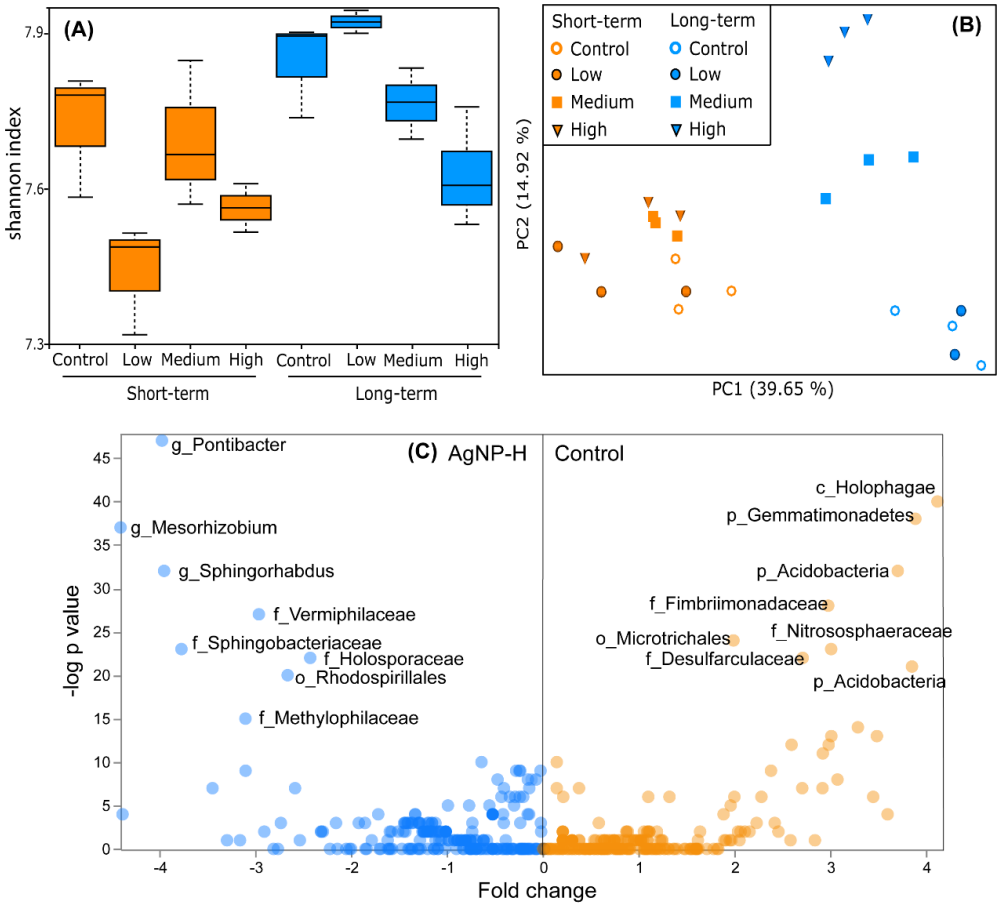

Figure 4.4. (A) Changes in the within community (α) diversity shown as the Shannon index; (B) Principal coordinate analysis (PCoA) of the bacterial community structure. (C) Featured taxa identified between the control and soil amended with 50 mg/kg AgNPs upon 63 d of incubation.

4.3.5 Correlation analysis of exposure conditions, soil pH, extractable Ag in soil, plant parameters, and soil bacteria communities

As shown from the map of Spearman's correlations (**Figure 4.5**), the soil pH was negatively correlated with exposure time but had no significant relationship with exposure concentration. The amount of DTPA-extracted Ag in both bulk and rhizosphere soil was significantly and 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 from root to shoot. No relationships were observed between exposure time or soil pH and extractable Ag in soil as well as Ag content in plants. On the contrary, the Shannon index of the soil bacterial community was positively correlated with exposure time but negatively correlated with soil pH and the BAFs of Ag in plants.

