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Behaviour of silver and silver sulfide nanoparticles in the environment: Effects on wastewater treatment processes and soil organisms

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Abstract

Manufactured silver nanoparticles (AgNPs) are the most commonly used manufactured nanomaterial in consumer products. They are incorporated into a vast array of products due to their strong, broad-spectrum anti-microbial activity. However, the anti-bacterial properties that render AgNPs desirable may also lead to increased environmental risks. This thesis examines that impact in wastewater treatment plants (WWTP) and terrestrial environments – the key risk pathways for AgNPs.

This thesis studied the life-cycle of released AgNPs, from their release to potential plant uptake, with a focus on their effects and fate in the environment. Four main experiments were undertaken to investigate 1) the effects of AgNPs on WWT processes, 2) the bioavailability of AgNPs and transformed AgNPs (Ag_2S-NPs) in soils to lettuce, 3) the effect of fertiliser addition on the bioavailability of AgNPs and Ag_2S-NP and 4) the effects of AgNPs and Ag_2S-NPs on soil microbial communities. The experiments were carried out to model realistic exposure concentrations and pathways (e.g. wastewater and soil cf. synthetic wastewater and hydroponic conditions, and Ag_2S-NPs cf. 'pristine' AgNPs).

The results from this thesis demonstrate that sub-dominant wastewater microbial communities can be affected by AgNPs. However, dominant microbial communities and key WWT processes, such as nitrification and methanogenesis, are unlikely to be affected by AgNPs at realistic exposure concentrations. During wastewater treatment it was found that AgNPs were almost completely transformed (> 95%) to sulfidised Ag species, predominantly as Ag-sulfide (Ag_2S-NPs).

The bioavailability of sludge-borne Ag_2S-NPs in soil was found to be very low. However, when thiosulfate fertiliser was added to soil, significantly more Ag was taken up by plants. Despite this increased uptake, the overall plant concentrations of Ag remained low; the Ag concentrations in edible plant parts (shoots) increased from 0.02% to 0.06% of the total amount of added Ag.

Finally, to assess the degree of risk that AgNPs and Ag_2S-NPs pose to soil microorganisms, a new molecular-based approach was developed to determine the effect on whole soil microbial communities. This new approach was used to calculate toxicity values for individual soil microbial populations following their exposure to Ag^+, AgNPs and Ag_2S-NPs. A combination of quantitative PCR (qPCR) and pyrosequencing-based analysis of the 16S rRNA gene region was used to develop dose-response curves for sensitive microbial populations. Based on pyrosequencing results, similar sequences were assigned to operational taxonomic units (OTUs); the abundances of which were then converted to absolute values. Toxicity values (EC_{20}) for sensitive soil OTUs were then plotted on a sensitivity distribution in order to calculate the Ag concentration that would theoretically protect a specified percentage of soil microorganism gene sequences (HC_x values). At the HC5 and HC10 values (95% and 90% of soil OTUs protected, respectively), there were no significant differences between Ag treatments, while at the HC20 (80% of OTUs protected), Ag_2S-NPs were significantly less toxic than AgNPs and Ag^+. The most sensitive OTUs (EC_{20} < HC5) were predominantly from the *Bacillaceae* family, with lower abundances of other families including *Frankiaceae*, *Comamonadaceae* and *Pseudonocardiaceae*.

In all experiments described in this thesis, the negative impacts of AgNPs and Ag_2S-NPs were less than or equal to the effects observed in ionic Ag (Ag^+) treatments. Overall, results from this thesis show that the risks associated with AgNPs and Ag_2S-NPs are overestimated (and conservatively covered) by the risk of ionic Ag^+ in terrestrial environments.

Declaration

I, Casey Doolette, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide.

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Publications arising from this thesis

Journal articles

Doolette, C.L, McLaughlin, M.J., Kirby, J.K., & Navarro, D.A. (2015) Bioavailability of silver and silver sulfide nanoparticles to lettuce (*Lactuca sativa*): Effect of agricultural amendments on plant uptake. *Journal of Hazardous Materials* (accepted pending revision)

Doolette, C.L, McLaughlin, M.J., Kirby, J.K., Batstone, D.J., Harris, H.H., Ge, H., & Cornelis, G. (2013) Transformation of PVP coated silver nanoparticles in a simulated wastewater treatment process and the effect on microbial communities. *Chemistry Central Journal* 7(1), 46-64.

Conference abstracts

Doolette, C.L., McLaughlin, M., Kirby, J., Navarro, D.G., & Gupta, V.G. (2014) The influence of sulfidised silver nanoparticles on soil nitrification processes and soil microbial communities. *Proceedings of: Society of Environmental Toxicology and Chemistry (SETAC) North America 35th Annual Meeting*, Vancouver, Canada.

Doolette, C.L., McLaughlin, M.J, Kirby, J., Navarro, D.G., Harris, H.H., & Cornelis, G. (2014) The influence of agricultural amendments on the bioavailability of silver sulfide nanoparticles in soil. *Proceedings of: 9th Society of Environmental Toxicology and Chemistry (SETAC) Asia Pacific 2014 Conference*, Adelaide, Australia.

Doolette, C.L., McLaughlin, M., Kirby, J., Harris, H.H., Navarro, D.G., & Cornelis, G. (2013) Will the environmental impacts of manufactured silver nanoparticles be more than a small problem? *Proceedings of: 3rd Society of Environmental Toxicology and Chemistry (SETAC) Australasia Conference*, Melbourne, Australia.

McLaughlin, M.J., Navarro, D.A., Langdon, K., Kirby, J.K., Kookana, R.S., **Doolette, C.L.**, Settimio, L., & Kumar, A (2013) Comparing the fate of ionic silver and nanometallic silver in soils - implications for ecotoxicity tests and risk assessment. *Proceedings of*: *SETAC North America 34th Annual Meeting*, Nashville, United States.

Doolette, C.L., McLaughlin, M.J., Kirby, J.K., Harris, H.H., Navarro, D., Cornelis, G., Batstone, D., & Ge, H. (2013) Will silver nanoparticles affect the efficient functioning of wastewater treatment plants? *Proceedings of*: *CSIRO Nanosafety Workshop for staff involved in nanosafety projects within the Advanced Materials Transformational Capability Platform*, Sydney, Australia

Doolette, C.L., McLaughlin, M.J., Kirby, J.K., Cornelis, G., Batstone, D.J., & Ge, H. (2012) Soil amendment with biosolids exposed to silver nanoparticles: is silver bioavailable? *Proceedings of: Joint SSA and NZSSS Soil Science Conference*, Hobart, Australia.

McLaughlin, M., Kirby, J., Batstone, D.J., **Doolette, C**., & Ge, H. (2012) Manufactured nanomaterials - An emerging issue for wastewater treatment and biosolids reuse. *Proceedings of: AWA Biosolids and Source Management National Conference*, Gold Coast, Australia.

Additional journal articles of related work not described in this thesis

Cornelis, G., Pang, L., **Doolette, C.**, Kirby, J.K., & McLaughlin, M.J. (2013) Transport of silver nanoparticles in saturated columns of natural soils. *Science of the Total Environment* 463–464, 120-130.

Cornelis, G., **Doolette, C.**, Thomas, M., McLaughlin, M.J., Kirby, J.K., Beak, D.G., & Chittleborough, D. (2012) Retention and dissolution of engineered silver nanoparticles in natural soils. *Soil Science Society of America Journal* 76(3), 891-902.

Statement of authorship

Components of the research described in this thesis have been published, or have been submitted for publication and are in review, or, are currently being prepared for publication. The contribution of each author to these works is described below.

Chapter 2: *Chemistry Central Journal;* 2013, **7**, 46 – 64.

Chapter 3: *Journal of Hazardous Materials*; 2015 (submitted).

Chapter 4: *The ISME Journal* (in preparation).

DOOLETTE, C.L. (Candidate)

Chapters 2 – 5: Experimental development; performed experiments; data analysis and critical interpretation; prepared the manuscripts; created all figures.

I hereby certify that the statement of contribution is accurate.

Signed Date

MCLAUGHLIN, M.J., KIRBY, J., NAVARRO, D.A. (Supervisors)

Chapters 2 – 5: Project design; experimental development; data analysis and critical interpretation of results; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date

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Signed Date

HARRIS, H.H. (Supervisor)

Chapter 2: Assisted and supervised XAS experiments; data interpretation; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date

CORNELIS, G. (Supervisor)

Chapter 2: Assisted with experimental design; data interpretation; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date 24 June 2015

BATSTONE, D.

Chapters 2 and 4: Assisted with project design and set-up; data analysis and interpretation; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date 25/6/15

GE, H.

Chapter 2: Set-up sequencing batch reactors; collected and analysed anaerobic sludge samples; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date 25/6/15

PAYNE, J.L.

Chapter 4: Co-wrote the R code; data analysis and critical interpretation of results; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date

GUPTA, V.V.S.R.

Chapter 4: Project design; data analysis and interpretation; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date

LU, Y.

Chapter 4: Assisted with project design; prepared the supplied DNA samples for pyrosequencing analysis; performed qPCR analysis; processed raw data from pyrosequencing and qPCR analysis; manuscript review.

I hereby certify that the statement of contribution is accurate.

Signed Date 25/6/15

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This thesis is dedicated to the memory of my Dad, Kevern Doolette, who encouraged me to take every opportunity to keep learning. More importantly, he taught me that it's the people you meet and friendships you make along the way that are the most rewarding.

Motivations and aims of the thesis

The past two decades have seen a dramatic increase in the use of silver nanoparticles (AgNPs) in consumer products. The widespread use of AgNPs can be attributed to their unique and enhanced physicochemical properties compared to the bulk material (> 100 nm). However, in the majority of AgNP-enabled consumer products, AgNPs are incorporated due to their broad-spectrum antimicrobial activity. It is this antimicrobial property that may pose a potential risk to the environment.

The main environmental exposure pathway of AgNPs is *via* soil application of AgNP-containing biosolids. When AgNP-containing products are washed, a portion of AgNPs are released into wastewater streams. Silver NPs – and ionic Ag (Ag^+) – can be released readily from AgNP-containing textiles, food containers and paints, and through the use of AgNP-coated washing machines. During wastewater treatment (WWT), the majority of AgNPs (or Ag^+) will adsorb to the biosolids (stabilised sludge) and depending on location, biosolids may then be applied to soil as an agricultural amendment to improve soil fertility and act as a soil conditioner. For example, in Australia, approximately 60% of biosolids are used in this way. Therefore, when considering the lifecycle of AgNPs and the potential environmental risks, the two main compartments that are susceptible to deleterious effects are WWT processes and soil environments.

In both WWT plants and terrestrial systems, microorganisms are responsible for a number of essential processes. Therefore, given the strong antimicrobial properties of AgNPs it is important to understand the fate and behaviour of AgNPs in soils and WWT plants: it is arguably more important to understand if the potential risks are over/under-estimated by the risks of Ag^+ in these two systems. A number of studies that have investigated the toxicity and behaviour of AgNPs have not included Ag^+ treatments; hence, it remains unclear whether the observed effects are nano- specific.

In the environment, the concentration of AgNPs is expected to be in the ng kg^{-1} to mg kg^{-1} range. Given the limited number of studies that have used realistic exposure concentrations, there is significant motivation to investigate the effects of AgNPs on environmental systems using realistic exposure scenarios. In addition to AgNP concentration, a number of other factors must also be considered to ensure that the risks of AgNPs are not overestimated, including the test matrix (e.g. synthetic wastewater vs. real wastewater), exposure times and AgNP transformation products.

During WWT, AgNPs may be transformed into nano- sized sulfidised Ag aggregates (Ag_2S-NPs) which are adsorbed by, or incorporated into, the biosolids. Therefore, in biosolids, AgNPs will not be present as 'pristine' AgNPs but will exist as Ag_2S-NPs. While studies that use pristine AgNPs are important for understanding the mechanisms of AgNP toxicity, such studies do not accurately predict the potential risks of AgNPs. Hence, to gain a more comprehensive understanding of the effects of AgNPs in WWT plants and soils, it is necessary to undertake experiments that use realistic spiking concentrations, test matrices, time scales and Ag species.

