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Fate of zinc and silver engineered nanoparticles in sewerage networks

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Abstract

Engineered zinc oxide (ZnO) and silver (Ag) nanoparticles (NPs) used in consumer products are largely released into the environment through the wastewater stream. Limited information is available regarding the transformations they undergo during their transit through sewerage systems before reaching wastewater treatment plants. To address this knowledge gap, laboratory-scale systems fed with raw wastewater were used to evaluate the transformation of ZnO- and Ag-NPs within sewerage transfer networks. Two experimental systems were established and spiked with either Ag- and ZnO-NPs or with their dissolved salts, and the wastewater influent and effluent samples from both systems were thoroughly characterised. X-ray absorption spectroscopy (XAS) was used to assess the extent of the chemical transformation of both forms of Zn and Ag during transport through the model systems. The results indicated that both ZnO- and Ag-NPs underwent significant transformation during their transport through the sewerage network. Reduced sulfur species represented the most important endpoint for these NPs in the sewer with slight differences in terms of speciation; ZnO converted largely to Zn sulfide, while Ag was also sorbed to cysteine and histidine. Importantly, both ionic Ag and Ag-NPs formed secondary Ag sulfide nanoparticles in the sewerage network as revealed by TEM analysis. Ag-cysteine was also shown to be a major species in biofilms. These results were verified in the field using recently developed nanoparticle *in situ* deployment devices (nIDDs) which were exposed directly to sewerage network conditions by immersing them into a municipal wastewater network trunk sewer and then retrieving them for XAS analysis.

Keywords

Silver, zinc oxide, nanoparticles, sewer, fate, transformations

1. Introduction

The rapid commercialisation of nanotechnology over the last decade has raised concerns about the potential environmental consequences of engineered nanoparticles used in consumer products. Silver (Ag) and zinc oxide (ZnO) are among the most common engineered nanoparticles used in this manner. For instance, nearly a quarter of the more than 1600 consumer products containing nanomaterials listed by the Project on Emerging Nanotechnologies (2013) claim to contain Ag nanomaterials, and it is reported that up to 500 tons/year of Ag nanoparticles (Ag-NPs) are produced and used worldwide (Gottschalk *et al.*, 2009; Sun *et al.* 2014). Silver nanomaterials are widely used as antibacterial and antifungal agents in a large variety of consumer products, as well as in food technology, textiles/fabrics, and medical products and devices. Unfortunately no clear data and statistics are available on the NPs presence in commercial/industrial products and on their usage by sectors. For instance, in the use of silver for biocidal applications it is reported by the NPIRS database (USA National Pesticide Information Retrieval System) that approximately 60% of these biocide products, surely or likely contain Ag-NPs. In this non-exhaustive list are included Ag additives, Ag disinfectant, Ag algacide and also silver impregnated water filters (Nowack *et al.*, 2011). In the case

of nanoscale ZnO, the estimated worldwide production in 2011 was greater than 33,400 tons/year (Future Markets Inc, 2012). Zinc oxide nanoparticles (ZnO-NPs) are mainly used as active ingredients in sunscreens and as industrial coatings to protect plastics, wood and textiles from UV exposure and microbial degradation, and they are also used in photoprinting and as thin conductive films in household appliances and solar cells (Lamb *et al.* 2004, Mu & Sprando, 2010).

The production, use, and disposal of these products leads to their release into various environmental compartments such as air, water and soil. The main exposure pathway for Ag and ZnO nanomaterials is through the wastewater system, and wastewater treatment processes have thus become one of the main research targets in the environmental risk assessment of these nanomaterials. Kim *et al.* (2010) reported the presence of Ag₂S nanoparticles in sludge from a wastewater treatment plant (WWTP), and the fate of engineered NPs within wastewater treatment plants has since been investigated and modelled in several studies (Hendren *et al.* 2013, Hou *et al.* 2012, Kaegi *et al.* 2011, Kiser *et al.* 2009). These studies have shown that Ag-NPs can be partly or totally converted to silver sulfide (Ag₂S) and suggested that this process occurs preferentially during anaerobic digestion (Lombi *et al.* 2013, Reinsch *et al.* 2012, Liu *et al.* 2011, Kaegi *et al.* 2011). However, Lowry *et al.* (2012) pointed out that pristine NPs are likely to undergo various transformations before reaching wastewater treatment plants and more attention should be paid to the fate of “aged” NPs.

