Bioavailability of silver from wastewater and planktonic food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*

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HIGHLIGHTS

- WWTP effluents were used to test the bioavailability of AgNP for fish.
- Bioavailability of Ag from AgNP in *O. mykiss* is reduced after wastewater treatment.
- Gills play a crucial role regarding the uptake of Ag into fish tissue.
- No transfer of Ag through the intestinal walls into other tissues could be detected.

GRAPHICAL ABSTRACT
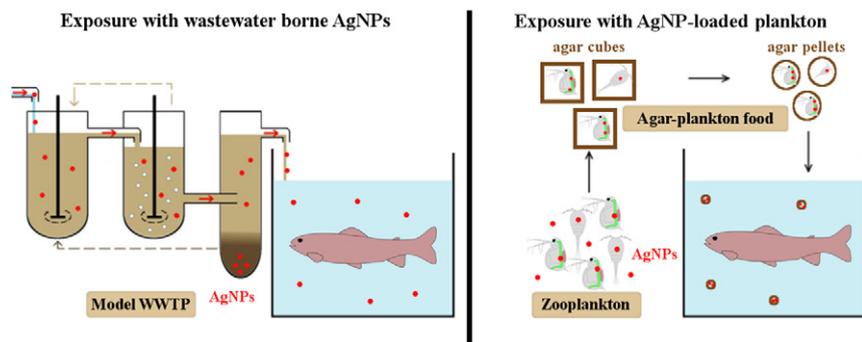
ARTICLE INFO

ABSTRACT

Silver nanoparticles (AgNPs) are present in a wide field of applications and consumer products and are likely to be released into the environment, mainly via urban and industrial sewage due to their extensive use. Even though AgNPs are mostly retained within the sludge of wastewater treatment plants (WWTPs), a small amount of mainly sulfidized particles still enters the aquatic environment, where they can be taken up by various aquatic organisms and transferred along the food chain. In this study, uptake and bioavailability of Ag from AgNPs following aqueous and dietary exposure were investigated in the rainbow trout *Oncorhynchus mykiss*. AgNPs in the effluent of model WWTPs and in tap water were used to perform aqueous exposure studies. No significant Ag uptake into the gills and carcass of the analyzed fish could be found for wastewater-borne AgNPs. However, when added to tap water at a concentration of $12.4\,\mu g\,L^{-1}$, a maximum total Ag tissue concentrations of around $100\,\mu g\,kg^{-1}$ and $50\,\mu g\,kg^{-1}$ in gills and carcass were measured, respectively. For the dietary exposure studies, freshwater zooplankton was exposed to AgNPs, and used for the preparation of food pellets with a total Ag concentration of $121.5\,\mu g\,kg^{-1}$. During the feeding study with rainbow trout significant total Ag concentrations up to $34.3\,\mu g\,kg^{-1}$ could be found in the digestive tract. However, only a limited transfer of Ag through the intestinal walls into the carcass could be detected. AgNPs in plankton and WWTP effluent were characterized by transmission electron micros- copy (TEM) in combination with energy dispersive X-ray spectroscopy (EDX) and found to be sulfidized. This

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transformation most presumably has led to their limited bioavailability for fish. The results emphasize the importance of realistic test conditions for the risk assessment of AgNPs by the use of environmental matrices.

1. Introduction

In the last decades, nanotechnology applications have entered nearly all fields of industry and production, creating new materials and concepts to improve processes in industries like water purification, agriculture, nanomedicine and energy storage (Roco et al., 2011). From the 1800 products which contained manufactured nanomaterials (MNMs) in 2018, silver nanoparticles (AgNPs) could be found in 443 of them (PEN, 2013). AgNPs are used in a wide field of products leading to an annual worldwide production volume of >10,000 t/year in 2010 (Piccinno et al., 2012). Due to their antimicrobial properties, AgNPs are predominantly used in textiles, cosmetics and coatings (Piccinno et al., 2012; Vance et al., 2015). During production, use and disposal of these products, MNMs can be released into the environment, e.g. by washing AgNP-containing clothes (Voelker et al., 2015). One of the major entry pathways of AgNPs into the environment is the release via wastewater treatment plant (WWTP) effluents (Kaegi et al., 2010; Sun et al., 2016). During sewage treatment, >90% of AgNPs adsorb to wastewater biosolids and have been shown to be transformed to Ag_2S within 2 h in a pilot WWTP (Kaegi et al., 2011). Although only a small fraction of the mostly transformed AgNPs leaves the WWTP, considerable AgNP concentrations of 0.7–11.1 ng L^{-1} could still be found in WWTP effluents (Li et al., 2016). Their discharge into receiving lake and river waters can lead to environmental concentrations which may have a significant impact on the aquatic life (Keller et al., 2013; Mueller and Nowack, 2008; Rozan et al., 1995). Gottschalk et al. (2009) predicted that the environmental concentrations of AgNPs in European surface waters are 0.76 ng L^{-1}. Peters et al. (2018) confirmed these findings by detecting average AgNP concentrations of 0.80 ng L^{-1} in Dutch surface waters. In general, the toxicity of metallic MNMs is driven by the release of ions, which is accompanied by physical damages (agglomeration, accumulation, attachment to organismic surface) caused by the physicochemical properties of the nanoparticles themselves (Asghari et al., 2012; Beer et al., 2012; Navarro et al., 2008). In fish, pristine AgNPs mostly affect the vitality of the organism via the gill tissue. Here, the nanoparticles agglomerate and adsorb to the tissue, leading to severe injuries, oxidative stress and may also release ions into the body via Na^+/K^+-ATPase ion channels in the gills and the blood circulatory system (Scown et al., 2010; Wood et al., 2004). However, in natural environments, several processes like sulfidation, dissolution, aggregation, adsorption and sedimentation influence the fate of AgNPs and may thus alter their effect on aquatic organisms (Furtado et al., 2016; Liu and Hurt, 2010). Even though sulfidized Ag species like Ag_2S show a reduced release of ions and are mostly resistant against oxidation and dissolution, they might still be bioavailable for aquatic organisms and thus potentially accumulate along the food chain (Azimzada et al., 2017; Choi et al., 2009; Kaegi et al., 2011; Kalman et al., 2015; Rozan et al., 2000). Kühr et al. (2018) assessed the chronic effects and bioavailability of AgNPs in the effluent from model WWTPs in the freshwater amphipod *Hyalella azteca*. Compared to pristine AgNPs supplied to tap water, wastewater borne AgNPs led to a reduced toxicity and bioaccumulation, which might be attributed to sulfidation or detoxification of the AgNPs by organic ligands like proteins or humic acids (Cedervall et al., 2007). Muth-Köhne et al. (2013) found an increased toxicity of AgNPs from WWTP effluents on fish embryos compared to pristine AgNPs and $AgNO_3$. Nevertheless, Bruneau et al. (2016) found no difference regarding the immunotoxicity of Ag^+ ions and AgNPs from wastewater effluents on the rainbow trout *Oncorhynchus mykiss*. Information on the bioavailability and fate of suspended MNMs in the aquatic

environment is still lacking but is fundamental for a detailed risk assessment. Once taken up or accumulated in the tissues of aquatic organisms, MNMs can be transferred across the food chain, an issue, which has been subject to little research so far (Cedervall et al., 2012; Chae and An, 2016; Skjolding et al., 2014; Wang and Wang, 2014; Zhu et al., 2010). Chae and An (2016) discovered that silver nanowires exhibit a significantly higher trophic transfer from algae, over daphnids to the zebrafish *Danio rerio* when the particles were smaller. This indicated that the size of the particles might be one of the main parameters facilitating the transfer along the food chain. Wang and Wang (2014) investigated the uptake of AgNPs by brine shrimp *Artemia salina* and the trophic transfer of AgNPs by feeding the AgNP-loaded shrimp to marine medaka *Oryzias melastigma*. Despite the low trophic transfer factors (TTF, Ag concentration in fish divided by Ag concentration in brine shrimp) of 0.009–0.011, a reduced growth and inhibition of Na^+/K^+-ATPase and superoxide dismutase activity could be detected, emphasizing the potential risk of AgNPs or Ag^+ released from them transferred across the aquatic food. Nevertheless, a comparison of the bioavailability of potentially transformed, wastewater-borne or planktonic food-borne AgNPs in fish is still missing. In a recent study, Vogt et al. (2019) analyzed the concentration of silver–containing nanoparticles in a prealpine lake that receives the discharge of a municipal WWTP. They did not find considerable AgNP levels neither in the WWTP effluent, in any of the measured lake water samples, nor in tissues from lake fish. However, Ag concentrations of 0.98 ± 0.62 μg g^{-1} were detected in the sediment of the lake in close proximity of the WWTP discharge point and also distributed throughout the lake, indicating a continuous sedimentation of low amounts of wastewater-borne Ag, that accumulates at the bottom of the lake (Vogt et al., 2019). From here, the sedimented Ag could be re-mobilized into the aquatic food chain by benthic organisms and a more detailed analysis of the bioavailability and trophic transfer of Ag in aquatic food webs is thus needed.