The main objective of this research was to carry out a comprehensive study of the behaviour of AgNPs in the environment; from their release into wastewater to their application to soil as Ag_2S-NPs in biosolids. Specifically, the aims of this thesis were to:

1. Investigate the fate of AgNPs during wastewater treatment and the changes in Ag speciation during this process;
2. Determine the effect of AgNPs on wastewater treatment processes using realistic experimental conditions;
3. Compare the bioavailability of transformed AgNPs to that of AgNPs and ionic Ag^+;
4. Determine the effects of agricultural amendments on the bioavailability of transformed AgNPs and AgNPs;
5. Investigate the effects of transformed AgNPs on soil nitrification processes; and,
6. Use genomic tools to develop a new method to quantify the ecotoxicity, and thus potential risk, of transformed AgNPs to whole soil microbial communities.

Structure of this thesis

This thesis is presented as a combination of papers that have been published, submitted for publication, as well as chapters that have not been submitted for publication.

Chapter 1 provides an overview of the literature on the behaviour of manufactured and naturally occurring nanomaterials in the environment with a focus on silver nanoparticles (AgNPs). Background information is also given for bulk Ag and the potential mechanisms of AgNP toxicity. Additional information that is specific to each study is presented in the introduction of each chapter.

Chapter 2 comprises a paper that is published in the *Chemistry Central Journal*. It describes the effects of AgNPs on wastewater treatment processes and assesses the chemical transformation of AgNPs during wastewater treatment.

Chapter 3 comprises a paper that has been submitted to the *Journal of Hazardous Materials* and has been accepted for publication pending revision. It describes two pot trials that were undertaken to examine the bioavailability of transformed AgNPs to lettuce. In the first pot trial, biosolids that were produced in **Chapter 1** and that contained transformed AgNPs (as Ag_2S-NPs) were applied to soil. In the second experiment, the effects of fertiliser application on the plant uptake of Ag were investigated.

Chapter 4 describes a soil incubation experiment that was carried out to investigate the sensitivity of soil microbial populations to AgNPs and transformed AgNPs. This experiment used molecular-based techniques to develop a new method that could be used to quantify the risks of AgNPs and Ag_2S-NPs to soil microorganisms. These results will be submitted for publication following submission of this thesis.

Chapter 5 provides a summary of the main outcomes of this thesis and includes recommendations for future research in this area.

Appendices 1 and **2** describe the method development for Ag_2S-NPs synthesis and for growing lettuce in a sandy soil.

Appendix 3 describes attempts to investigate the impact of fertiliser application on Ag speciation in soil using synchrotron based X-ray absorption spectroscopy analysis.

CHAPTER 1

The behaviour of manufactured silver nanoparticles in the environment:
A review of the literature

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The behaviour of manufactured silver nanoparticles in the environment: A review of the literature

1. Introduction

Rapid expansion of the nanotechnology industry has occurred over the previous two decades. Manufactured nanomaterials (MNMs) are commonly defined as materials that have at least one dimension < 100 nm (10^{-7} m) [2]. Materials in this size range (1 – 100 nm) exhibit unique and enhanced electronic, optical and chemical properties compared to the bulk material. As a result of these enhanced properties, MNMs have a variety of applications across many industries. Such applications include pharmaceutical and medical products, consumer goods, microelectronics, and automotive products [3]. Nanomaterials can be manufactured in a wide variety of forms. However, for clarity, they can be divided into two main groups 1) nano-sized particles (having at least two dimensions < 100 nm) and 2) materials that are not particulate but have features that are specific to their nano- size [4]. The former group, nanoparticles (NPs), are the most common type of MNM and include carbon-based fullerenes, quantum dots and metal NPs. Of the metal NPs, silver NPs (AgNPs) are the most commonly used in consumer products [5].

Despite their extensive use, the environmental risks associated with AgNPs remain unclear. Studies have demonstrated that when AgNP-containing products are washed, a portion of the embedded AgNPs will be released into wastewater streams [6-8]. Therefore, wastewater treatment (WWT) processes are susceptible to the potentially deleterious effects of AgNPs [9-11].

It is likely that the majority (> 90%) of AgNPs entering a WWT plant will be removed from the wastewater fraction and adsorb to the biosolids [12]. Depending on location, biosolids may then be applied to soil as an agricultural amendment; a common practice in Australia. Given this scenario, it is likely that soils will act as a sink for AgNPs. Currently, little is known about the behaviour, fate and toxicity of AgNPs in terrestrial systems.

This chapter reviews the literature regarding the fate of AgNPs in the environment: specifically, their mechanisms of toxicity, their effects on WWT processes and terrestrial organisms, and, their behaviour in soil. This chapter also provides background information on the behaviour of natural NPs in the environment and the factors that control their behaviour.

2. Nanomaterials

The definition of NMs is widely debated and it may be inadequate to use one upper size value to classify all NMs [13]. Nanomaterials may exist in single, aggregated or agglomerated forms and have various shapes, for example, tubular or irregular. Materials in this size range (Figure 1) are of great interest as they have enhanced physicochemical, biological and optical properties compared to the bulk material. The unique properties of NMs are primarily due to their small particle size (not an intrinsic nanoscale property) [14, 15] and can be explained by two main factors: surface area and quantum effects.

As particle size decreases, the surface area to volume ratio increases dramatically. This exposes an exponentially increasing proportion of surface molecules (Figure 2) and, as a result, more surface atoms are susceptible to reaction [16]. Due to their high surface reactivity, MNMs are used in a number of applications, yet their enhanced reactivity also renders them undesirable in many cases (e.g. cellular dysfunction and toxicity) [16].

As the size of NMs decrease, quantum effects also begin to control their behaviour. This is because electrons become spatially confined and as a result their normal motion is limited. Electron confinement gives rise to new electronic confirmations [17-19].

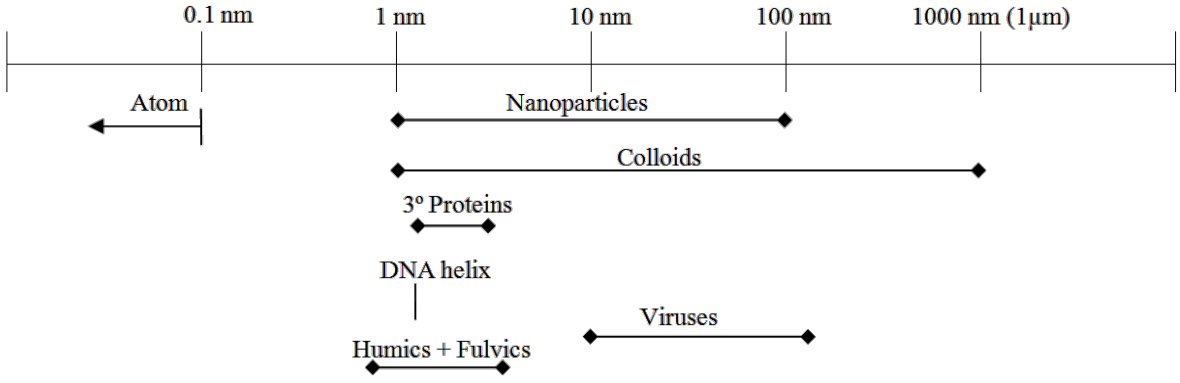

Figure 1. Log scale comparison of nanoparticles and colloids with biological and chemical entities

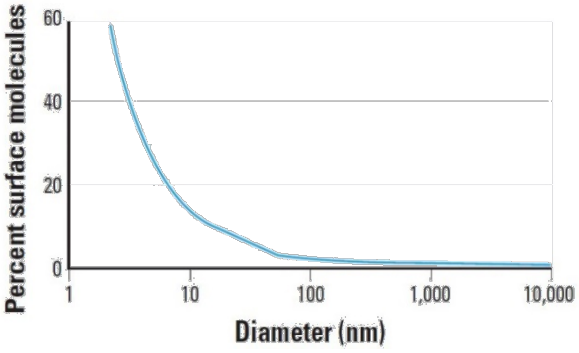

Figure 2. The number of exposed surface molecule increase exponentially as particle size decreases below 100 nm [16].

Consequently, the thermodynamic properties of NMs (e.g. specific heat capacity) can vary greatly from that of the bulk material [20].

3. Manufactured nanomaterials

The term 'manufactured nanomaterial' specifically defines NMs that have been intentionally created and do not occur naturally [21, 22]. Australia's working definition of MNMs, as defined by NICNAS[1], is in line with other international definitions and is stated below:

> *"…industrial materials intentionally produced, manufactured or engineered to have unique properties or specific composition at the nanoscale, that is a size range typically between 1 nm and 100 nm, and is either a nano-object (i.e. that is confined in one, two, or three dimensions at the nanoscale) or is nanostructured (i.e. having an internal or surface structure at the nanoscale)."* [23]

Whilst some NMs are the result of anthropogenic activities (e.g. particulates emitted from car exhausts) it is important to note that such materials are not termed MNMs as their production is not deliberate. Manufactured NMs can be classified as either nano-objects (e.g. carbon nanotubes) or nanoparticles (NPs); having two or three dimensions < 100 nm, respectively [2]. Depending on their composition and form, MNMs can be further categorised into seven main classes: carbonaceous nanomaterials; semiconductors (e.g. quantum dots) metal oxides; nanopolymers (e.g. dendrimers); nanoclays; emulsions (e.g. acrylic latex used in paints); and, metals (e.g. silver) [4]. Due to the many possible forms of MNMs, they are used in numerous industries, for example, food and agriculture, electronics, medical and pharmaceutical, construction, aerospace, automotive and consumer products [24]. Currently, there are 1806 nanotechnology-enabled consumer products that are publically available (December 2014, http://www.nanotechproject.org) and approximately 250 new products are added each year. Of all the types of MNMs that are available, NPs are most commonly used in consumer products and will be the focus of this chapter.

4. Nanoparticles in the environment

Nanoparticles naturally exist in the environment. In soil and aquatic systems, the term "colloid" is used to define particles with at least one dimension between 1 nm to 1 µm size [25]. In the atmosphere, particles of this size are considered part of the 'ultrafine particle fraction' (UFP) [26]. Regardless of the terminology, NPs have always naturally occurred in the environment – in air, water and soil compartments.

4.1. Atmospheric nanoparticles

Nanoparticulate matter can be released into the atmosphere by natural processes including volcanic eruptions, forest fires, terrestrial dust storms and erosion. Simple combustion also releases NPs into the atmosphere and to this effect humans have been releasing NP into the atmosphere for thousands of years. Currently, the primary contributor to atmospheric nano- and micro - particles is from diesel and motor vehicle exhausts [27].

4.2. Aquatic nanoparticles

In natural surface waters, colloidal particles may be generated from various sources, including biota, external sources (e.g. waste disposal), precipitation from supersaturated solutions and from the mobilisation of soil colloids [28]. Aluminosilicate colloids are the most abundant colloids in aquatic environments and enter aquatic systems via the weathering of aluminosilicate rocks [28]. Other natural aquatic colloids include iron oxides, manganese oxides, particulate natural organic matter (humic and fulvic compounds) and carbohydrates that are excreted by algae, bacteria and phytoplankton [28]

4.3. Soil nanoparticles

In soil systems, colloids include clays, humic substances, mobile colloids and, mineral precipitates (Al, Mn and Fe oxides and hydroxides) [29]. Soil NPs are the most chemically active components of the soil and greatly influence plant nutrient uptake [32]. Soil NPs also participate in many ecological processes including; the regulation of element cycling, acting as a source or sink of organic carbon, adsorption of contaminants, and the transport of metals and organic contaminants through the soil column [29]. However, for the latter process to be environmentally significant, specific conditions are required (e.g. the contaminant must desorb slowly but adsorb strongly to mobile NPs) [29].

The behaviour of soil colloids and NPs has been investigated for many decades [33], yet the process of separating naturally occurring soil NPs from manufactured NPs remains a significant analytical challenge [34-36].

