Silver Nanoparticles in Sewage Sludge: Bioavailability of Sulfidized Silver to the Terrestrial Isopod *Porcellio scaber*

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Abstract: Silver nanoparticles (AgNPs) are efficiently converted during the wastewater-treatment process into sparingly soluble Ag sulfides (Ag₂S). In several countries, sewage sludge is used as a fertilizer in agriculture. The bioavailability of sulfidized Ag to the terrestrial isopod *Porcellio scaber* was investigated. Sewage sludge containing transformed AgNPs was obtained from a laboratory-scale sewage-treatment plant operated according to Organisation for Economic Co-operation and Development (OECD) guideline 303a. The results of transmission electron microscopy with energy dispersive X-ray of sludge samples suggest that AgNPs were completely transformed to Ag₂S. Adult isopods were exposed to OECD 207 soil substrate amended with the AgNP spiked sludge for 14 d (uptake phase) followed by an elimination phase in unspiked soil of equal duration. Most of the Ag measured in *P. scaber* at the end of the uptake phase was found in the hindgut (71%), indicating that only a minor part of the estimated Ag content was actually assimilated by the isopods with 16.3 and 12.7% found in the carcass and hepatopancreas, respectively. As a result of this, the Ag content of the animals dropped following transition to unspiked sludge within 2 d to one-third of the previously measured Ag concentration and remained stable at this level until the end of the elimination period. The present study shows that Ag₂S in sewage sludge is bioavailable to the terrestrial isopod *P. scaber*. Environ Toxicol Chem 2018;37:1606–1613. © 2018 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

Silver nanoparticles (AgNPs) are used in a range of different products, such as clothing, cosmetics, and personal care products, because of their antibacterial properties [1]. Nanoparticles may be released to the environment through sewage sludge, wastewater, and waste incineration [2]. The predicted concentrations of AgNPs in sewage sludge from European sewage-treatment plants are in the order of a few milligrams per kilogram [3]. Removal efficiencies for Ag during wastewater treatment can reach levels from 92 to 99% [4], with evidence demonstrating that sewage sludge represents a major sink for AgNPs within the wastewater-treatment plant [5,6]. Sewage sludge is used worldwide as fertilizer in agriculture, albeit many countries don’t apply sludge to land [7–10]. The total sludge production in Germany was approximately 1.9 million tons in 2010, and approximately 30% of this sludge has been used for agriculture [11]. Thus, there is a potential for transfer of AgNPs into the terrestrial environment, resulting in increased Ag concentrations within agricultural soils.

Metallic AgNPs are mainly transformed into Ag sulfides (Ag₂S) during wastewater transport and treatment [5,12–17]. The toxicity of Ag strongly depends on its speciation [18], and sparingly soluble Ag₂S is less toxic compared with ionic silver (Ag⁺) released from pristine AgNPs [19,20]. However, recent studies have demonstrated that Ag₂S nanoparticles are bioavailable to some extent and may cause toxic effects in different organisms [21–24]. Therefore, transformed AgNPs may still be toxic to terrestrial organisms [26].

Bioaccumulation approaches can provide important information on the bioavailability of metals. Because of their strong ability to accumulate metals, terrestrial isopods have been used for bioaccumulation studies with zinc [25–27], cadmium [26–29], and lead [26–28,30]. The primary storage location of the metals are the S-cells of the hepatopancreas [31]. Investigations of the accumulation of copper, zinc, and AgNPs in *Porcellio scaber* and *Porcellionides pruinosus* also...
showed metal accumulation within digestive gland cells, which was explained by the assimilation of dissolved ions [32–34]. However, information on the bioavailability of sparingly soluble sulfidized AgNPs in sewage sludge is lacking for terrestrial isopods.

A test system with terrestrial isopods [35] was adapted for testing sewage sludge from laboratory-scale sewage-treatment plants (STPs). Silver nanoparticles and their transformation products in sewage sludge were characterized by transmission electron microscopy (TEM). Fresh sewage sludge containing sulfidized AgNPs was mixed with soil after drying and milling the sludge to a fine powder. The Ag contents in contaminated soil and test animals collected during the uptake and elimination phases were measured with inductively coupled plasma mass spectrometry (ICP-MS). Investigations on tissue distribution were conducted to determine the location of accumulated Ag in the terrestrial isopods.