In this study, we investigated the uptake and bioavailability of Ag from AgNPs in the rainbow trout *Oncorhynchus mykiss* that are exposed via the water and their diet. Several model WWTPs were conducted according to OECD TG 303A to investigate the bioavailability of Ag from AgNPs in WWTP effluent compared to pristine particles suspended in tap water. To assess the bioavailability of MNMs contained in feed, fresh zooplankton was exposed to AgNPs. The AgNP-loaded plankton was embedded in agar cubes, cut into pellets and fed to *O. mykiss*. Following aqueous and dietary exposure, tissue samples of *O. mykiss* were analyzed for total Ag contents by quantitative inductively coupled plasma - mass spectrometry (ICP-MS). AgNPs in stock dispersion, WWTP effluents and AgNPs loaded in zooplankton were characterized by transmission electron microscopy (TEM) in combination with energy dispersive X-ray spectroscopy (EDX).

2. Material and methods

2.1. Ag nanomaterial characterization and stock dispersions

The aqueous silver nanomaterial dispersion NM-300K was used for the aqueous and dietary exposure studies and stipulated by the OECD repository of representative manufactured nanomaterials (Totaro et al., 2016). The nanoparticles have a main particle size of 15 nm with a narrow size distribution (99% <20 nm) according to TEM analyses and are dispersed by an aqueous matrix of 4 wt% each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan monolaurate (Tween® 20) (Klein et al., 2011). The nominal silver

concentration (10.16% w/w) and the particle number were shown to be stable for at least 12 months according to ICP-OES and UV-VIS analyses (Klein et al., 2011). The hydrodynamic diameter (DZ) of NM-300K was characterized in ultrapure water, copper ion-reduced tap water, pond water and WWTP influent by dynamic light scattering (DLS) using a zetasizer (Zetasizer Nano Series, Malvern Instruments Ltd., UK) (see Supporting Information).

The AgNP stock dispersion used for the production of WWTP effluents was prepared by adding 8 mL of ultrapure water to glass vials containing 2 mL NM-300K dispersion, resulting in a total Ag concentration of 20 g L^{-1}. The vials were thoroughly shaken by hand for 1 min and sonicated for 15 min in a sonication bath (160 W, Sonorex Super RK 510, Bandelin electronic GmbH & Co. KG, Germany). Subsequently, 15 mL of the dispersions were added to a polyethylene container with 6 L of ultrapure water to achieve a nominal Ag concentration of 0.05 g L^{-1} in the stock solution.

AgNP stock solutions for the preparation of the spiked test media applied in the aqueous exposure study were prepared by adding 300 µL NM-300K dispersion to a 50 mL polypropylene vial with 30 mL ultrapure water. The resulting dispersions (1 g Ag L^{-1}) were thoroughly shaken by hand and sonicated for 10 min (2 min effective, 200 W, pulsation pause ratio of 0.2/0.8) by indirect probe sonication (Cup Horn BB6, Bandelin electronic GmbH & Co. KG, Germany).

2.2. Model WWTPs

Two lab-scale WWTP units (behrotest® Laboratory Sewage Plant KLD 4 N, behr Labor Technik GmbH, Düsseldorf, Germany) were conducted according to OECD Guideline 303A (OECD, 2001) to simulate the transformation of nanomaterials during sewage treatment. A single WWTP unit (total volume 10 L) consists of a non-aerated reactor, an aerated reactor and a secondary clarifier to simulate denitrification, nitrification and sedimentation processes of a full-scale WWTP (see Supporting Information, Fig. S1). The units were conducted as previously described (Kampe et al., 2018; Kühr et al., 2018) and initially fed with activated sludge (2.5 g dry mass L^{-1}) from a municipal WWTP in Schmallenberg, Germany (51°09′N 8°16′E). The sludge inoculum was sieved (≤2 mm) on the first day of the study. The dry matter of the sludge was determined with a moisture analyzer (HB43-S, Mettler Toledo, USA) and did not exceed 3.0 g dry mass L^{-1} during the study. Both units were operated in a temperature controlled room (20–25 °C) and continually fed with artificial wastewater (30 mg L^{-1} urea, 28 mg L^{-1} K_2HPO_4, 7 mg L^{-1} NaCl, 4 mg L^{-1} $CaCl_2 2H_2O$, 2 mg L^{-1} $MgSO_4 7H_2O$, meat extract, peptone) according to OECD Guideline 303A (OECD, 2001). In contrast to the guideline, the concentrations of peptone and meat extract in the artificial wastewater were raised to 150 and 180 mg L^{-1}, respectively, in order to avoid the formation of bulking sludge. The stock solutions of artificial wastewater and the AgNP dispersions (both prepared 10-fold concentrated) were freshly prepared every 3–4 days and stored at 4 °C. Via a tube system (PLP 33; SP04/3.5 K, behr Labor-Technik, Germany), wastewater and AgNP dispersions were diluted 1:10 with tap water and pumped into the denitrification reactor with a continuous flow of 750 mL h^{-1} (total influent volume of 288 L during the study), leading to a retention time of 6 h within the WWTP units. One WWTP unit was running as control and one unit was exposed to AgNPs with a concentration of 5 mg L^{-1} via the influent. An adaptation phase without the addition of AgNPs lasting six days was carried out until processes within the WWTP units reached stable conditions which were characterized by a dissolved organic carbon (DOC, measured with TOC-V CPH Total Carbon Analyzer, Shimadzu, Japan) elimination rate >80% and constant concentrations of ammonium, nitrite and nitrate in the effluent (OECD, 2001). After this phase, AgNPs were added to the WWTP influent for a total of 6 days. After four days, significant Ag concentrations could be found in the effluent. From this day on, the effluent was collected for 40 h and stored as one composite sample per treatment (one effluent sample containing AgNPs, one with control effluent) in a 30 L polyethylene (PE) container (Züchner GmbH, Köln, Germany) in the dark at 4 °C for 4 days. During the WWTP study, concentrations of ammonium, nitrite and nitrate in the effluents were measured photometrically (NANOCOLOR® 500D, Macherey-Nagel, Germany) at least once per week. The pH values in the denitrification (7.66 ± 0.06 and 7.59 ± 0.05) and the nitrification reactor (7.34 ± 0.17 and 7.26 ± 0.11) of the WWTPs without and with AgNPs, respectively, were measured daily. The oxygen concentration in the nitrification reactor was monitored and kept in the range between 2 and 4.5 mg L^{-1}.

2.3. Exposure studies with O. mykiss

2.3.1. Fish maintenance

Juvenile rainbow trout (Oncorhynchus mykiss) were obtained from Fischzucht Störk (Bad Saulgau, Germany) and maintained in a flow-through system in 200 to 250 L tanks with constant aeration at 14 ± 2 °C. Fish were kept in copper ion-reduced tap water (for physicochemical properties see Supporting Information, Table S2) under a 16:8 light-dark cycle until the start of the study. The fish were fed with a commercially available food for fish breeding (Inicio Plus®, BioMar, Denmark).

