LONG-TERM EFFECTS OF SULFIDIZED SILVER NANOPARTICLES IN SEWAGE SLUDGE ON SOIL MICROFLORA

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Abstract: The use of silver nanoparticles (AgNPs) in consumer products such as textiles leads to their discharge into wastewater and consequently to a transfer of the AgNPs to soil ecosystems via biosolids used as fertilizer. In urban wastewater systems (e.g., sewer, wastewater treatment plant [WWTP], anaerobic digesters) AgNPs are efficiently converted into sparingly soluble silver sulfides (Ag2S), mitigating the toxicity of the AgNPs. However, long-term studies on the bioavailability and effects of sulfidized AgNPs on soil microorganisms are lacking. Thus we investigated the bioavailability and long-term effects of AgNPs (spiked in a laboratory WWTP) on soil microorganisms. Before mixing the biosolids into soil, the sludges were either anaerobically digested or directly dewatered. The effects on the ammonium oxidation process were investigated over 140 d. Transmission electron microscopy (TEM) suggested an almost complete sulfidation of the AgNPs analyzed in all biosolid samples and in soil, with Ag2S predominantly detected in long-term incubation experiments. However, despite the sulfidation of the AgNPs, soil ammonium oxidation was significantly inhibited, and the degree of inhibition was independent of the sludge treatment. The results revealed that AgNPs sulfidized under environmentally relevant conditions were still bioavailable to soil microorganisms. Consequently, Ag2S may exhibit toxic effects over the long term rather than the short term. Environ Toxicol Chem 2017;36:3305–3313. © 2017 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 as broad-spectrum antibiotics in various consumer products such as textiles [1], cosmetics [2], and medical devices [2]. Thus the release of AgNPs into sewer systems and consequently wastewater treatment plants (WWTPs) is to be expected [3]. Based on modeled and experimental results, the AgNPs accumulate to a large extent in sewage sludge [3–5]. Biosolids are used as agricultural fertilizers because of their high content (and good plant availability) of macro- and micronutrients, especially phosphorous, which is a limited resource. Several techniques such as anaerobic digestion can be used for sewage sludge treatment. During the transport of the AgNPs along the sewer and during wastewater and sludge treatments, AgNPs are subjected to various transformations, the most important of which is the formation of silver sulfides (Ag2S) [6–11]. The formation of sparingly soluble Ag2S is considered a natural antidote to the toxicity toward single cellular and multicellular organisms [12–14]. Reinsch et al. [12] demonstrated that the degree of sulfidation determines the observed ecotoxic effects. Schlich et al. [15] reported that with increasing incubation time (100–140 d) the effect of AgNPs, spiked to a laboratory-scale WWTP and applied via biosolids to soils, on nitrification was comparable to the effect of the pristine AgNPs. This finding suggested that long-term ecotoxic effects from sludge-applied AgNPs cannot be excluded. However, in that study, the sludge was used without further pretreatment, and a transformation was expected but not analytically proved. The delayed effects were discussed as a masking function of the sludge, which has first to be degraded before effects become visible.

In the present study, we focus on the influence of sewage sludge pretreatment on the bioavailability of AgNPs used as fertilizer on agricultural land. According to International Organization for Standardization (ISO) guideline 17402 [16], the portion of a contaminant that causes an effect must be bioavailable. For that purpose, realistic exposure pathways of an AgNP were simulated by spiking pristine AgNPs to a laboratory-scale WWTP, followed by both anaerobic and aerobic sewage sludge treatments before the biosolids were mixed with the soil matrix. Size, shape, and elemental composition of the AgNPs were determined during the process. We conducted our WWTP experiments with the recommended composition of the artificial wastewater [17] and in addition an increased sulfur concentration in the influent to make sure that the sulfidation of AgNPs was not limited by the available sulfur. Ecotoxicity to soil microflora was studied by addressing the sensitive transformation step from ammonium to nitrite [18] performed by ammonium-oxidizing bacteria as part of the nitrification process.

MATERIALS AND METHODS

Silver nanomaterial

The AgNPs (NM-300K) from the Organisation for Economic Co-operation and Development (OECD) Sponsorship Programme were used for the experiments. The NM-300K is a
colloidal silver suspension with a nominal silver concentration of 10% (w/w) and a particle size of approximately 15 nm with a narrow size distribution. A second particle size of 5 nm, which is much less abundant, was identified by transmission electron microscopy (TEM). The NM-300K stabilizing agent is NM-300K DIS, comprising 4% (w/w) each of polyoxyethylene glycol triolate and polyoxyethylene sorbitan monolaurate (Tween-20). Further characterization data, such as information on stability, solubility, and electron microscopy provided by various laboratories are listed by Klein et al. [19].

AgNP stock suspension

The AgNPs were added to the WWTP as a stock suspension. First, 2000 mg NM-300K, containing 200 mg Ag, was suspended in 8 g H2Odeion in glass vials, thoroughly shaken by hand for 1 min, and sonicated for 15 min at 35 kHz in a sonication bath (Bandelin RK 510). Then 3 mL of this suspension was added to 6 L H2Odeion in polyethylene vessels to obtain an AgNP concentration of 10 mg/L. For each WWTP, 18 L of stock suspension was used.