MATERIALS AND METHODS
Standards and reagents

As required by the Organisation for Economic Co-operation and Development (OECD) sponsorship programme NM-300K AgNP dispersion was used [36]. The colloidal Ag dispersion has a nominal Ag concentration of 10% (w/w) and a particle diameter of approximately 15 nm. As stabilizing agents, NM-300K contains 4% (w/w) of polyoxymethylene glycerol trioleate and 4% (w/w) of polyoxymethylene sorbitan monolaurate (Tween-20) [37]. Hydrochloric acid (purity 30%) was purchased from J.T. Baker. Nitric acid (purity 69%) was obtained from Roth.

Laboratory-scale STP

Sewage treatment was conducted according to guideline OECD 303a [38]. A laboratory-scale STP (behrotest® Laborkläranlage KLD 4N; Labor-Technik) with a denitrification and nitrification reactor and a secondary clarifier was used. The sewage sludge to start the laboratory-scale STP was obtained from a municipal STP. An STP run was conducted including 1 system with continuous addition of 1 mg/L of NM-300K to the influent and a control system without AgNP addition. The oxygen level in the nitrification reactor was maintained between 2 and 4.5 mg/L. The STP was operated with a hydraulic retention time of 6 h and a continuous influent flow rate of 750 mL/h. Experiments were initiated by introducing the sewage sludge into the laboratory-scale STP and adding tap water to adjust the dry matter content to approximately 2.5 g/L. After a 10-d establishment phase, NM-300K was added for 16 d to the laboratory-scale STP. The influent consisted of a mixture of synthetic sewage according to OECD guideline 303a [38], tap water, and NM-300K and was delivered to the denitrification reactor via a tube system. The dissolved organic carbon of the influent was 100 mg/L. The pH of the sewage sludge in the denitrification and in the nitrification reactors was measured continuously. The dry matter content of the sewage was determined periodically. If the content exceeded 3 g dry matter/L, the amount of sludge in the STP was reduced accordingly to maintain a content of ≤3 g dry matter/L in the test system. The dissolved organic matter was measured daily in the influent and effluent.

TEM

Samples of the stock suspension (NM-300K) were prepared by diluting 20 µL of NM-300K in 1 mL of doubly deionized water containing 0.1% FL70 (Fischer Scientific) as dispersion agent. Silver nanoparticles were deposited onto the TEM grid by placing 50 µL of this suspension on a holey carbon-coated TEM grid (Plano) and drawing the suspension through the grid using a paper tissue. Sludge samples were prepared by dispersing approximately 20 mg of freeze-dried sludge in 1 mL of doubly deionized water containing 0.1% FL70, followed by 1 min of ultrasound treatment (100% amplitude, 50% cycle; VialTweeter; Hielser Ultrasonics). The suspension was diluted 1:10 000 in doubly deionized water and centrifuged (1 h at ~ 25 000 g) directly onto carbon-coated TEM grids (Quantifoil Micro Tools). Samples were investigated using a scanning TEM (HD2700Cs; Hitachi) operated at 200 kV. For image formation a high-angle annular dark field (HAADF) detector was used, and elemental analyses of selected particles were conducted with an energy-dispersive X-ray (EDX) analysis system (EDAX). The average diameter of the AgNPs was derived from TEM images using image analysis tools (ImageJ, Ver 1.51n).

Preparation of soil

The sludge derived from the laboratory-scale STP was added to test soil as a fine powder. In brief, the sludge was centrifuged (Beckman Coulter; Avanti J-26S XP) for 10 min at 7000 rpm (9000 g) and dried at 65 °C for 72 h. Afterward, the dry sludge was ground for 4 min into a fine powder with a ball mill (Retsetch RM100). The measured Ag concentration in the dry powder was 4.02 g/kg_dry (dry wt). The soil substrate consisting of 70% sand, 20% kaolin, and 10% humus (sphagnum peat) was prepared according to OECD guideline 207 [39] and mixed with the dried sludge powder. The sludge to soil ratio in all tests was 4 g/kg_soil (dry wt) to cover the food requirements of the animals. The water content of the soil was adjusted to 25% (v/v) at the beginning of the present study. Each test container was filled with 100 g soil (dry wt) containing 400 mg sludge powder. The same batch of amended soil mixture was used for the uptake and elimination study as well as the accompanying investigations on tissue distribution.

