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Environmental Significance Statement

It is still unclear how SRB affects the bioavailability and phyto-uptake of NPs. In this study, we found that although the occurrence of SRB can reduce the bioaccumulation of Ag ions, they can significantly enhance the uptake of Ag^0-NPs in a wetland plant, by transforming Ag^0-NPs into secondary Ag sulfide-NPs. The secondary biogenic NPs were in the size range of less than 10 nm formed by a dissolution-diffusion-reprecipitation process, showing more significant phyto-bioaccumulation relative to their initial pristine Ag^0-NPs. The present study is the first to elucidate the enhanced phyto-uptake of Ag-NPs in the presence of SRB.

Sulfate-reducing bacteria (SRB) can enhance the uptake of silver-containing nanoparticles in a wetland plant

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Abstract

The presence of sulfate-reducing bacteria (SRB) can reduce the bioavailability of toxic metal ions (e.g., Ag^+) to plants via mediating the formation of metal sulfide precipitates; it remains largely elusive if SRB can also affect the phytouptake of metal nanoparticles (e.g.,

Ag^0-NPs) however. In the current study, the bioavailability of Ag^0-NPs to a model wetland plant, *Scirpus triqueter*, was investigated in the presence/absence of SRB. Comparative experiments were conducted using 0.01-10 mg/L Ag0-NPs and silver ions. In addition to quantifying the total dissolved Ag concentrations, we analyzed the average sizes and particle concentrations of Ag-containing NPs (Ag-NPs) in plant tissues, including both roots and stems, after the designated treatments. The results show that although the presence of SRB can reduce the uptake of total Ag by 37% during the exposure of the plant to Ag ions, it can significantly enhance the uptake of total Ag during exposure of the plant to Ag^0-NPs, likely by transforming Ag^0-NPs into Ag-sulfide NPs with smaller particle sizes. Transmission electron microscopy data revealed that biogenic secondary Ag-sulfide particles smaller than 10 nm in size form in the vicinity of pristine Ag^0-NPs. These NPs are likely generated from the parent Ag^0-NPs via a dissolution-diffusion-sulfidation process. Moreover, the phytouptake of Ag^0-NPs of various sizes (i.e., 20, 40 and 80 nm) in the presence/absence of SRB also confirmed a size dependent pattern, with more silver identified in the plants in exposure to smaller Ag-NPs. The combined results suggest that the enhanced bioavailability of Ag-NPs to *Scirpus triqueter* in the presence of SRB is mainly attributed to the formation of secondary biogenic NPs with minute size. This result points to the importance of the complex, coupled interactions between aqueous solutions, bacteria, plants, and labile NPs.

Keywords: Sulfate-reducing bacteria (SRB); silver nanoparticles(Ag-NPs); availability; *Scirpus triqueter*

1. Introduction

Silver nanoparticles (NPs) are of particular commercial interest due to their unique optical and antibiotic properties[1, 2] and are widely used as antibiotics in many nanotechnology-enabled products, such as food packaging, clothes and medical devices.[3, 4] Of the 1814 products containing nanomaterials listed in the United States nanotechnology consumer products inventory in 2015, 435 products (accounting for 24% of the entire inventory) were obtained using Ag-NPs.[5] As a result, particular focus has been placed on the environmental and health-related risks caused by Ag-NPs. Once released into the environment, nearly all NPs undergo some series of

environmental modifications, such as homo/heteroaggregations, surface coatings, and structural/compositional transformations. The transformation of Ag^0-NPs under different environmental conditions, including different types of coatings and various sizes, has been studied, and oxidative dissolution and sulfidation are considered the most common transformations.[6-8] Sulfidation as a function of particle size[9] and sulfur-to-Ag^0-NP ratio[10] is considered the dominant transformation process of Ag^0-NPs under anaerobic conditions. For example, Ag_2S can account for more than 90% of the silver in wastewater treatment plant (WWTP) sludge, and many of these silver particles are nano-sized.[7, 11] The formation of Ag_2S-NPs, which have a notably lower solubility than Ag^0-NPs, has been considered an antidote to the toxicity of Ag^0-NPs toward organisms because the toxicity of silver is dominated by the release of Ag ions.[12] However, several recent studies have revealed that Ag_2S-NPs are available for soil microbes[13] and aquatic plants[14] and thus pose potentially toxic effects over the long term. It is important to note that Ag_2S-NPs could also be formed in the environment through biological processes, for example, in the presence of sulfate-reducing bacteria (SRB). SRB, which are ubiquitous in aquatic environments, particularly sediments, are known to convert metal ions into sulfide NPs via the reduction of sulfate.[15] Traditionally, it is believed that SRB can "fix" toxic metals and reduce the availability of these metal ions for plants,[16] whereas nanosized metal sulfide can be formed through dissimilatory bacterial sulfate reduction and is potentially available for plants due to its small size.[14, 17, 18] However, most previous studies on the transformation and availability of Ag-NPs for plants have focused on abiotic chemical transformation, and very few studies have addressed the impact of microbes, particularly SRB, on the availability of Ag-NPs for plants.

Therefore, the present study aimed to study the transformation of Ag^0-NPs in the presence of SRB and to test the availability of the resulting NPs for plants. For biological uptake in our experiments, we chose to study the pioneer species in many wetland areas, *Scirpus triqueter*. This plant has been studied for decades due to its ecological functions and its use in bioremediation. The abundance of *Scirpus triqueter* in wetlands provides an extensive habitat for shorebirds[19] and also leads to a high rate of autotrophic and heterotrophic processes through interactions with other organisms.[20] Specifically, a recent study revealed that SRB were concentrated in *Scirpus triqueter*

rhizosphere sediments and associated with metal sulfides,[16] and the minute size of metal sulfide NPs suggests that these biogenic NPs might be available for plants.[16] Nevertheless, the Ag concentrations in our previous studies of *Scirpus triqueter* were under the detection limit of ICP-MS. Therefore, this study provides an excellent first opportunity to trace the behavior and phytoaccumulation of Ag-NPs/SRB products in this important plant species.

In this study, we studied the uptake of Ag^0-NPs by *Scirpus triqueter* roots and stems in the presence/absence of SRB during a 1-week period. The specific objectives of this study were to (1) compare the total Ag concentrations in plant tissues after exposure to Ag^+ and Ag^0-NP in the presence and absence of SRB, (2) determine the differences in the particle sizes and concentrations of Ag-NPs taken up by plant tissues during the various treatments through SP-ICP-MS analysis, and (3) identify and characterize the Ag-NPs and further understand the impact of SRB on the transformation and phytoaccumulation of Ag^0-NPs using transmission electron microscopy techniques.

2. Materials and methods

Silver ions and silver NPs

Silver nitrate ($AgNO_3$) was obtained from Sigma-Aldrich (Lot # BCBR2756V). Different dilutions of $AgNO_3$ were prepared in Milli-Q water (18 MΩ•cm, Millipore). Polyvinylpyrrolidone (PVP)-coated Ag^0-NP standards were purchased from nanoComposix (San Diego, CA, USA), and the mass fractions of silver and PVP were >95% and <5%, respectively.

SRB isolation and culture

A sulfate-reducing *Enterobacter sp.* was isolated from *Scirpus triqueter* rhizosphere sediments collected at the Yangtze Estuary, China. This type of SRB is widely found in aquatic environments and is commonly used in the bioremediation of heavy metal-contaminated soil and wastewater.[21] The SRB was enriched and cultivated according to a previously published method[22] with minor modifications. The detailed description of the methods used for isolation and identification of the bacteria are provided in the Supporting Information (SI). A pure culture of this identified bacterium was conducted in LB medium at 37 ºC under anaerobic conditions in the presence of introduced nitrogen gas, and the cultured bacteria were used in the subsequent exposure experiments.