Notably, sewers can contain sections with anaerobic conditions, which typically result in the formation of hydrogen sulfide and other malodorous organic sulfides (Sharma *et al.* 2014). Thus, it is likely that Ag- or ZnO-NPs undergo transformations

before reaching the WWTP. Despite this, the fate and transformations of NPs during their transport through sewers remains relatively unexplored, although a recent study by Kaegi *et al.* (2013) demonstrated a partial sulfidation of Ag-NPs both in batch studies and when Ag-NPs were spiked into a real sewer system. However, these results could have been affected by the presence of high initial oxygen concentration (above 5 mg/L in both the experiments), unusually low concentrations of sulfides in the batch tests and short retention time in the sewer. As for ZnO-NPs, to the best of our knowledge, no literature reporting their transformations in sewerage systems is available.

The aim of this study was to investigate the transformations that can occur in the sewer before Ag- and ZnO-NPs reach WWTPs. To this end, a lab-scale sewerage system was set up and operated for 3 months (to establish biofilm) prior to spiking with Ag-NPs and ZnO-NPs for 3 consecutive days. A control system was run in parallel using equivalent amounts of Ag and Zn spiked in dissolved forms instead of as NPs.

2. Materials and methods

2.1. Nanoparticles synthesis and characterisation

Citrate stabilised Ag-NPs and uncoated ZnO-NPs were used in this study. Ag-NPs were prepared immediately before the trials, while commercial ZnO-NPs were used as received (Micronisers Pty Ltd., VIC, Australia). The latter product is the same as the OECD sample NM-112 (Singh *et al.*, 2011). Ag-NPs were prepared according to a modified version of the classic wet chemical procedure (Turkevich *et al.*, 1951) using

sodium borohydride (NaBH_4 , Sigma Aldrich, 98%) as the reductant and trisodium citrate ($\text{Na}_3\text{C}_3\text{H}_5\text{O}(\text{COO})_3$, Ajax Finechem, 99%) as a stabilizer. Ten mL of 1.0 mM silver nitrate (AgNO_3 , Sigma Aldrich, 99.99%) was added dropwise to 30 mL of 2.0 mM NaBH_4 solution under vigorous stirring conditions in an Erlenmeyer flask at room temperature. The solution was brought to boiling point (100°C) in 15 minutes and 1 mL of 1% trisodium citrate in ultrapure water was quickly injected. The reaction was kept at boiling point and stirred for 1 h, then stirred without heating for another 30 minutes. The synthesised Ag-NPs were stored in the dark until use.

Morphological characterization of both Ag- and ZnO-NPs was performed using Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The hydrodynamic diameter and zeta potentials of the NP samples were measured with a Nicomp 380 ZLS (Particle Sizing Systems Inc., FL, USA). A Quanta 450 Field Emission SEM (FEI Company, OR, USA) and a Philips CM 200 TEM (Royal Philips, Eindhoven, The Netherlands), both coupled with an energy dispersive X-ray detector (EDX) for elemental identification, were used to image and analyse the NPs. For SEM analysis, graphite discs were mounted on to standard aluminium stubs, and the NPs were deposited on the discs. In case of Ag-NPs, the discs were pre-treated with poly-L-lysine in order to create a positively charged surface and exposed to a solution of Ag-NPs pre-ultrasonicated for 5 minutes. In this way, the negatively charged Ag-NPs were attached to the surface for analysis. For ZnO-NPs a silicon wafer substrate was used on to which, NPs were deposited drop-wise as methanol solutions after 5 minutes of ultrasonication. All samples were then dried under nitrogen and in a dark atmosphere. TEM samples were prepared using the protocol used

for wastewater samples (see details below). All SEM/TEM images were analysed using ImageJ (National Institutes of Health, MD, USA) using a semi-automatic procedure involving manual processing of the images and automatic counts using the Analyse Particles tool (Mateos & Pérez, 2013).