[1] NICNAS: National Industrial Chemicals Notification and Assessment Scheme

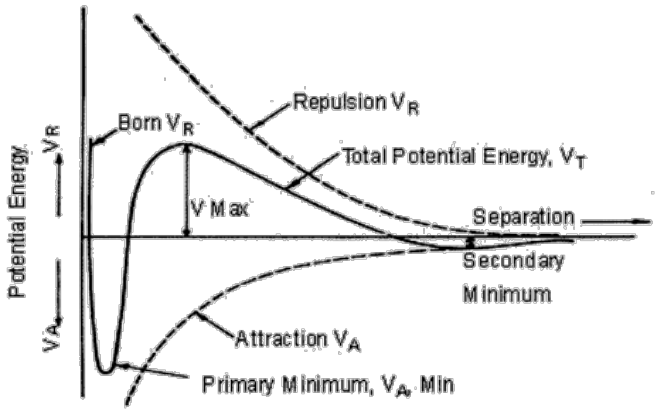

Figure 3. Change in potential energy as the distance between two particles increases. Where V_T = total potential energy, $V_{A=}$ Van der Waals energy and V_R = repulsive energy [1].

4.4. Behaviour of nanoparticles in simple solution phases

A number of studies have investigated the behaviour of colloids in aqueous solutions; however, few studies have focused on terrestrial systems. This can be attributed to the complexity, polydispersity (the property of having many non-identical components in the dispersed phase of a colloidal system) and, spatial and temporal variability of soils [34]. The behaviour of manufactured NPs is likely to mimic that of natural colloids; therefore, it is important to understand what factors influence the behaviour of natural colloids [4]. The following discussion describes the main factors that influence the behaviour of nanoparticles (both natural and manufactured) when in solution. From this point forward 'NP' will be used to signify manufactured NPs unless otherwise stated.

Particle stability has a major influence on the environmental behaviour of NPs, specifically their fate, bioavailability and transport. Two important phenomena that influence NP stability are aggregation and dissolution [4, 42]

4.5. Aggregation processes

It is well documented that the behaviour of NPs in solution is controlled by aggregation [43-45]. During aggregation, NPs gradually associate and increase in size until they form larger particles (> 1 µm). Once they reach this size, their transport will be controlled by sedimentation (in solution phases). Before interacting with natural NPs, manufactured NPs may associate with each other (homocoagulation) [46] and once in the environment, they may coagulate with particles of a different type (e.g. clay colloids – heterocoagulation [47]). It is widely accepted that in the natural environment, heterocoagulation between manufactured NPs and colloids will control the behaviour of the former as the concentrations of colloids will far outweigh that of manufactured NPs [42].

For the aggregation of two NPs to occur, an energy barrier must be overcome (Figure 3). As two particles approach each other, the total potential energy curve reaches a maximum, V_{max}, and when this barrier is overcome the particles will attract strongly, leading to aggregation.

This aggregation/repulsion phenomenon can be explained by classic colloidal chemistry theories, including Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. Details of these theories will not be discussed, but the suitability of DLVO theory for predicting NP behaviour in the environment is briefly summarised below.

4.5.1. Suitability of DLVO theory for predicting nanoparticle behaviour in environmental systems

The behaviour of NPs in environmental systems is complicated by the presence of organic colloids (e.g. humic substances). As a result, it is impractical to predict the behaviour of NPs using DLVO theory alone [48]. If organic colloids are smaller than the manufactured NPs, they may form a thin coating on the MNP surface. Conversely, if the organic macromolecules are larger, they may bind to several NPs and induce aggregation by a 'bridging' mechanism [43]. In both cases, aggregation kinetics may be altered considerably compared to those predicted by DLVO theory alone. To overcome this, several studies have used extended DLVO theory which takes into account non-DLVO interactions (e.g. steric [50] and acid-base interactions [51]).

4.5.2. Factors affecting nanoparticle aggregation

Colloid aggregation is influenced by a number of factors, including; composition of the electrolyte, pH, ionic strength, concentration of suspended particles, presence of co-contaminants, physical properties of the particle (e.g. size, density and rigidity) [52] and chemical properties of the particle surface which influences binding strength between particles [42]. It should be noted that the enhanced properties of NPs may affect their aggregation kinetics differently [42]; this is why it is essential to investigate the behaviour of NPs in environment systems.

The mobility of NPs in environmental systems is greatly dependent on their ability to remain suspended [53]. For example, in aquatic systems, continued aggregation of colloids leads to the sedimentation of large aggregates and their removal from the bulk

solution phase. Trace metals may adsorb to sedimenting colloids in a process termed 'colloidal pumping' [54-56]. In aqautic systems, manufactured NPs may mimic this behaviour, with sedimentation being their eventual fate [34]. Less is known about the behaviour of manufactured NPs in soils: NP aggregation may still occur but their attachment to soil surfaces is likely to have more of an effect on NP fate (see **9.1***)*. In environmental systems, aggregation and sedimentation processes decrease the bioavailability of NPs by removing them from the bioavailable pool. However, other processes, such as NP dissolution, can have the opposite effect and increase bioavailability.

4.6. Dissolution processes

Dissolution is the process by which a particle goes into the solution phase, forming a homogeneous mixture [57]. This process may produce products that are toxic to organisms or the environment if they accumulate e.g. dissolution of AgNPs produces toxic Ag ions (Ag^+). During the dissolution process, molecules move from the NP surface to the bulk solution. The rate at which this occurs slows as equilibrium is established between concentrations in the bulk solution and the diffuse layer. The main factors that influence NP dissolution are 1) NP solubility, and 2) the concentration gradient between the bulk solution phase and the particle surface [57]. Nanoparticles, if uncoated, are expected to dissolve faster than the bulk material for these reasons, but also because the thickness of the electric double layer decreases with particle size.

The behaviour of manufactured NPs in the environment will be controlled primarily by aggregation, sedimentation and dissolution processes. The influence of each process will be dependent on the specific environmental system (e.g. soils, aquatic systems etc.), extrinsic factors (e.g. solution pH, ionic strength, temperature) and intrinsic NP properties (e.g. solubility, chemical composition, surface morphology, surface charge, nature of capping agents).

5. Silver

5.1. Physico-chemical properties of silver

As stated in the Introduction, AgNPs are one of the most commonly used MNMs in consumer products, accounting for 24% of all nanotechnology-enable consumer products [58]. Whilst their high electrical conductivity means that they are used in a variety of electrical goods, the primary reason for incorporating AgNPs into consumer products is for their broad spectrum anti-microbial activity [59]. In the following section, the physicochemical properties of bulk Ag will be summarised, followed by a discussion of AgNPs.

Silver has the highest thermal and electrical conductivity of all metals and the lowest contact resistance. The metal is very reactive and can exist in four oxidation states; Ag^0, Ag^+, Ag^{2+} and Ag^{3+}. Metallic Ag (Ag^0) and monovalent Ag^+, are the most environmentally abundant species [60]. In the natural environment, Ag is rare, with an abundance of ~ 0.07 mg Ag kg^{-1} in the Earth's crust [61]. Silver is extracted from Argentite (Ag_2S) – the most common Ag mineral ore – and to a lesser extent from lead ores [62].

Silver can be released into the environment by natural geological processes (weathering of rock) or by anthropogenic activities such as smelting, mining of Ag_2S, waste from the photographic industry [61] and release from AgNP-containing products. In the environment, Ag^+ exists together with sulfide, bicarbonate or sulfate or adsorbed onto particulate matter in the aqueous phase with sulfates or chlorides [63]. In freshwater and soils under oxidising conditions, Ag compounds primarily occur with halides, specifically bromides, chlorides and iodides [64]. The interaction of AgNPs with these ions will also play a role in the stability and fate of AgNPs in terrestrial systems.

Elemental Ag is insoluble in water but soluble when in the metallic salt form (e.g. $AgNO_3$ and Ag_2SO_4). The solubilities of other Ag compounds are shown in Table 1. Silver, being a highly polarisable metal and soft acid does not form stable complexes with highly electronegative atoms (e.g. O-containing functional groups). Instead, it forms stable complexes with ligands that contain atoms of low electronegativity (e.g. N or S); where the interaction involves significant covalent character. Therefore, in soil, the interactions between Ag^+ and O containing groups (e.g. COOH) of soil organic matter (SOM) are less relevant than N or S binding [65].

Table 1. Solubility product constants (K_{sp}) for various silver compounds at 25°C. All values are from [66], except Ag_2S [67].

Silver compound	Formula	K_{sp} (at 25°C)
Silver(I) acetate H	$AgCH_3COO$	1.94×10^{-3}
Silver(I) carbonate	Ag_2CO_3	8.46×10^{-12}
Silver(I) bromide	AgBr	5.35×10^{-13}
Silver(I) chloride	AgCl	1.77×10^{-10}
Silver(I) iodide	AgI	8.52×10^{-17}
Silver(I) phosphate	Ag_3PO_4	8.89×10^{-17}
Silver(I) sulfide	Ag_2S	6.30×10^{-50}

6. Silver nanoparticles

6.1. Historical uses of silver nanoparticles

Over the previous decade, research interest in AgNPs has dramatically increased, but the use AgNPs is not new [68]. In 1889, Lea described the synthesis of a citrate-stabilised Ag colloid [69]. Then, in 1897 a AgNP/Ag-colloidal preparation sold as "Collargol" was manufactured for medical applications [70]. In the following decades, numerous AgNP preparations were

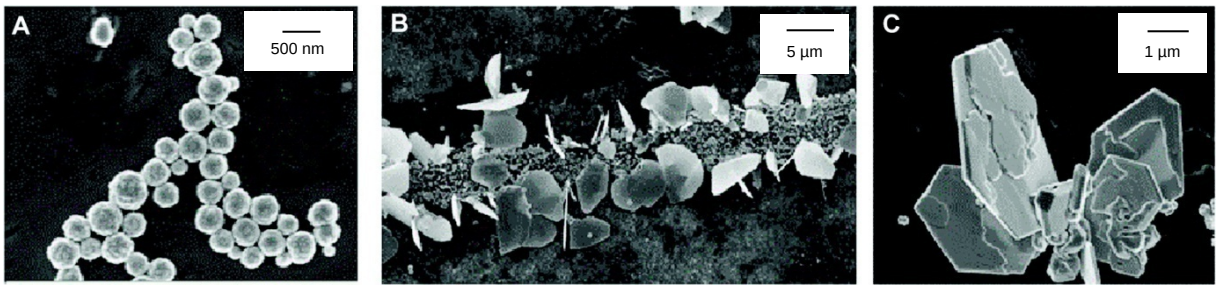

Figure 4. Silver nano- and micro- structures of varying size and shape: A) colloidal synthesised particles deposited on a titanium surface, B) silver flakes C) higher magnification of a silver flake cluster [86]

developed and marketed chiefly as anti-bacterial products. The inventors of the first AgNP containing products understood the importance of NP size [71, 72]. In contrast to other MNMs (e.g. fullerenes), AgNPs are not new materials; instead they have been used for many decades but over recent years the nomenclature has changed [68]. Although the use of AgNPs is not novel, the synthesis techniques and wide range of applications that use AgNPs are.

6.2. Forms of manufactured silver nanomaterials and classification

The key determinants of AgNP toxicity are: NP shape [90, 91]; particle size [87]; and, surface properties [75, 88, 89]. For example, triangular AgNPs (Figure 4) have been shown to be more toxic to Gram-negative bacteria than spherical or rod-shaped AgNPs [90]. Regardless of their specific properties, AgNPs are incorporated into products primarily for their strong anti-microbial activity [85].

Products containing AgNPs have been categorised by the US-EPA into three main groups: 1) Ag-impregnated water filters; 2) Ag algaecides and disinfectants; and 3) Ag biocidal additives [68]. Silver-impregnated water filters have been used since the 1970s and usually consist of activated carbon impregnated with AgNPs [68]. Algaecides and disinfectants have been used in direct water applications since the 1950s (e.g. swimming pools). In this case, AgNPs are stabilised in solution and then added to water. Silver biocides are currently the most common application of AgNPs. Consumer products that utilise the broad-spectrum antimicrobial properties of AgNPs include; textiles (e.g. clothing), appliances (e.g. refrigerators), personal care products (e.g. hair brushes), food preparation products (e.g. chopping boards) and medical products (e.g. bandages).