Experimental plants

Scirpus triqueter samples were collected at the Yangtze Estuarine wetland in April 2017. Specifically, whole plants were collected using sterile shovels and placed into plastic bags on ice. Immediately after being transported to the laboratory, the plants were rinsed with sterile Milli-Q water and precultured in 1.63 g/L Hoagland Modified Basal Salt Mixture (Phyto Technology Laboratories, Shawnee Mission, KS, USA) containing sulfate (approximately 180 mg/L) for 3 days prior to further experiments.

Toxicity of silver ions and NPs to SRB in Hoagland solution

The toxicity of Ag ions and 20-nm Ag^0-NPs toward SRB was investigated in Hoagland solution prior to the exposure experiments. The SRB were cultured in Hoagland solutions with different concentrations of silver ions (0, 0.01, 0.1, 1 and 10 mg/L) and 20-nm Ag^0-NPs (0, 0.01, 0.1, 1 and 10 mg/L) under culture conditions that were completely consistent with the subsequent plant exposure experiments. Suspension samples were collected at 0, 2, 4, 8, 12 and 24 h, and the culturability percentage of SRB, i.e., SRB colony numbers of different treatment groups to those of the control (0 h), at every time point was measured by plate counting after dilution with sterilized Milli-Q water.

***Scirpus triqueter* culture and treatment**

Precultured *Scirpus triqueter* specimens of similar sizes were transferred to 100-mL conical flasks containing 50 mL of Hoagland solution (1.63 g/L), SRB and different concentrations of Ag ions or 20-nm Ag^0-NPs. In each conical flask, 15 plants were cultured with their roots in the culture solution. The same treatments were also performed in the absence of SRB. All the sample groups were then incubated in an incubator with a 16-h light/8-h dark cycle to allow the plants to grow. The temperature was maintained at 20 ± 1 ℃. To provide adequate nutrition for *Scirpus triqueter* growth and ensure the dispersion of SRB, the culture solutions with constant concentrations of silver species and SRB, were replaced every day. The treatment groups included the following: (1) control (Hoagland solution only); 0.01, 0.1, 1 and 10 mg/L Ag ions in the presence (2)/absence (3) of SRB; and 0.01, 0.1, 1 and 10 mg/L 20-nm Ag^0-NPs in the presence (4)/absence (5) of SRB. For each treatment, plants were cultured in three conical flasks as replicates, and from each conical flask, three plants were randomly collected after 1, 3, 5 and 7

days of exposure. The culture solutions after 1 day of exposure were also collected for ICP-MS and SP-ICP-MS analysis. The collected plant samples, including stems and roots, were thoroughly cleaned with Milli-Q water using an ultrasonic cleaner immediately to remove the adsorbed Ag species before further analysis.

Measurements of total silver concentrations

To determine the total Ag concentrations (mg/kg) in *Scirpus triqueter*, all the samples were digested according to the method described by Wang et al.[23] with minor modification. In brief, whole freeze-dried plants were separated into stem and root tissues and ground using a tissue grinder (SCIENTZ-48). For the root tissues, both the amount of silver attached to the exterior of the roots and the amount of internalized silver were measured. After weighing, the tissue powder was digested with concentrated nitric acid overnight at a temperature over 70 ºC, and then 30% hydrogen peroxide was added into the sample after cooling and the samples were digested at a temperature over 70 ºC for at least 2 h. The remaining solution was diluted with Milli-Q water, filtered with a 0.22-μm sterile syringe filter and analyzed by ICP-MS(PerkinElmer, NexION 350D, USA). The dissolved Ag in Hoagland solution was measured after centrifugation at 14,000 r/min for 10 min according to the method described by Su et al.[24] Moreover, a silver standard sample was spiked into the homogenized plant sample as a quality control in triplicate for the acid digestion and ICP-MS analysis, and the recovery of the total Ag concentration was 101 ± 2 %.

SP-ICP-MS analysis

Macerozyme R-10 is a multicomponent enzyme mixture containing cellulose, hemicellulose and pectinase, and its enzymatic activity is greater than 3000 units/g. Therefore, this enzyme mixture can be used to digest *Scirpus triqueter* tissues and release NPs. A previous study demonstrated that the application of Macerozyme R-10 causes no measurable effect on the size distribution of Ag-NPs, as determined by SP-ICP-MS.[25] Therefore, we selected this enzyme for the extraction of Ag-NPs from *Scirpus triqueter*. To determine the size and concentration (particles/g) of Ag-NPs in plants, the samples were enzymatically digested as described by Dan et al.[26] In brief, whole *Scirpus triqueter* plants were separated into stem and root tissues, and these tissues were ground using a tissue grinder (SCIENTZ-48) and homogenized in 8 mL of 2 mM citrate buffer with a pH of approximately 6, in accordance with the manufacturer's

recommendations. After homogenization, the samples were treated with 2 mL of enzyme solution (prepared by the addition of 1 g of enzyme powder in 20 mL of Milli-Q water) and shaken at 37 ºC for 24 h. The samples were settled and diluted using Milli-Q water prior to SP-ICP-MS analysis. The Ag-NPs in Hoagland solution were measured directly after dilution with Milli-Q water. A NexION 350D ICP-MS with a Syngistix Nano Application module from PerkinElmer was used for the SP-ICP-MS analysis, and 30-, 60- and 100-nm Au-NP standards were used for particle calibration to measure the particle size of Ag-NPs in the samples. The instrumental conditions were optimized for maximum sensitivity for ^{107}Ag. The dwell time and sampling time were set to 100 μs and 60 s, respectively. The transport efficiency of the particle concentration was 3.5%. Particle size detection limits were determined to be 10-12 nm for Ag-NPs (including Ag^0-NPs and Ag_2S-NPs). Standard Ag^0-NP and Ag_2S-NP samples with known particle sizes and concentrations were spiked into the homogenized samples separately and independently for quality control purposes when performing enzymatic digestion and the SP-ICP-MS analysis. The measured particle sizes matched well with the spiked NP sizes, and the recoveries of Ag^0-NP and Ag_2S-NP concentrations were 84 ± 0.2% and 83 ± 1%, respectively.

Electron microscopy and Zetasizer characterization

The morphology, size, elemental composition and crystal structures of Ag-NPs were investigated using a transmission electron microscope (TEM, JEOL 2100 TEM, Tokyo, Japan) coupled with an energy dispersive X-ray spectrometer system (EDS) and selected-area electron diffraction (SAED). The 20-nm Ag^0-NP standard solution and culture solutions after 24 h of exposure were homogenized using an ultrasonic instrument. Approximately 7 μL of solution was dropped onto a TEM grid (CF300-Cu, Electron Microscopy Science), and the TEM grid was dried in a desiccator prior to TEM analysis. The plant root samples were freeze-dried, homogenized in Milli-Q water and attached to the copper grid for TEM investigation. The hydrodynamic diameter and Zeta-potential of 20-nm Ag-NPs in Hoagland solution were measured using a Zetasizer Nano (Malvern, UK).

Statistical analysis

The statistical analyses were conducted using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). The differences between different treatments were assessed through a normality test

followed by one-way analysis of variance (ANOVA) and then a post hoc multiple comparison test (Tukey's honestly significant difference test, HSD). For the data not normally distributed, the non-parametric ANOVA analyses (Kruskal-Wallis test) were performed. $P < 0.05$ was considered to indicate a significant difference.

3. Results and Discussion

3.1. Characterization of Ag^0-NPs and their toxicity to SRB

Ag^0-NPs with particle sizes of 20 nm were spherical, and the average TEM size was consistent with the expected sizes, as shown in Fig. S1A. Both EDS and SAED analyses indicate that 20-nm Ag^0-NPs are metallic Ag.[27] Detailed characterization data are provided in SI. Ag^0-NPs with a size of 20 nm dispersed in the culture solutions were also characterized using a Zetasizer, including the hydrodynamic diameters and the Zeta-potential. The average hydrodynamic sizes were approximately 46-56 nm in Hoagland solution without SRB, while these values increased to 64-75 nm in the presence of SRB (Table S2), which could be partially attributed to the aggregation of Ag-NPs caused by extracellular polymers produced by SRB [28] The average hydrodynamic diameter of 20-nm Ag^0-NPs determined in Hoagland solution is larger than the primary TEM size due to the aggregation of Ag^0-NPs in the solution. Moreover, the absolute Zeta-potential values of Ag^0-NPs in the culture solution were significantly higher in the presence of SRB than those without SRB. As shown in Table S2, taking the 10 mg/L 20-nm Ag^0-NP-treated group for example, the Zeta-potential values of particles were -9.17 mV for the group treated with SRB and -4.82 mV for the group treated without SRB, indicating that SRB can improve the stability of the NP solution.