2.2. Experimental Setup

Two identical bench-scale sewerage transfer systems were set-up and run simultaneously, one spiked with Ag- and ZnO-NPs and the other with equivalent concentrations of metal ions (Ag^+ as AgNO_3 and Zn^{2+} as $\text{Zn}(\text{NO}_3)_2$) to function as controls. Both the systems were built according to the OECD guidelines for testing of chemicals (OECD, 2008). They consisted of three sewer sections equipped with gas collection apparatus, pH and redox probes, plus inlet and outlet systems (each equipped with storage tank, pumps, re-circulators, samplers and monitoring probes). The sewer sections were kept in the dark and mainly left undisturbed, except during the pre-pumping and pumping events when they were mechanically stirred by an orbital shaker at 170 rpm to completely remix them in order to minimise the accumulation of sediments (Figure 1). The total volume throughout the simulated sewer was 2 L and the hydraulic retention time was 8 hours. The diameter of the sewer sections was 105 mm and the area/volume ratio (A/V) was calculated as $61.9 \text{ cm}^2/\text{cm}^3$, assuming that almost the entire internal surface of the sections were in contact with the sewage with the exception of a small head space on top of each section that allowed for the potential collection of biogas. Peristaltic pumps were used to intermittently feed the sewage into the system with a

feeding pattern consisting of 18 pumping events per day. The sewer systems were fed daily with municipal wastewater collected from a sewer pipeline before the Bolivar Sewage Treatment Works (SA, Australia), which collects and processes the largest portion of the municipal wastewater produced in Adelaide's metropolitan area. The systems were run for approximately 3 months before spiking with Ag and Zn began, in order to allow enough time to develop an active biofilm and the typical environmental conditions encountered in sewers.

The spiking trials lasted for 144 hours. The sewerage system was monitored for the first 24 hours to establish the baseline for all parameters (detailed below), before spiking the sewage samples either with Ag-NPs and ZnO-NPs (Sewer "SA") or with equivalent concentrations of dissolved Ag^+ and Zn^{2+} (Sewer "SB"). The target concentration of Zn was $700 \mu\text{g L}^{-1}$, approximately double the background concentration in the wastewater as this would facilitate good differentiation of the contribution of added Zn in the XAS spectra. In the case of Ag, since the background concentration was very low ($<10 \mu\text{g L}^{-1}$), $90 \mu\text{g L}^{-1}$ was selected to facilitate greater reliability of ICP-MS quantification and suitably good statistics in terms of XAS data quality. Moreover, lithium chloride (LiCl , $700 \mu\text{g L}^{-1}$) was added as a tracer, to evaluate residence/flow behaviour and performance of the sewerage systems. During the trials, physico-chemical parameters such as pH, oxidation-reduction potential (Eh) and temperature were constantly monitored in order to acquire real-time data of the systems' behaviours. A GteK datalogger (GteK Computers, SA, Australia) was used to record these parameters every 15 minutes. Dissolved oxygen (DO) was also measured few times with a portable

DO oxygen meter (mod. 407510, Extech Instrument Corp., MA,USA) to periodically check that the low DO condition typical of sewer were maintained during the test.

Influent and effluent sewage samples were collected from the inlet and outlet sampling units (ISU and OSU – see Figure 1) after each cycle (8 hours) and characterized as described in the next section.

2.3. Wastewater characterisation

Total suspended solids (TSS), total volatile solids (VSS), chemical oxygen demand (COD), suspended and dissolved sulfides, chloride, sulfate, lithium and heavy metals were measured for all sewage samples collected during the trial. TSS and VSS were measured gravimetrically according to the 2540d APHA method (Eaton & Franson, 2005). The COD was determined using high-range Hach Lange vials for dichromate digestion according to standard methods (USEPA, 1983; Eaton & Franson, 2005) and a DR890 colorimeter for measurement (Hach Lange, Co-USA). Suspended and dissolved sulfides were determined immediately after sampling using the methylene blue method (USEPA 1978; Eaton & Franson, 2005). Inorganic anions were determined on filtered samples (0.45 μm) by ion chromatography (Dionex ICS 2000, Thermo Scientific, Ca - USA) in accordance with US EPA Method 300.0 (USEPA, 1993). For Li and metal measurement, wastewater samples were digested in reverse aqua regia using a microwave assisted digestion system (MARS 6, CEM Corp., NC, USA), according to the standard digestion method 3015a (USEPA, 2007). Metal concentrations were then determined

using an Agilent 8800 Triple Quadrupole ICP-MS (Agilent Technologies, CA, USA). Two replicate analyses were performed for each sample.

All TEM samples were prepared according to a protocol similar to the one suggested by Kim *et al.* (2010). Two mg of dry sewage was hydrated with 40 μ l of ultrapure water, mixed with 1.96 ml of methanol and then sonicated for 1 hour in an ultrasonic bath set at 20 °C and 43 ± 2 kHz (model 250HD, Soniclean, Australia). Single drops of these suspensions were released onto 100-mesh copper grids with carbon coating on Formvar resin film (GSCU100C-50 grids, ProSciTech Pty Ltd, Australia) and dried under vacuum.