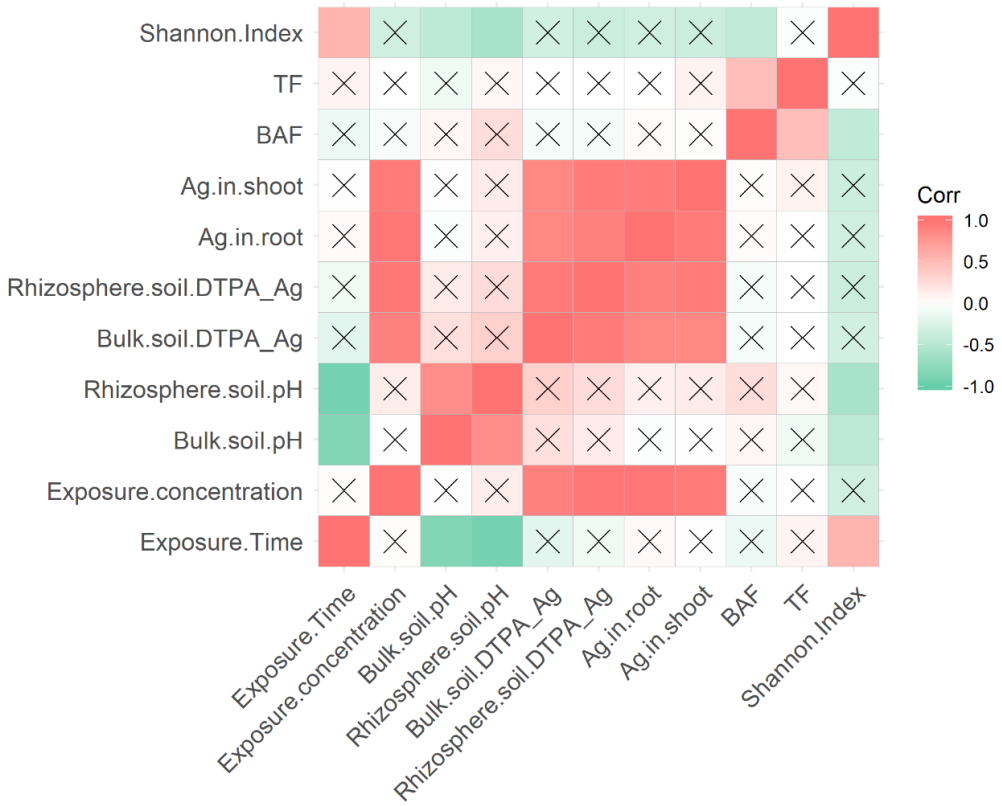

Figure 4.5. Spearman correlation map between the tested parameters including exposure conditions, soil pH, extractable Ag in soil, plant related parameters and Shannon index of soil bacterial community. Correlations with $p > 0.05$ fill with ×. Significant negative correlations (corr < 0) are given in gradations of green. Positive correlations (corr > 0) are given in gradations of red.

4.4. Discussion

Overall this study enhanced the understanding of how the dynamic dissolution of AgNPs in soil affects their bioavailability in the rhizosphere-lettuce interface and of long-term impacts of AgNPs on 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 4.1**A). This statistically significant increase is in line with the results of previous studies observing an increase of soil pH after amending soil with metallic nanoparticles[138,246]. The alterations of the soil metabolite profiles as well as the abundance and composition of root exudates can change soil pH[245,246]. For example, Zhang et al.[246] suggested that the increase of 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[248] as it can consume the H^+ in the system or release OH^- into soil following the stoichiometry below:

$2\ Ag(s) + 1/2\ O_2 + 2H^+ \rightarrow 2\ Ag^+ + 2\ H_2O$[256]

or:

Ag_2O (oxide layer formed on the surface of AgNPs) + $H_2O \rightarrow 2\ Ag^+ + 2\ OH^-$ [231].

However, the soil pH decreased after increasing the incubation time to 63d and no significant differences were observed between treatments after 63d of incubation. This pattern was similar to the findings of Das et al.[257], who also reported the soil pH increased at the initial exposure period but decreased after long-term exposure. The findings suggest that the aging of AgNPs in soil neutralized the pH changes between treatments.

The dissolution of metallic nanoparticles is known to be the dominant process governing the availability of metals derived from metallic nanoparticles[248]. 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 the previous studies[49,258], which also revealed the very low lability/release of Ag from AgNPs amended soil. Interestingly, a gradual increase of