To provide adequate nutrition for *Scirpus triqueter* growth and ensure the dispersion of SRB, the Hoagland solution and SRB in the conical flask were replaced every day during silver exposure experiments. Therefore, the dose- and time-dependent culturability percentages of SRB to the presence of silver ions and 20-nm Ag^0-NPs were measured for a duration of 24 h in Hoagland solution (Fig. S2). As expected, Ag ions (especially) and 20-nm Ag^0-NPs had strong lethal effects on SRB. Almost all of the SRB could not survive after 24 h in culture except for the SRB exposed to the 0.01 mg/L 20-nm Ag^0-NPs. However, at 12 h, the culturability percentages of SRB were generally over 100% in culture after 12 h, except for the SRB exposed to 1 and 10

mg/L Ag^+. Notably, at relatively low concentrations of Ag^+ (0.01, 0.1 mg/L) and Ag^0-NPs (0.01, 0.1 and 1 mg/L), the culturability percentages of SRB showed a trend of increasing first and then decreasing. At a concentration of 10 mg/L 20-nm Ag^0-NPs, the culturability percentage of SRB decreased for the first 4 h, increased in the following 8 h, and then decreased rapidly in the last 12 h. Moreover, SRB showed a trend of growth recovery after the growth inhibition generally, and the time points of growth recovery were later for the SRB exposed to high concentrations of silver than those exposed to low concentrations of silver. This could be attributed to the fact that SRB need longer time to adapt to the stress caused by the higher concentrations of silver. Overall, in the presence of Ag^+ and Ag^0-NPs, SRB still showed the ability to survive for several hours, which was enough time for their interaction with Ag^+, Ag^0-NPs and plants and the generation of silver sulfide, because the SRB-sulfate reaction is a continuous process starting with SRB being cultured in a sulfate-containing environment.[29]

3.2 Impact of SRB on the phytoaccumulation of total silver

Phytoaccumulation of total Ag through exposure to Ag^+ in the presence/absence of SRB

The total Ag concentration (mg/kg) accumulated by *Scirpus triqueter* tissues exposed to Ag^+ with and without SRB were measured at days 1, 3, 5 and 7, respectively, and the results are shown in Fig. 1. Clearly, the concentration of total Ag in *Scirpus triqueter* tissues, especially for roots, generally increased with increasing exposure concentration of Ag^+ and exposure time. Compared with those in the plant tissue collected at day 1, the concentrations of total Ag increased by several-fold in *Scirpus triqueter* roots and stems at day 7. The uptake of Ag ions by plants is a rapid and continuous process. This is consistent with the previous results[30] that silver (Ag^+) could be taken up quickly by the plant roots and transported to the other tissue in a time-dependent manner after a short period of exposure.

Significantly, in the presence of SRB, the accumulation of total Ag in *Scirpus triqueter* was reduced at different exposure concentrations and times compared with those without SRB, as shown in Fig. 1. Taking the 10 mg/L Ag^+ treated *Scirpus triqueter* collected on the 5th day for example, the concentrations of total Ag in plant tissues were approximately 545 mg/kg for roots and 11 mg/kg for stems in the presence of SRB, while the corresponding concentrations were 1248 and 26.6 mg/kg for roots and stems, respectively, without SRB. To better understand

whether SRB can enhance/reduce the uptake of Ag by plants, total Ag concentrations in the whole plants treated with SRB (+) were divided by those without SRB (-) for all treatment groups (i.e., $R_{+/-}$). If $R_{+/-} > 100\%$, it suggests that SRB can enhance the uptake of Ag by plants. Conversely, ($R_{+/-} < 100\%$), SRB can reduce the uptake of Ag by plants. As shown in Table S3, when plants were exposed to Ag^+, the $R_{+/-}$ values ranged from 18.97% - 98.10% (62.71% averagely), suggesting that the phytoaccumulation of total Ag by the whole plants exposed to Ag^+ are averagely reduced by 37.29%, under the effect of SRB. Moreover, we investigated the dissolved Ag in Hoagland solutions both treated with and without SRB (Fig. S3A). The results show that in general, the dissolved Ag concentrations in the culture solution with SRB were significantly lower than the group treated without SRB ($P < 0.05$). We thus conclude that the presence of SRB can reduce the concentrations of dissolved Ag in culture solution and consequently inhibit the uptake of silver by plant roots. These results support the traditional conclusion that SRB can "fix" metal and effectively reduce the availability of metal for plants when the metal is in ionic form.[16, 31] Previous studies have also found that organic root exudates released by plants can stimulate the metabolic activity of SRB, thus promoting sulfate reduction into sulfide by SRB under anaerobic conditions.[32] The enhanced SRB activity in Hoagland solutions can promote the sulfidation of Ag ions into Ag sulfide particles, thereby reducing the available Ag^+ for plants significantly. Additionally, studies have found that microorganisms have an intrinsic ability to adsorb metal ions on the cell surface and sequester them in biologically inactive forms in response to environmental stress via detoxification mechanism, e.g., the overproduction of metal-binding proteins.[33-35] Therefore, the presence of SRB cells may also provide extra sorption sites for the adsorption of silver ions, resulting in the decrease of free Ag^+ concentrations.

The ratio of total Ag concentrations in stem versus those in root (translocation ratio, i.e., TR) was introduced to evaluate the translocation of Ag in plants. It is noteworthy that for the Ag^+ treatment group without SRB, TRs ranged from 0.07 to 0.29, suggesting the strong accumulation of Ag associated with roots and low translocation of Ag from roots to stems. Our results are consistent with a previous conclusion that the majority of Ag accumulated in the root and only a small portion of Ag transferred from the root to other tissues.[36] It is likely that there is limited apoplastic translocation for the upward translocation of silver to other tissue regions.[37]

Furthermore, with the participation of SRB, the translocation ratios of Ag, ranging from 0.02 to 0.13, were significantly lower than those without SRB ($P < 0.05$). Studies have found that highly soluble metal species showed greater translocation in plants than those with less solubilites.[14, 38] In this study, Ag_2S-NPs with low solubility could be formed in the Ag^+ treatment group with the presence of SRB.[17, 39] These Ag_2S-NPs could aggregate and be not easily transported through the plant tissue, from roots to stems. Moreover, a previous study also confirmed that the translocation ratios of Ag ions were higher than those of Ag-NPs in wheats.[40]

Phytoaccumulation of total Ag through exposure to Ag^0-NPs in the presence/absence of SRB

The uptake of 20-nm Ag^0-NPs by *Scirpus triqueter* was studied by analyzing the total Ag concentrations in plants collected on days 1, 3, 5 and 7. The results are shown in Fig. 2. Similar to the treatment of Ag^+, the concentration of total Ag in plants also increased generally with increasing concentration of Ag^0-NPs and exposure time. However, silver concentrations in plants exposed to 20-nm Ag^0-NP were 1-2-fold lower than those in the groups treated with Ag ions, which suggests that Ag ions are more readily accessible to the plants than the NP forms of Ag.[14] Moreover, the translocation ratio when exposed to 20-nm Ag^0-NP was low, with TRs of 0.02 - 0.31 for the plants treated with SRB and 0.01 - 0.35 for the plants treated without SRB. The results suggest that the participation of SRB had no significant effect on the translocation ratio of silver in the form of NP.