2.3.2. Aqueous exposure study

The aqueous exposure study with O. mykiss was performed according to OECD guideline 305 (OECD, 2012a). Juvenile rainbow trout (40 fishes per tank, 40 L medium, 1 tank per treatment) were exposed over 14 days (uptake phase) to (i) effluent from model WWTP with AgNP-treated influent (see Section 2.2), (ii) effluent from control WWTP manually spiked with pristine AgNPs and (iii) dilution water spiked with pristine AgNPs. Copper ion-reduced tap water was used as dilution water (see Supporting Information, Table S2). All tested WWTP effluents were 10-fold diluted with dilution water to simulate the dilution effect of the receiving waters of a full-scale WWTP (for physicochemical properties of undiluted WWTP effluents see Supporting Information, Table S2). Based on the measured total Ag concentrations of 14.7 µg L^{-1} in the diluted WWTP effluent, the nominal test concentrations in all treatments was set to 15 µg L^{-1}. Following the uptake phase, the test organisms were transferred to new tanks containing dilution water without any test item. The depuration phase lasted 14 days. A control group was kept in dilution water over the entire experimental period of 28 days. The study was performed in a semistatic approach (change of media at 48 h intervals) and the fish were fed daily with commercially available fish food (Inicio Plus®, pellet size 1.1 mm, BioMar, Denmark) at a rate corresponding to 1.5% body weight per day, according to the OECD guideline 305 (OECD, 2012a). During the entire study the fish were kept under a 16:8 light-dark cycle and all tanks were aerated continuously (oxygen saturation >60%). The vitality of the organisms, the water conditions [concentrations of ammonium (0.1–4.7 mg L^{-1}), nitrate (18–22 mg L^{-1}) and nitrite (0.0–1.4 mg L^{-1}), pH (7.4–8.5) and water temperature (14 ± 2 °C)] were monitored periodically. Prior to the test start, an adaptation period of 14 days was performed to verify that the test animals were in good condition. Before the start of the test, feeding was suspended for 24 h. At test start, two times 40 animals were randomly distributed to the experimental tanks. The mean wet weight of 20 fish, which were randomly selected from each tank was measured (4.6 ± 1.3 g). During the complete test period, groups of 4 animals were taken periodically from each treatment (day 0, 2, 6, 10, 12, 14, 15, 17, 20, 23, 27) and euthanized with 5 g L^{-1} Chlorobutanol (Merck, Germany) followed by the dislocation of the neck according to the directive of the EU on animal welfare (EU, 2010). Subsequently, the digestive tract, gills (from day 6 on) and carcass were dissected on ice, snap frozen in liquid nitrogen and stored at −20 °C until further processing. On day 0, the digestive tract was rinsed with ultrapure water to exclude possible contaminations by the fish food.

2.3.3. Preparation of plankton food

Stoten_135695Fresh zooplankton was chosen as feed component within the dietary exposure study to simulate the natural freshwater food chain. The plankton was collected in a natural pond at the fish farm "Fischzucht Riegger" in Ettenheim, Germany (48°26′N 7°83′E) in a rural environment close to the Black Forrest. The collection was done by a rope winching device, which pulled a plankton net (200 µm) in parallel lines through the water. The condensed zooplankton was transferred to 30 L PE containers filled with 25 L pond water (see Fig. 1). Subsequently, 7.5 µL NM-300K were added to the containers to achieve a nominal Ag concentration of 30 µg L^{-1}. The exposure concentration of 30 µg L^{-1} was chosen to ensure a sufficient uptake of silver by the plankton organisms. Exposure concentrations in the range of 4–64 µg L^{-1} were used by Vincent et al. (2017), who exposed natural plankton communities with differently coated AgNPs. Additional containers without the addition of AgNPs were used as a control. After gentle stirring of the test containers, live zooplankton was exposed for 5 h with hourly measurements of the oxygen concentration. Subsequently, the plankton was drained by pouring the previously exposed samples through a plankton net (mesh size 65 µm, Conatex Lehrmittel, Germany), transferred into 6 L freezer bags (QuickPack, Germany) and frozen at −20 °C.

Control and AgNP-loaded plankton were embedded into agar pellets, to achieve an experimental diet suitable for fish that still preserves the natural plankton structure (see Fig. 1). The homogenous distribution of the test substance and the sufficient stability of the agar pellets avoiding the release of the test substance into the surrounding water were confirmed in a preliminary study, where the agar pellets were added to 500 mL dilution water and kept under static conditions for 7 days in the absence of fish. No significant total Ag concentrations (below background of 0.04 µg L^{-1}) were found in the surrounding medium (see Supporting Information, Table S3). Agar pellets (5 replicates, 200 mg each) were analyzed for total Ag content. The results show that total Ag concentrations (121.5 ± 0.1 µg kg^{-1}) were homogenously distributed in the experimental diet. For the preparation of the agar pellets, the frozen plankton was lyophilized (Alpha 1–2 LDplus, Christ, Germany). Afterwards, 250 mg agar (Agar-Agar, Kobe I, Carl Roth GmbH, Germany) were dissolved in 15 mL ultrapure water under heating in a microwave, mixed with 600 mg dried plankton and 800 mg ground fish food pellets (Inicio Plus®, Biomar, Denmark) and poured finally into small cube moulds (1 cm^3 each cube, silicon mould, Belmalia, Germany). The cubes were dried overnight and pressed through a nylon net (mesh size 3 mm) to achieve small pellets of a suitable size to be eaten by juvenile fish. A pre-test with rainbow trout was carried out to exclude toxic effects induced by the AgNP loaded plankton food pellets.

2.3.4. Characterization of plankton and food pellets

The species composition of the collected zooplankton was analyzed using an identification key (Streble et al., 2002). Subsamples of the frozen plankton plates were carefully defrosted and species were determined microscopically. The zooplankton consisted of 65% Copepoda (44% Cyclopoida, 20% Calanoida, 1% Nauplius larva) and 35% Branchiopoda (24% Bosmina longirostris, 11% Daphnia spec.). All of the identified species and taxa were also detected when analyzing the food pellets clearly showing that the integrity of the plankton material was maintained throughout the feed preparation process.

The nutritional value of the previously described plankton food pellets was determined by quantitative analysis of the lipid according to Smedes (1999). For lipid extraction, pellets (50 mg) were transferred to glass vials, mixed with 200 µL extraction solution I (55% cyclohexane/ 44% isopropyl alcohol (v/v)) and homogenized (Potter homogenizer, B. Braun, Germany). Subsequently, 2.75 mL ultrapure water were added, the solution was centrifuged (12 min, 543 ×g, Mega Star 1.6R, VWR, USA) and the organic phase was transferred to glass vessels (previously stored over night at 65 °C and weighed). The remaining aqueous phase was mixed with 2.5 mL extraction solution II (87% cyclohexane/ 13% isopropyl alcohol (v/v)), centrifuged again (same conditions) and the resulting organic phase was combined with the organic phase from the first extraction step. The extract was dried with nitrogen gas, stored over night at 65 °C and weighed to calculate the lipid content.

The total lipid of the plankton food pellets (dry weight) was 3.0 ± 0.4%.

2.3.5. Dietary exposure study with O. mykiss

The bioavailability of AgNPs contained in fish feed by O. mykiss was assessed by performing a dietary exposure study following the principles of OECD guideline 305 (OECD, 2012a). Since the lipid content of the plankton pellets (3.0 ± 0.4%) was 5-fold lower than in commercially available fish food (17.5 ± 0.2%, Inicio plus®, Biomar, Denmark), the daily ration was raised from 1.5% (as recommended by OECD guideline 305) to 7.8% of fish body wet weight. The feeding was performed once per day and the pellets were eaten immediately. Feces and potential feed residuals were removed 1 h after feeding by vacuuming through a glass tube. Juvenile rainbow trout (45 fish per tank, 75 L medium, 1 tank per treatment) were exposed over 14 days (uptake phase) to AgNP via the diet. A control group was fed with unfortified plankton pellets under otherwise identical experimental conditions. After the uptake phase, the test organisms in all tanks were fed with control pellets for another 14 days (depuration phase). The study was performed under flow-through conditions with a flow rate of 15.6 L h^{-1} equivalent to a 5-fold water renewal per day. The experimental conditions within the test system such as oxygen saturation (>60%), concentrations of
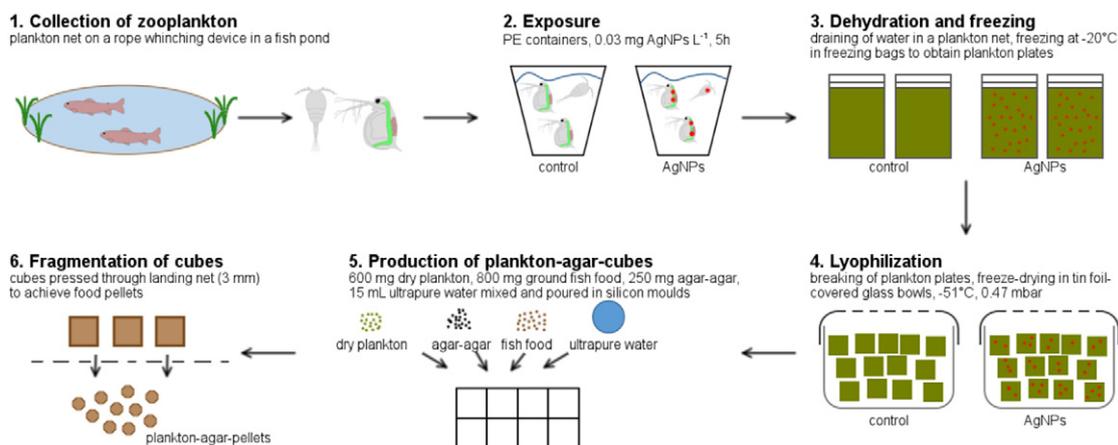

Fig. 1. Scheme of the production of plankton-agar-pellets. Zooplankton was collected in a fish pond and exposed to AgNPs in PE containers, 5 h. After freezing and freeze-drying, plankton was embedded in agar matrix enriched with commercial fish food. Natural structure of plankton organisms was maintained during processing.

nitrate (8–10 mg L^{-1}), nitrite (<0.01 mg L^{-1}) and ammonium (0.01–0.03 mg L^{-1}), pH (7.1–7.8) and water temperature (14 ± 2 °C) were monitored periodically. During the test, groups of 5 fish were taken periodically (day 0, 7, 14, 15, 17, 19, 22, 25, 28) from each treatment and euthanized as described in Section 2.3.2. Similar to the method described in Section 2.3.2, the digestive tract and carcass (all body parts apart from the digestive tract) of the fish were dissected on ice, snap frozen in liquid nitrogen and stored at −20 °C until further processing. Additionally, groups of 3 fish were taken from each treatment (on day 0, and on day 28) to analyze the lipid content of the fish (Smedes, 1999).