Test system and study design

The test system for bioaccumulation studies recently described by Kampe and Schlechtriem [35] was modified for the application of soil substrate enriched with sewage sludge. Plastic boxes (16 × 12 cm, 1 L) grouted with 0.5 cm of plaster cast (Danogips), to maintain the humidity in the test boxes during application, were used as test containers. A Petri dish cover (5.8 cm diameter) coated with aluminum foil, with an entrance...
hole on 1 side, was placed in each test box on the soil to provide a shelter for the experimental animals. The plaster bottoms of the test containers were moistened with 20 mL water at the beginning of the present study. All boxes were covered with perforated translucent test box covers to allow gas exchange. The temperature and air humidity were measured weekly in the test containers. The mean (+ standard deviation [SD]) temperature during the test was 19.0 ± 4 °C, and the mean air humidity was 95 ± 6%. The mean light intensity measured at the beginning of the experiment was approximately 100 lux. Every container was filled with 100 g (dry wt) of sewage sludge-amended soil. The soil was evenly distributed in the container on top of the plaster cast lining, resulting in a 2-cm-thick soil layer.

Porcellio scaber from the established stock culture of the Fraunhofer Institute for Molecular Biology and Applied Ecology IME were used as experimental animals. The culturing procedure was carried out according to Kampe and Schlechtriem [35]. To reduce the risk of unwanted reproduction during the experiments, only male isopods and females without a marsupium (brood pouch) were selected. The uptake and elimination study involved 24 test containers with a total of 120 isopods. Every test container (replicate) was stocked with 5 isopods. In addition, a control group with 3 replicates was established with nonspiked powdered sludge. The test containers were checked daily for dead animals, which were removed from the systems. If more than 1 animal died during the study, the whole replicate was considered invalid and removed from the study. Every day 5 mL of water was added to each test container to maintain a constant humidity in the test system. No additional food was added to the containers during the experiment. The results showed that the soil substrate was of sufficient nutritional value to maintain growth of the animals over the experimental period.

The experiment ran for 28 d and included 14 d for uptake, followed by 14 d for elimination. Following the exposure period, animals were transferred into new test containers holding soil mixed with noncontaminated powdered sludge. During the uptake and elimination phase, groups of animals were collected to measure the Ag concentration in the animal tissue. During the uptake and the elimination phases, samples were collected on days 0, 2, 4, 8 (10), and 14. Every sample included 3 replicates (containers) with 5 isopods each. At the beginning of the experiment 3 replicates of 5 isopods were collected to determine the background concentration of Ag in the animal tissue. The animals in each replicate were analyzed as a group. The animals were weighed and frozen at −20°C for the subsequent Ag analysis by ICP-MS. The guts of the test animals were not emptied before sampling. The total Ag concentration measured in the test animals may thus reflect, to a certain extent, the Ag load in the intestinal contents.

Tissue distribution of Ag in P. scaber

The distribution of accumulated Ag in the tissue of P. scaber was investigated in animals that were kept under the same experimental conditions as applied during the uptake and elimination study. Animals from the same batch of isopods were used. Three replicates including 5 isopods each were exposed to the same soil–sewage sludge mixture as applied in the main test. After 14 d of exposure, the animals were weighed and the 3 tissue fractions—hepatopancreas, hindgut, and carcass (body minus hepatopancreas and hindgut)—were separated as described by Kampe and Schlechtriem [35]. The tissue fractions were stored at −20°C for subsequent Ag analysis by ICP-MS.