Interestingly, we observed that compared with the treatment without SRB, the uptake of total Ag by *Scirpus triqueter* was enhanced by SRB at different exposure concentrations of 20-nm Ag^0-NPs with different exposure times, as shown in Fig. 2. This phenomenon is in contrast to the finding for the effect of SRB on the Ag ions. The concentration of total Ag in *Scirpus triqueter* tissues (primarily in/on root) treated with Ag^0-NPs in the presence of SRB were generally significantly higher than those treated only with Ag^0-NPs, without SRB. Taking the exposure concentration of 0.01 mg/L 20-nm Ag^0-NP as an example, the concentrations of total Ag in the root and stem tissue after 7 days of exposure were approximately 26 mg/kg and 4.29 mg/kg, respectively, under the effect of SRB, whereas these values were only 9.4 mg/kg for root and 3.28 mg/kg for stems in the corresponding group without SRB. However, in most treatment groups, the enhancement of SRB on the phytoaccumulation of total Ag in plant stems exposed to 20-nm

Ag^0-NPs was not significant. Thus, this result indicates that SRB can effectively enhance the uptake of Ag by plants, especially for roots, when the plants are exposed to 20-nm Ag^0-NPs. Moreover, as shown in Table S3, the $R_{+/-}$ values were in the range of 103.63% - 247.90% (155.84% averagely) for the 20-nm Ag^0-NP treatment groups, suggesting that the phytoaccumulation of total Ag by plants exposed to 20-nm Ag^0-NPs is enhanced by 55.84% on average under the effect of SRB.

Traditionally, a significant enhancement of metal uptake has been attributed to increases in dissolved metal ion concentrations. However, by analyzing dissolved Ag concentrations in the culture solution, we found that the dissolved Ag concentrations were lower in culture solution containing both Ag^0-NPs and SRB than those containing only Ag^0-NPs, without SRB (Fig. S3B). This result indicates that the enhanced uptake of Ag by plants when exposed to Ag^0-NP in the presence of SRB is not attributed to the release of Ag ions directly. Possible reasons for this finding are discussed in the following sections.

3.3 Impact of SRB on the Phytoaccumulation of Ag-NPs

Contrary to the finding that SRB can reduce the uptake of total silver when exposed to Ag ions, they can significantly enhance the phytoaccumulation of total silver when exposed to 20-nm Ag^0-NPs. To elucidate the mechanism, *Scirpus triqueter* tissues, including both roots and stems, were enzyme digested and analyzed by SP-ICP-MS for the particle concentrations and average sizes of Ag-NPs. Although the efficiency of the enzymatic hydrolysis method (used to extract nanoparticles in plant tissue for SP-ICP-MS analysis) on different plants is discrepant,[26] it is reliable to use this method for the same type of plant samples to compare the differences of Ag-NPs at different treatments in this study. To ensure adequate detection concentrations, plant samples treated with 10 mg/L Ag ions and Ag^0-NPs, with and without SRB, were selected. In addition, SRB could survive for several hours under the pressure of 10 mg/L Ag ions and Ag^0-NPs. Although the culturability percentage of SRB decreased significantly in the first 4 h, SRB showed a rebound trend after 8 h for the 10 mg/L Ag^0-NP-treated group (Fig. S2). Studies have found that biotransformation processes of Ag^0-NPs could occur once particles interact with organisms, including SRB, in aquatic environments.[41] Therefore, in spite of the lethal pressure by silver, SRB could interact with silver and plants, making the dissimilatory sulfate reduction at 10 mg/L

concentrations of Ag^+/Ag^0-NPs.

It is worth noting that the average size of Ag-NPs based on the SP-ICP-MS technique is calculated based on two hypotheses: (1) all the particles are spherical; (2) known molecular formula, and known ratios of different chemical compositions and corresponding density of certain metal-NPs,[42] such as Ag-NPs in this case. A previous study found that SRB could convert metal ions, including Ag ions, into sulfide NPs via the reduction of sulfate.[15] Moreover, because many biomolecules in plants serve as reducing agents for the biosynthesis of NPs,[43, 44] Ag-sulfide NPs could also be formed in *Scirpus triqueter* when exposed to Ag^+ or Ag^0-NPs without SRB. This finding was confirmed by our TEM investigations of the plant roots, as shown in the following section. A recent study also found that both Ag_2S and Ag^0-NPs were formed in plants exposed hydroponically to either Ag^+ or Ag^0-NPs based on X-ray absorption spectroscopy (XAS) analysis, in the absence of SRB.[14] Furthermore, for the condition in the presence or absence of SRB, we are not able to determine the detailed composition ratios of different Ag-NPs, including Ag^0-NPs and Ag_2S-NPs, for both Ag^+ and Ag^0-NPs treated groups. The average sizes of Ag-NPs in plants and the culture solutions were therefore calculated based on Ag^0 and Ag_2S, respectively. Additionally, the detection limit of the SP-ICP-MS technique for the average size of NPs is about 10 nm. However, Zetasizer and TEM (as described below) analysis showed that the majority of Ag-NPs in culture solutions and plants were aggregated as shown in Table S2 and Fig. S5. Therefore, it is reliable to use this technique to measure Ag-NPs, with (aggregation) sizes larger than 10 nm in culture solutions and plants, and to compare the differences of Ag-NPs at different treatments in this study.

SP-ICP-MS analysis of Ag-NPs through exposure to Ag^+ in the presence/absence of SRB

The results of SP-ICP-MS analysis for the plants exposed to Ag ions after 1, 3, 5 and 7 days of exposure are shown in Fig. 3A. The average sizes of these Ag-NPs in plants, including roots and stems, were generally smaller in Ag^+ treated groups with SRB than in groups without SRB. Moreover, the particle concentrations of Ag-NPs in plants treated without SRB were generally higher than those treated with SRB. As shown in Fig. 3A, taking the 3rd day samples for the example, if all the Ag-NPs were Ag^0-NPs, the average size of Ag-NPs in the plants exposed to Ag^+ with SRB were 23 nm for stem and 27 nm for root, while the average particle sizes were 26

nm for stem and 31 nm for root if all of the Ag-NPs were assumed to be Ag_2S-NPs. Because of the uncertain composition ratio of different Ag-NPs, including Ag^0 and Ag_2S, the average sizes of Ag-NPs in *Scirpus triqueter* treated with Ag^+ when SRB were presented were therefore in the range of 23-26 nm for stem and 27-31 nm for root, and the corresponding sizes in the plants treated without SRB were in the range of 27-31 nm and 42-48 nm for stem and root, respectively. In addition, the plants exposed to both silver ions and SRB contained Ag-NP concentrations (5.93×10^8 particles/g for stem and 1.73×10^9 particles/g for root) only about a sixth to a ninth of those exposed to silver ions without SRB (5.71×10^9 particles/g for stem and 1.16×10^{10} particles/g for root).

The results suggest that when the plants are exposed to Ag ions, SRB can reduce the uptake of silver into plants, not only in the total silver concentration but also in the Ag-NP concentrations. Large particle accounts of Ag-NPs were found in *Scirpus triqueter* tissues treated without SRB, suggesting that plants can synthetize Ag-NPs in their tissues via a natural reduction process after being exposed to silver ions.[37, 45] Many biomolecules in all parts of plants, such as flavonoids, saccharides, and proteins, play a dual role in biosynthesis of reducing and protective agents.[43] Moreover, when exposed up to 10 mg/L Ag^+ with/without SRB, the concentration of total Ag in the plants, especially for roots, increased with the exposure time as shown in Fig. 1D, while their corresponding Ag-NP concentrations did not show an increasing trend with time. This finding can probably be attributed to the aggregation of Ag-NPs associated with roots.