7. Toxicity mechanisms of silver nanoparticles

Despite their widespread use as biocidal agents, the toxicity mechanisms of AgNPs are only partially understood; there is ongoing debate as to their exact mode of action [85, 92]. Furthermore, it is not known to what extent AgNP dissolution drives toxicity i.e. does released Ag^+ cause toxicity or is it a specific AgNP effect? The majority of studies suggest that AgNP dissolution is the most important factor controlling toxicity [93-98]. However, some studies [99, 100] have shown that released Ag^+ is not responsible for all of the observed toxic effects of AgNPs [92].

The primary mechanisms of AgNP toxicity are likely to be a combination of oxidative stress, lipid peroxidation, membrane damage and direct uptake effects [92]; whereby oxidative stress is considered to be the main cause [101]. A simplified diagram showing potential toxicity mechanisms is shown in Figure 5.

Recently, Ivask et al. extensively reviewed the toxicity mechanisms of AgNPs to various organisms [92]. Of all the published articles that the authors cited, they found that 69% of studies focused on bacteria (*Escherichia* coli), 19% on mammalian cell cultures and less than 4% investigated environmentally relevant organisms (e.g. fish and algae) – soil organisms had not been investigated. This highlights the knowledge gap regarding the effects of AgNPs on terrestrial organisms.

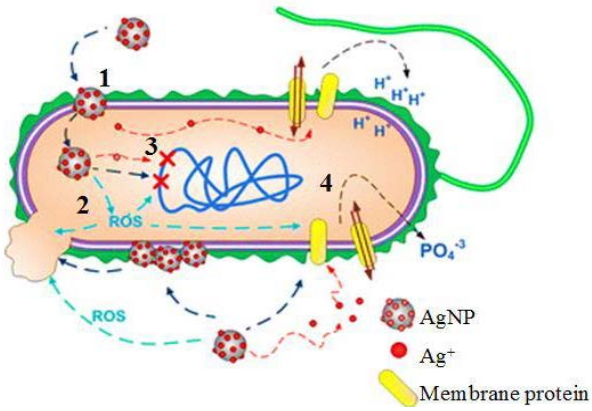

Figure 5. Summary of the interactions of AgNPs with bacterial cells. Silver NPs may 1) release Ag^+ and generate reactive oxygen species inside the cell (ROS); 2) disrupt functioning of membrane proteins and cause proton efflux [102, 103]; 3) generate ROS, release Ag^+ and affect DNA; and 4) release Ag^+ disrupting cell phosphate balance [104]. Alternatively, AgNPs may not be taken up directly by the cell and instead, released Ag^+ may directly affect the cell membrane. Adapted from [73].

8. Fate and effects of silver nanoparticles in the environment

8.1. Release of silver nanoparticles into the environment

Silver NPs enter wastewater streams [105] upon their release from AgNP-containing products such as textiles [6, 8, 106], outdoor paints [107], plastics [10] and washing machines [7]. During wastewater treatment (WWT), AgNPs may be converted to Ag^+ [6], agglomerate [108], complex with ligands [89] or remain as NPs [10]; however, the most likely outcome is *in situ* formation of Ag-sulfide NPs (Ag_2S-NPs) [12, 109]. The majority of AgNPs (and their transformation products) will be captured by biosolids (> 90%) [10, 110, 111] due to strong complexation with dissolved organic carbon and numerous ligands, such as chloride and sulfide (log K = 9.7 and 49, respectively [112]).

The end use of biosolids varies greatly between locations. In Switzerland, all biosolids are incinerated (for cement production or in industrial furnaces) [113], whereas in the EU, US and Australia, a proportion is applied soil as an agricultural amendment to improve soil fertility (55% [10], 63% [114] and 55% [115] respectively). As is the case for all environmental systems [116-118], predicting the soil concentration of Ag in biosolids-amended soil is challenging [119]. However, current models suggest that Ag concentrations in biosolids-amended soil will be in the µg Ag kg^{-1} range; with an annual increase of ~ 500 – 1500 ng kg^{-1} y^{-1} [9]. There is insufficient data to quantify whether such concentrations would pose a risk to soil organisms. It should be noted that these predictions are based on broad assumptions and data extrapolation. Regardless of the specific Ag concentrations, it is essential to understanding the behaviour of manufactured AgNPs in WWT plants and terrestrial systems in order to perform accurate risk assessments.

8.1.1. Impacts of silver nanoparticles on wastewater treatment processes

Successful treatment of wastewater is dependent on the activity of a diverse range of microbial communities. These wastewater communities are responsible for a variety of processes such as nitrification, denitrification and enhanced biological phosphorus removal. Therefore, it is possible that AgNPs, being strongly anti-microbial, may affect functional wastewater microbial communities and as a result impair the operation of WWT plants [10]. However, the impacts of AgNPs on WWT processes remain unclear [120-122].

Since the research reported in this thesis commenced (2011), the effect of AgNPs on WWT plants has been reviewed [122] and it was found that results are often conflicting. For example, some studies have shown no effect on wastewater nitrification [110], whereas other studies have shown varying degrees of inhibition [123-125] following AgNP addition to WWT plants or bioreactors. Only a limited number of studies have investigated the effects of AgNPs on wastewater microbial communities, but results from these studies are also conflicting. This is most likely due to differences in the test media (synthetic vs real wastewater), AgNP properties, exposure concentrations and the different analysis techniques (e.g. culture methods, qPCR, next-generation sequencing). For example, Yang et al. [126] exposed activated sludge samples to AgNPs (35 nm) at a relatively high concentration (40 mg Ag L^{-1}) and found a significant decrease in microbial diversity and abundance. Conversely, at the same Ag concentration (40 mg L^{-1}), but in a different study, methanogenic communities were unaffected by exposure to AgNPs (30 nm) [127].

Results from a recent sequencing batch reactor experiment [128], showed that AgNPs affect wastewater communities in a dose-dependent manner. At low Ag sludge concentrations (0.1 mg Ag L^{-1}), the populations of some functional bacterial increased (e.g. *Bacteroidetes* or *Inhella* genera). However, at higher concentrations (0.5 mg Ag L^{-1}), AgNPs negatively affected these populations and caused a greater shift in the bacterial community composition compared to the control. One of the key points to consider when carrying out such studies is the speciation of Ag. As with most metals, speciation will control stability and mobility and, therefore, toxicity.

8.1.2. Transformation of silver nanoparticles during wastewater treatment

Prior to the commencement of the research reported in this thesis, little was known about the transformation of AgNPs during WWT. This paragraph details the knowledge gaps prior to 2011; the year that the reported research commenced. It has been suggested that during WWT, AgNPs will be transformed to sulfidised Ag species (primarily to Ag_2S) [109,129]. However, it is unknown at what stage of the WWT process sulfidation will occur. Broadly speaking, WWT processes can be classified as either aerobic or anaerobic. Both processes are usually used in modern WWT plants that service large cities, whereas smaller, rural WWT plants may only use one process or the other. The order of treatment may also differ between WWT plants e.g. aerobic treatment (activated sludge processes) may be used before or after anaerobic digestion. Therefore, given that WWT plants differ in their set-up, investigation is needed in order to understand at what stage of WWT AgNP sulfidation occurs. For example, if sulfidation does not occur during aerobic processes, the biosolids and effluent produced from WWT plants that only use aerobic treatment may pose a greater risk than those produced from other WWT plants that employ both processes.

Silver sulfide species may also exist in the nano-size range in sludge; however, it is unclear if AgNPs are directly sulfidised to Ag_2S-NPs or if they dissolve and then re-precipitate as nano- sized Ag_2S aggregates [130]. Whilst the extent of sulfidation and the time taken for it to occur are still debated, recent studies have suggested that it may occur to completion (>90%) relatively quickly; again, this is dependent on the set-up of the WWT plant [12, 109, 129].

There is very limited information on the chemical and physical properties of Ag_2S-NPs in different environments. However, similar to other metal sulfide complexes [131], Ag_2S-NPs are very stable under aerobic conditions [89]. This stability is due to the low solubility of Ag_2S (K_{sp} = 6.3 x 10^{-50}) which makes it relatively resistant to oxidation and dissolution. Several studies have demonstrated that sulfidation reduces the toxicity of AgNP to bacteria [89, 132, 133]. The extent to which sulfidation decreases the toxicity of AgNPs to wastewater bacteria has been debated recently [134]. Overall, the toxicity, fate and behaviour of biosolids-borne sulfidised Ag-NPs in terrestrial systems have not been investigated.

9. Behaviour of silver nanoparticles in terrestrial systems

9.1. Fate of silver nanoparticles in soil

Very few studies have investigated the behaviour of AgNPs in soil – the majority of studies have focused on aquatic systems. Given that soils are a likely sink for AgNPs, it is essential this knowledge gap is addressed. The fate and bioavailability of manufactured NPs (not AgNPs specifically) has recently been reviewed [46, 135, 136]. Cornelis et al. [46] also described the processes that will control the fate of manufactured NPs in terrestrial systems (Figure 6).

A number of factors will influence the behaviour of AgNPs (and Ag_2S-NPs) in soil, including; soil pH, soil clay content, concentration of soil dissolved organic matter (DOM), soil salinity and AgNP properties (e.g. charge, size, Ag speciation). The interaction of AgNPs with DOM is one of the most important factors. Soil DOM can have two opposite effects; it can either stabilise or destabilise AgNPs in soil [46, 137]. A recent study [138] suggested that Ag concentration will influence whether DOM promotes AgNP stability or aggregation. The authors found that uncoated AgNPs adsorbed to DOM in soil solution extracts at Ag concentrations of 0.5, 5 and 10 mg L^{-1}. At the lower concentrations (0.5 and 5 mg L^{-1}), adsorption of AgNPs to DOM caused instability and AgNP aggregation resulted, while at 10 mg Ag L^{-1}, DOM promoted stabilisation and AgNP aggregates were smaller. Stabilisation of AgNPs by DOM can occur *via* the electrostatic repulsion of DOM hydroxyl groups [139, 140] or due to steric effects. The concentration of dissolved organic carbon has also been shown to affect the toxicity of Ag^+ (not AgNPs) to soil nitrification processes [141]. The behaviour of AgNPs in soil can also be affected by clay content as demonstrated by Cornelis et al. [142]. This study calculated the non-equilibrium retention factors (K_r) of AgNPs in 16 Australian soils and found that clay content was the only parameter that correlated with *K*r values. The fate and behaviour of AgNPs in soils will determine their toxicity and bioavailability to terrestrial organisms.

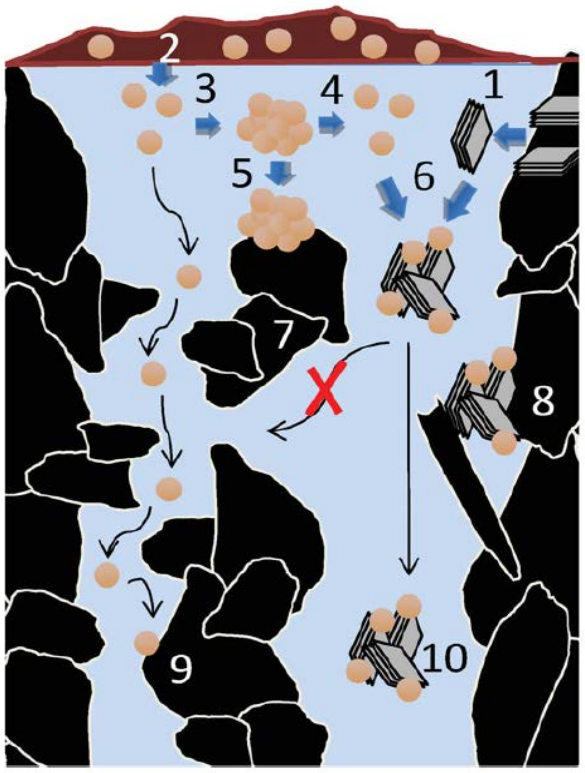

Figure 6. The main fate-determining parameters of colloids and manufactured nanoparticles (MNPs) in soil. 1. Colloid generation. 2. MNP leaching from biosolids. 3. Homoaggregation. 4. Fragmentation. 5. Sedimentation. 6. Heteroaggregation. 7. Size exclusion. 8. Straining. 9. Deposition 10. Convective transport. [46].