DTPA extractable Ag in rhizosphere soil was observed during the cultivation period from 3 to 15 d (**Figure 4.3**A), indicating the gradual dissolution of AgNPs in soil. However, the extractable Ag in rhizosphere soil decreased as the cultivation period increased from 15 to 63 d. Also, the Ag concentration extracted from the AgNPs amended soil after 63 d of cultivation was slightly lower than that after 3 d and 7 d of cultivation. There are several possible explanations for this observation of declining Ag concentrations. Firstly, the dissolution of AgNPs might have become slower after 15 d of cultivation, or the AgNPs dissolution reached saturation over a longer exposure duration. There is a plethora of information revealing the two-phase dissolution behaviour of AgNPs, containing a short but rapid initial release phase and a longer but slower second release phase[259,260]. Secondly, 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 4.3**A and 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 soil solid matrix, as well as the transformation of AgNPs in soil[24]. After long-term exposure in soil, AgNPs have a large potential of being transformed to silver sulphide or other sulphur-bound Ag forms[49,261], 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 two weeks and to six months, and evidenced S groups-bound Ag was the predominant form after the soil was amended with soluble Ag[262], so-called aging processes. The changes of extractable concentration of Ag over time suggest that the single or fixed exposure duration cannot capture the actual dissolution and accumulation process of AgNPs in soil[245], 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 4.2**D), suggesting that the effect of lettuce on the dissolution of AgNPs was limited. The $CaCl_2$-extractable amount of Ag followed the order of unplanted soil > bulk soil > rhizosphere soil (**Figure 4.2**A). 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.[245] also suggested that DTPA-extraction is a better indication for metallic nanoparticle dissolution in soil while $CaCl_2$ extraction provides a more accurate prediction of the uptake of nanoparticles by plants. The observed Ag in plant shoot tissues shows the translocation of Ag from root to shoot. In addition, the Ag accumulation and translocation were positively correlated with the extractable amount of Ag in soil (**Figure 4.5**) and the trend of Ag accumulation in the plants over time was similar to the dissolution of AgNP in soil (**Figure 4.3**). 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 AgNP in short-term exposure (7d) regardless of exposure concentration. However, after long-term exposure (63 d), the Shannon index decreased, and the bacterial communities separated from control with increasing exposure concentration. This suggests that the effects of AgNPs concentration on the diversity and composition of the rhizosphere bacterial community varied over time. Moreover, 8 upregulated bacterial taxa and 8 downregulated featured taxa were observed in the treatment of 50 mg/kg AgNPs after 63 d of exposure, which contributed the most in inducing the differences between AgNPs treatment and the control. Previous research stated that the soil microbiome can shift its composition by increasing the Ag-tolerant taxa in response to AgNPs stress[137,263]. Similarly, the abundance of Ag resistant and sensitive genus *Mesorhizobium*[264] 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[264,265], and *Sphingorhabdus* and *Sphingobacteriaceae* that are related with the degradation of a variety of recalcitrant organic compounds[247,246,266]. In addition, *Pontibacter* (strongly associated with the N fixation gene nifH)[146], *Mesorhizobium*[261] and *Rhodospirillales* (containing free-living N_2-fixing bacteria)[247,263] were also promoted, indicating that long-term exposure of AgNPs stimulate the bacterial taxa related to nitrogen cycling. Besides these upregulated bacterial taxa, several bacteria were significantly inhibited upon long-term exposure to high concentration of AgNPs such as Acidobacteria and

Desulfarculaceae[246,267,268]. These bacteria are involved in carbon usage, sulphur reduction, and iron reduction. The alterations of the identified featured taxa highlight the potential disruption of agricultural systems because of AgNPs exposure by affecting the functional bacterial groups associated with nutrient acquisition, stress tolerance and biogeochemical elements cycling (such as C, N and S). Moreover, the changes of the diversity and structure of the rhizosphere soil bacterial community over time also emphasize the importance of investigating the dynamic impacts of nanoparticles on the rhizosphere bacterial communities.

4.5. Conclusions

Overall, the presence of the lettuce played a limited role in affecting AgNPs dissolution in soil as the extractability of Ag in unplanted and planted soil was similar at 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 AgNPs 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 concentration of AgNPs. These results highlight the importance of taking in consideration time-resolved dynamics of the soil-plant system in response to nanoparticle exposure. The slow but continuing dissolution of AgNPs in soil can provide a sustaining antimicrobial effect against plant pathogens (phytopathogenic fungi, bacteria, and viruses). This implies that the 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.