Additionally, Ag-NPs in Hoagland solution, after treating plants for 1 day, also showed a similar distribution pattern as those in plants (Fig. S4A). SRB-related NPs were generally smaller with less particle concentrations than those without SRB. This is consistent with the finding of Ag-NPs in plant tissues. Specifically, root exudates are probably also responsible for the formation of Ag-NPs in culture solution without SRB. Plant roots can release large amounts of organic matter, such as acetic acid, propionic acid, lactic acid and saccharides,[46] which could reduce Ag^+ and protect the stability of the Ag-NPs in solution.[43, 47] Additionally, the average sizes of Ag-NPs in Hoagland solution are generally comparable with those in plants. Therefore, in addition to phytosynthesis in the plant tissue, Ag-NPs might also be synthetized in culture solution under the influence of root exudates and in the presence of SRB and further taken up by *Scirpus*

triqueter.

SP-ICP-MS analysis of Ag-NPs through exposure to Ag^0-NPs in the presence/absence of SRB

The average size and particle concentrations of Ag-NPs in plants exposed to 10 mg/L 20-nm Ag^0-NPs in the presence/absence of SRB are shown in Fig. 3B. The particle concentrations in the plants showed an increasing trend with the exposure time. Importantly, with SRB in the culture solution, the average sizes of Ag-NPs in plants, including both roots and stems, were significantly smaller than those without SRB on the 1st and 3rd days. Although these differences were not significant on days 5 and 7, the average sizes of these Ag-NPs in both roots and stems were also smaller in the Ag^0-NPs treated groups with SRB than those without SRB. Moreover, the particle concentrations of Ag-NPs in plants treated with SRB were generally higher than those treated without SRB. Taking 3rd day treatments at 10 mg/L Ag^0-NPs in the presence and absence of SRB as an example, the average sizes of Ag-NPs in the plants exposed to 20-nm Ag^0-NPs in the presence of SRB were 17-19 nm for stems and 22-25 nm for roots, and the corresponding sizes in the plants treated without SRB were in the range of 26-29 nm and 27-32 nm for stem and root, respectively. In addition, Ag-NPs concentrations in the plants exposed to Ag^0-NPs without SRB (1.01×10^9 particles/g for stem and 1.35×10^{10} particles/g for root) were lower than those with SRB (1.27×10^{10} particles/g for stem and 1.38×10^{10} particles/g for root). Notably, significant differences were found for Ag-NP concentrations associated with roots between groups treated with and without SRB on days 5 and 7, but not for those on days 1 and 3. Ag-NPs could have been absorbed in the root, and/or they could also have been adsorbed onto the root surface, causing detection uncertainty, although ultrasonic cleaning was applied to wash the plants in this study. Because it is difficult to remove all the attached Ag^+/Ag-NPs from plant roots, as found by previous studies.[14, 40, 48]

Compared with the conditions without SRB, those with SRB showed relatively high particle concentrations of Ag-NPs with smaller sizes in *Scirpus triqueter* when exposed to Ag^0-NPs. As discussed before, the dissolved Ag concentrations were lower in culture solution containing both Ag^0-NPs and SRB than those containing only Ag^0-NPs, without SRB (Fig. S3B), suggesting that the enhanced uptake of silver by plants was not attributed to the dissolve Ag ions. Notably, Ag-NPs with smaller sizes were formed in culture solution with SRB than those formed without

SRB when exposed to Ag^0-NPs (Fig. S4B). Therefore, we conclude that secondary Ag sulfide NPs with smaller sizes relative to the pristine Ag^0-NPs could be formed by SRB, and these tiny NPs were more available and easily taken up by the plants.

3.4 Electron microscope analysis of Ag-NPs and the formation of secondary NPs

The findings of the SP-ICP-MS analysis suggest that SRB might promote the transformation of Ag^0-NPs into silver sulfide NPs with smaller particle sizes and further be taken up by plants. To test this hypothesis, TEM analysis was performed for Ag-NPs in culture solution treated with 10 mg/L 20-nm Ag^0-NPs in the presence and absence of SRB after 1 day of culture. As shown in Fig. 4, without the participation of SRB, no visible change was observed for Ag^0-NPs with a primary size of approximately 20 nm (Fig. 4A). When SRB were introduced, new NPs with sizes less than 10 nm were produced adjacent to the Ag^0-NPs (Fig. 4B, C and D). EDS analysis showed that sulfur and silver elements appeared in the secondary nanosized particles, indicating that the new nanoparticles could be biogenic Ag-sulfide NPs. The strong Cu signal according to the EDS analysis can be attributed to the Cu-based TEM grids, and the Si signal came from the Hoagland solutions. Moreover, Si can also be released into the culture solution through the plant roots.[49] According to the high resolution (HR)-TEM (Fig. 5), the newly generated secondary NPs were identified as acanthite (Ag_2S) with a *d*-spacing of 2.58 Å, corresponding to the (0 2 2) planes.[50] The original particles were identified with *d*-spacing of 2.35 Å corresponding to the (1 1 1) lattice planes for metallic silver.[51] A previous study, based on XAS and TEM analysis, found that most biogenic silver sulfides were poorly crystalline, which clearly differed from standard abiotic chemical synthesis of Ag_2S-NPs with fully crystalline.[52] In this study, although the secondary Ag sulfide NPs seems to be less crystallinity compared to their pristine Ag particles as shown in Fig. 4 and 5, we cannot prove that these Ag_2S-NPs are poorly crystalline. The less crystallinity of Ag_2S-NPs shown by TEM image could be attributed to the minute size of these biogenic NPs, and under TEM, it could be not right along the lattice fringe axis of the crystal structure. Notably, as described previously, the increasing hydrodynamic size of Ag-NPs in culture solution when SRB was added is attributed, at least in part, to the attachment of secondary NPs. Moreover, studies have found that Ag_2S-NPs were available for plants and could be absorbed through their direct uptake by plants, especially for the tiny Ag_2S-NPs (< 10 nm).[14, 53, 54] So compared with Ag^0-NPs,

Ag sulfide NPs detached from the original NPs could be more easily absorbed by the plants due to their smaller size. Therefore, SRB can enhance the uptake of Ag^0-NPs in plants by promoting the transformation of Ag^0-NPs to Ag sulfide NPs with smaller size. Additionally, a large number of Ag-NPs with various sizes and shapes associated with plant roots treated with Ag^+ or Ag^0-NPs with/without SRB were identified based on the TEM technique (details are shown in SI). Numerous Ag_2S-NPs were found to be associated with plant roots treated in the presence of SRB, as shown in Fig. S5.

In this study, the sulfate in the culture solution was reduced to sulfide (S^{2-}) due to the presence of SRB. The Ag^0-NPs could then undergo the following conversions: (1) Ag^0-NPs can be sulfurized directly, which could generate a core-shell structure of $Ag@Ag_2S$[14] and/or (2) Ag_2S-NPs can be precipitated by Ag^+ released from Ag^0-NPs.[6, 55] Here, we investigated the time-dependent concentrations of dissolved silver in culture solution (without plants) treated with Ag^0-NPs in the presence/absence of SRB. As shown in Fig. S6, in the absence of SRB, the concentrations of dissolved Ag^+ increased during the 24-h experimental period. In the presence of SRB, the dissolved Ag^+ concentrations in Hoagland solution generally increased slowly or did not increase. Moreover, as previously demonstrated in the TEM analysis, Ag sulfide NPs formed on or near the pristine Ag^0-NPs, and no core-shell structured $Ag@Ag_2S$ was found. Therefore, a dissolution, diffusion and reprecipitation process in the presence of S^{2-} from the SRB is most likely involved in the formation of the biogenic Ag sulfide NPs in the present study. Interestingly, the oxidative dissolution of the Ag^0-NPs could supply electrons needed by the SRB for sulfur reduction. Moreover, the presence of SRB accelerated and enhanced this dynamic behavior (dissolution, diffusion and reprecipitation) probably. In the present study, we found incidental Ag-NPs which were produced from engineered Ag^0-NPs with the response of bacteria, unintentionally. Our finding showed some intriguing similarities to the study conducted by Glover et al.,[6] who characterized the production of incidental silver NPs in the vicinity of the source Ag^0-NPs at three distinct stages: dissolution, diffusion and reduction.