9.2. Plant uptake of silver and silver sulfide nanoparticles

The bioavailability of AgNPs to plants has been investigated to a limited degree. The majority of studies have used solution media and not natural soils [136]. All plant phytotoxicity studies of AgNP have used hydroponic growing conditions or exposed plant seeds to AgNPs in a petri-dish. A hydroponic experiment using zucchini found a reduction in plant biomass and transpiration when plants were exposed to AgNPs at 250 mg L^{-1} [143]. In another study, that exposed crop seeds – specifically flax (*Linum usitatissimum*), ryegrass (*Lolium perenne*) and two-rowed barley (*Hordeum vulgare*) – to suspensions of AgNPs (0 – 100 mg Ag L^{-1}), germination was adversely affected at 10 mg Ag L^{-1} [144].

The bioavailability of bulk Ag_2S to a variety of crops (corn, lettuce, oat, turnip and soybean) has been investigated by Hirsch [145]. The author spiked a natural soil with biosolids and bulk Ag_2S at 12 and 106 mg Ag kg^{-1} and measured the plant uptake of Ag. In the edible plant parts (e.g. lettuce leaves, corn kernels, oat grains) lettuce was the only crop that accumulated significantly more Ag ($p \leq 0.05$) than plants grown in the control soil (soil amended with biosolids – no added Ag_2S). A recent mesocosm study [146] that used a natural soil, exposed several plant species (*Carex lurida, Juncus effusus, Lobelia cardinalis, Microstegium vimineum,* and *Panicum virgatum*) to a sludge slurry that was spiked with

AgNPs to give a soil concentration of 0.14 mg Ag kg^{-1}. Whilst the plant concentrations of Ag were not reported, there were no effects on plant biomass, with the exception of *Microstegium vimeneum*. The effects on the soil microbial communities in this experiment are discussed in detail in Section 11.3. There is a clear knowledge gap regarding the bioavailability of Ag_2S-NPs, the realistic form of AgNPs in the environment, to plants. However, it is not only higher organisms (e.g. plants, soil invertebrates) that may be affected by Ag_2S-NPs, soil microbial communities are also at risk. Just as microorganisms control WWT processes, they are also vital for soil nutrient cycling processes.

10. Effect of manufactured silver nanoparticles on soil microbial communities

10.1. Classification and role of soil microorganisms

Soil microorganisms represent a large fraction of global genetic diversity and are the largest group of organisms in soil [147]; both in terms of the number of different species and overall biomass. Soil microbes range in size from 0.5 to 100 μm for bacteria and nematodes, respectively. This size range relative to soil pore size is illustrated in Figure 7.

The four main types of soil microorganism are bacteria and archaea (both prokaryotic) and fungi and algae (both eukaryotic). Regardless of cell type, all soil microorganisms can be classified based on their physiological characteristics. For example, if a species uses light as their energy source, they are termed phototrophs, whereas if they derive energy chemically, they are termed chemotrophs. The second order of classification relates to carbon source:

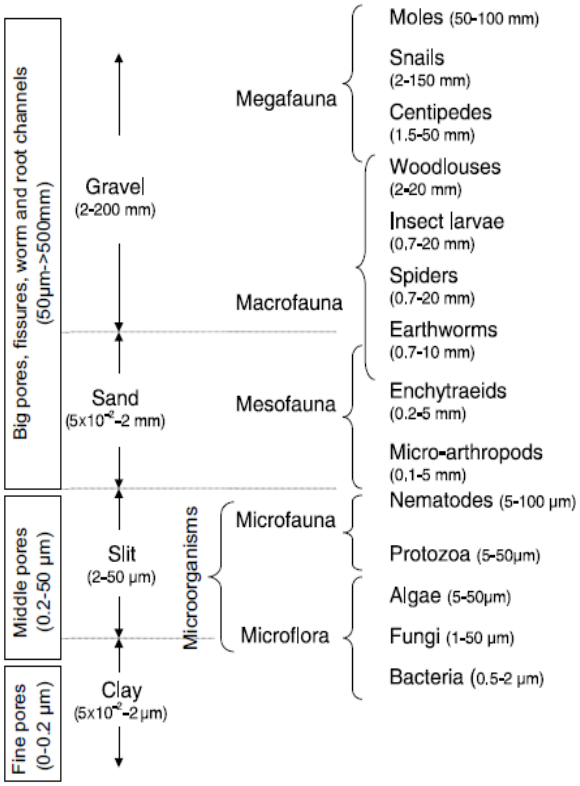

Figure 7. Classification of soil biota in relation to size (in [148], adapted from [149])

organisms that use CO_2 are classified as autotrophs, whereas those that use organic compounds are heterotrophs (Figure 8).

In soil, the vast majority of microorganisms are bacteria and fungi, with most species being chemoorganoheterotrophs. However, other important soil microorganisms, such as those from the *Nitrobacter* genus, are chemolithoheterotrophs. The difference between the two being that the latter obtains energy from inorganic compounds whereas the former uses organic compounds.

Soil microorganisms have a number of important functions in soil including: weathering [150]; formation and stabilisation of soil aggregates (reviewed by [151]); transformation of organic matter [152];
carbon cycling [153]; regulation of plant diversity [154]; and acquisition and cycling of nutrients ([155] and [156, 157], respectively). Given the numerous roles of soil microorganisms, any changes to the soil microbial community structure will also affect ecological processes.

Metal NPs may affect soil biota in two ways 1) effects due to NP size, commonly referred to as a "nano-specific" effects and/or 2) effects that are attributable to the release of metal ions [158]. It is very difficult to determine if effects are caused by released metal ions or if they are "nano-specific", particularly for AgNPs [159-162]. Consequently, debate is still ongoing as to the most important mechanisms that control toxicity. As a result, in the sections that follow, there is no distinction between mechanisms of toxicity or uptake; i.e. the literature that is reviewed is based on experiments that have *exposed* organisms to MNPs, regardless of whether the effects are "nano-specific" or not.

10.2. Uptake of manufactured nanomaterials by soil microorganisms

It is very difficult to predict how metal NPs enter prokaryotic cells. When considering bacteria, the main barrier that prevents NP uptake is the cell wall. The structure of bacterial cell walls varies depending on the type of bacteria (Gram-positive or Gram-negative). Gram-positive bacteria have only one outer layer that consists of peptidoglycan (20 – 80 nm) that is very rigid yet porous. Gram-negative bacteria have two outer layers that are chemically more complex than Gram-positive bacteria; the peptidoglycan layer is thinner (10 – 15 nm) and is encased by an outer membrane (phospholipid bi-layer). The toxicity of MNPs cannot be predicted based on bacterial cell wall properties alone. For example, although the thicker peptidoglycan layer found in Gram-positive bacteria can increase their resistance to NP permeation, the tightly packed lipidpolysaccharide molecules in Gram-negative bacteria can also be an effective barrier against NPs [163]. Despite the differences in cell wall structure, the surface of all bacteria are negatively charged due to deprotonation of carboxylate and phosphate groups [164]. Therefore, positively charged AgNPs may readily attach to bacterial cell walls and induce toxicity. Conversely, negatively charged

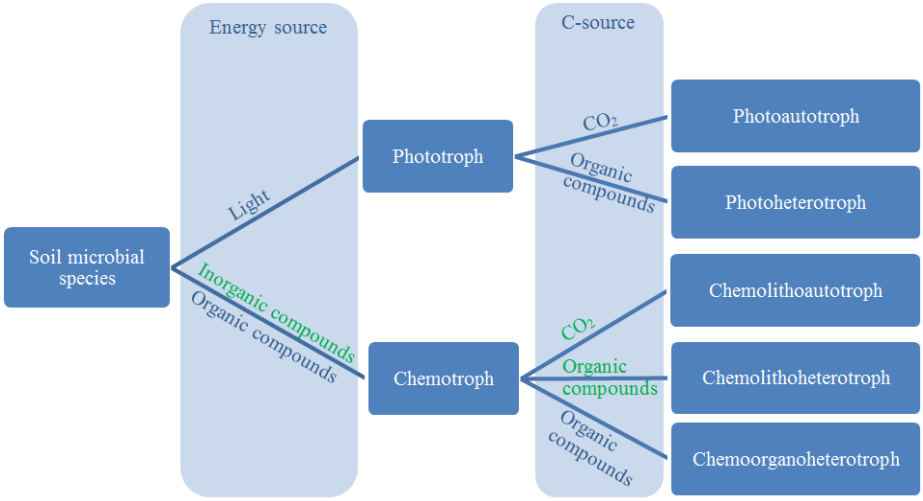

Figure 8. Classification of microbial species based on metabolism and nutritional requirements

AgNPs will experience electrostatic repulsion and as a result may be less toxic to bacteria cells [165].

If the bacteria cell wall is intact, it is unlikely that large NPs (> 16 nm) will directly enter the cell. Instead, it is believed that NPs may migrate into bacteria cells by damaging the cell wall [166, 167]. The direct contact of AgNPs with bacterial membranes has been shown to increase the toxicity of AgNPs toward certain Gram-negative and Gram-positive bacteria [168]. Neal et al. [169] demonstrated similar results when they exposed the soil bacterium *Cupriavidus necator* to zinc oxide NPs: the authors attributed toxicity principally to cell membrane damage.

Despite these studies, the precise mechanism of NP uptake by bacterial cells is still unclear [92, 136]. Even less is known about the uptake of AgNPs by soil microorganisms. However, it is likely that multiple uptake mechanisms are responsible, including: non-specific membrane damage (e.g. *via* ROS production following NP contact with the cell wall [166]); NP dissolution at the cell membrane and subsequent release of metal ions; non-specific diffusion (e.g. through porins) [170]; and, specific uptake [170]. Although there is limited research on the uptake mechanisms of MNPs, the toxicity of NPs has been studied in greater depth. Silver NPs in particular have been the focus of much research due to the possibility of toxicity towards non-targeted bacteria species i.e. beneficial bacteria.

10.3. Toxicity of manufactured silver nanoparticles to specific soil microorganisms

The toxicity of AgNPs to soil bacteria has been investigated in more depth than has occurred for higher organisms (e.g. plants). However, toxicity testing has rarely been carried out using a natural soil, instead, pure culture solution assays are usually used. Fajardo et al. [171] exposed pure cultures of two common soil bacteria, *Bacillus cereus* and *Pseudomonas stutzeri* to AgNPs. They reported significant toxicity for *B. cereus* and *P. stutzeri* following exposure to AgNPs at concentrations of 0.5 and 5 mg AgNP L^{-1}, respectively. Toxic effects of AgNPs to rhizosphere bacteria have also been reported [172]. Rhizosphere bacteria were isolated from a natural soil and cultivated in culture media and exposed to AgNPs. The minimum inhibition concentrations (MIC)[2] of 7 isolated *Bacillus* bacteria were between 1 and 20 mg AgNP L^{-1} [172]. In one of the few studies that have used a natural soil to investigate the effects of AgNPs on soil bacteria, the potency of AgNPs was reduced by soil components (e.g. clay) [173]. More complex methods for toxicity testing were used by Kumar et al. [174] in their experiments that investigated the impact of AgNPs on arctic soil bacterial assemblages. Their methods included differential respiration, phospholipid fatty acid analysis, PCR-denaturing gradient gel electrophoresis and DNA sequencing. The authors found that soil microbial communities were affected by AgNPs when exposed to AgNPs at concentrations of 0.066% and 6.6% (w/w). They also demonstrated that *Pseudomonas* and *Janthinobacterium* bacteria were more resistant to AgNPs than bacterial species from other genera.

Although these examples demonstrate the importance of investigating the effects of AgNPs on specific soil microorganisms, the majority of recent ecological microbiology research has focused on the effect on community composition. As stated in a review by Schimel and Schaeffer [175], the question in relation to soil microbiology has changed from "who's there?" and "what are they doing?" to now "does who's there matter?". The rapid development of new molecular methods (e.g. PCR amplification) for the analysis of soil bacterial communities over the previous 5 – 10 years has changed the way that microbial communities are investigated.

[2] The lowest concentration of an antibacterial agent that inhibits visible growth of a bacterium.