Wastewater treatment, sewage sludge digestion, and soil experiments

Sewage sludge. Activated sludge (recirculated sludge) was collected from the WWTP in Schmalkenb (Germany). The sewage sludge met the requirements of the German Sewage Sludge Ordinance [20] regarding the heavy metal content for sewage sludge assumed to be applied as agricultural fertilizer. Sludge Ordinance [20] requires that sewage sludge be anaerobic, it was also inoculated with 10 mL of digested sewage sludge from the digestion tank of the WWTP Maunke, Lennestadt (Germany) and saturated with N2 at the beginning of the experiments and once every third day to generate an O2-free atmosphere. After 2 d, when the sludge was assumed to be anaerobic, it was also inoculated with 10 mL of digested sludge from the digestion tank of the WWTP Maunke, Lennestadt (Germany) and saturated with N2 5 times.

Laboratory-scale WWTP

The WWTP experiments were conducted in accordance with OECD guideline 303A [17] in 4 10-L laboratory WWTPs (Laboratory Sewage Plant KLD 4N, behr Labor-Technik) consisting of denitrification and nitrification reactors and a secondary clarifier. The experiments were conducted with 2 control units and 2 units fed with 1 mg/L AgNP nominal influent concentration. The concentration in the influent was selected to achieve an Ag concentration in soil by addition of the amount of biosolids that results in effects. Previous experiments (not published) have shown that this influent concentration of NM-300K led within the 10-d application period to silver concentrations in the sewage sludge (~3.5 mg Ag/g dry matter sewage sludge) that cause significant adverse effects in soil (~6 mg Ag/kg dry matter soil). One pair consisted of a control and an AgNP-treated unit and was operated with regular artificial wastewater. A second pair was operated with an increased sulfate concentration in the artificial wastewater. The room temperature was kept at 20 to 25 °C, and the oxygen concentration in the nitrification reactor was kept between 2 and 4.5 mg/L. Each WWTP was inoculated with 25 g dry matter of sewage sludge, resulting in 2.5 g dry matter/L per WWTP. The WWTPs were fed with artificial wastewater composed of meat extract, peptone, urea, K2HPO4, NaCl, CaCl2, and MgSO4 [17]. The influent dissolved organic carbon (DOC) was adjusted to 150 mg/L by a higher amount of meat extract (147 mg/L) and peptone (180 mg/L) to reduce the formation of bulking sludge. For the experiments conducted with increased sulfate concentrations, Na2SO4 (248 mg/L in influent corresponding to 200 mg SO42-/L) was added. Artificial wastewater and AgNPs were prepared as stock solutions/suspensions, concentrated 10-fold, and stored at 4 °C in a refrigerator. The concentrated solutions/suspensions were added continuously to the WWTPs with pumps (PLP 33; SP04/3.5 K, behr Labor-Technik) and diluted with tap water in the tube lines. The influent flow rate was 750 ± 20 mL/h, leading to a mean hydraulic retention time of 6 h in the nitrification reactor.

Four days of feeding with synthetic wastewater were needed to adapt the sewage sludge to laboratory conditions, as indicated by steady ammonium, nitrite, and nitrate concentrations. After this period AgNPs were added to the WWTP over 10 d. The DOC was monitored during the experiment to assess the effects of AgNPs on microbial activity. The DOC elimination was calculated from the DOC concentrations measured in the influent and effluent.

Sewage sludge treatment

After 10 d of addition of AgNPs into the WWTP, the sludge was collected separately in polyethylene containers. To reduce the water content, 80 mL of 0.2% (v/v) cationic polyacrylamide (Sedifloc 154, Kemira Germany) was added as a flocculant before the water was decanted. The flocculant was applied in accordance with the instructions of the local sewage treatment plant. Samples for TEM analysis were collected before addition of the flocculant.

After the water was decanted, the sludges were split into 2 halves. One half was further dewatered by centrifugation (15 min at 10000 g; Beckmann Coulter Avanti 265 XP) and mixed with soil matrix. The other half of the sludge was digested anaerobically for 35 d before centrifugation and mixing into soil. The digestion was carried out by stirring in an anaerobic incubation chamber at 33 to 35 °C. The chamber was evacuated 5 times to 600 mbar and saturated with N2 at the beginning of the experiments and once every third day to generate an O2-free atmosphere. After 2 d, when the sludge was assumed to be anaerobic, it was also inoculated with 10 mL of digested sludge from the digestion tank of the WWTP Maunke, Lennestadt (Germany) and saturated with N2 5 times.

Soil experiment: Application and incubation

Taking into account the German Sewage Sludge Ordinance [20], 1.67 g dry matter sludge was added to 1 kg dry matter soil (Supplemental Data S1; corresponding to 5 t/ha/3 yr; soil density: 1.5 g/cm3; tillling depth: 0.20 cm). The dewatered slurries were first floated in 50 mL H2Odeion and thereafter evenly distributed over 1 kg dry matter test soil before mixing thoroughly using a scoop. The mixed soils were adjusted to 50% of the maximum water-holding capacity using H2Odeion. The incubation was carried out in glass vessels with perforated lids at 20 ± 1 °C for 140 d. The soil–biosolid mixtures were turned every second week to avoid anaerobic conditions. The loss of water was adjusted with H2Odeion. After 60, 90, and 140 d, effects on soil microorganisms were determined with an AgNP-sensitive potential ammonium oxidation test [18,21].