2.4. Transmission electron microscopy

The AgNPs from WWTP effluents used in this study were extracted by cloud-point extraction and characterized by scanning transmission electron microscopy (STEM) in combination with high-angle annular dark-field (HAADF) and energy-dispersive x-ray (EDX) detectors as part of a parallel study carried out by Hartmann et al. (2019). AgNPs in the NM-300K stock dispersion and the AgNP-loaded, lyophilized zooplankton were characterized by TEM and the elemental composition of selected NP was assessed using an EDX system attached to the microscope. The stock dispersions (NM-300K) were diluted 1:10⁶ in deionized water and 1 mL of this was directly centrifuged (1 h, ~14,000 ×g) on TEM grids. For the plankton spiked with NM-300K, 30 mg of freeze dried material was added to 1 mL of 0.2% FL-70 (Thermo Fisher Scientific, USA) and sonicated for 1 min in a Vial Tweeter (Hielscher Ultrasonics GmbH, Germany). The resulting dispersion was diluted 1:1 in deionized water and directly centrifuged on TEM grids using the same conditions as for NM-300K. As the AgNP carried a negative surface charge, the TEM grids were functionalized with Poly-L-Lysine (PLL, 0.1% (w/v) in H_2O, Sigma Aldrich) to enhance AgNP deposition on the TEM grids. The preparation of the TEM grids is described in more detail in Uusimaekia et al. (2019). The TEM grid was investigated using a dedicated scanning transmission electron microscope (STEM, HD2700Cs, Hitachi, Japan), operated at an acceleration voltage of 200 kV. For image formation the HAADF signal was used. Elemental analyses were conducted using an EDX system (EDAX, USA) and the spectra were recorded and processed using Digital Micrograph (v.1.85, Gatan Inc., USA).

2.5. Determination of Ag concentrations

2.5.1. Preparation of aqueous samples

During generation of WWTP effluents and the course of the aqueous exposure study, aliquots of the media were sampled periodically for determination of total silver concentrations. For WWTP studies, aliquots were taken daily in duplicate from the effluent following the addition of AgNPs to the influent of the WWTPs. Effluent samples were taken for 4 days and analyzed for total Ag content until stable Ag concentrations were found in the effluent. Over the course of the aqueous exposure study with *O. mykiss*, water samples were collected from fresh (0 h) and aged media (24 h and 48 h old) at least twice per week and within the dietary exposure studies at the beginning and the end of uptake and depuration phase to exclude that leaching of test item into the surrounding medium occurred. For total silver analysis, 5 mL of the aqueous sample were transferred to 15 mL vials and mixed with 5 mL *aqua regia* and 5 mL ultrapure water. Nitric acid (69%, Suprapur®, Carl Roth, Germany) and hydrochloric acid (30%, Suprapur®, Baker, The Netherlands) were mixed 1:3 for the preparation of *aqua regia*. Water was purified using an ELGA Pure Lab Ultra water purification system (>18 MΩcm).

2.5.2. Preparation of tissue samples

Carcass samples, taken during the exposure studies with rainbow trout, were ground manually in a zirconium oxide mortar under constant cooling by liquid nitrogen followed by lyophilisation (Alpha 1–2 LDplus, Christ, Germany). Digestive tract and gill samples were not ground since they were small enough to be processed completely. Subsamples of 200 mg ground carcass as well as digestive tract and gill samples were mixed with 5 mL of nitric acid (69%) and digested in a microwave (UltraClave II, MLS GmbH, Germany, 25 min heating up to 220 °C, 30 min on 220 °C, 95 min cooling, max pressure 80 bar). Further, a certified reference material standard (Oyster tissue NIST® SRM® 1566b, Merck, Darmstadt, Germany) was treated equally to validate the digestion process. Plankton and food samples were prepared identically but were mixed with 8 mL *aqua regia* instead of nitric acid. After sample digestion, samples were filled up to 15 mL with ultrapure water.

2.5.3. Quantitative silver analysis by ICP-MS

The measurement of total silver concentrations in the previously prepared media and tissue samples was performed by using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700, Agilent Technologies, Waldbronn, Germany) set on isotope ^{107}Ag in collision gas mode with helium. In order to detect and compensate for instrument drifts, a rhodium standard (Merck KGaA; Certipur®) was used. The matrix adjusted calibration standards were prepared with commercially available silver ICP standard solutions (Merck Certipur® 1000 mg L^{-1} Ag in 3% (v/v) nitric acid, Merck, Darmstadt, Germany). The calibration function as well as the limit of detection (LOD) were calculated by the ICP-MS software (Agilent MassHunter workstation) using a linear regression. The limit of quantification (LOQ) was calculated as three times the LOD. In all measurement series, all samples were measured in triplicate (internal triplicate measurement). Further, certified aqueous reference materials (TMDA 70.2, Environment Canada) and quality control samples were prepared independently from the calibration samples (e.g. from multielement standard Merck IV, Merck, Darmstadt, Germany) and measured in the same measurement series for validation.

2.6. Statistical analysis

All statistical analyses were performed with OriginPro 2017 (OriginLab Corp., Northampton, MA, USA). The α-level for all tests was set to 0.05 (Zar, 2009). All data sets with at least 4 replicates were checked for outliers by using the Grubb's test (SQS 2013 Version 1.00 by J. Klein and G. Wachter). Time weighted average concentrations (TWA) of Ag in test media of the different treatments were calculated according to OECD 211 (OECD, 2012b) for the aqueous exposure studies with *O. mykiss*.

3. Results

3.1. Total Ag concentrations in test media and plankton food

The total Ag concentrations measured in the test media and in the plankton and food samples are shown in Table 1. For the studies with the model WWTPs, AgNP stock dispersions of 50 mg L^{-1} were prepared and diluted 10-fold before being added to the WWTP via the influent, as described previously (see Section 2.3.). An Ag concentration of 202.6 µg L^{-1} was measured in the effluent, which was collected 4 days after the initiation of AgNP dosing to the influent. After a storage time of 4 days, the effluents were 10-fold diluted before they were used in the aqueous exposure studies, resulting in a time weighted average Ag concentration of 14.8 µg L^{-1}. Control effluents and dilution water which were manually supplemented with pristine AgNPs showed total Ag concentrations of 16.3 µg L^{-1} and 12.4 µg L^{-1}, respectively.

Live zooplankton was exposed to AgNP dispersions of 29.8 ± 0.3 µg L^{-1} (Table 1). The lyophilized plankton showed a homogenous Ag concentration of 1639.6 ± 43.3 µg $kg^{-1}$and was used to produce agar-plankton pellets with a total Ag concentration of 121.5 ± 0.1 µg kg^{-1}. The food pellets contained agar, plankton and commercial fish food. Since it turned out that the commercial food itself contained

Table 1

Composition of test media and food. Aqueous samples were measured in duplicate; plankton and food samples were measured in quintuplicate. TWA = time weighted average Ag concentration. LOQ < 7 ng L^{-1}.