Ag analysis

Silver analysis of isopods, isopod tissues, sludge, and soil–sludge samples was carried out according to the following procedure, fulfilling the EN ISO16174.2012 method [40]. Prior to digestion in a microwave system (CEM Discover SP-D), 15 mL of aqua regia (HNO₃ [69%] and HCl [30%] in a ratio of 3:1) was added to the samples. The heating time was 12 min with an initial energy of 200 W. The samples were heated to 175°C at a maximum pressure of 30 bar. The maximum temperature was kept constant for 10 min for the extraction in aqua regia and afterward cooled for approximately 8 min to 20°C. After microwave digestion, each sample (~15 mL) was diluted with 35 mL HNO₃ (0.5 M). The Ag analysis was performed by ICP-MS (Agilent Technologies; 7700 Series). Silver was quantified according to Wasmuth et al. [41] using isotope 107Ag. A rhodium standard (Merck KGaA; CertiPUR) was used as an internal standard. All concentrations were evaluated using data recorded in no-gas mode of the analytical device. Integration time was set to 0.1 s, and plasma conditions were adjusted to 1500 W. Gas flows were 0.96 L/min for carrier gas and 0.13 L/min for dilution gas. The limit of detection (LOD) was 0.56 ng/L, and the limit of quantification (LOQ) was 1.31 ng/L. The recovery of external reference material (Merck XXI, Merck KGaA; CertiPUR) was 99.1 ± 4.1 and 97.3 ± 1.0% (Lake Ontario fortified water TM 26.2) for all measurements. The LOD and LOQ were calculated by ICP-MS (reported by the Agilent MassHunter Workstation software).

Statistical analyses

Tissue concentrations measured during the uptake and elimination phase as well as temperature, light intensity, survival rates, and percentage distribution estimates are presented as means ± SD. Results of the tissue distribution experiment were compared by t tests. Differences were reported as statistically significant when p < 0.05 (SigmaStat 3.5; Systat).

RESULTS

Experimental parameters

The mean (± SD) fresh weight of the exposed isopods was 42.6 ± 4.2 mg, with a mean survival rate and water content of 95.0 ± 10.6 and 66.5 ± 2.5%, respectively. The mean fresh weight (± SD) of the control isopods was 41.1 ± 4.5 mg, with a mean survival rate and water content of 93.3 ± 11.6 and
69.3 ± 2.1%, respectively. The Ag content of the isopods sampled at the beginning of the test and that of the control sludge were below the detection limit.

**TEM results**

The particles were spherical, as revealed by TEM of the starting material. The average diameter was 15 ± 2.5 nm \((n = 93)\). High-resolution TEM imaging and EDX analysis conducted on selected particles confirmed that the AgNPs were metallic (Figure 1). Images from sewage sludge containing AgNPs suggested that the AgNPs preserved their spherical shape during sewage treatment and were still of comparable size as the AgNPs observed in the NM300K starting suspension. The AgNPs in the samples were well dispersed. The images were recorded with an HAADF detector, whose signal intensity scales with roughly the square of the atomic weight [42]. The HAADF images therefore reflect a contrast in the atomic number, and Ag\(^0\) would appear much brighter next to Ag\(_2\)S particles of comparable sizes. However, neither contrast differences between individual particles nor core-shell structures on individual particles were observed, suggesting that AgNPs were completely transformed. This hypothesis is supported by elemental analyses of selected particles from both samples revealing substantial amounts of S in addition to Ag \((n = 24)\), Ag/S ratio ranging from 1.2 to 2.1 with an average of 1.6; Figure 2). The most likely transformation product is Ag\(_2\)S, and the lower Ag/S ratios were likely caused by additional S present in the sludge next to the AgNPs. Analysis by TEM can only reveal the particulate Ag, and Ag complexed to organic matter would not be detected. However, based on the comparable size of the AgNPs and considering results from previous studies where Ag was dominantly transformed Ag\(_2\)S [5,12,43], we also assume that in this experiment the AgNPs were mainly present as Ag\(_2\)S after the transformation procedure in the STP.