Microbial determinations

Determination of sulfate-reducing bacteria. Sulfate-reducing bacteria were considered to be important, because they are involved in the sulfidation process. These organisms reduce sulfate to S2−, which reacts with Ag+ to form Ag2S. The presence and diversity of sulfate-reducing bacteria in the sewage sludge were determined at the beginning of the experiments (before AgNP application), at the end of the WWTP simulation and after 35 d of anaerobic digestion by
extraction of deoxyribonucleic acid (DNA) followed by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). The DNA was extracted with the PowerFecal DNA Isolation Kit as specified by the manufacturer (Qiagen). From each treatment, 50 mL of sludge was placed into polyethylene vials and centrifuged at 10 000 g for 5 min. The supernatant was discarded, and approximately 500 mg of sludge was weighed into PowerBead Tubes.

The primers (dsrF-GC-2: 5'-GGC GGC GCC GCC GCC AAC CGT CG-3' / dsrF-GC-5: 5'-GGA CTG GTG GTA GGA AGA AGG CAA GAA CCG-3') were designed with the CLC Sequence Viewer 7.0.2 based on the dsrB gene sequences from 7 sulfate-reducing bacteria, all of which are ubiquitous in European sewage and soil habitats.

Amplification of the dsrB gene sequence was conducted with 0.25 µL HotTaq-polymerase, 1 µL forward primer (10 pmol), 1 µL reverse primer (10 pmol), 2.5 µL HotMaster Taq buffer (10×), 19.25 µL H2Odeion, and 1 µL DNA template. The PCR was carried out as follows: 5 min initial denaturation of DNA at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 55.5 °C, and 1 min elongation at 72 °C. Amplification was completed by a final elongation step at 72 °C for 5 min. For DGGE a denaturing gradient of 30 to 80% (v/v) denaturants (100% denaturants is a mixture of 42 g/L urea and 40% [v/v] formamide) was used in 9% (v/v) polyacrylamide gel. Electrophoresis was performed in Tris–acetate–ethylene diamine tetraacetic acid (EDTA) buffer at 60 °C, at a constant voltage of 120 V for 16 h. Afterward the gels were incubated in an SYBR® Gold (Invitrogen) solution for 30 min and then rinsed in H2Odeion. GENE FLASH station (SYNGENE) was used to photograph the gels. Individual bands were isolated and extracted with the Gen Ellute Gel extraction Kit (Sigma-Aldrich) according to the manufacturer’s protocol for DNA sequencing. Sequencing was performed at Fraunhofer Institute of Molecular Biology and Applied Ecology (Aachen, Germany).

Potential ammonium oxidation activity

To examine effects on soil microorganisms, a short-term potential ammonium oxidation test was performed. The intent of this method is to measure the ammonium oxidation potential, which provides an indication of the activity of the ammonium-oxidizing bacterial population.

In accordance with ISO guideline 15685 [21], 4 250-mL Erlenmeyer flasks/treatment were filled with 25 g dry matter of soil biosolid mixture. Then 100 mL of mineral test medium consisting of KH2PO4 (0.56 mM), K2HPO4 (1.44 mM), NaClO3 (5 mM), and (NH4)2SO4 (1.50 mM) was added. The slurries were incubated on an orbital shaker at 25 ± 1 °C (Multitron, Infors HT). After 2 and 6 h, 5-mL samples were taken, supplemented with 5 mL of 4 M KCl, and centrifuged for 3 min. For DGGE analysis, the supernatants were stored under N2 atmosphere until further processing at the Swiss

Determination of Ag concentrations

The extraction procedure for soil sewage sludge and sludge samples was performed according to the German Institute for Standardization (DIN) ISO 11466 and DIN European Standard EN 13346. Prior to digestion, the soil or sludge was dried at 105 °C and ground for 3 min with a mortar grinder (Retsch RM 100). Then 3 g of the homogenized soil or 0.5 g of sludge was mixed with 28 mL aqua regia (21 mL HCl 30%, J.T. Baker) and 7 mL HNO3 (69% Suprapur, Carl Roth) in 250-mL tubes and incubated at room temperature (~ 20 °C) for 16 h without agitation. The mixtures were then heated to 140 °C by a trace metals digestion unit (SMA20A, Gerhardt) under reflux for 2 h. The aqua regia extraction mixtures were cooled to room temperature and then filled up to 100 mL with 3% HCl. Mixtures were filtered before analysis (0.45-µm syringe filter, PET membrane, Graphic Controls). Certified reference material (CRM) 026 050 (RTC, Laramie, Wyoming) and blanks were each digested at 3 different positions, along with the soil sewage sludge samples to verify the digestion procedure.