Study	Matrix	TWA	
Aqueous exposure study with O. mykiss	Effluents from MNM treated WWTPs	14.8 μg L^{-1}	
	WWTP effluent supplemented with MNMs	16.3 μg L^{-1}	
	Dilution water supplemented with MNMs	12.4 μg L^{-1}	
	Control effluent without MNMs	0.0 μg L^{-1}	
Study	Matrix	Total Ag	St. Dev.
Preparation of plankton food	Exposure medium	29.8 μg L^{-1}	0.3 μg L^{-1}
	Lyophilized plankton	1639.6 μg kg^{-1}	43.3 μg kg^{-1}
Dietary exposure study with O. mykiss	Plankton food pellets	121.5 μg kg^{-1}	0.1 μg kg^{-1}
	Control plankton food pellets	14.7 μg kg^{-1}	1.0 μg kg^{-1}
	Commercial fish food	37.3 μg kg^{-1}	10.9 μg kg^{-1}

a total Ag concentration of 37.3 ± 10.9 μg kg^{-1}, Ag was also found in the control plankton food pellets (14.7 ± 1.0 μg kg^{-1}) which were fed to the fish during the second half of the study and to the control group during the entire study.

During the dietary exposure study, water samples taken from the experimental tank were measured for total Ag concentrations to check for AgNPs or Ag$^+$ species that might have leached from the fortified food pellets. However, no significant Ag concentrations (>LOQ) were found.

3.2. DLS and TEM results

The results of the DLS measurements of AgNPs in different media (ultrapure water, dilution water, pond water, WWTP influent) are shown in the Supporting Information, Table S1. The DZ of the particles was in the range of 30–60 nm when high AgNP concentrations (100 or 1000 μg L^{-1}) were dispersed in the media. When low Ag concentrations (1 and 10 μg L^{-1}) were used, the DZ increased to very high values (max. 1017 nm). These exorbitant values were most presumably caused by the low quality of the measurement at low concentrations as it is shown by the high attenuator values (9–11).

The AgNPs in the stock dispersion were well dispersed on the TEM grids (Fig. 2A). A particle size distribution was derived from multiple images using the NanoDefine-Particle sizer (Wagner, 2016) and revealed a mean particle size of 18.3 nm with a standard deviation (1σ) of 3.1 nm (Fig. 2A, inset). Elemental analyses of individual particles showed a dominant signal of Ag (Lα), next to Cu (Kα) which was related to signals resulting from the Cu grid (Fig. 2B).

STEM analyses of AgNPs from WWTP effluents showed that the particle size was not changed during the passage of the WWTP, while the EDX revealed an association of wastewater-borne AgNPs with sulfur, indicating their sulfidation (see Hartmann et al., 2019).

By using high-angle annular dark field (HAADF) images of the plankton samples, AgNPs appeared as bright spots incorporated in a light grey fuzzy matrix representing the planktonic matrix (Fig. 2C). The low Ag concentration in the plankton and thus the rather low particle number concentration of the AgNPs did not allow the establishment of a particle size distribution of the AgNPs. However, individual AgNPs that were detected in the planktonic matrix were always of a very comparable size to the particles observed in the NM-300K stock dispersion. Elemental analyses of individual AgNPs, however, always revealed a considerable sulfur (S) signal intensity, next to the Ag signal, suggesting that the AgNPs were sulfidized to a large extent (Fig. 2D).

3.3. Aqueous exposure of O. mykiss

During the aqueous exposure study no mortalities or diseases were observed. The mean wet weight of the rainbow trout in the control group increased during the study by 51% (30% during uptake phase, 16% during depuration phase) from 4.7 ± 1.0 g on day 0 to 7.1 ± 2.2 g on day 28. The evolution of total Ag concentrations measured over the uptake and elimination period in carcass, gills and digestive tract are shown in Fig. 3. At test start, fish were sampled to assess the background Ag concentration in the investigated tissues. For carcass and gut the concentrations were found to be 2.4 ± 0.4 μg kg^{-1} and 11.6 ± 1.5 μg kg^{-1}, respectively (Fig. 3A). The initial total Ag concentrations measured in gill tissues were below LOQ.

The carcass of the fish (whole fish without gut and gills) showed no significant uptake of Ag following exposure to AgNPs from WWTP effluents. The highest total Ag concentration in this treatment of 4.0 ± 1.1 μg kg^{-1} was found on day 14. Similarly, the exposure to AgNPs supplemented to control effluents resulted in a non-significant increase of the carcass burden to a level not higher than 7.5 ± 1.3 μg kg^{-1} (day 14) within the uptake phase of the study. In contrast, significant Ag concentrations of 9.1 ± 1.0 μg kg^{-1} and 10.7 ± 6.5 μg kg^{-1} were detected during the course of the depuration phase on day 17 and 20, respectively. Afterwards, the total Ag concentrations decreased to 3.8 ± 1.1 μg kg^{-1} on day 28. The highest Ag concentrations in the carcass of the tested fishes were found after exposure to AgNPs supplemented to dilution water. During the uptake phase, a significant Ag uptake could be detected on days 10, 12 and 14 with 18.7 ± 1.6 μg kg^{-1}, 23.9 ± 3.9 μg kg^{-1} and 40.0 ± 3.6 μg kg^{-1}, respectively. At the beginning of the depuration phase, this tendency continued until day 17 (45.7 ± 6.6 μg kg^{-1}), followed by a steady decrease of Ag concentrations until day 28 (18.2 ± 0.9 μg kg^{-1}). A complete depuration of previously accumulated Ag (decrease to the initial value measured on day 0) was not observed until the end of the test period. The total Ag concentration in the carcass of the control group fish slightly decreased from day 0 (2.4 ± 0.4 μg kg^{-1}) to day 28 (1.0 ± 0.0 μg kg^{-1}).

The gill tissue was analyzed separately (Fig. 3B). After exposure to AgNPs present in or added to WWTP effluent, the gills of both treatments exhibited Ag concentrations of 13.4 ± 7.4 μg kg^{-1} (day 12) and 10.6 ± 3.0 μg kg^{-1} (day 14) towards the end of the uptake phase, followed by a decrease to 1.6 ± 0.5 μg kg^{-1} and 2.2 ± 0.7 μg kg^{-1} after 13 days of depuration, respectively. In contrast, Ag concentrations in the gill tissues of fish exposed to AgNPs supplemented to dilution water were 10.5 ± 2.7 μg kg^{-1} on day 6, increased significantly to 90.9 ± 17.5 μg kg^{-1} on day 12 and remained stable at that level until day 20 (98.9 ± 14.4 μg kg^{-1}) after 6 days of depuration. The tissue concentrations decreased to 56.9 ± 14.5 μg kg^{-1} until the end of the depuration phase and did not reach the initial tissue concentration measured on day 0. The Ag concentrations in gills of the control group animals stayed on a very low level (2.4 ± 1.5 μg kg^{-1}, 0.2 ± 0.2 μg kg^{-1} and 0.3 ± 0.1 μg kg^{-1} on days 6, 14 and 28, respectively).