4.6 Supplementary Information

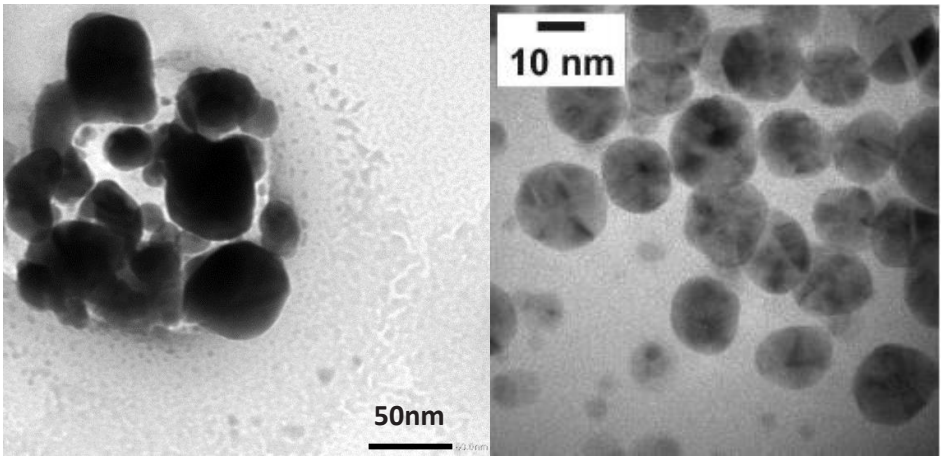

Figure S4.1 TEM picture of AgNP

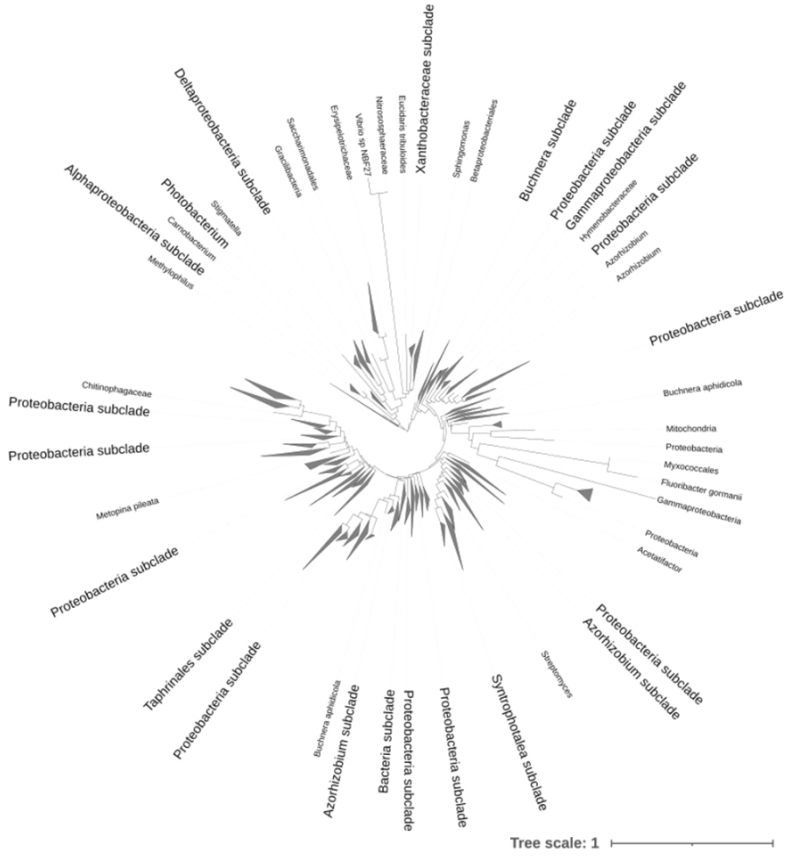

Figure S4.2 Phylogenetic tree of the rhizosphere bacterial community generated using align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin.