Influent and effluent samples were preserved according to DIN 38402-11 (2009). First, 20 mL of liquid was filtered with syringe filters (0.45 µm, PET membrane, Graphic Controls,) and acidified with 200 µL HNO3 Suprapur (69%; Carl Roth) immediately after sampling. Samples collected at the beginning and end of the WWTP simulation were measured after fusion with aqua regia. Aqua regia (5 mL) was added to samples for 24 h. Afterward they were poured into 50-mL poly vials and storage vials were rinsed twice with 10 mL aqua regia to prevent sorption to vials and therefore an understimation of Ag concentration (as observed in previous studies). Samples remained for 24 h without further treatment prior to filling up to 50 mL with purified water.

Silver concentrations in aqueous samples of digested soils were measured by inductively coupled plasma (ICP)–mass spectrometry (Agilent 7700, Agilent Technologies) set on isotope 107Ag in collision gas mode with helium. Samples of the digested influent and effluent as well as the extracted sludge samples were measured by ICP–optical emission spectrometry (Agilent 720, Agilent Technologies) at a wavelength 328.068 nm. All samples were measured in triplicate (internal triplicate measurement). Dilutions of certified standards were used for establishment of a calibration function. In both measurement series, quality control samples were measured to correct or to identify potential instrument drift.

The nitric acid was of Suprapur® quality (Carl Roth), and water was purified using an ELGA Pure Lab Ultra water purification system (puriﬁed water resistivity > 18 MΩ cm). A commercially available silver ICP standard containing 1000 mg/L Ag in 10% (v/v) nitric acid was used to prepare appropriate stock and calibration solutions. All prepared standard solutions had a final HNO3 concentration of 10% (v/v). The analytical method was verified using a multielement Merck IV standard.

TEM

The AgNPs in biosolid and soil–biosolid samples were investigated using TEM. Measurements were carried out on biosolid samples after the sewage sludge treatment (dewatering without flocculant) and on soil–biosolid samples after 140 d of incubation. The minimum concentration number for this method is addressed in the Supplemental Data (S2). Approximately 3 g fresh weight of each sample was freeze dried and stored under N2 atmosphere until further processing at the Swiss
Federal Institute of Aquatic Science and Technology (Dübendorf, Switzerland).

Samples were ground to a fine powder using an agate mortar and a pestle. Ten milligrams of biosolid or 100 mg of soil–biosolid powder were weighed into a 1.5-mL vial (polypropylene, Eppendorf), filled up to 1.5 mL with H$_2$O$_{deion}$ and sonicated with a VialTweeter sonotrode (6 × 10 s sonication/10 s pause; 6–7 W; ultrasonic processor UP200ST, Hielser). Samples were diluted 1:10 000 (biosolid) and 1:100 (soil–biosolid) in 1:10 steps, alternating with sonication between steps. Coarse sand particles in soil–biosolid samples quickly deposited on the bottom of the vials and were not included in further dilution steps.

The TEM grids (carbon-coated Cu grids, Quantifoil, or holey carbon Cu grids, Plano) were made hydrophilic using a glow discharge system (ELMO [10–1 mbar, 2.5 mA, 30 s], Cordouan Technologies), and particles from the final suspensions were deposited on grids by centrifugation and drop deposition. Suspensions were centrifuged with a swinging bucket rotor at 14 000 g, at 15 °C for 1 h. For the drop deposition, 5 drops of suspension were applied and then drawn through the grid using a paper tissue. After deposition, the TEM grids were washed with 3 drops of H$_2$O$_{deion}$ to prevent the formation of salt crystals. The samples were investigated with a scanning TEM (HD-2700-Cs, Hitachi). Recorded images were further processed using Fiji [22]. Elemental spectra of individual particles were recorded with a Digital Micrograph (Gatan) using an energy dispersive X-ray (EDX) system (EDAX) coupled to the microscope.

RESULTS

WWTP and sludge treatment

Characterization of starting material. The AgNPs were characterized using dynamic light scattering (DLS) and electron microscopy. The pristine NM-300K had a number-weighted mean hydrodynamic diameter of 62 ± 47 nm (polydispersity index = 0.511). The particle size calculated from TEM images using the image analysis software Fiji revealed a mean diameter of 14.3 ± 2.8 nm (n = 218; Supplemental Data, Figure S1). Smaller particles (<10 nm) were also occasionally observed, in agreement with the characterization reported by Klein et al. [19].

Mass balance of total Ag. Representative Ag concentrations analyzed in the influent of the WWTP units were in the range of 0.734 to 0.853 mg/L (Supplemental Data, Table S1). In the effluent, 0.015 to 0.043 mg/L was measured independent of the sulfate addition to the influent, corresponding to a mean removal of 96.2 ± 1.3% Ag in the WWTPs. In the control units the Ag concentrations were below the detection limit.

In the sludge samples Ag concentrations were approximately 6 μg Ag/g in the directly applied sludge and 7.8 μg Ag/g in the digested sludge. The control sludge had a concentration of less than 0.01 μg Ag/g. The controls were assumed to have the same amount of Ag and thus were analyzed representatively in the directly applied sludge without sulfur addition (Table 1).

Microbial degradation activity

To examine the effects of AgNP spiking on microbial activity in activated sludge, DOC elimination was determined. No effect on microbial activity as a result of the AgNPs or sulfate was observed in terms of DOC elimination. When the AgNP addition was initiated, the DOC elimination was between 96 and 97% in all WWTPs and remained constant (data not shown).