2.4. nano *In situ* Deployment Devices (nIDDS)

Together with the *ex situ* experiment (bench-scale systems), another trial was conducted *in situ* in a real municipal sewerage network using Ag-NPs immobilised on nIDDS (nano *In situ* Deployment Devices). These devices have been recently developed by our group as an approach to facilitate the *in situ* study of engineered nanomaterials in the environment (Sekine *et al.*, 2013).

nIDDS were immersed into two consecutive sections of a main gravity-based sewer for four different exposure times. One exposure position ('Globe Derby') was 5 km before the Bolivar WWTP and the other ('Bolivar') was just before the intake to the WWTP. The exposure times were 2, 4, 8, and 16 hours for both locations. Wastewater pH, oxidation reduction potential (Eh), dissolved oxygen and temperature were recorded

at each sampling time. After deployment, the devices were immediately rinsed with ultrapure water, dried with N₂ and kept under an inert atmosphere.

2.5. X-ray Absorption Spectroscopy (XAS)

Bulk XAS analysis was performed at the XAS beamline at the Australian Synchrotron (AS, Victoria, Australia) and at the Materials Research Collaborative Access Team (MRCAT) beamline 10-ID at the Advanced Photon Source (APS), Argonne National Laboratory (ANL, IL, USA). Sewage samples were centrifuged at 4000×g for 20 min and the pellets were freeze dried on a Modulyo D freeze drying machine (Thermo Fisher Scientific Inc, MA-USA). The recovered solids were kept under a N₂ atmosphere until analysis.

Silver K-edge XAS analysis was performed at the AS, with the synchrotron operating at 3 GeV and 200 mA in top-up mode. A liquid N₂ cooled monochromator equipped with Si(311) crystal pairs was used to select the incident photon energies, and a platinum-coated mirror was used for harmonic rejection. Zinc analysis was performed at the APS, with the synchrotron operating at 7 GeV in top-up mode, and a Si(111) double crystal monochromator was used to select the energies. The monochromators were calibrated by assigning the first derivative inflection point of the absorption K-edge of Ag and Zn metal foils to 25,514 eV and 9,659 eV respectively.

All the samples were positioned 45° to the incident beam; each sample was scanned at room temperature in 3 different spots and one spot was scanned twice in order to check for photon-induced damage. Sample spectra were acquired in fluorescence

using a 100-element (AS) or 13-element (APS) detector. Reference X-ray spectra for Ag or Zn metal foils (collected congruently with the sample spectra), were used for energy calibration and spectral alignment. Standard spectra were acquired from pellets containing different Ag and Zn standards at *ca.* 200 mg/kg in granular cellulose (see further details in section 3.3). XAS data extraction was performed using Sakura (Kappen and Ruben, 2013) while background subtraction and normalisation was completed using Athena (Ravel and Newville, 2005). Linear combination fitting (LCF) of the XANES (X-ray Absorption Near-Edge Structure) regions of each spectrum was also performed using Athena. The selected fitting region was -25 to 100 eV for Ag, and -20 to 100 eV for Zn.

3. Results and Discussions

3.1. Nanoparticles characterization

The ζ -potential (average of 15 measurements) was -39.0 ± 0.2 mV for Ag-NPs and $+23.7 \pm 0.2$ mV for ZnO-NPs. The pH values of the stock solutions were 8.4 and 7.9 respectively.

The number-weighted particle size distributions for both the Ag- and ZnO-NPs were used to determine the modal sizes. The Ag-NPs showed a mono-modal distribution with average hydrodynamic diameters of 26 ± 2 nm and a dispersity index value of 0.384 (Figure A1-a). SEM analysis on secondary electron images confirmed this monomodal distribution, with average modal diameters of 25.6 ± 0.4 nm (Figure A8). In agreement

with the other measurements, TEM analysis showed an equivalent sphere diameter (ESD) for Ag-NPs in the range of 18 nm with 68.2% of the particles less than 20 nm and 96.3 % of the particles less than 40 nm (Figure A8).