In the gut tissue (Fig. 3C), significantly elevated Ag levels were detected during the uptake phase reaching total Ag concentrations of 27.4 ± 10.6 μg kg^{-1}, 46.7 ± 17.0 μg kg^{-1} and 69.7 ± 27.8 μg kg^{-1} following exposure to AgNPs from WWTP effluents, AgNPs supplemented to control effluents and AgNPs supplemented to dilution water, respectively. During the depuration phase the Ag concentration decreased in all matrices (Ag concentrations on day 28 were 10.8 ± 1.7 μg kg^{-1}, 11.3 ± 2.1 μg kg^{-1}, 30.1 ± 4.8 μg kg^{-1} for WWTP effluent, supplemented effluent and supplemented dilution water, respectively). The measured Ag concentrations in the digestive tract of the organisms were generally higher than the measured Ag concentrations in the carcass. The control group animals displayed constantly low Ag concentrations (8.2 ± 2.8 μg kg^{-1}, 7.1 ± 3.2 μg kg^{-1} and 8.7 ± 1.9 μg kg^{-1} on days 6, 14
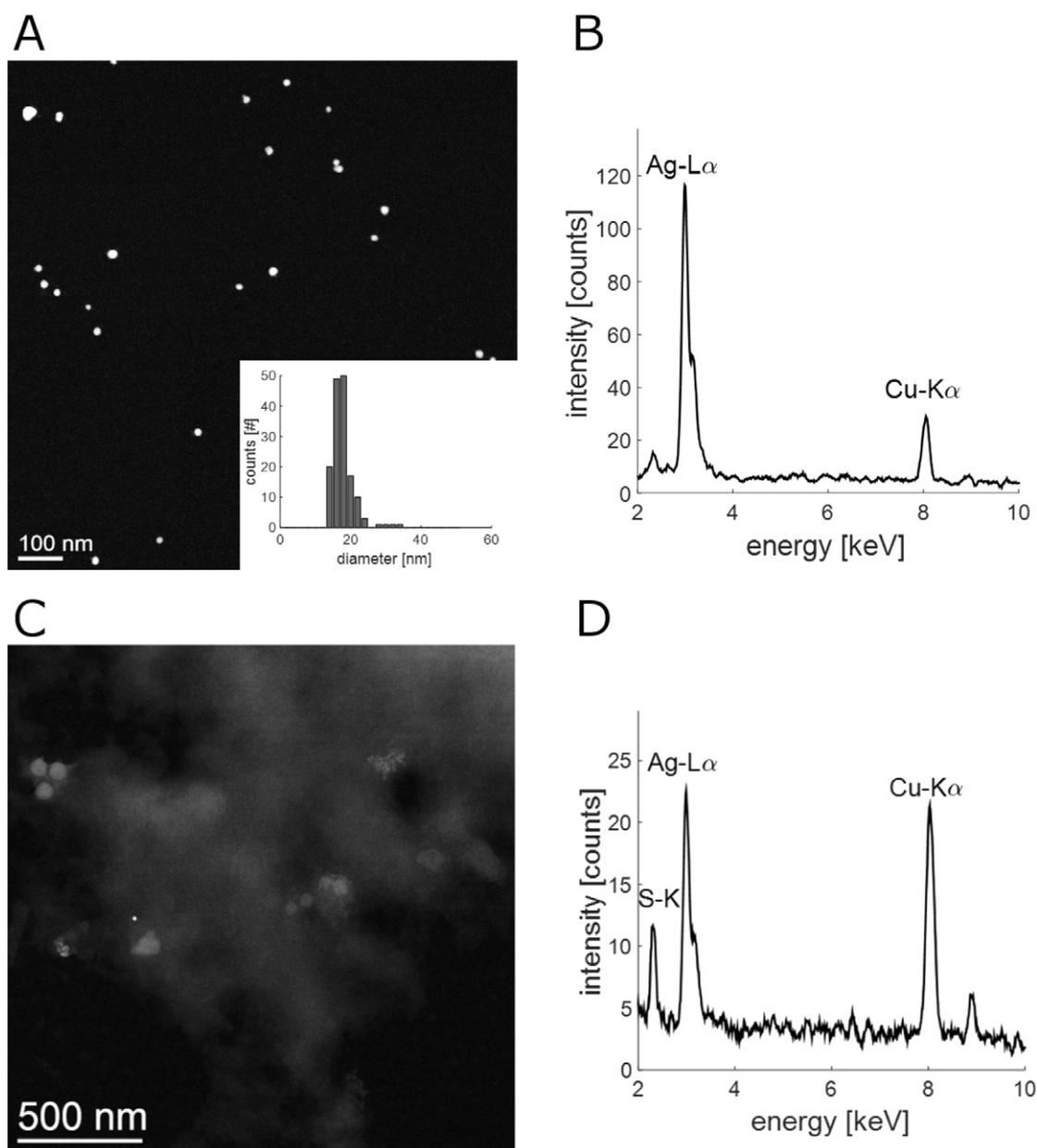
Fig. 2. High-angle annular dark field (HAADF) image with an energy dispersive X-ray (EDX) spectrum of AgNP from the stock dispersion (A, B) and freeze-dried zooplankton containing AgNPs with the corresponding EDX spectrum (C, D). The particle size distribution of AgNPs ($n = 153$) derived from several HAADF images is shown as an inset in (A).

and 28, respectively) in the digestive tract until the end of the test period.

3.4. Dietary exposure of O. mykiss

The wet weight of the rainbow trout in the control group increased during the dietary exposure study by 150% (91% during uptake phase, 31% during depuration phase) from 2.0 ± 0.5 g on day 0 to 5.1 ± 0.3 g on day 28. On day 0, the total lipid content of the test fish was $5.0 \pm 0.7\%$ and decreased during the study to $2.9 \pm 1.1\%$. During the study, no behavioural changes, mortalities or physical damage were observed.

The total Ag concentrations in digestive tract and carcass of the fish collected during the dietary exposure study are shown in Fig. 4. On day 0, 7 and 14 Ag concentrations of $8.18 \pm 1.0 \,\mu g\, kg^{-1}$, $27.9 \pm 17.7 \,\mu g\, kg^{-1}$ and $34.3 \pm 13.8 \,\mu g\, kg^{-1}$ were measured in the digestive tract,

respectively. AgNP-free food pellets were fed from day 14 onwards. Thus, the Ag concentrations decreased to $4.8 \pm 1.7 \,\mu g\, kg^{-1}$ until day 17 and stayed at this level until the last sampling on day 28 ($3.3 \pm 0.8 \,\mu g\, kg^{-1}$). The Ag concentrations in the digestive tract of the control group, stayed on a low level until the end of the study with $1.2 \pm 1.5 \,\mu g\, kg^{-1}$, $1.1 \pm 1.4 \,\mu g\, kg^{-1}$, $3.3 \pm 3.7 \,\mu g\, kg^{-1}$ on days 7, 14, 28, respectively. Throughout the entire study, no significant uptake of Ag was observed in the carcass of the control fish. On day 0, an Ag concentration of $2.9 \pm 0.2 \,\mu g\, kg^{-1}$ was measured. The background Ag concentration level decreased to $1.0 \pm 0.2 \,\mu g\, kg^{-1}$ on day 7 and stayed on this level until the end of the test with $1.5 \pm 0.6 \,\mu g\, kg^{-1}$ measured on day 28. In contrast, the Ag concentration in the digestive tract stayed relatively constant during exposure to AgNP-containing food pellets ($2.9 \pm 0.2 \,\mu g\, kg^{-1}$, $2.2 \pm 0.4 \,\mu g\, kg^{-1}$ and $3.0 \pm 0.7 \,\mu g\, kg^{-1}$ on days 0, 7 and 14, respectively) and even kept that level until day 28 ($2.2 \pm 1.1 \,\mu g\, kg^{-1}$).

4. Discussion

4.1. Aqueous exposure of O. mykiss

Significant amounts of Ag were found in the carcass but especially the gills of the rainbow trout after exposure to AgNPs that were added to copper-ion reduced tap water. However, when exposed to AgNPs in WWTP effluent (added prior or after the passage through the WWTP), Ag levels in carcass and gills remained at a low level below 10 µg kg^{-1}. This difference might be due to the transformations that AgNPs undergo in WWTP effluents. Many studies have shown that AgNPs adsorb to biosolids, are sulfidized to a large extent and transformed to Ag_2S (Fletcher et al., 2019; Georgantzopoulou et al., 2018; Kaegi et al., 2013, 2011; Kim et al., 2010; Levard et al., 2012; Ma et al., 2014). Hartmann et al. (2019) performed multi-generation studies with *Daphnia magna*, where the animals were exposed to the same WWTP effluents which were also used in the present study. Likewise, they confirmed that AgNPs in WWTP effluents were probably sulfidized according to EDX analyses. This transformation process takes <2 h (Kaegi et al., 2011), which might explain the similar bioavailability of Ag from AgNPs present in or supplemented to the WWTP effluent matrix. Fletcher et al. (2019) analyzed the release of Ag$^+$ from AgNPs at various levels of sulfidation and found that even if sulfide concentrations in the media are low, sulfidation limits the release of Ag$^+$ ions. However, it is not yet clear to what extent Ag$^+$ ions and the particles themselves, contribute to the toxicity and bioaccumulation potential of Ag from AgNPs in aquatic organisms (Behra et al., 2013). The results of our studies may highlight a primary role of Ag$^+$ uptake from AgNPs in fish. Moreover, they are in accordance with the results of Kühr et al. (2018) where a limited bioavailability of Ag from AgNPs for *Hyalella azteca* was observed when the AgNPs were spiked into WWTP or in the effluent of control WWTP and were thus presumably sulfidized.