Sulfate-reducing bacteria

The presence of sulfate-reducing bacteria was determined at the beginning and end of the WWTP experiment and after anaerobic digestion (Supplemental Data, Figure S2). The community structure at the beginning of the WWTP experiment was altered after 10 d of wastewater treatment and after 35 d of anaerobic digestion. The initial bands disappeared and new ones emerged. Only the lowest DNA band, identified as Desulfovibrio sp. by sequencing, was present at all sampling dates. Differences between the various treatments (with vs without sulfate addition and control treatment vs AgNP treatment) were visible after the wastewater treatment and the digestion process.

Characterization of AgNPs in biosolids

The TEM images of AgNPs in biosolids after sludge treatment revealed no differences between the AgNPs in the differently treated sludges (direct treatment vs anaerobic digestion, with vs without sulfate addition). The AgNPs were evenly distributed and predominantly present as individual particles (Figure 1A) with a spherical shape and a mean

| Identification | Ag conc. in biosolids (mg/g) | Ag conc. in soil biosolid mixtures (mg/kg) |
|----------------|-----------------------------|------------------------------------------|
| DL/LOQ (μg/L)  | 0.00865/0.02595              | 0.00030/0.0009                           |
| DL/LOQ biosolid (mg/g) | 0.173/0.519                  | 0.00001/0.00003                          |
| Soil biosolid mixture (mg/kg) samples |                          |                                          |
| Pristine soil (C$_{soil}$) |                  | 0.05 ± 0.00                              |
| Pristine soil for the anaerobic approach (C$_{soil\_anaerobic}$) |               | 0.05 ± 0.00                              |
| Control (C) | Below DL*                   |                                           |
| Control with sulfur addition (C+S) | 0.10 ± 0.01             |                                           |
| Control, anaerobic digested (C$_{anaerobic}$) | n.d.                | 0.06 ± 0.00                              |
| Control with sulfur addition, anaerobically digested (C+S$_{anaerobic}$) | n.d.                | 0.08 ± 0.02                              |
| AgNP (AgNP) | 3.77*                      | 4.80 ± 0.10                               |
| AgNP with sulfur addition (AgNP+S) | 3.59*                | 5.18 ± 0.08                               |
| AgNP, anaerobic digested (AgNP$_{anaerobic}$) | 4.69*                | 8.34 ± 0.58                               |
| AgNP sulfur addition, anaerobically digested (AgNP+S$_{anaerobic}$) | 4.65*                | 7.93 ± 0.25                               |
| Trace metals sandy loam 9 CRM026-052; Ag 0.57 mg/kg |                  | 0.58 ± 0.01                              |

*Single determination because of limited amount of biosolids.

DL = detection limit; LOQ = limit of quantification; n.d. = not determined.
diameter of approximately 15 to 20 nm (Figure 2A). This closely matched the size and shape of the pristine AgNPs. In addition, smaller particles (<10 nm) were occasionally observed (Figure 2B). The EDX spectra of selected AgNPs always showed considerable S signal intensities, mostly with an Ag:S intensity ratio of 2:1 (Figure 2C), suggesting complete transformation of the AgNPs into Ag₂S-NPs.

Long-term incubation experiments in soils

Silver concentrations in pristine and in sludge-amended soils. As shown in Table 1, the pristine soil (C_soil) had a concentration of 0.05 mg Ag/kg dry matter. The Ag concentration in the control treatments (no AgNP addition) ranged from 0.06 mg Ag/kg dry matter in the control with sulfur addition (C+S) to 0.12 mg Ag/kg dry matter in the digested control with sulfur addition (C+San aerobic). Silver concentrations in the soils mixed with dewatered sludge (AgNP; AgNP+S) were approximately 5.00 ± 0.21 mg Ag/kg dry matter, and the Ag concentrations in soils mixed with digested sludge (AgNP anaerobic, AgNP+San aerobic) were 8.91 and 8.18 mg Ag/kg dry matter, respectively.

Characterization of AgNPs in soil–biosolid mixtures

During long-term incubation experiments the size distribution of the AgNPs became wider, and considerably larger AgNPs (~50 nm) were found. The EDX spectra of selected AgNPs again showed an Ag:S intensity ratio of 2:1, indicative of the formation of Ag₂S. Occasionally, we observed that the particles changed their morphology under the electron beam, possibly an indication for the formation of weakly crystalline or amorphous Ag-S phases in addition to the crystalline Ag₂S particles. In addition to the Ag₂S particles, AgCl particles and partly sulfidized particles with a metallic Ag fraction (Figure 3) were also detected. However, these particle types were observed considerably less often compared with the Ag₂S particles.

Based on the comparison between the secondary electron image, reflecting the topography of the sample and the high-angle annular dark-field image, reflecting a mass/thickness contrast, it can be concluded that the AgNPs were mostly incorporated into the soil organic matter rather than attached to its surface (Supplemental Data, Figure S3).