**Investigations on uptake and elimination**

The measured Ag concentration in the soil–sewage sludge mixture \((mean ± SD)\) was 13.68 ± 1.87 mg/kg\(_{soil}\) (dry wt; 10.26 ± 1.40 mg/kg [fresh wt]). The uptake and elimination of silver in *P. scaber* from soil mixed with powdered sludge containing AgNPs are shown in Figure 3. After 2 d of exposure, a concentration of 4.87 ± 1.46 mg Ag/kg fresh weight was measured in the animals. Concentrations measured in the animals further increased within 4 d (10.56 ± 5.87 mg Ag/kg fresh wt). Lower concentrations were measured after 8 and 14 d, with 4.00 ± 0.98 and 6.04 ± 2.55 mg Ag/kg fresh weight, respectively. After 2 d of elimination, the Ag concentration in the collected isopods decreased to 1.42 ± 0.10 mg/kg fresh weight and remained fairly constant until the end of the study, with 1.26 ± 0.16, 1.93 ± 0.52, and 1.52 ± 0.13 mg/kg fresh weight, measured after 4, 10, and 14 d, respectively.

**FIGURE 1:** High-resolution transmission electron microscopy (TEM) with energy dispersive X-ray (EDX) of stock suspension (NM300K). (A) A TEM image of a silver nanoparticle (AgNP; NM-300K). Lattice fringes indicate the polycrystalline structure of the particles. (B) The EDX spectrum of metallic AgNPs used as starting materials. The small silicon signal intensity observed in the spectra originates from contaminations on the TEM grid and was also present in EDX spectra from background locations. (C) High-angle annular dark field (HAADF) image of AgNPs deposited on TEM grids. (D) Particle size distribution derived from several HAADF images \((n = 93)\). Si = silicon.
Tissue distribution of silver in P. scaber

The mean survival rate (± SD) in the replicates used for the investigations on tissue distribution was 93.3 ± 11.6%. The tissue concentration and distribution of Ag in P. scaber which were exposed for 14 d to the soil–sewage sludge mixture containing 13.68 mg Ag/kg soil (dry wt) are shown in Figure 4. Concentrations of Ag measured in the tissues of P. scaber collected during the experiment were 0.96 ± 0.47 (carcass), 9.73 ± 0.96 (hepatopancreas), and 67.28 ± 55.08 ng/mg fresh weight (hindgut). The contribution of the different tissues to the total Ag content in the animals was 16.3 ± 3.78 (carcass), 12.7 ± 10.69 (hepatopancreas), and 71.0 ± 12.77% (hindgut).

DISCUSSION

The Ag concentration tested in the present study was much higher than expected for agricultural fields treated with sewage sludge. The Ag concentration in sewage sludge is currently in the order of a few tens of milligrams per kilogram total suspended solids, although concentrations as high as 850 mg/kg total suspended solids have been reported [44]. Predicted AgNP concentrations in sewage sludge are even lower (1.31–4.44 mg/kg [Europe], 1.29–5.86 mg/kg [United States]) [3] and thus several orders of magnitudes below the AgNP concentrations of the present study (4.02 g/kg sludge). However, the exposure scenario still provided the opportunity to elucidate the bioavailability of Ag$_2$S in the terrestrial isopod P. scaber exposed to soil enriched with sewage sludge.

Elemental analyses of stock suspension (NM-300K) and individual AgNPs in sewage sludge conducted using TEM indicated that AgNPs transformed completely into Ag$_2$S during the wastewater-treatment process. The transformation of the AgNPs in the wastewater matrix is consistent with results from batch experiments conducted in real wastewater matrix, suggesting that AgNP transformation to Ag$_2$S occurs rapidly under nonaerated conditions within less than 2 h [5]. Furthermore, recent studies on the transformation kinetics of AgNPs reported half-life times ranging from a few minutes to a few hours, depending of the availability of bisulfide [43,45].

Pipan-Tkalec et al. [34] explained the presence of Ag in the tissue of terrestrial isopods exposed to nano-Ag as a result of the assimilation of dissolved Ag$^+$ ions. In contrast, well crystalline Ag$_2$S is only very sparingly soluble under relevant environmental conditions [46], leading to the assumption that the transformation of AgNPs to Ag$_2$S in the urban wastewater system is mitigating the bioavailability and thus the toxicity of the AgNPs. However, Pradas del Real et al. [47] reported the formation of a small fraction of amorphous Ag$_2$S on sulfidation of AgNPs in sewage sludge. Amorphous or poorly crystalline Ag$_2$S was also observed by Levard et al. [48]. This amorphous Ag$_2$S phase may show a higher degree of solubility compared with the well crystalline Ag$_2$S, which might explain the bioavailability of sulfidized AgNPs observed in the present study.