10.4. Next generation DNA sequencing tools to investigate the impact of manufactured nanomaterials on bacterial communities: the role of 16S rRNA gene sequencing

The use of advanced techniques to detect and identify archaea and bacteria in soil samples avoids the problems that are associated with traditional culture-based methods. Such difficulties include isolation of only the most abundant species, bias towards those species that flourish in a particular growth medium, dormant spores that become dominant once cultured and the process is time consuming and laborious [176]. Furthermore, it has been estimated that 99% of soil bacteria species are unculturable [177]. In addition to avoiding these problems, the main advantage of new molecular based techniques (i.e. PCR-based methods) is that bacteria and archaea species are identified *in situ* [176]. By investigating the diversity and structure of soil bacterial communities, a better understanding of the interactions between environmental factors and ecosystem functions can be obtained [178].

Molecular methods involve a broad range of techniques that are based on the analysis of microbial DNA which can be extracted *via* numerous methods as summarised by multiple reviews [179-182]. Target regions of the extracted DNA can then be amplified using polymerase chain reaction (PCR). Next generation sequencing then targets specific regions; for prokaryotes (bacteria and archaea), the most commonly targeted region is the 16S ribosomal[3] (rRNA) gene sequence. This region is often targeted for four reasons 1) it is universally present in all prokaryotes 2) it has a highly conserved region 3) the function of the gene evolves slowly over time and 4) it contains a hypervariable region which leads to species specific sequences which enables taxonomic classification [183]. Analysis of 16S rRNA gene sequences leads to an understanding of the community structure, diversity, and phylogeny of microorganisms in terrestrial environments [184].

10.5. Effect of manufactured silver nanoparticles on soil microbial communities

The long-term impacts of AgNPs on soil microbial communities remain unclear [136, 158, 189]. An incubation study by Hänsch and Emmerling [187] dosed soil at lower AgNP concentrations (3.2 – 320 µg Ag kg soil^{-1}) and found that soil microbial biomass decreased with increasing AgNP concentration and that it was significantly less than the control ($p \leq 0.05$). However, AgNPs did not significantly affect enzymatic activity, soil pH or concentration of soil organic carbon.

Since the research in this thesis commenced, Shah et al. [188] investigated the effect of AgNPs, applied at 0.0625 mg AgNPs kg soil^{-1}, to soil microbial communities using DNA extraction and pyrosequencing analysis (Gray28F and Gray519r primers). After 30 days, AgNPs significantly changed the structure of the soil bacterial community ($p \leq 0.05$). The authors concluded that the effects were not toxic because compared to the control, species richness and the abundance of nitrifiers both increased. In AgNP treated soil, the proportion of organisms from *Bacillus* and *Geobacter* genera increased to double that of the control soil, whilst the abundance of other genera (e.g. *Rudaea*) decreased after 120 days. An Ag^+ control was not used in this study so it is unclear if the effects were caused by AgNPs or released Ag^+.

A similar study by Carbone [186] also used PCR-DGGE (polymerase chain reaction denaturing gel electrophoresis) to investigate the impact of AgNPs on microbial communities in a forest soil. They reported that AgNPs applied to soil at 100 mg Ag kg^{-1} significantly decreased heterotrophic bacteria after 60 days of incubation ($p \leq 0.05$). Furthermore, after 90 days, there was a significant shift in microbial community structure compared to the control soil; there was an increase in the number of microorganisms that are known to be resilient to harsh conditions (e.g. *Dyella* spp. strains). An Ag^+ control was not used in this study, so the effects of AgNPs and released Ag^+ cannot be separated.

During the previous four years, several studies have assessed the short term effects [146, 174, 185-188] of AgNPs on soil microorganisms. A recent study [146] investigated the impact of AgNPs on soil microbial communities by dosing established mesocosms with AgNPs, Ag^+ (control treatment) or a 'slurry-only' control (at 0.14, 0.56 and 0.02 mg Ag kg^{-1}, respectively). The effects on three microbiological properties were investigated: soil microbial biomass, extracellular enzyme production and microbial community composition. Whilst each parameter was significantly affected by AgNPs ($p \leq 0.05$), there were several caveats in the study. For example, after one day, the microbial composition of the AgNP treated soil was significantly different ($p \leq 0.05$) to that of the slurry-only control but not to the Ag^+ treated soil. Moreover, after 50 days, bacterial community composition was the same across all Ag treatments.

A microcosm study using arctic soils suggested that AgNPs could significantly change the structure of soil microbial communities [174]. A high concentration of AgNPs was added to soil (600 mg Ag kg^{-1}) which was then subjected to temperature changes in order to simulate normal seasonal variation. Pyrosequencing analysis of 16S rRNA genes was used to group bacteria into orders. Silver NPs significantly decreased ($p \leq 0.05$) the number of bacterial orders present in soil compared to the control soil and soil dosed with larger Ag particles (7500 nm). At the genus level, one of the most susceptible genera was *Rhizobium*[4] which had a 370-fold decrease in the number of sequences reads in AgNP treated soil. Conversely, the sequence reads for the order

[3] In prokaryotes, 16S ribosomal RNA is a structural component of the small subunit (30S) that makes up the ribosome.

[4] Rhizobiales represent many beneficial plant-associating bacteria.

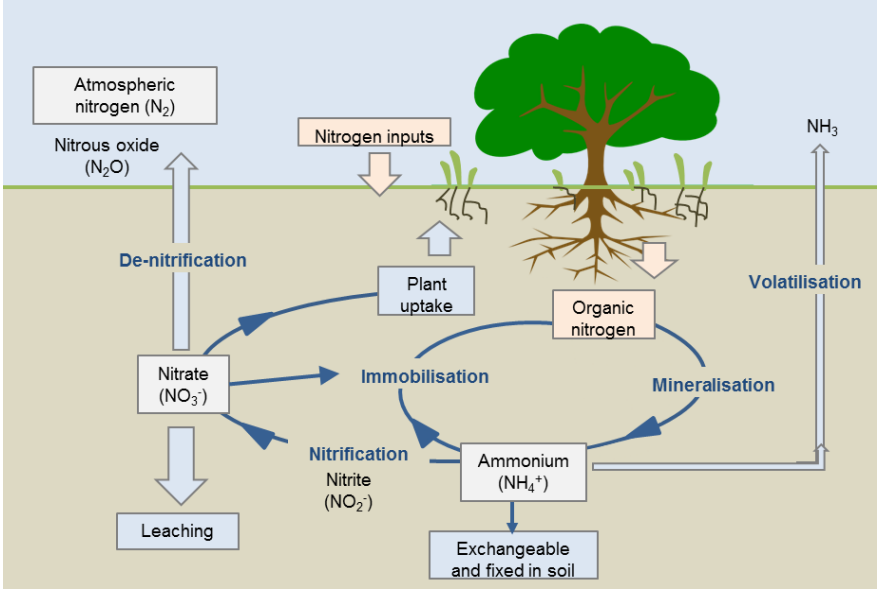

Figure 9. Soil nitrogen (N) cycle showing nitrification. Source: Government of Western Australia, Department of Agriculture and Food.

Nitrosomonadales[5] actually increased compared to the control soil.

It is difficult to make firm conclusions from these studies because in each study, different Ag concentrations were used and more importantly, so were different soils. Soil physico-chemical properties are the main determinant of AgNP toxicity in soil, whereby soil organic matter (SOM) is particularly important [46, 189] (see **9.1**) Despite the differences between studies, the results demonstrate that AgNPs can affect soil microbial communities. Consequently, soil processes that are controlled by microbial communities may also be affected by AgNPs [158], nitrification is one such process.

10.5.1. Effects of silver nanoparticles on soil nitrification processes

Soil nitrification is an important step in the nitrogen transformation process (Figure 9). Nitrification is a two-step process that converts ammonium (NH_4^+) to nitrite (NO_2^-) to nitrate (NO_3^-). Microorganisms are responsible for each step. Specifically, ammonium oxidising bacteria (AOB) (*Nitrosomonas* species) for the conversion of NH_4^+ to NO_2^-, and nitrite oxidising bacteria (NOB) (*Nitrobacter* species) for NO_2^- oxidation to NO_3^- [190]. The process is particularly sensitive to metal contamination and as a result is often used to predict the potential risk of metals in soil. For both soil nitrification and wastewater nitrification, results are conflicting as to whether Ag^+ or AgNPs are more toxic.

Silver NPs have been shown to inhibit the growth and abundance of nitrifying bacteria in wastewater at 1 mg Ag L^{-1} [77]. In soil, Masrahi et al. found that PVP coated AgNPs (15 nm) could suppress soil nitrification processes more than Ag^+ when added to a soil slurry at 1 mg Ag L^{-1} [191]. Conversely, Yang et al. [192] showed that Ag^+ was between 20-fold to 48-fold more toxic than AgNPs to three nitrogen-cycling bacteria.

Ammonia oxidising bacteria in soil will generally be more sensitive to toxicants (i.e. AgNPs) than NOB [125, 192, 193]. Therefore, conversion of NH_4^+ to NO_2^- is more susceptible to AgNPs than the conversion of NO_2^- to NO_3^-. In addition to the negative effects on nitrification, Ag has been shown to have a stimulatory effect on soil nitrification at low Ag concentrations (< 10 mg Ag kg^{-1}) [141]. Langdon et al. found considerable hormesis in a variety of soils that were exposed to $AgNO_3$. The main factors that influenced the toxicity of Ag to soil nitrification process were soil properties, specifically soil pH and organic carbon concentration. Another study has also recorded a stimulatory effect on nitrification in the presence of Ag [192]. Yang et al. reported upregulation of nitrifying genes in the nitrifier *N. europaea* at low Ag^+ and AgNP concentrations (2.5 µg L^{-1}). The effect of sulfidised AgNPs on soil nitrification processes has not been investigated.

11. Summary and aims of the thesis

Although considerable research has been devoted to the behaviour of 'pristine' AgNPs, rather less attention has been paid to transformed AgNPs (Ag_2S-NPs); the realistic form of AgNPs in the environment. This is of concern given that the environmental loadings of Ag-based NPs are predicted to increase over the coming years. The two main systems that are potentially at risk from AgNPs are WWT processes and soil organisms. Consequently, the effects of 'pristine' AgNPs and transformed AgNPs on these two systems were investigated in this thesis.

Wastewater treatment is dependent on the efficient functioning of microorganisms and as such is particularly susceptible to the anti-microbial effects of Ag-based NPs. The majority of studies that have investigated the effects of AgNPs on WWT processes

[5] Many genera belonging to this order are responsible for ammonia oxidation in soil.

have used unrealistically high Ag concentrations and simple exposure media instead of real wastewater. Therefore, to ensure that the potential risks of Ag-based NPs are not overestimated, studies that use realistic exposure concentrations and exposure matrices (i.e. real wastewater) are required. Accordingly, this thesis investigated the effects of AgNPs on WWT process and microorganisms using realistic exposure conditions.

Biosolids that contain Ag-based NPs (e.g. Ag_2S-NPs) may be applied to soils as an agricultural amendment and therefore soil organisms may also be exposed to Ag-based NPs. So far, studies on the behaviour of AgNPs in soils have been confined to 'pristine' AgNPs; the behaviour of transformed AgNPs (Ag_2S-NPs) in soils has not been investigated. Accordingly, the second focus of this thesis was to investigate the bioavailability of Ag_2S-NPs (the realistic form of AgNPs) to plants and, the effects of transformed AgNPs on soil microorganisms.

Addressing the knowledge gaps that are outlined above will enable a more accurate risk assessment of AgNPs in the environment; this was the overall aim of this thesis.

The specific aims of this thesis are stated on **page xi** and an outline of the life cycle of AgNPs and the stages that were investigated in this thesis are illustrated on **page 22**.