The ZnO-NPs distribution appeared to be bimodal. The average hydrodynamic diameters were 64 ± 4 and 240 ± 17 nm respectively, but the dispersity index was calculated as 0.262, indicating that the particle distribution tended towards monodispersion (Figure A1-b). In fact, the second modal group only represented around 1% of the number-weighted distribution, and the detection of larger particles was due mainly to aggregation of nanoparticles into small clusters, as revealed by SEM. The SEM analyses for the ZnO-NPs confirmed the presence of polydisperse distributions of particles; aggregated and agglomerated particles were present with average ESD of 41.2 ± 1.7 nm. The SEM ZnO-NPs distribution appeared tailed towards particles of one order of magnitude larger sizes (200-250 nm) with relatively low but consistent frequencies (Figure A7). TEM analysis of the ZnO-NPs resulted in an average ESD of 22.9 ± 0.9 nm.

The differences observed between sizing techniques are related to the intrinsic characteristics of the measured parameters and to measuring issues. DLS measures the intensity fluctuations of the light scattered by particles and by analysing the autocorrelation function (intensity vs time) calculates the translational diffusivity coefficient, which is then used to compute the particles diameter using the well-known Stokes-Einstein relation. The particle size calculated by DLS, also called the hydrodynamic diameter, tends to be larger than the core diameters measured using electron microscopy techniques. SEM and TEM both give information about the core size

of the NPs, however, TEM can give more accurate information about the particle core size and shape.

The discrepancies found between the sizes as determined by SEM and TEM are due to the fact that in TEM ZnO primary particles size were more easily discriminated during the semi-automatic counting and sizing procedure while in SEM aggregated particles were analysed as larger, single particles by the software. Overall the ESD and Feret particle size reported from SEM analysis showed that the particles/grains are generally made by two or more primary particles aggregated together. These results are similar to those reported by Singh *et al.*, (2011) in the OECD publication on the NM-112 ZnO-NP certified material.

3.2. Sewerage system parameters during simulation

The NPs were spiked into the sewage influent for sewer SA, and equivalent concentrations of dissolved Ag and Zn were spiked into the influent for sewer SB (control). Both systems were co-spiked with LiCl as a tracer and monitored for 3 consecutive days. The data analysis showed that the sewerage systems: (a) had no significant operational differences between them, and (b) effectively simulated the chemical conditions usually encountered in pressurised sewers. During the trials the Eh was on average ca. -475 mV in both the systems (Figures A11, A14), consistent with the anaerobic conditions commonly encountered in sewerage channels with completely filled pipes (Hvitved-Jacobsen, 2002; Vollertsen *et al.*, 2005; Jelic *et al.*, 2015). The average temperatures were similar in both the systems with a very small increase inside the

sewers, and were 21.7, 22.9 and 22.6 °C in the influent, sewer and effluent respectively (Figure A12). Under these conditions some hydrolysis and acidification is expected, and the average wastewater pH decreased from around 7.9 in the influent containers to approximately 7.1 in the effluents (Figure A13). The DO measured during the test was ranging between 0.25 and 0.60 mg/l. No gas was collected in the biogas collection bags, suggesting that the temperature and hydraulic retention time (8 hours) were not suitable for methanogenesis.

The influent TSS was 375 ± 20 and 387 ± 11 mg/L respectively in sewers SA and SB, and the overall mass balance of the TSS was close to 100% for both the sewers, despite the effluent concentration showing a greater variability (especially in the case of SB; see Figure A9, Table A1). Similar results were found for VSS and COD, with differences of less than 10% (i.e. in the range of the experimental error). Interestingly, soluble phosphate increased from 4-9 mg/L in the influents to 18-20 mg/l in the effluents, possibly due to pH variation and hydrolysis during sewage transit through the systems. On the other hand, sulfate content decreased from around 75 mg/L in the influents of SA and SB to 34 ± 4 mg/L in the effluents (Figure 2). In turn, the total sulfide content (TS) increased from an average of 0.64 mg/L S in the influent to 7.42 and 7.59 mg/L in the SA and SB effluent, respectively (Figures 3, A20). For greater clarity, however, it needs to be stressed that total sulfide measurements do not include other insoluble metal sulphides that are likely to form like, as example, CuS. Similarly, the dissolved sulfide (DS) was about 0.24 mg/L in the influent but reached average values of 6.09 and 6.40 mg/L in the SA and SB effluents respectively. These results reflect the presence of reducing conditions and sulphate reducing bacteria in the simulated sewers (Barton, 1995; Okabe,

1999). Overall, the findings described above demonstrate the successful simulation of conditions similar to those found in real sewerage pipelines. Under these conditions, the transformations of Ag- and ZnO-NPs during transportation in the sewer could be properly investigated.