Potential activity of ammonium oxidation

The activity of potential ammonium oxidation measured after 60, 90, and 140 d is shown in Figure 4, and the calculated inhibitions (compared with the respective control treatments) derived from the activity data are given in Figure 5. The activity of the aerobic control treatments with and without sulfur addition (C+S, C) was similar and within the range of 97 h and 112 ng NO₂⁻-N/g dry matter/h (Figure 4). The activity of the 2 aerobic AgNP treatments (AgNP, AgNP+S) was slightly lower than the respective control treatments after 60 d. This activity was substantially decreased from approximately 100 ng NO₂⁻-N/g dry matter/h after 60 d to 49 ng NO₂⁻-N/g dry matter/h (AgNP) and 46 ng NO₂⁻-N/g dry matter/h (AgNP+S) after 140 d. The differences between the activities measured for the AgNP treatments and their control treatments were statistically significant. However, additional sulfur (+S) did not result in statistically significant differences compared with the experiments conducted without additional sulfur. A comparable trend was observed for the approach with anaerobically digested sludge. However, the microbial activities of the control treatments were at a lower level on day 60.

The activities measured in the anaerobic control treatments showed no differences between the 2 sulfur levels.
throughout the whole experiment. In addition, the data for anaerobic AgNP treatments indicated no difference between the 2 sulfur levels. After 60 d, the activity was 43 ng NO₂⁻-N/g dry matter/h (AgNP_{anaerobic}) and 38 ng NO₂⁻-N/g dry matter/h (AgNP + S_{anaerobic}) and was thus comparable to the respective control treatments. Afterward, NO₂⁻ production of AgNP_{anaerobic} increased up to approximately 60 ng NO₂⁻-N/g dry matter/h after 90 d but then sharply decreased to less than 10 ng NO₂⁻-N/g dry matter/h after 140 d. The activity of AgNP + S_{anaerobic} showed a comparable trend.

Ammonium oxidation in the AgNP and AgNP + S treatments was inhibited by less than 10% after 60 d of incubation (Figure 5). However, inhibition increased to approximately 30% after 90 d and reached almost 55% at the end of the experiment after 140 d. Inhibition of the ammonium oxidizing process in the anaerobic treatments (AgNP_{anaerobic}) increased from 13% after 60 d, (AgNP + S_{anaerobic} 30%) to more than 40% after 90 d and reached more than 90% after 140 d. Inhibition of the process in the anaerobic treatments was thus much stronger than the inhibition observed for the aerobic treatments.

DISCUSSION

Characterization of starting material

The size distribution of the AgNPs (NM-300K) was in agreement with the results reported by Klein et al. [19]. Thus, the results from the present study can be compared with other experiments using the same material. The larger particle sizes obtained from DLS measurements (62 ± 47 nm) compared with the mean size extracted from TEM images (14.3 ± 2.8 nm) are possibly because of a few agglomerates present in the suspension. This also explains the rather high polydispersity index (0.511) observed in DLS measurements. In addition, the substantial amounts of macromolecules (8% w/w) used as dispersant in the NM-300K suspension may have led to a considerable hydration shell around the particles, which is included in the DLS, but not in the TEM measurements.

WWTP and sludge treatment

Silver mass balance. The low influent concentration of approximately 80% of the nominal concentration corresponds to the observations of M. Hoppe (Federal Institute for Geosciences and Natural Resources, Hannover, Germany, personal communication), who found a recovery of 79% (n = 9) in studies on colloidal stability. These differences are probably because of losses of AgNP to the walls of the sample containers. Although the representatively measured influent concentrations differed slightly from each other, most likely because of the pumping systems, the total amount of stock suspension was delivered to both WWTPs (AgNP spike, AgNP + S spike) by the end of the experiment, because the complete amount of stock solution had been pumped into each WWTP.
Approximately 95% of the Ag was removed in the WWTP. These results are consistent with the findings of Shafer et al. [23], who found removal of Ag from the wastewater stream of 92 to 99%, and the findings of Kaegi et al. [24], who spiked AgNPs (NM-300K) to a pilot WWTP and found 5% of the total Ag in the effluent, 85% in the excess sludge, and 5% still in the WWTP (mass closure 95%).

Microbial degradation activity

No impact as a result of the AgNP addition on the elimination and degradation of DOC in the WWTP was observed. Even in previous experiments conducted with AgNP concentrations as high as 16 mg/L, DOC elimination was not affected (data not shown). However, DOC elimination provides no information on possible effects at the level of individual species, and this factor may thus have been overlooked. In experiments where the microbial community structure of sewage sludge was addressed, it was demonstrated that species involved in nitrogen removal like *Nitrosomonas* or *Chloroflexi* were inhibited by AgNPs (35-nm AgNP; 40 ppm), which had an impact on nitrogen removal and floc structure [25].

Sulfate-reducing bacteria

As obligate anaerobic bacteria, sulfate-reducing bacteria are ubiquitous in European sewage and soil habitats. The establishment of this bacterial group in the present experiment supports the assumption that the conditions were representative for WWTPs, that sulfidation of AgNMs was possible, and that consequently the results regarding fate and effect can be transferred to the environment. Although the various treatments show that the DNA of different sulfate-reducing bacteria groups was affected by the AgNPs and influenced by the sulfur amount, sulfate-reducing bacteria were present and active at all sampling dates, enabling the formation of Ag$_2$S. Despite the antimicrobial and growth-inhibitory properties of Ag$^+$ and AgNPs, we still observed emerging DNA bands indicating growth and thus activity of sulfate-reducing bacteria. In addition, the *Desulfovibrio* sp. band was observed at all sampling dates. This band represented DNA from an active sulfate-reducing bacteria group; otherwise the DNA would have been degraded rapidly by deoxyribonuclease [26] in the sewage environment.