In our study with rainbow trout, AgNPs were most likely adsorbed to the gill epithelia where Ag$^+$ was potentially released. Ionic silver is known to disturb the ionoregulation in the gills by competing with other cations for anionic binding sites and this is able to block the Na$^+$/K$^+$-ATPase activity and cause respiratory stress (Bianchini et al., 2002; Bilberg et al., 2010; Janes and Playle, 1995). Dissolved Ag species can be transported to other organs via the circulatory system, especially the liver, the central organ of detoxification. As a result, Wood et al. (1996) found the highest Ag tissue concentrations in the liver of adult rainbow trout after exposure to AgNO$_3$. Similar observations were made by Scown et al. (2010) and Joo et al. (2013) after exposure of rainbow trout to AgNPs. In our test design, the liver was not analyzed individually but as part of the carcass. While the Ag concentrations in the gills reached a maximum of around 100 µg kg^{-1} at the end of the exposure phase with AgNP supplemented dilution water, the Ag levels in the carcass developed at a lower level but were still rising after 14 days of exposure. The difference in tissue concentrations might be explained by metallothioneins (MTs), cytosolic proteins, that could be found in a wide range of organisms especially in gill tissue (Kaegi and Schaeffer, 1988; Kaji et al., 1993). By binding free metal ions, MT shows a detoxifying effect (Schlenk et al., 1999). The MT gene expression is upregulated by various metals like Cd, Au, Pt and Ag and the MT reaction has been shown to correlate well with increasing body burdens of the metals (Lansdown et al., 2001, 1999, 1997). Due to its high amount of cysteine (approx. 30%), MT shows a high binding affinity and capacity of 12 mol Ag per mol MT (Lansdown, 2002; Nielson et al., 1985). Chowdhury et al. (2005) showed that an exposure to Cd resulted in an 8.2-fold increased MT level in the gills and 400 and 15 times higher Cd level in gills and carcass if compared to control, respectively. Heerden et al. (2004) described, that the upregulation of MT expression in gills of *O. mykiss* could be used as a more sensitive biomarker for metal exposure than the mere accumulation of the metals in the tissue. Regarding this, the delayed or limited uptake and accumulation of Ag in the carcass, compared to the gills, may be the result of MTs in the gill tissue
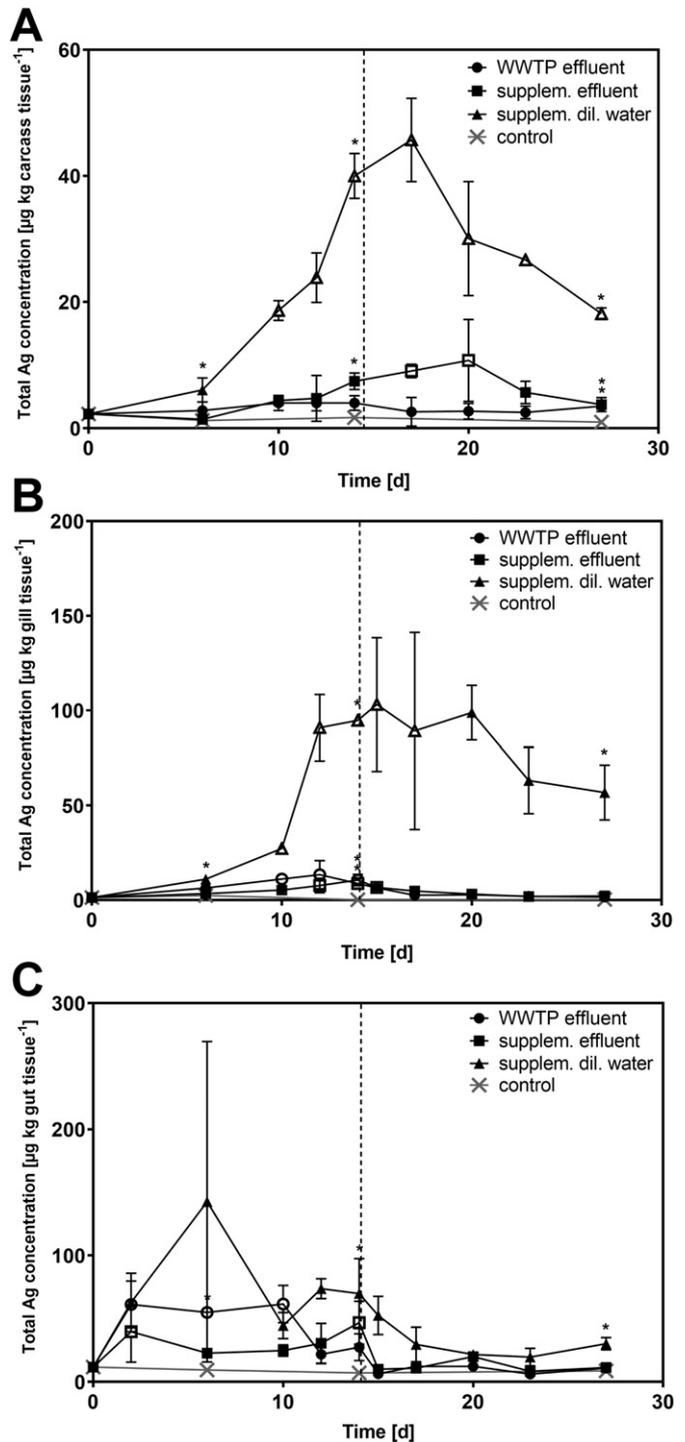

Fig. 3. Total Ag concentrations in carcass (A), gill tissue (B) and gut (C) of *Oncorhynchus mykiss* during 14d exposure to AgNPs present in or supplemented to WWTP effluent, and added to water, followed by 14d exposure to dilution water without test item. Ag concentrations are presented as µg/kg wet weight. Data points are mean ± standard deviation of four organisms. Asterisks and empty symbols show statistically significant differences ($p \leq 0.05$) relatively to the respective control group value and the respective start value on day 0, respectively, according to one-way ANOVA followed by Dunnett's post-hoc test. Dotted lines represent change of media (with and without test item, respectively) after 14d.

trapping Ag$^+$ released from AgNPs. The MTs in the gills may bind a high proportion of Ag$^+$ before it can reach other tissues like the carcass. When the loading capacity of the MTs for Ag is reached in the gill tissue additional Ag$^+$ may reach the carcass unimpeded. This may explain that during the 14d exposure to AgNPs no saturation of Ag tissue
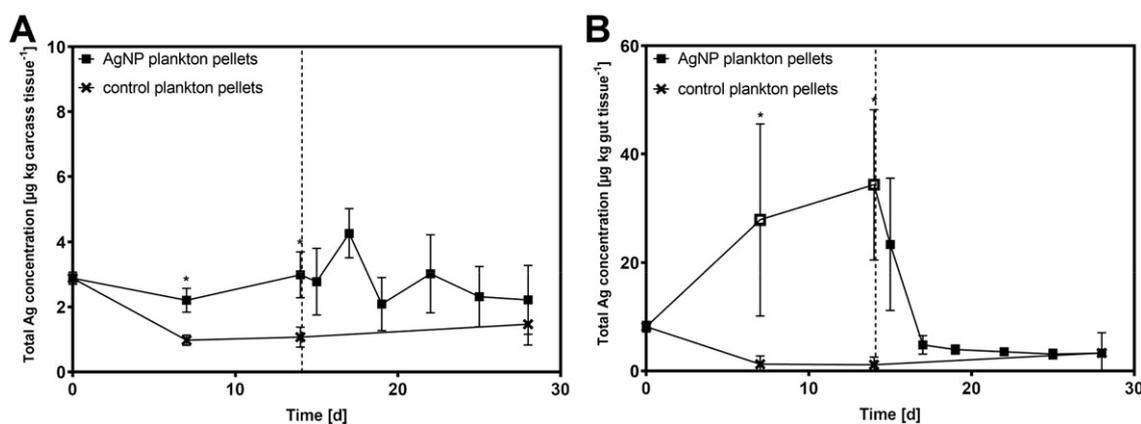
Fig. 4. Total Ag concentration in carcass (A) and digestive tract (B) of *Oncorhynchus mykiss* during 14d dietary exposure to plankton pellets with/without AgNPs followed by 14d exposure to control plankton pellets. Ag concentrations are presented as µg/kg wet weight. Data points are mean ± standard deviation of five organisms. Asterisks and empty symbols and show statistically significant differences ($p \leq 0.05$) relatively the respective control group value and the respective start value on day 0, respectively, according to *t*-test. Dotted lines represent change of food (with and without test item, respectively) after 14d.

concentrations in the carcass could be observed, due to the slow transfer and distribution of dissolved Ag species from the gills via the circulatory system. However, even after the end of the exposure phase, Ag concentrations in the rainbow trout carcass were still increasing, which might be attributed to the further release of Ag^+ ions from AgNPs, that were previously adsorbed to the gills or the intestinal walls.

MTs may also be the reason for the slow and incomplete elimination of Ag in the gills and carcass during the depuration phase. Norey et al. (1990) observed a retention of Cd after intraperitoneal injection to rainbow trout. They found that 98% of the retained metal was localized in the tissue of liver, kidney and gills and no significant elimination of the metal was observed during the following 98 days of depuration. They also showed that the Cd was bound to the MT that they isolated from the tissues. MTs are also discussed as an explanation for an incomplete elimination of Ag from the fresh water mussels *Corbicula fluminea* that were exposed to $AgNO_3$ for 144 h (Sebastian Kühr, pers. com), showing comparable kinetics of Ag tissue concentrations during the depuration phase as observed in this study.