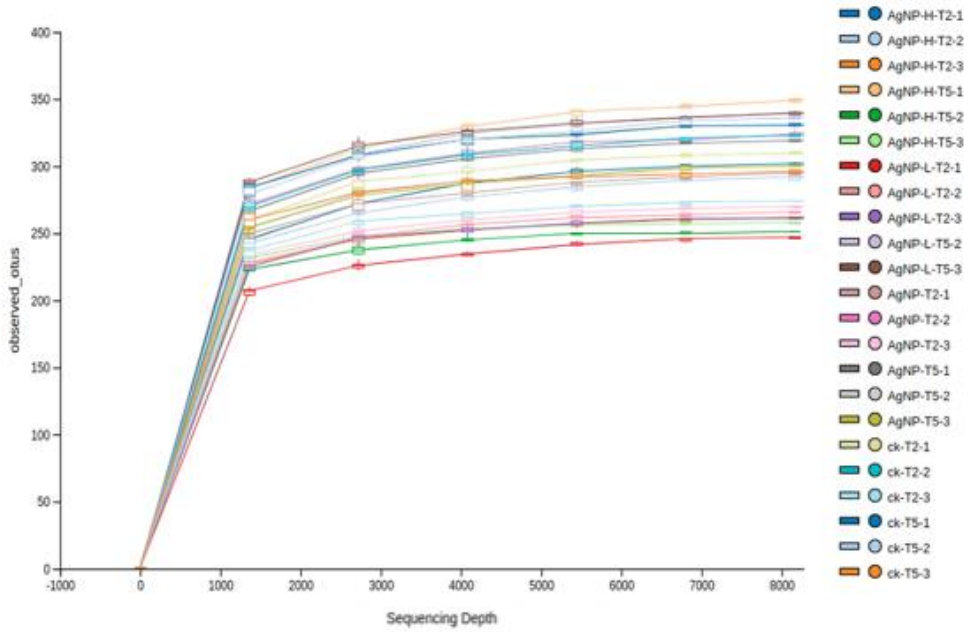

Figure S4.3 Rarefaction curves of rhizosphere bacterial communities in the soil samples of control and different AgNPs treatments.

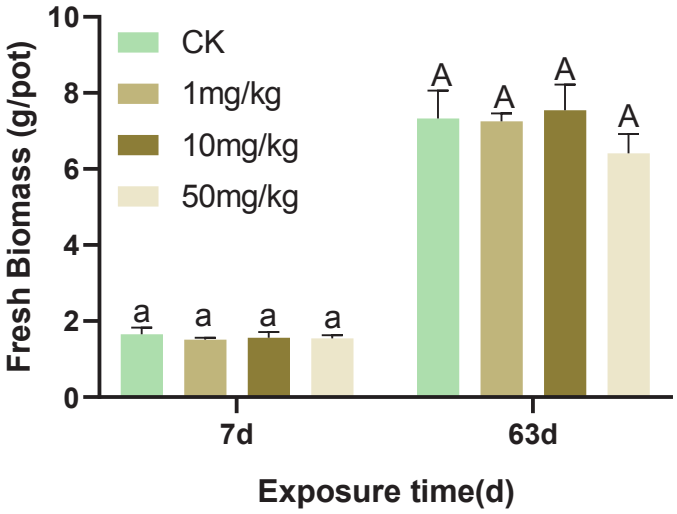

Figure S4.4 The biomass of lettuce exposed to different concentrations of AgNPs upon 7d or 63 cultivation.