As shown in Figure 4, Li concentrations over the trial period followed the typical profile of a plug-flow system (i.e. a sequence of three completely mixed sections in this case). Silver and zinc profiles appear to be somehow related to that of Li, but also related to the variability in the TSS output (Figure A9). This suggests that, even when dosed as soluble ions, Ag and Zn were immediately sorbed to the suspended solids in the sewer or immediately precipitated as nanoscale colloids and attached to the TSS as suggested by Kaegi *et al.* (2013). The mass balances for Li and both Ag and Zn indicated a well closed system with residual errors of ± 8 % showing that the measurements were consistent and accurate (Table A2).

3.3. XAS analysis and speciation of Ag and Zn

XAS data from the collected samples were analysed using linear combination fitting (LCF) and revealed information regarding the speciation of the elements under study. For Zn, LCF was performed using Zn species that are likely to be present in these environmental conditions as components, including Zn oxide, Zn sulfide, Zn phosphate, and Zn carbonate species. Moreover, Zn bound to organic ligands was represented by cysteine-, acetate-, citrate- and humic acid- bound Zn. Similarly, components used in the

Ag fitting, in addition to the Ag-NPs, included Ag sulfide, Ag chloride, Ag phosphate and Ag sulfate, as well as cysteine-, histidine- and humic-acid-bound Ag (Figure 5).

Selected samples from key compartments and times were analysed by XAS (as listed in Table 1). These samples originated from each of the 3 spiking days and from both the sewer systems (SA and SB), at the inlets and at the outlets (Figure 6). In addition, in the case of Zn, unspiked background samples were also analysed in order to take into account the large natural abundance of Zn in wastewater. All the results are reported here as the average of 3 spectra (Tables 2, 3).

The analysis of the wastewater Zn background showed that in both the system inlets Zn was largely present in reduced sulfidized forms, with Zn sulfide and Zn cysteine values in the range of 77 % and 10 % respectively (Table 2). Zn citrate and Zn phosphate were also identified with values close to 9 % and 10 % respectively. The LCF fits did not improve significantly by the addition of more standard components.

At the outlet of the sewers, sulfidic forms of background Zn dominated due to the increasingly anaerobic conditions. The Zn citrate content tended to decrease slightly, while the phosphate forms of Zn disappeared completely, in line with the increase in free phosphate as measured by ion chromatography (Table A1). No detectable presence of humic acid bound to Zn was found in any of the XAS samples analysed (Table 2). This indicates that this organic ligand, often present in wastewater as a result of organic matter degradation processes, may have a lower affinity for Zn than it does for other metals under these conditions.

On average the ZnO-NPs spiked into system SA contributed 20 % of the total Zn species found in the inlet samples. After transit through the system, all of the ZnO-NPs

added were completely transformed to sulfidic forms. The amount of Zn cysteine and citrate forms remained almost constant at the outlet, and Zn phosphate underwent a complete transformation as already observed in the background analysis. In the control system (SB) about 80 % of the spiked Zn was determined to be Zn sulfide in the inlet sample and this percentage increased to 90% at the outlet; cysteine forms decreased from 19 % to 6 %, while the citrate form increased slightly from 2 % to 4 %. However, it should be noted that due to the insensitivity of the LCF process when working with complex matrices like wastewater solids, the role of components with values less than 10% should not be over-interpreted. Spectra of the bio-film/sediments attached on both of the sewers confirmed the high presence of reduced sulfur forms and confirmed that the citrate forms tended to remain constant.

Silver XAS revealed that NPs underwent a fast and complete transformation in the sewer system. Kaegi *et al.* (2013) reported an incomplete sulfidisation of 100 nm Ag-NPs in a batch system over 5 h. However, given that the size distribution of the Ag-NPs in the current study showed a large amount of NPs lower than 30 nm and reaction rates depend on size (Zhang *et al.*, 2011; Levard *et al.*, 2012), it is likely that the particles in this study were transformed more quickly. Further transformation may also have occurred during the time required for sample freezing and the extraction of the solid phase from the wastewater (approx. 2-4 h); for this reason this preparation/extraction time was also accounted by adding 4 h to the retention times in the sewer system.