12. References

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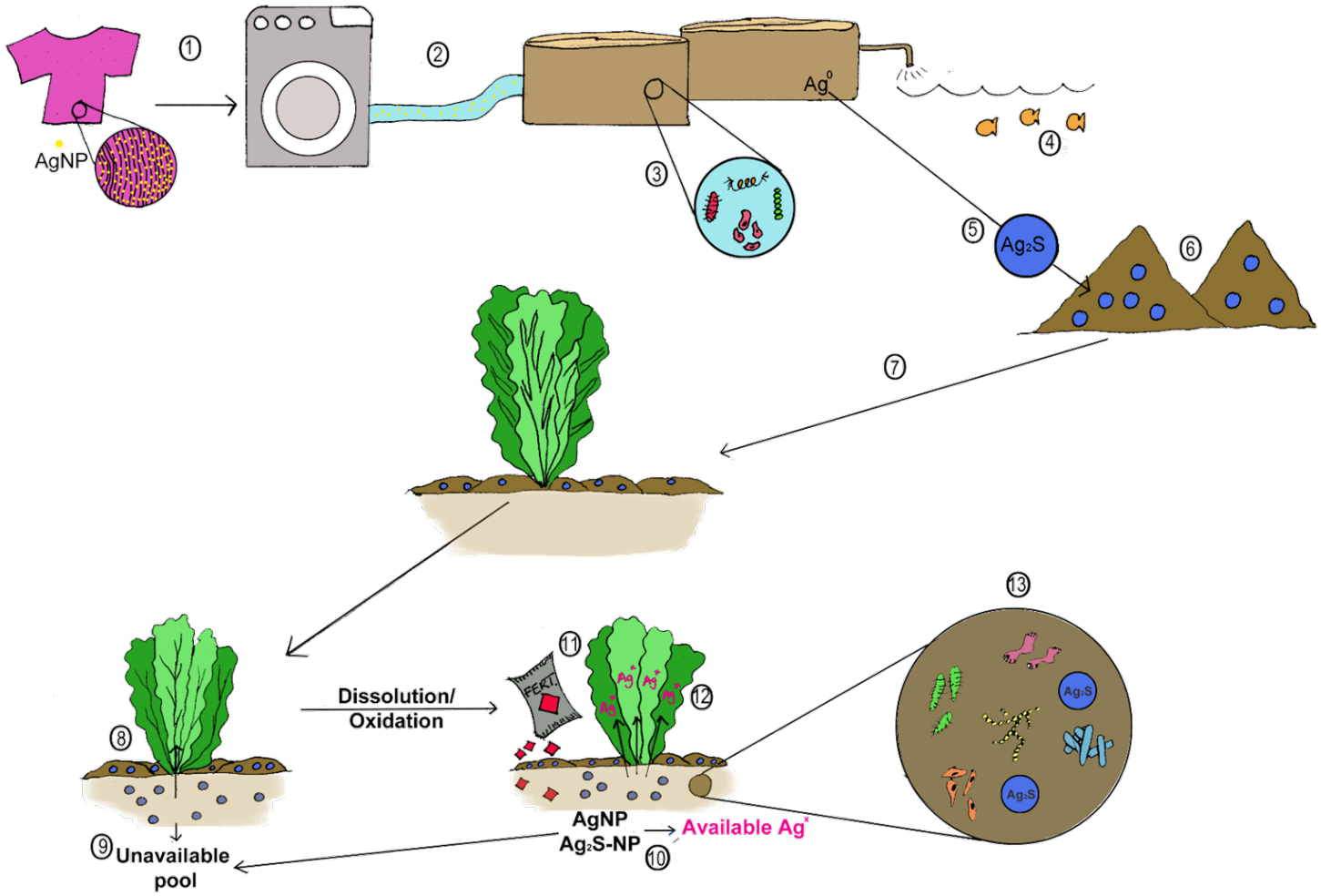
The fate of released silver nanoparticles (AgNPs). Numbers in bold indicate processes that were investigated in this thesis: (1) Release of AgNPs (yellow particle) or ionic Ag (Ag^+) from AgNP-containing products (2) AgNPs transported to wastewater treatment (WWT) plants. **(3)** Wastewater microorganism may be affected by Ag. (4) A portion of AgNPs may be released with effluent. **(5)** During WWT, AgNPs may be sulfidised (Ag_2S-NP – blue particle). **(6)** Majority of Ag_2S-NPs will adsorb to the biosolids. (7) Biosolids containing Ag_2S-NP are applied to soils. **(8)** Ag_2S-NPs (or released Ag^+) may be taken up by plants or become unavailable (9). Dissolution of Ag-based NPs can release Ag^+ (10). Fertiliser application may increase Ag_2S-NPs dissolution and therefore the uptake of Ag by plants **(11)**. Concentrations of Ag in plant parts can be measured to determine bioavailability of Ag_2S-NPs **(12)**. Ag-based NPs may negatively affect soil microorganisms **(13)**



CHAPTER 2

Transformation of PVP coated silver nanoparticles in a simulated wastewater treatment process and the effect on microbial communities

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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RESEARCH ARTICLE Open Access

Transformation of PVP coated silver nanoparticles in a simulated wastewater treatment process and the effect on microbial communities

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Abstract

Background: Manufactured silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials in consumer goods and consequently their concentrations in wastewater and hence wastewater treatment plants are predicted to increase. We investigated the fate of AgNPs in sludge that was subjected to aerobic and anaerobic treatment and the impact of AgNPs on microbial processes and communities. The initial identification of AgNPs in sludge was carried out using transmission electron microscopy (TEM) with energy dispersive X-ray (EDX) analysis. The solid phase speciation of silver in sludge and wastewater influent was then examined using X-ray absorption spectroscopy (XAS). The effects of transformed AgNPs (mainly Ag-S phases) on nitrification, wastewater microbial populations and, for the first time, methanogenesis was investigated.

Results: Sequencing batch reactor experiments and anaerobic batch tests, both demonstrated that nitrification rate and methane production were not affected by the addition of AgNPs [at 2.5 mg Ag L^{-1} (4.9 g L^{-1} total suspended solids, TSS) and 183.6 mg Ag kg^{-1} (2.9 g kg^{-1} total solids, TS), respectively].
The low toxicity is most likely due to AgNP sulfidation. XAS analysis showed that sulfur bonded Ag was the dominant Ag species in both aerobic (activated sludge) and anaerobic sludge. In AgNP and $AgNO_3$ spiked aerobic sludge, metallic Ag was detected (~15%). However, after anaerobic digestion, Ag(0) was not detected by XAS analysis. Dominant wastewater microbial populations were not affected by AgNPs as determined by DNA extraction and pyrotag sequencing. However, there was a shift in niche populations in both aerobic and anaerobic sludge, with a shift in AgNP treated sludge compared with controls. This is the first time that the impact of transformed AgNPs (mainly Ag-S phases) on anaerobic digestion has been reported.

Conclusions: Silver NPs were transformed to Ag-S phases during activated sludge treatment (prior to anaerobic digestion). Transformed AgNPs, at predicted future Ag wastewater concentrations, did not affect nitrification or methanogenesis. Consequently, AgNPs are very unlikely to affect the efficient functioning of wastewater treatment plants. However, AgNPs may negatively affect sub-dominant wastewater microbial communities.

Keywords: Silver nanoparticles, Silver sulfide, Wastewater treatment, STEM HAADF, Sequencing batch reactor, Nitrification, Microbial communities, Pyrotag sequencing, Silver speciation, XAS, Synchrotron, Biosolids

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Background

Rapid expansion of the nanotechnology industry has occurred over the previous decade. Manufactured nanomaterials (MNMs) encompass a variety of engineered materials, which can be divided into two groups for the sake of clarity: nano-sized particles (having at least two dimensions < 100 nm) and secondly, materials that are not particulate but have nano-sized properties [1] (i.e. enhanced electronic, optical and chemical properties compared to the bulk material). Silver (Ag^0) nanoparticles (NPs) are the most widely used NPs in both consumer products and in medical applications [2]. The anti-bacterial properties that render AgNPs desirable may lead to increased risks to human and environmental health following release into the environment. The primary exposure pathway of AgNPs into the environment is via wastewater streams. Silver NPs may enter wastewater through the washing of Ag nano-containing textiles [3,4] or plastics [5], or as a result of the use of nano-enhanced outdoor paints [6] and washing machines [7].

Several authors have investigated the fate of manufactured AgNPs in wastewater treatment plants (WWTPs) and have reported that the majority (> 85%) of AgNPs will be captured by biosolids (stabilised sludge) [5,8-11]. Accordingly, the predicted effluent concentrations of AgNP are very low (ng L^{-1}) [11], whereas AgNP concentrations in sludge are predicted to be much higher (1 – 6 mg Ag kg^{-1}) [11]. Both concentrations are likely to increase as the AgNP producing industry expands. Given this scenario, and the strong anti-bacterial effects of AgNPs, the stages of WWT that are likely to be affected by AgNPs are those that are dependent on the efficient functioning of microbes. Such stages are the aerobic activated sludge process and anaerobic digestion, which proceeds the former process in most WWTPs. There are very few studies that have investigated the impact of AgNPs on both processes in a sequential manner. Given that the transformation of AgNPs is likely during WWT [8,12], it is crucial to understand at what stage transformation occurs so accurate risk assesments can be conducted using AgNPs in realistic forms.

During the activated sludge process, organic nitrogen and phosphorus are removed by various microbial communities. Several studies have investigated the impact of AgNPs on nitrification [9,13-15] and the effects on microbial populations that perform these processes [16,17]. However, results from nitrification studies are divergent with no inhibition [9] and varying degrees of inhibition [14,15,18] observed on nitrification following AgNP addition in WWTPs or bioreactors at concentrations between 0.4 and 1 mg Ag L^{-1}.

The observed variation is most likely explained by the differences in input variables. A number of parameters differ between studies, all of which are known to influence AgNP fate and toxicity e.g. intrinsic AgNP properties (size, coating), Ag concentration, sludge/wastewater properties (temperature, ionic strength (IS)), total suspended solids (TSS) and dissolved organic carbon, (DOC)), the type of sludge/wastewater used (realistic or artificial) and general experimental set-up (e.g. light intensity and wavelength which may cause photocatalytic reduction of Ag^+ and AgNP).

The impact of AgNPs on anaerobic digestion has been less studied than that of nitrification. Methanogenic microorganisms are generally less sensitive to toxicants than aerobic communities. Silver NPs have been shown to have no effect on biogas and methane production at concentrations of 40 and 85 mg Ag L^{-1}, [19] and [13], respectively.

The bactericidal mechanism of AgNPs (and Ag^+) to organisms is only partially understood and debate is ongoing as to the exact means of action [20]. However, there is concern that the same properties that render AgNPs useful as an antimicrobial may also impact WWTP performance by affecting sludge microbial populations. A high diversity of bacterial populations in WWTPs is crucial for successful removal of BOD/COD, SS and biological phosphorus and nitrogen.

The effects of AgNPs on sludge microbial communities have been investigated by a limited number of studies. The model nitrifying bacteria *Nitrosomonas europae* has been shown to be adversely affected by AgNPs at concentrations of 0.3 mg Ag L^{-1} [16] and 2 mg Ag L^{-1} [17]. These are much higher Ag concentrations than would normally be found in the environment at present. The microbial communities found in anaerobic systems generally have a different response to toxicants compared to aerobic communities and are usually more sensitive to surface active and homeostatic inhibitors and less sensitive to metabolic inhibitors [21] . For example, at very high Ag concentrations (40 mg L^{-1}), methanogenic communities (*Methanosaeta* and *Methanomicrobiales)* have been shown to be largely unaffected by AgNP exposure [19]. So far, however, there has only been analysis of dominant microbes, through relatively insensitive techniques such as qPCR, without assessing the impact on subdominant populations as allowed by next generation techniques such as t-RFLP.

This study was undertaken to (i) investigate the effects of Ag and polyvinylpyrrolidone coated (PVP) AgNPs on organic nitrogen removal from wastewater (nitrification) (ii) examine the fate of Ag^+ and AgNPs during various stages of WWT (iii) investigate the effects of transformed Ag^+ and AgNP on anaerobic digestion efficiency, and (iv) to determine if dominant and niche microbial community structures in aerobic and anaerobic sludge are impacted by exposure to transformed Ag^+ and AgNPs using pyrosequencing.