Characterization of AgNPs in biosolids before application to soil

Essential for the sulfidation of AgNP to Ag$_2$S is the presence of sulfate-reducing bacteria and a sufficient amount of reducible sulfur. The efficient sulfidation of AgNPs in wastewater systems [10] and in a pilot WWTP [11,24] has been reported, and Ag$_2$S nanoparticles were found in sludge samples from a full-scale municipal WWTP [6]. We found predominantly sulfidized AgNPs in biosolids after all sludge treatments prior to the long-term incubation experiments. This is in agreement with results from previous studies, where the sulfidation of AgNPs in wastewater/sludge has been addressed [10,11,24,27,28]. A study addressing the transformation of 4 silver/silver chloride nanoparticles during anaerobic sludge treatment revealed that AgNPs transformed into Ag$_2$S during anaerobic digestion and Ag$_2$S remained stable for up to 6 mo during sludge postprocessing under oxidative conditions [29].

In the biosolids samples, sulfidized AgNPs showed a spherical morphology and had an average size comparable to the size of the pristine AgNPs (NM-300K). The similarity between the pristine and the transformed AgNPs thus indicated that the transformation process occurred via direct sulfidation, rather than via a dissolution–reprecipitation pathway. This correlates with the results of Kent et al. [30], who examined the sulfidation mechanism of AgNPs in a wastewater environment and reported a direct sulfidation of the AgNPs without substantial dissolution. It also correlates with the findings of Liu et al. [31], who determined direct oxy sulfidation as the dominant reaction at high sulfide concentrations in laboratory experiments.

Soil long-term incubation

**Total silver concentrations.** Compared with the directly treated biosolids and the subsequent biosol–soil mixtures, Ag concentrations increased after anaerobic digestion (Table 1) because of degradation of organic matter resulting in an accumulation of AgNPs in the sludge. The Ag concentrations in the digested biosolids and biosol–soil mixtures increased by factors of 1.3 and 1.8, respectively. These concentrations (~8.5 mg Ag/kg dry matter soil) were approximately a factor of 100 higher than the predicted environmental concentrations (PECs) of AgNPs in sludge-amended soils after 50 yr of permanent amendment [3]. Although the high test concentrations exceeded the PEC, this approach is considered to be justified, because only experiments with concentrations in the effect range of AgNPs reveal whether the sulfidation of these NPs really results in less toxic Ag$_2$S during wastewater treatment.

Characterization of AgNPs in soil–biosolid mixtures

The AgNPs were still spherical after 140 d of incubation and also the size was comparable to the pristine AgNPs, although we also observed considerably smaller particles as well as sulfidized AgNPs with diameters up to 50 nm. Both of these particle types were not found in the pure sludge samples, but particles <5 nm were present in the pristine material. Thus we assume that these particles, especially the larger ones, formed during incubation in the soil. The larger particles were often unstable under the electron beam and changed their morphology during imaging. A comparable behavior was observed in a study addressing the sulfidation kinetics of AgNP in the presence of humic acid [32]. The authors speculated that these radiation-sensitive so-called Ag$_2$S particles may represent a weakly crystalline–amorphous Ag–S phase, with different physical–chemical properties compared with their well-crystallized counterpart. Amorphous structures of particles were also detected using dark-field TEM after sulfidation of AgNPs in HS- and in a full-scale WWTP [30]. Levard et al. [7] reported that more than 60% of the sulfidized AgNPs were amorphous. The fraction of Ag-thiol and/or amorphous Ag–S of the total Ag in biosolids (anaerobically digested sludge) was approximately 24% and increased by up to 36% in biosolid-amended soil directly after the biosolids were mixed into soil [28]. This suggests that the sulfidation of AgNPs under realistic conditions results in the formation of a so-called amorphous Ag–S phase in addition to crystalline Ag$_2$S, which may exhibit a higher solubility than expected for crystalline Ag$_2$S and thus lead to a higher release rate of Ag$^+$ during the exposure experiments.

Donner et al. [33] reported a higher fraction of labile Ag (12–30%) in immediately archived biosolids stored for several years in glass bottles than in biosolids stockpiled under oxidative conditions (3–6%). These results indicate that a proportion of the labile Ag leached from the biosolids during stockpiling, leaving less soluble Ag$_2$S species behind.
Potential activity of ammonium oxidation