XAS analysis revealed a very high presence of sulfidic forms in the inlet of the system spiked with Ag-NPs; unfortunately it was not possible to quantitatively discriminate between the transformations occurred during the time NPs remained in the

inlet with those occurred during the sample preparation, but the contribution from the both are likely to be significant. In fact, in the inlet, more than 80% of the Ag-NPs converted rapidly (i.e. 2-4 h) into sulfidic forms and LCF indicated the remainder to be in the form of Ag chloride, Ag sulfate and Ag bound to histidine (Table 3). It should be noted that Ag- sulfates are unlikely to occur under aqueous environmental conditions (Levard *et al*, 2012): the LCF output indicating Ag-sulfate is likely due to Ag found in a similar environment (i.e. surrounded by oxygen atoms) that can produce similar XANES spectra (e.g. Ag_2SO_4 vs Ag_2CO_3). At the outlet, the sulfide form decreased by 20% of the total Ag while the cysteine, histidine and chloride forms increased consistently, with average values of 12, 15 and 11% respectively. At the control system inlet (SB), the ionic Ag behaved similarly to the Ag in the Ag-NP system (SA) with a large portion of Ag present as sulfide (3-day average was 62 %) and also as cysteine (3-day average was 20 %) and histidine (12 % average). At the outlet, an average increase of the Ag-cysteine form up to 32 % was observed, and an increase of up to 18 % for Ag-histidine, while an almost proportional decrease in the reduced sulfur forms of Ag was indicated.

When looking at the biofilm/sediments attached to the sewer walls of SA (Table 3), it was estimated that about 57 % of Ag was present as cysteine-bound Ag and 28% was linked to histidine (a non-sulfurated amino acid). Furthermore, Ag chloride was found to be in the order of 15 % of the total Ag present, while Ag sulfide was not identified at all. Similarly the SB biofilm/sediments were also found to contain close to 50 % of the total Ag as Ag-cysteine, while the histidine forms were more represented than in the NP sewer system film (41 %). In the control effluent AgCl was found but at lower values than in the NP sewer system (8 %), along with a very small percentage of

Ag sulfide (3 %). It should be stressed that XAS spectra of Ag sulfide and Ag cysteine appear quite similar and in some cases, it can be difficult to discriminate between them. However, by looking at the XANES over an extended region in k-space, it is possible to discriminate between them the undulation at $k \sim 4$ shifts slightly to the left in the Ag sulfide standard spectrum compared to the Ag-cysteine standard as shown in Figure 7. The spectra from the biofilms do indeed align to that of cysteine, while the wastewater samples (e.g. A4in and A4out) align with that of Ag sulfide, supporting the discrimination observed between the two compartments.

Therefore, in light of these results it can be argued that cysteine could play a prominent role acting both directly and indirectly as a Ag complexing agent in the presence of nanoparticulate and ionic forms. In fact, during their growth and development, microorganisms may take advantage of the cysteine originating from protein degradation, or most probably actively produce it in extracellular polymeric substances (EPS), using it for its dual functional capacity as i) a structural stabilizer and ii) a binding agent. The stabilization effect on EPS has been underlined in several studies (e.g. Sheng *et al.* 2010; Sheng *et al.* 2011; Xie *et al.* 2013). The present study indicates that cysteine, temporarily binding Ag to EPS, may represent an intermediate alternative step to the sulfidation process of NPs, converting Ag towards less toxic, less bioavailable and more stable species through ligand exchange mechanisms. In fact the EPS contains a small but considerable and well documented presence of amino acids and proteins with different metal binding capacity due to the presence of thiol groups (Neu *et al.*, 1999; Verdugo *et al.*, 2004; Navarro *et al.*, 2008; Miao *et al.*, 2009). Silver is known to have a

range of affinities towards various amino acids, with thiol and amine containing side chains (Shoeib *et al.*, 2002; Blaske *et al.*, 2013).

In regards to the role of histidine, our study suggests that this amino acid play a role in Ag-microbial interactions, perhaps in defence mechanisms exerted by bacterial communities growing in the presence of Ag. Previous studies have identified some periplasmic Ag-binding proteins synthesized only during bacterial growth in Ag contaminated media which are able to bind selectively to Ag(I) (Gupta *et al.*, 1999; Silver, 2003).