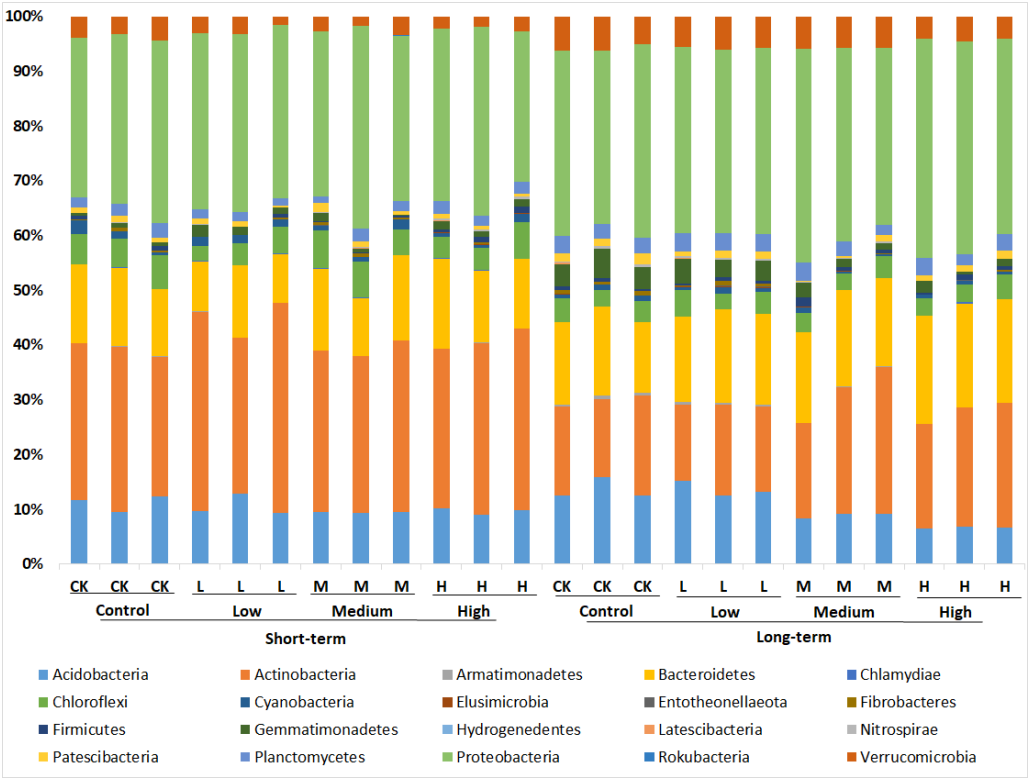

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Figure S4.5 The composition of the rhizosphere soil bacterial communities at the phylum/class levels.

Table S4.1. Hydrodynamic Diameter and Zeta-Potential of AgNPs Suspensions in 1/4 Hoagland solution

Nominal concentration	Hydrodynamic diameter (nm)			Zeta-potential (mV)		
	1 h	24 h	48 h	1 h	24 h	48 h
1 mg/L	246±26	692 ±64	1190±262	-16±0.4	-15.1±0.6	-15.3±0.5
10 mg/L	43±1	62±23	71.1±27	-15.2±0.5	-14.8±0.4	-12.1±0.4
50 mg/L	35±7	35±2	37±1	-12.0±0.7	-10.3±0.3	-9.5±0.5

Physicochemical properties of the soil used in this study[269]

The physicochemical properties of the soil used in this study are reported in **Table S4.2**.

• The pH was determined according to the method reported by Slattery et al. (1999)[270]. The soil: water and soil: KCl ratio was 1:2.5 for both measurements.

• Organic carbon was analysed according to the method reported by Walkley and Black (1934)[271].

• The cation exchange capacity (CEC) and exchangeable cation content were determined according to the method reported by Hendershot and Duquette (1986)[272]. Al, Ca, K, Mg and Na were extracted with 0.1 M $BaCl_2$, and the concentrations were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (PerkinElmer Optima 4300 DV, PerkinElmer, Waltham, MA, USA).

• The total metal contents were extracted with a mixture of HNO_3 and HCl (1:3 v/v) in Teflon reactors under 10 bar, 180 °C and 35 min as operational conditions of the microwave oven. The concentration in the extracts was determined by ICP-OES as above.

Table S4.2. Soil characteristics (standard error)

	Units	Soil
pH_{H2O}	-	8.35 (0.04)
pH_{KCl}	-	7.43 (0.04)
Organic C	%	2.16 (0.09)
CEC	$cmol^{(+)}\ kg^{-1}$	0.386 (0.009)
Na^{+}		0.054 (0.001)
K^{+}		0.032 (0.001)
Ca^{2+}		0.062 (0.001)
Mg^{2+}		0.102 (0.002)
Al^{3+}		0.137 (0.003)
Element		**Total concentration***
Ag	$mg\ kg^{-1}$	udl
As		udl [76]
Cd		udl [13]
Co		udl [190]
Cr		1.41 (0.24) [180 (Cr^{3+}) – 78 (Cr^{6+})]
Cu		2.24 (0.03) [190]
Fe		8284 (435) [niv]
Mn		172.91 (5.84) [niv]
Ni		30.53 (10.61) [niv]
Pb		udl [530]
Ti		357.7 (23.6) [niv]
Zn		8.97 (1.48) [720]

CEC: Cation Exchange Capacity, udl: under detection limit. []: Intervention values for soil remediation in Netherlands (niv: no intervention value). (1.VROM - Ministry of Housing, Spatial Planning and the Environment (2013) Circular on target values and intervention values for soil remediation. The Netherlands).

Table S4.3. Experimental design

Exposure time	Treatment	plant	Concentration ($mg\ L^{-1}$)
0d	AgNPs1	planted	1
	AgNPs10	planted	10
	AgNPs10	unplanted	10
	AgNPs50	planted	50
7d	AgNPs0 (ck)	planted	0
	AgNPs1	planted	1
	AgNPs10	planted	10
	AgNPs10	unplanted	10
	AgNPs50	planted	50
63d	AgNPs0(ck)	planted	0
	AgNPs1	planted	1
	AgNPs10	planted	10
	AgNPs10	unplanted	10
	AgNPs50	planted	50
3d	AgNPs10	Planted	10
15d	AgNPs10	Planted	10
28d	AgNPs10	Planted	10