In this study, a gill total Ag concentration of around $100\,\mu g\,kg^{-1}$ was observed after 14d exposure to AgNPs supplemented to water at a level of $12.4\,\mu g\,L^{-1}$. In contrast, Scown et al. (2010) found an Ag tissue concentration in the gills of juvenile rainbow trout of approx. $300\,\mu g\,kg^{-1}$ after 10d exposure to AgNPs at $10\,\mu g\,L^{-1}$ (Scown et al., 2010). This might be explained by the fact, that Scown et al. (2010) did not feed the rainbow trout during exposure. AgNPs have a tendency to adsorb to natural organic matter like food or feces and might therefore be predominantly taken up by ingestion rather than being adsorbed to the gills. This might also explain the ambiguous results we observed for total Ag concentrations in gut tissue. When exposed to AgNPs in tap water, the Ag concentration in the intestinal tract varied significantly during the exposure phase, which might be attributed to the ingestion of agglomerated AgNPs or particles adsorbed to the food pellets. Nevertheless, these variations in gut tissue concentrations had obviously no effect on the Ag concentrations in the carcass, which indicates a limited or no transfer of Ag species through the intestinal wall.

4.2. Dietary exposure of O. mykiss

A comparison of the dietary uptake and bioavailability of wastewater-borne and planktonic food-borne AgNPs in fish is still missing. Hence, the dietary uptake pathway was also assessed in this study. Zooplankton collected from a natural pond and exposed to AgNPs was chosen as experimental diet for the exposure studies with rainbow trout to simulate a natural aquatic food chain. Based on the total Ag analysis, it could be shown that the exposure of plankton to AgNPs led to considerable Ag concentrations in the zooplankton. However, it

remained unclear, whether the AgNPs were ingested by the planktonic organisms or if they were attached to the carapace of the plankton organisms. Total silver concentrations in the experimental diet were comparable to concentrations as found in planktonic organisms exposed to silver from environmental sources (Eisler, 1997). The AgNP-loaded plankton was not ground to maintain the original structure of the plankton matrix within the food pellets. Freshwater zooplankton from fertilized earthen ponds contain around 73–79% and 10–14% of proteins and lipids, respectively (Mitra et al., 2007; Schlechtriem et al., 2003). Due to the agar matrix and the accompanying high water content of the food pellets, the lipid values were considerably lower with the final food pellets containing 3.0% lipids in the fresh material. Although the total lipid content of the experimental diet was quite low, the wet weight of the fish doubled (2.0 to 4.3 g) during the study, indicating that the quality of the plankton pellets which were supplemented with ground commercial fish food provided a sufficient amount of nutrients to maintain growth in the experimental fish. Interestingly, the commercial fish food, which was used for the maintenance of the fish prior to the study and also to enrich the plankton food matrix, showed a considerable total Ag concentration of $37.3\,\mu g\,kg^{-1}$. Since this fish food was also part of the control plankton pellets, they also showed a total Ag concentration of $14.7\,\mu g\,kg^{-1}$. Moreover, Ag species released from the commercial fish food before test start may have been bound to the mucus layer and thus may have contributed to the measured Ag concentrations of 11.6 and $8.18\,\mu g\,kg^{-1}$ in the intestine tissue on the first day of the aqueous and the dietary exposure studies. This effect was already described by Khan et al. (2017) who analyzed the intestinal uptake of Ag in rainbow trout. It was shown, that most of the Ag was bound to the mucus layer of the intestine, while only 8 to 15% were transferred to the blood compartment (Khan et al., 2017). During the uptake phase intestinal Ag levels increased to $34.3\,\mu g\,kg^{-1}$ due to the exposure to the AgNP-loaded plankton food followed by a significant decrease of Ag concentrations to around $3\,\mu g\,kg^{-1}$ during the depuration phase. The control group was exposed to control plankton pellets throughout the test period leading to a decrease in intestinal Ag concentrations from $8.2\,\mu g\,kg^{-1}$ to $1.2\,\mu g\,kg^{-1}$ within the first 7 days. Taking into account the decrease of intestinal Ag concentrations after exposure to AgNP-loaded plankton food, the Ag species of both fish foods (commercial fish food applied before test start and AgNPs in the plankton pellets applied in the study) were eliminated from the digestive tract very quickly. The decrease of Ag concentrations in the carcass of the control group from 2.9 to $1.0\,\mu g\,kg^{-1}$ within the first 7 days was related to the lower Ag concentrations in the control plankton pellets than the commercial fish food ($14.7\,\mu g\,kg^{-1}$ and $37.3\,\mu g\,kg^{-1}$, respectively). In contrast, the Ag concentration in the carcass remained constant when fish were exposed to the AgNP-containing plankton pellets

although they contain a considerably higher Ag concentration ($121.5\ \mu g\ kg^{-1}$) than the commercial fish food, indicating a limited bio-availability of the AgNPs from the experimental diet.

Elemental analyses of the AgNPs in the lyophilized zooplankton by EDX showed that the particles kept their original size and shape but were mainly sulfidized. It can be only speculated at which stage of the feed preparation process, during exposure or during the freezing and lyophilization process, the sulfidation of the particles happened. Sulfidation might have been the main reason why only a limited Ag transfer from AgNPs-loaded plankton through the intestinal walls could be detected as it was already shown after exposure to AgNP-loaded WWTP effluents. Nevertheless, the fact that a limited bioavailability of Ag was observed for sulfidized AgNPs that were exposed via the food, underlines our assumption that the gills are the major uptake route of Ag from AgNPs and that the intestinal tract of *O. mykiss* only plays a negligible role.

4.3. Environmental implications

Our studies investigated the bioavailability of Ag from AgNPs in WWTP effluents or loaded live zooplankton. Our results indicate, that Ag from AgNPs is hardly bioavailable for fish under the given conditions. As described before, the uptake of Ag from the surrounding medium and its distribution in fish tissues is mainly mediated by the adsorption of the AgNPs to the gill lamellae, where Ag^+ ions are released that may enter the circulatory system of the organism. The results of our aqueous exposure study indicate, that this only occurs when fish are exposed to pristine AgNPs, that are likely to release Ag^+ ions. Presumably, due to the transformation of Ag from AgNPs to the insoluble Ag_2S within WWTP effluents (Georgantzopoulou et al., 2018; Hartmann et al., 2019) no significant uptake could be shown for AgNPs added to or present in WWTP effluent. Likewise, only a limited uptake of Ag into the carcass of the fish could be shown, when the fish were exposed to AgNP-loaded plankton. Within the freshwater plankton matrix, the Ag at the AgNP surface was also transformed to Ag_2S as it could be shown by EDX analyses, leading to a limited bioavailability in the digestive tract as previously shown for this Ag species when exposed via the surrounding medium. Our results indicate a low bioavailability of Ag from AgNPs from natural matrices, regardless if exposed via WWTP effluent or via loaded zooplankton, and thus explain why fish collected from a prealpine lake (Vogt et al., 2019) did not contain considerable AgNP levels although clear concentrations of Ag were detected in the sediment of the lake. However, a potential impact of AgNPs on the aquatic environment still cannot be entirely excluded. Despite the limited uptake of wastewater-borne AgNPs by the gills, the exposure can still have an impact on this organ on the molecular level by decreasing the epithelial integrity and increasing the oxidative stress (Georgantzopoulou et al., 2018). Bruneau et al. (2016) stated, that AgNPs from WWTP effluents have an impact on the immune system and cause inflammation, despite the fact that they are 2.3 times less bio-available to rainbow trout than $AgNO_3$. Hence, even though our results indicate that the uptake and distribution of AgNPs from WWTP effluents and zooplankton within the aquatic food chain might not be a major concern, the chronic impact of these Ag species on the aquatic environment should not be underestimated. This is especially true, when taking into account, that other aquatic organisms like e.g. zooplankton might be even more receptive to AgNP toxicity, which could thus indirectly affect the fish population.

5. Conclusion

Our studies provide clear indications that the bioavailability of AgNPs (NM-300K) in fish is reduced following wastewater treatment with the gills still playing a crucial role regarding the uptake of Ag into fish tissue. However, no transfer of Ag through the intestinal walls into other tissues could be detected.

Further studies are required to elucidate the impact of further Ag species (Ag^+ ions, nanoparticles, Ag_2S) on Ag uptake via the gills and the digestive tract. As shown in this study, the distribution of tested Ag species within the tissues and organs of fish should be analyzed to be able to draw conclusions on how synthetic AgNPs may influence the aquatic food chain.

Declaration of competing interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135695.

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