From these results it can be inferred that biofilms could play a role in the interaction between microbial community and NPs mainly in two ways: physical and chemical. First of all, biofilms provide an effective screening against NPs physically isolating and protecting the living organism from the toxicants. At the same time, the chemical interaction of the biofilm with dissolve Ag⁺ ions (and NPs) could also occur. In fact, as it can be observed from the mass balance (Table A2), the amount of Ag directly involved in interaction with the biofilm is only 1-2% of the total Ag-NPs dosed in the sewer system which could be indicative of its effectiveness in sorbing Ag⁺. This would explain the speciation observed in the biofilm. .

Another interesting observation is that some Ag sulfides that had already formed in the inlet wastewater seem to be further “transformed” during/after transit through the system. One of the reasons could be related to the non-uniformity of the sulfidation process on the surface on the nanoparticle cores, with several sites potentially exposed to both inorganic and organic ligands, as pointed out by Kent *et al.* (2014). The local variations in physical, chemical and microbial conditions may induce competition, and in

some cases, promote thiolation more than sulfidation. However, this is an issue that requires further investigation.

3.4. TEM analysis

The microscopic images coupled with EDX spectra of the samples showed that most likely Ag in the sewer SA was at least partially sulfidised even at the inlet and further sulfidation may have occurred after the transit in the sewer (Figures A15, A16). Silver sulfide nanoparticles have been observed previously by Kim *et al.* (2010) in sludge and by Kaegi *et al.* (2013) in batch studies. Other elements such as O, Si, Al, Fe, Ca and K are also evident in the EDX spectra probably linked to the presence of aluminosilicates in the solid phase. Due to the high background level of P, S, K, Cl and Fe it is not possible to conclude Ag sulphidation from TEM/EDX alone even though it is likely that this is the case.

Similar behaviour was observed in the control sewer (SB) with sulfidation processes evident right from the influent stage. Furthermore, TEM analysis also revealed the presence of NPs in the control system (Figures A17, A18, A19), despite the fact that Ag was added to this system as dissolved Ag. Previous work has hypothesized that Ag-NPs can be formed under appropriate environmental conditions (Akaighe *et al.*, 2011) and it is likely that Ag sulfide was precipitated as incidental NPs in the sewer system during treatment. Such neo-formation of NPs may occur during the transit into the sewer network but the prevalence of this process is unknown. This result is very interesting as it

highlights the analytical challenge of discriminating between engineered nanoparticles in environmental samples and those forming naturally within environmental matrices.

3.5. nIDDS trial results

The chemical parameters were similar in both the sewer trunks in which nIDDS were deployed; pH and redox potential were 7.3 and -322 mV in the Globe Derby section and 7.11 and -260 mV in the Bolivar section respectively. The DO levels were on average around 0.21 and 0.08 mg/l during all the sampling times (Table A3, A4). Rapid sulfidation was recorded on the nIDDS deployed in both sewer sections, as revealed by XAS analysis. In fact, this trial confirmed that within 2 hours all of the samples were almost completely sulfidised. This again differs significantly from the results of Kaegi *et al.* (2013). One of the advantages of the nIDDS is that they are not subject to ongoing transformations once they have been retrieved and removed from the reactive environment. Therefore, it appears that under the conditions of this study Ag-NPs were predominantly transformed to sulfides in both the laboratory system and field exposure experiments.

4. Conclusions

Relatively little data are available showing the environmental conversion of nanoparticles during the transit from their initial use and release point to where they become part of the wastewater treatment plant influent. In this study, slight differences were observed in the

behaviours of NPs and metal salts but the final transformation products were quite similar. The sulfidic forms represent one of the predominant endpoints in the transformation of dissolved and nanoparticulate forms of Ag and Zn in the wastewater stream but thiolation also plays a role.

Zinc was transformed almost exclusively to sulfidic species regardless of its original form, while for Ag the dominant endpoint in the sewer was also as sulphide minerals, but with a larger contribution of cysteine and histidine bound forms. It should be stressed that accessibility, interactions and competition between other dissolved metals and NPs for binding sites like cysteine and sulfide could slightly modify the transformation pathways and delay the kinetics of transformation of the NPs. Microbial processes play a direct role in terms of speciation not only through driving changes in redox conditions but also by producing materials such as cysteine and histidine that can efficiently complex Ag in biofilms. Thiolation seems to be a key process in situation where due to the low (or absent) oxygen concentrations other mechanisms are inhibited or slowed down like oxidative dissolution/precipitation or direct sulfidation. Given the differences observed with microbial interactions, further research into microbe-mediated transformations under different sewer conditions (e.g. DO levels) is recommended to identify any longer term consequences to their fate..

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