The different strengths of effects between the direct treatment (~55%) and the digested treatment (~90%) were the result of the different total Ag concentrations (~5 mg Ag/kg [direct treatment] vs ~9 mg Ag/kg [digested treatment]). Schlich et al. [15] conducted potential ammonium oxidation experiments with NM-300K with concentrations up to 9 mg Ag/kg dry matter applied via biosolids and observed almost complete inhibition at a concentration of 5.2 mg/kg after 140 d. The calculated effect concentration with 10% effect (EC10) values were 0.5 mg Ag/kg dry matter after 28 d for the pristine NM-300K (directly mixed into soil, without biosolid application). The EC10 values were thus in the same range as the EC10 values derived for effects of AgNO₃ on nitrification (OECD guideline 216) in an aeronsol soil with comparable properties [34]. Results from experiments conducted with pristine AgNPs and AgNPs applied via biosolids (10 d of WWTP simulation, biosolids direct applied) revealed comparable effects after an extended incubation time of 140 d [15]. The additional digestion procedure in the present study did not further reduce the bioavailability and toxicity of AgNPs for ammonium oxidation in long-term exposure experiments. This is very surprising, because various studies have demonstrated that the sulfidation of AgNPs leads to dramatically reduced toxicity [13], that Ag₂S is stable over extended periods [35], and that Ag₂S in stockpiled biosolids is essentially nonlabile [33]. However, at the same time, it is also reported that exposure to sulfidized AgNPs can still result in toxic effects. Levard et al. [13] demonstrated reduced but still existing toxicity to zebrafish, killifish, nematodes, and duckweed even after exposure to AgNPs with partial sulfidation. They explained the remaining toxicity by the fact that the Ag⁰⁰ fraction remained in the core of the particles. Effects of nanosized Ag₂S on nematodes (Caenorhabditis elegans) were considerably reduced compared with AgNPs and AgNO₃, but sulfidation did not result in complete detoxification of the AgNPs [14].

Judy et al. [36] investigated the effects of different Ag species on tomato plant growth, mycorrhiza fungi, and microbial community structure in biosolids-amended soil. The Ag₂S NPs were found to be less toxic than polyvinyl pyrrolidone (PVP)-coated AgNPs and Ag⁰⁰ to plants, fungi, and microbial communities. Although a dose–effect relationship was not observed for Ag₂S, there was still an impact at environmentally relevant application procedures even at low Ag concentrations (microbial biomass; 1 mg Ag/kg dry matter). Another study with sludge-amended soil at environmentally relevant concentrations revealed 4 times stronger impacts of at least partly sulfidized AgNPs on terrestrial plants and microbial communities than AgNO₃ [37]. Pradas del Real et al. [28] observed reduced root growth for monocot and dicot crops in pots filled with sludge-amended soil induced by transformed AgNPs (mainly Ag₂S and organic and/or amorphous Ag₅S). It was demonstrated that root exudates and certain biomolecules could also enhance dissolution of Ag and that trace levels may be sufficient to cause toxicity [38]. These studies demonstrated that the sulfidation of AgNPs does not completely detoxify the AgNPs, which is in agreement with the results of the present study showing effects of sulfidized AgNPs on ammonium oxidizing process.

The strong inhibitory effect on ammonium oxidation in the present study is surprising considering the nearly complete sulfidation of AgNPs during the wastewater treatment and the low solubility of crystalline Ag₂S. The following 3 reasons may explain the substantial inhibition of ammonium oxidation. First, there was still a small remaining fraction of Ag⁰⁰ that can easily release ions and AgCl, which is less stable than Ag₂S; both were observed in TEM analyses. Second, there was a large amount of small Ag₂S particles (<5 nm) possibly representing secondary particles (formed by a dissolution–precipitation process). Because of their size, the small particles are able to penetrate cells [39]. The particles could be oxidized by intracellular reactive oxygen species and may cause direct cell damage. Third, amorphous or labile so-called Ag-S species with possibly higher solubility compared with crystalline Ag₂S could cause the effects. Different studies have demonstrated a significant amount of amorphous structures or lability of sulfidized particles [7]. This was especially the case when the sulfidation took place under environmentally relevant conditions during wastewater or sludge treatment [28,30]; an impact on soil microorganisms, fungi [36], and root development [28] was also demonstrated. The changing morphology of sulfidized AgNPs observed under electron microscopy is in line with an amorphous structure of the sulfidized AgNPs.

CONCLUSIONS

Sulfidation of Ag is an environmentally relevant process in wastewater and sludge treatment leading to nearly complete sulfidation of AgNPs. However, the transformed AgNPs seem to be at least partially amorphous, resulting in so-called Ag₅S species with properties (e.g., solubility) different from those of the crystalline Ag₂S counterpart. The increased release of Ag ions from amorphous Ag₅S species may explain the impacts on ammonium oxidation observed after 90 and 140 d. Sludge treatment in an anaerobic digester and a surplus of sulfate did not result in reduced toxicity of the transformed AgNPs. Therefore, it can be concluded that treatment in WWTPs followed by anaerobic digestion does not necessarily result in nontoxic substances. Adverse effects of Ag₂S on soil microflora cannot be excluded over the long term. The present study demonstrated that detailed knowledge about the structure of transformed Ag species in biosolids is required to assess the potential risk related to the increased use of AgNPs. Short-term experiments, and experiments conducted with AgNPs transformed under artificial conditions, may therefore underestimate the toxicity of sulfidized AgNPs that were sulfidized under environmentally relevant conditions. Further experiments should provide more information as to why sulfidized AgNPs still release significant amounts of Ag⁺, whereas for Ag₂S, low or nontoxic properties have been summarized in a review [40]. Clarification is needed as to whether the ion release is typically just for AgNP sulfidized via WWTPs or whether chemically sulfidized AgNPs show the same effect.

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