The most important route for metal uptake in terrestrial isopods is known to be the digestive tract [49]. Bioaccumulation studies on metallic oxide nanoparticles showed that metal ions and not particulate NPs are assimilated by the digestive gland cells [32,33]. It can be assumed that also in the present study Ag$^+$ ions were assimilated following oral uptake of sulfidized AgNPs. However, the dissolution behavior of sulfidized Ag in the gut of...
the isopods is still not understood and requires further investigations. Micromorphological investigations including electron microscopy on isopods exposed to contaminated soil or food may deliver additional information on the behavior of sulfidized AgNPs during their passage through the digestive tract.

Isopods are in constant contact with the soil and porewater. Therefore, dissolved Ag might be also taken up through the uropods by capillary action [50]. Metals taken up by this route are distributed in the entire body via the hemolymph.

Another potential route of Ag uptake is via the isopod cuticle. However, the cuticle consists of chitin hardened by calcium salts [51] and is assumed to be relatively impermeable to toxic elements. Studies have shown that metal adsorption to the isopod surface is negligible and that it is not likely that the subsequent passage through the membrane is the rate-limiting step for metal uptake in *P. scaber* [49].

Concentrations of Ag in the test animals showed a high variability during the uptake phase. Most of the Ag measured in *P. scaber* at the end of the uptake phase was found in the hindgut, indicating that the Ag load in the intestinal contents had a major impact on the total Ag concentrations. This finding is in tune with the observation that Ag concentrations of the animals dropped within 2 d to one-third of the previously measured Ag concentration following transition to unspiked sludge. The remaining Ag concentration remained stable until the end of the elimination period. A similar effect was shown by Golobić et al. in a study on exposure of isopods to CuNPs, where Cu in *P. scaber* was efficiently depurated from the gut and the carcass but remained accumulated in the hepatopancreas [33]. Similarly, the inability of isopods to excrete Cd, which is stored in the hepatopancreas, was described [52,53]. The hepatopancreas is an efficient storage organ for metals, which may contain up to 90% of the metals accumulated in the isopod’s body [54]. It can only be speculated whether the stable residual Ag content measured during the present study was immovably stored in the hepatopancreas or remobilized and redistributed within the animals during the elimination phase. Further investigations on the uptake and depuration dynamics of sulfidized Ag in terrestrial isopods are required.

A frequently used approach to provide a relative assessment of metal bioavailability from the soil is the calculation of a bioaccumulation factor (BAF) [26]. However, a BAF for sulfidized Ag, as the ratio between Ag accumulated by isopods and metal concentration in the soil, could not be calculated because of the high intestinal contents masking the fraction of truly accumulated Ag. Calculating an approximated BAF, as the ratio of the Ag concentration in the carcass (0.96 ng/mg fresh wt) of the animals collected at the end of the exposure period and the Ag concentration in the soil substrate (13.7 ng/mg), a low estimate of 0.07 is obtained, giving clear indications for a true BAF which is well below 1. However, also a limited accumulation of Ag by terrestrial isopods in agricultural fields treated with sewage sludge supports the mobilization of transformed AgNPs, making the Ag available for higher trophic levels. Long-term studies on the effects of sulfidized AgNPs on soil organisms are required.
CONCLUSIONS

The present study confirmed that AgNPs are efficiently converted during the wastewater-treatment process into Ag₂S as revealed by microstructural investigations of sewage sludge samples using TEM in combination with EDX. The bioaccumulation approach showed that sparingly soluble Ag₂S is bioavailable to the terrestrial isopod Porcellio scaber. Further studies are required to elucidate the link between uptake of Ag₂S by soil organisms and their accumulation and/or effects within the organisms.

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Data availability—Essential data are presented in the text. Raw data are available on request from the corresponding author (christian.schlechtriem@ime.fraunhofer.de).

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