3.5 Size-dependent Uptake of Ag^0-NPs by Plants

To elucidate the size-dependent uptake of Ag^0-NPs by plants, *Scirpus triqueter* samples were exposed to 0.1 mg/L Ag^0-NPs with average sizes of 20-, 40- and 80-nm in the

presence/absence of SRB. Information regarding the TEM characterization of 40- and 80-nm Ag^0-NPs is shown in SI (Fig. S1B and C). The total Ag concentrations, Ag-NP sizes and particle concentrations in the plants and culture solutions were measured according to the previously described method. Similar to the treatment with 20-nm Ag^0-NPs, the concentrations of total Ag in plants, particularly plant roots, after exposure to 0.1 mg/L 40- and 80-nm Ag^0-NPs in the presence of SRB, were generally significantly higher than those obtained after treatment with Ag^0-NPs in the absence of SRB (Fig. S7). Based on the $R_{+/-}$ values shown in Table S4, the phytoaccumulation of total Ag by the whole plants were on average increased by 42.24%, 41.27% and 37.01% after the 0.1 mg/L 20-, 40-, and 80-nm Ag^0-NP treatments, respectively, in the presence of SRB compared with the amounts found in the control groups without SRB. Moreover, the 0.1 mg/L 20-, 40- and 80-nm treatment groups with SRB generally resulted in the detection of more Ag-NPs with smaller sizes in the plants compared with those obtained in the absence of SRB, as shown in Fig. S8. In addition, the average sizes of Ag-NPs formed in culture solution with SRB were smaller than those formed in the absence of SRB. Taking the 0.1 mg/L 40-nm Ag^0-NP treatment group as an example, the average sizes of Ag-NPs in the culture solution without/with SRB were 39-45 and 31-35 nm, respectively, which is comparable to the average sizes in the plants treated with 0.1 mg/L 40-nm Ag^0-NPs. This finding suggests that secondary Ag-sulfide NPs with smaller sizes could be formed by SRB despite the size of the pristine Ag^0-NPs. We therefore conclude that SRB can enhance the uptake of Ag^0-NPs of different sizes by plants, largely by transforming Ag^0-NPs into Ag-sulfide NPs with smaller particle sizes, which show increased availability and are more easily taken up by plants.

Notably, the analysis of all the Ag^0-NP treatment groups with different sizes in the presence/absence of SRB could be ranked based on the total Ag concentrations associated with plant roots as 20-nm treatment group > 40-nm treatment group > 80-nm treatment group (Fig. S7), which indicated that plant roots exposed to Ag^0-NPs with smaller sizes can accumulate Ag more easily than plant roots exposed to larger-sized Ag^0-NPs. This result is consistent with the previous finding that cucumber seedlings treated with Ce-NPs with a smaller size exhibit a significantly higher total Ce concentration than those exposed to Ce-NPs with a larger size.[56] Moreover, a recent study have found that Ag-NPs with the size of 7.6 nm were more easily absorbed by

hydroponic rice (*Oryza sativa L.*) than Ag^{+}.[57] However, according to our study, for *Scirpus triqueter*, the availabilities of Ag^{0}-NPs with different sizes were all lower than that of Ag ions. This is could be resulted from the different experimental conditions (e.g., the plant species).[38, 40, 58] However, it is confirmable that the smaller Ag-NPs, especially for the Ag-NPs with size less than 10 nm, can be more easily absorbed by plants than the those with larger sizes. According to the total Ag concentrations associated with roots, we calculated the average total Ag uptake rate (mg/kg/day) for the plant roots after 7 days of exposure (Fig. S9), and the results show that the uptake rates of smaller-sized Ag^{0}-NPs by plant roots were higher than those of larger-sized Ag^{0}-NPs, regardless of the presence/absence of SRB. This finding indicates that plant roots exposed to smaller-sized NPs could accumulate Ag^{0}-NPs directly and can also absorb Ag ions released by pristine Ag^{0}-NPs at a higher rate compared with the findings obtained during exposure to larger-sized Ag^{0}-NPs. Moreover, the total Ag uptake rates of *Scirpus triqueter* roots treated with 20-, 40- and 80-nm Ag^{0}-NPs in the presence of SRB (5.82, 5.28 and 2.57 mg/kg/day, respectively) were higher than those obtained in the absence of SRB (3.44, 3.08 and 2.38 mg/kg/day, respectively), which suggests that the presence of SRB can increase the root uptake rate of Ag during plant exposure to Ag^{0}-NPs of different sizes. Due to the low translocation ratio, the plant stems did not show a significant particle size-dependent uptake of Ag^{0}-NPs.

4. Conclusions and Environmental Implications

SRB are an effective tool for the bioremediation of metalloids and toxic metals in various contaminated sites, such as areas of acid-mine drainage.[59] Biogenically produced sulfide can react with dissolved metals to form metal sulfide precipitates because the solubilities of most toxic metal sulfides are generally very low. In this study, numerous biogenic Ag sulfide NPs were found in the culture solution with SRB and Ag ions, which suggests (a suggestion that has been verified in this study) that although Ag ions can be precipitated as sulfide NPs, these NPs are phytoavailable due their minute size. Further studies should focus on the potential mobility/bioavailability of these biogenic metal sulfide NPs, which are traditionally considered stable or immobile. Furthermore, during exposure to Ag^{0}-NPs, SRB can significantly enhance the uptake of silver by *Scirpus triqueter*, a typical wetland plant, by transforming Ag^{0}-NPs into secondary silver-sulfide NPs. Notably, these secondary biogenic NPs, formed through a

dissolution-diffusion- sulfidation process, were smaller than 10 nm in size and thus showed increased availability for and phytoaccumulation in plants compared with the initial pristine PVP-coated Ag^0-NPs with an average size of 20 nm. An interesting finding reported by Glover et al.[6] showed that newly formed Ag^0-NPs could be released from larger particles and even macroscale sources of silver via chemical- and/or photoreduction. Based on the results obtained in our study, we hypothesize that NPs, and even large macroscale particles, might become bioavailable through the formation of secondary ultrafine particles in the presence of microbes, such as SRB.

NPs smaller than 10 nm in size could be phytotoxic through their direct accumulation in plant tissue[53] and might thereby cause potentially adverse eco-effects to the food chain via trophic transfer. This study encourages further studies aiming to directly investigate the potential biotoxicity of these nanoparticle reactions to wetland plants, such as *Scirpus triqueter*, and the interaction of Ag^0-NPs with a diverse array of common soil microbes and other contaminant metals.

Supporting Information

Additional explanations, tables, and figures.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figures:

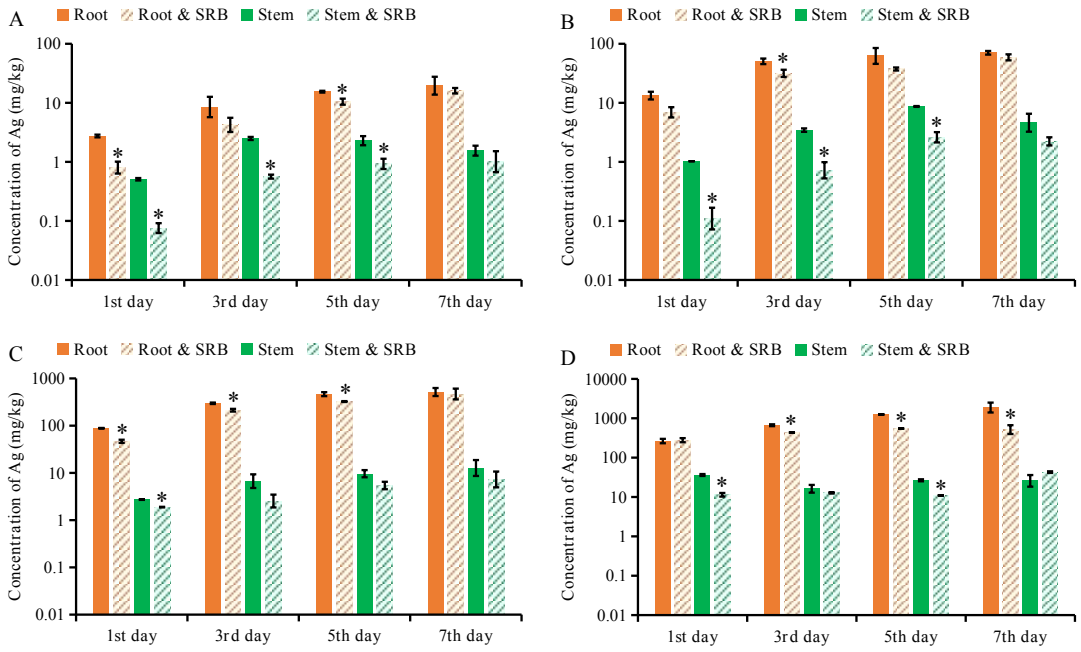

Figure 1. Total Ag concentrations in *Scripus triqueter* treated with silver ions. *: Significant difference, $P < 0.05$. A: 0.01 mg/L; B: 0.1 mg/L; C: 1 mg/L; D: 10 mg/L. Root/Stem & SRB: Root/Stem of *Scirpus triqueter* treated in Hoagland solution in the presence of SRB; Root/Stem: Root/Stem of *Scirpus triqueter* treated in Hoagland solution in the absence of SRB.

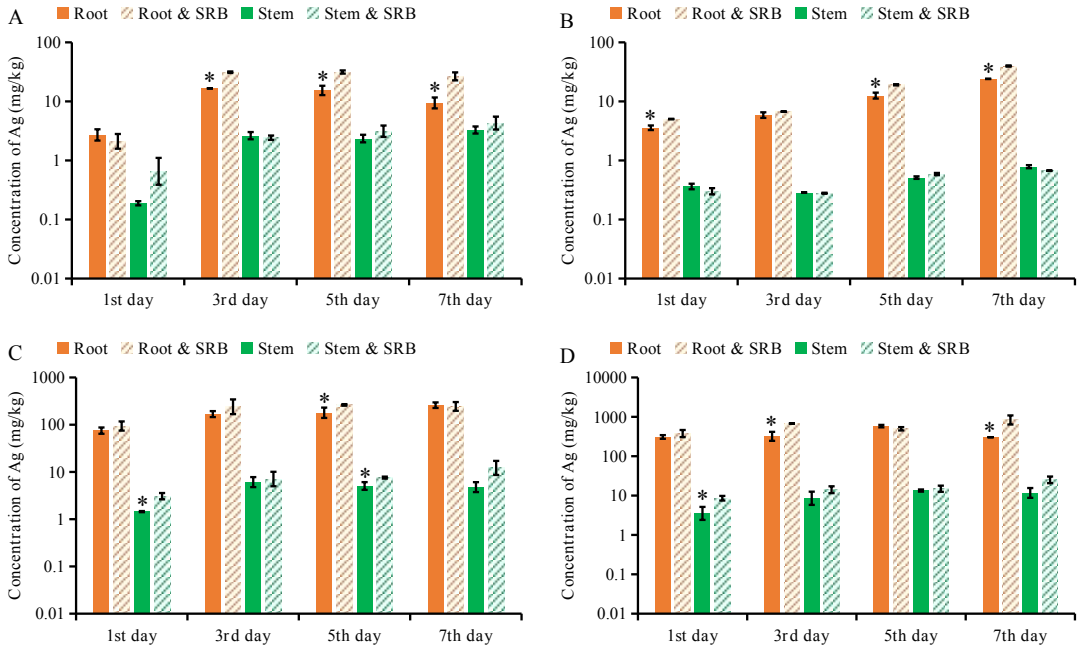

Figure 2. Concentrations of total Ag in *Scirpus triqueter* treated with 20 nm Ag^0-NPs. *: Significant difference, $P < 0.05$. A: 0.01 mg/L; B: 0.1 mg/L; C: 1 mg/L; D: 10 mg/L. Root/Stem & SRB: Root/Stem of *Scirpus triqueter* treated in Hoagland solution in the presence of SRB; Root/Stem: Root/Stem of *Scirpus triqueter* treated in Hoagland solution in the absence of SRB.

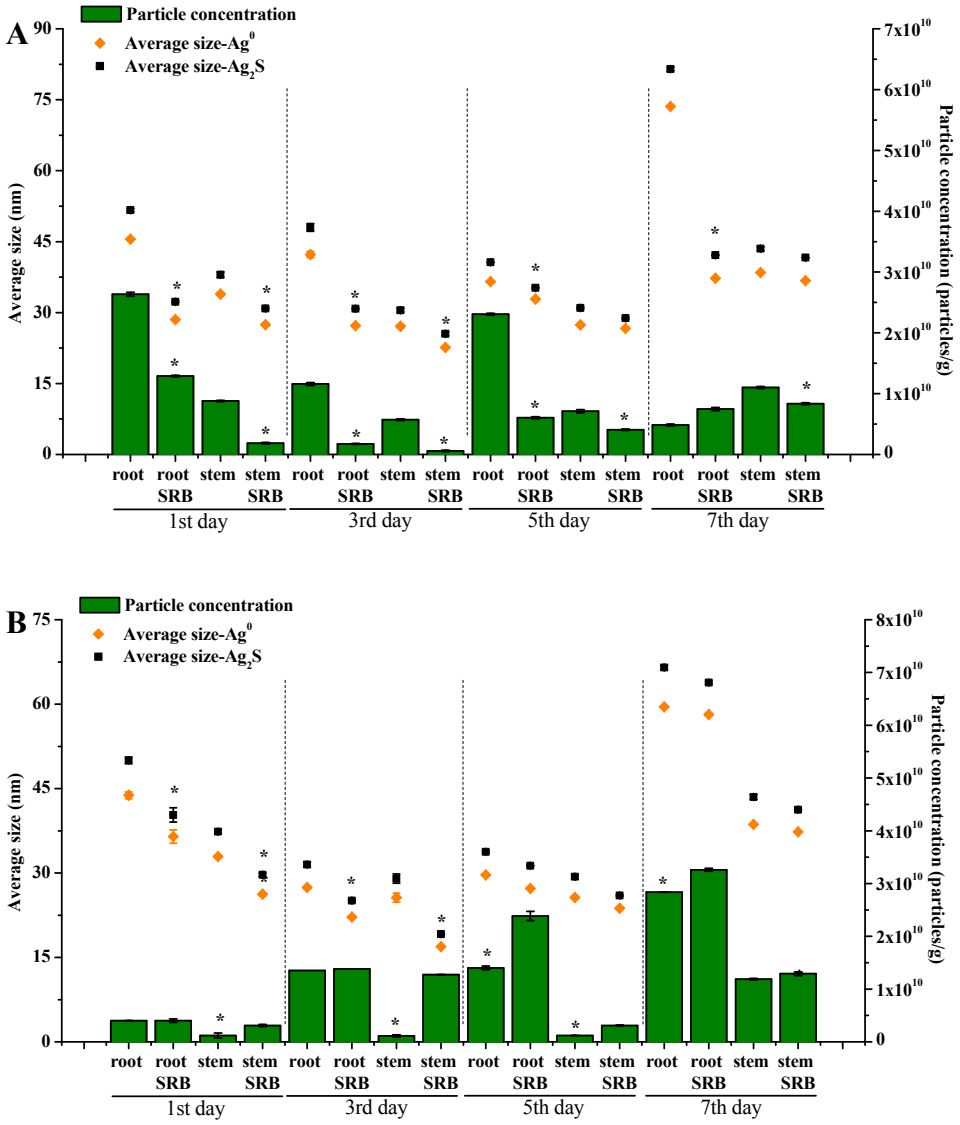

Figure 3. Particle concentration and size of Ag-NPs in silver ions (A) and 20 nm Ag^0-NPs (B) treated *Scirpus triqueter*. Root/Stem SRB: Root/Stem of *Scirpus triqueter* treated in Hoagland solution in the presence of SRB; Root/Stem: Root/Stem of *Scirpus triqueter* treated in Hoagland solution in the absence of SRB. Average size-Ag^0: the average sizes were calculated based on Ag^0; Average size-Ag_2S: the average sizes were calculated based on Ag_2S. *: Significant difference, $P < 0.05$.

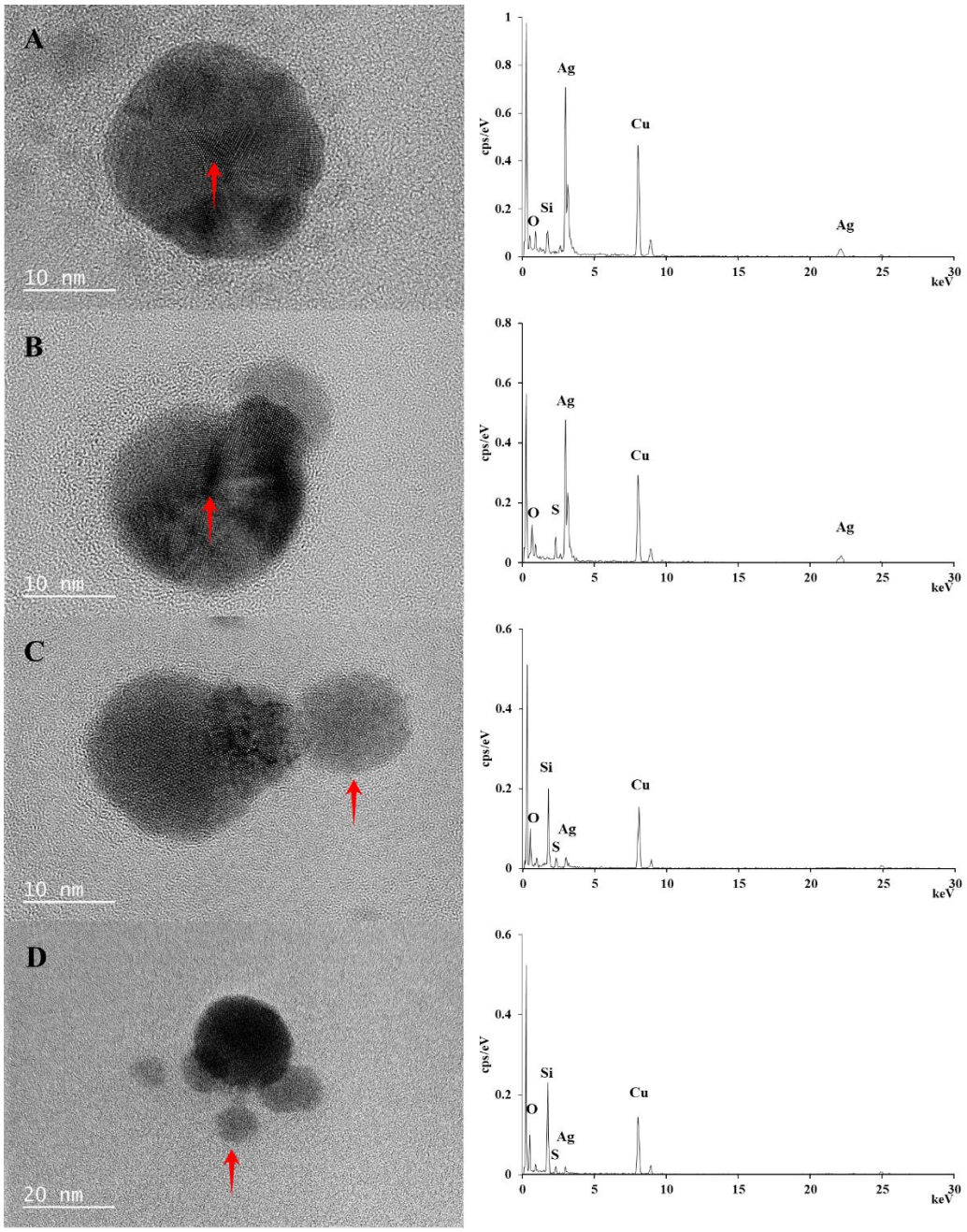

Figure 4. Transmission electron microscopy (TEM) images and the corresponding energy dispersive X-ray spectrometer system (EDS) analysis of Ag-NPs in Hoagland solution with 20-nm Ag^0-NPs after 1 day exposure. A: treated without SRB; B, C and D: treated with SRB. Red "↑": regions for EDS.

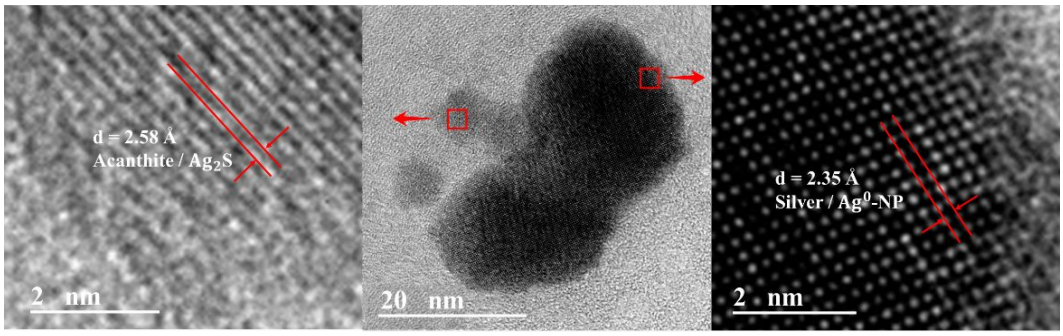

Figure 5. HR-TEM images (left and right images) of Ag-NPs in Hoagland solution treated with 20-nm Ag^0-NPs and SRB as seen in the middle image. Red □: regions for HR-TEM analysis. Right HR-TEM image shows a Ag^0-NP. Left HR-TEM image shows a secondary Ag_2S-NP.

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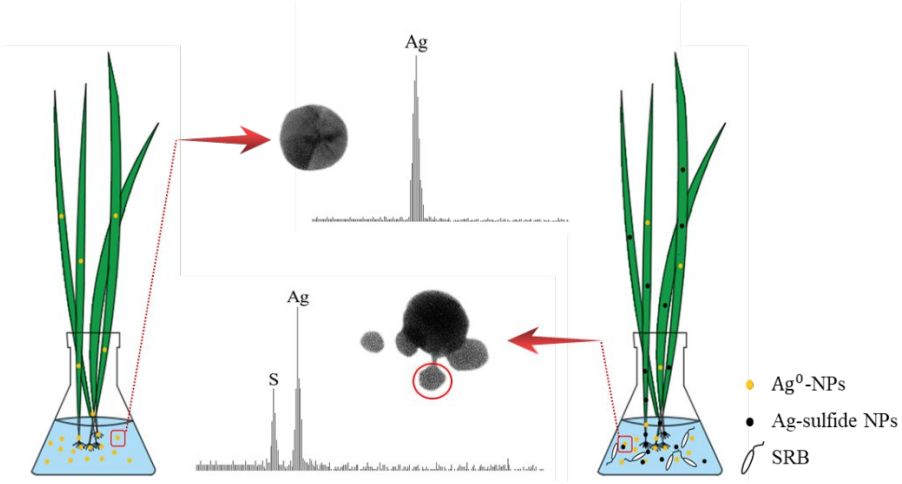

The enhanced phyto-uptake of Ag-NPs in the presence of SRB, by transforming Ag^0-NPs into secondary Ag sulfide-NPs.