Results and discussion

Silver nanoparticle partitioning in the sequence batch reactor process

Measured concentrations of Ag as a function of time in the mixed liquor and effluent are shown in Figure 1. Silver concentrations in the mixed liquor of each sequencing batch reactor (SBR) increased non-linearly during the 10 d aerobic stage. The cumulative concentration of Ag in the mixed liquor was less than the nominal value (taking into account Ag losses with effluent) possibly due to losses of mixed liquor that occurred during sampling for nitrification analysis and during decanting. In addition, Ag losses may have been due to sorption/complexation of Ag/AgNPs onto SBR tubing and container walls.

The effluent concentrations of Ag in the SBR spiked with AgNPs varied from 0.5 μg L^{-1} (day 5) to 7.7 μg L^{-1} (day 1). This corresponds to between 0.1% (for days 5 –8) and 5.4% (day 1) of the total amount of Ag in the mixed liquor (nominal) being removed with the effluent. Similarly, in the Ag^+ dosed SBR, between 0.1% (days 5 – 8) and 1.1% (day 1) of Ag was released with the effluent. Surprisingly, the Ag concentration range of the effluents collected from the control SBR [0.3 μg L^{-1} (days 2 and 7) to 4.7 μg L^{-1} (day 8)] were within the same range as the effluents collected from the Ag^+ dosed SBR; 0.3 μg L^{-1} (days 7 and 8) to 4.7 μg L^{-1} (day 4). This can be explained by background Ag concentrations in the influent wastewater (15.0 ± 7.6 μg Ag L^{-1}) and activity sludge mixed liquor. Overall, the average (n = 8 days, where day 1 and 8 are the first and last days of Ag addition) percentages of Ag in the effluents ± standard deviation (SD) were 0.8 ± 0.1%, 0.4 ± 0.4%, and 2.0 ± 2.8%, for the AgNP, Ag^+ and control SBRs, respectively. There is large variation in the control as the background Ag concentrations were close to inductively coupled plasma-mass spectrometry (ICP-MS) instrumental detection limits (0.05 μg/L). The results demonstrate that the majority of Ag spiked into SBRs, as AgNPs or ionic Ag^+ was retained by the sludge.

The partioning results in this study are in agreement with previous studies which have shown that the majority of AgNPs in wastewater will be partitioned to the sludge fraction following wastewater treatment [8-10]. However, the degree to which AgNPs are removed from wastewater has varied between each study. In a pilot WWTP experiment [8], 2.5% of spiked Ag (added as AgNPs stabilised by polyoxyethylene fatty acid ester) was released from the WWTP with the effluent, whereas in a 15 d simulated SBR experiment (0.9 L working volume), citrate coated AgNPs were found to be completely removed from the wastewater [9]. In the literature, the lowest removals of AgNPs from wastewater (88 ± 4%) were recorded from a SBR experiment using synthetic wastewater and AgNPs with an unspecified polymer coating [10].

The observed variations in removal efficiency of AgNPs from the above studies may be due to a number of factors including; the intrinsic properties of the NP (i.e. size, surface charge and capping agent) which in turn are influenced by additional parameters (e.g. mixed liquor pH, chloride concentrations, etc.), method of spiking [16] and perhaps most importantly, the characteristics of the influent wastewater and activated sludge. The TSS content of the influent and activated sludge determines the initial mixed liquor TSS. In the current study, the TSS content (4.5 ± 0.6 g TSS L^{-1}) was greater than that used in other studies [8-10](3, 2.4 and 1.8 g TSS L^{-1}, respectively). This may explain the high removal efficiency of AgNPs from wastewater (> 99%) which we observed in this study. Most NPs in WWTP sludge is likely to be heteroaggregated with bacteria [10,22-24] but NPs can also be associated with iron oxides or other inorganic particles [23].

At the conclusion of the SBR experiment, sludge Ag concentrations were 418, 168 and 6 mg Ag kg^{-1}(TS) for the AgNP, Ag^+ and control treatments, respectively. The high concentration of Ag in the AgNP sludge is due to the higher Ag concentrations in the AgNP spiking suspensions (mean ± SD; 39 ± 6 mg Ag L^{-1}, n = 11) compared to the Ag^+ solution (mean ± SD; 19.4 ± 0.1 mg Ag L^{-1}, n = 3); rather than a greater removal of Ag from the wastewater fraction. Due to logistical limitations, the concentrations of each AgNP suspension could not be determined before spiking as the homogenised NP suspensions degrade after 24 h.

The Ag concentrations of the prepared AgNP suspensions were higher than we had previously achieved and therefore higher than the nominal spiking concentration. The total mass of Ag added to the AgNP and Ag^+ SBRs, not including background inputs from effluent, was 12.7 mg and 6.4 mg, respectively. The results from the SBR experiments show that the majority of AgNP is partitioned to the solid phase.

Silver nanoparticle transformation during the sequence batch reactor process as determined using STEM analysis

Numerous bright regions were observed in sludge collected from the AgNP dosed SBR (Figure 2a) using scanning transmission electron microscopy (STEM) analysis in high-angle annular dark field (HAADF) mode. Further analysis of the bright spots by energy dispersive X-ray analysis (EDX) confirmed that these regions contained Ag (Figure 3). The STEM image shows aggregates of Ag approximately 100 – 120 nm in diameter (Figure 2b). The higher magnification image (Figure 2b.) shows that each aggregate appears to consist of smaller agglomerated spherical NPs of approximately 40 – 50 nm diameter. EDX analysis of this agglomerate showed that each region consisted of Ag and S with varying ratios. Two regions in the 100 – 200 nm aggregate had a Ag/S ratio of 2:1 (spot 1 and 2), whereas

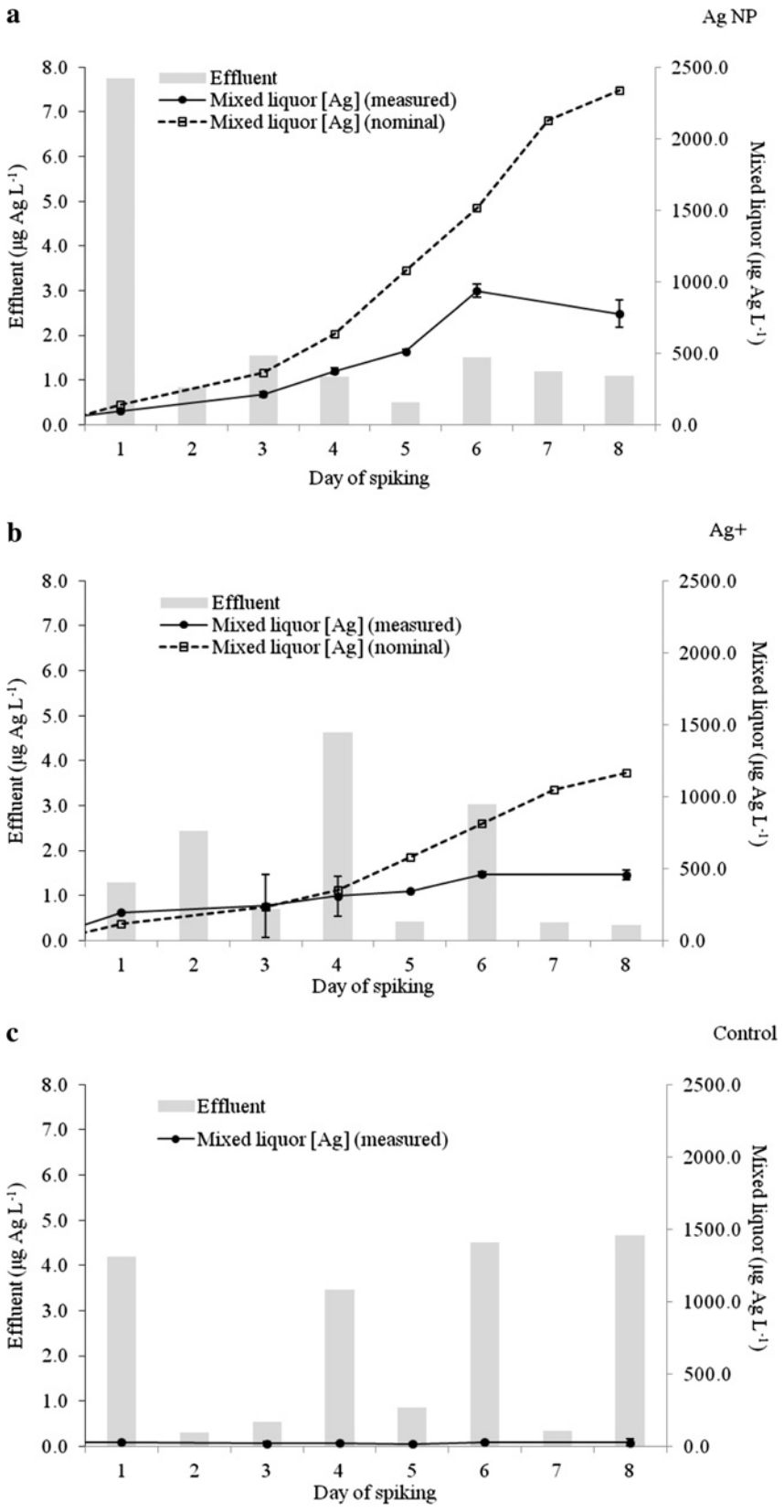

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 Silver concentrations in the effluent and the total mass of Ag added to the AgNP dosed (a); Ag^+ dosed (b); and, control (c) SBRs. Less than 1% of added Ag was found in the effluent. Nominal Ag concentrations were calculated from measured Ag spiking solution concentrations. Error bars represent one standard deviation (n = 3).

one region contained Ag/S with a 1:1 ratio (spot 3) (Figure 3). The specific cause of NP aggregation requires further investigation. However, it may be attributable to a number of factors including the ionic strength of the mixed liquor and the presence of organic chlorides and minerals. Ionic strength in domestic wastewater is typically < 0.1 M, whereas in anaerobic digesters IS is < 1 M, this may cause NP homocoagulation (see Additional file 1: Table SI.2 for wastewater elemental analysis). Conversely heterocoagulation of NPs may arise from the interaction of AgNPs with organic chlorides and minerals.

Silver sulfide (as α-Ag_2S) in the nano- size range has previously been identified in sludge [8,12]; however, to determine the crystal phase of the nano-sized particles in this study, further crystallographic investigation is required. At temperatures < 173°C, the monoclinic crystalline form of silver sulfide (α-Ag_2S) dominates (acanthite). For this phase to exist in the current study, Ag(0) in the original AgNP must be first oxidised to Ag^+ [25,26].

A recent study [25] provided evidence for the direct conversion of AgNPs to Ag_2S via an oxysulfidation mechanism which was dependent on the presence of small amounts of dissolved O_2. In the SBR experiment, residual O_2 did remain during the 110 min anoxic phase. Interestingly, EDX analysis showed that S was present in all nanosized Ag particles identified in the sludge despite the very short anoxic phase relative to the sulfidation reaction times of AgNPs (i.e. > 5 h [8]). Given the very short anoxic phase (105 min), the results suggest that sulfidation of AgNPs in mixed liquor may occur more rapidly than previously shown. Alternatively, AgNP sulfidation in the SBR may have been a gradual process that occurred during successive anoxic phases; i.e. during each anoxic phase a fraction of AgNPs may have been sulfidised until all AgNPs were sulfidised. This pathway is unlikely though as 'fresh' AgNPs were added each day.

To the best of our knowledge, the results are the first to identify sulfidised Ag nanosized aggregates in aerobically generated sludge. A previously study that identified Ag_2S in 'aerobic' mixed liquor of a pilot WWTP was not truly representative of an aerated sample because the mixed liquor was first subjected to anaerobic treatment [8]. We therefore suggest that Ag_2S identified in that aerobic sludge would have been produced during the initial anaerobic treatment because once formed, Ag_2S is very resistant to oxidation and dissolution of Ag (analogous to other metal oxides [27]). Overall, the results show that in WWTPs, the sulfidation of AgNPs may occur during activated sludge treatment prior to anaerobic digestion.

Silver speciation in wastewater, activated sludge and anaerobic digestate as determined by synchrotron studies

Principal component analysis (PCA) and target transformation identified six standard compounds suitable for the fitting of Ag in sludge samples: Ag_2S NPs, Ag-acetate,

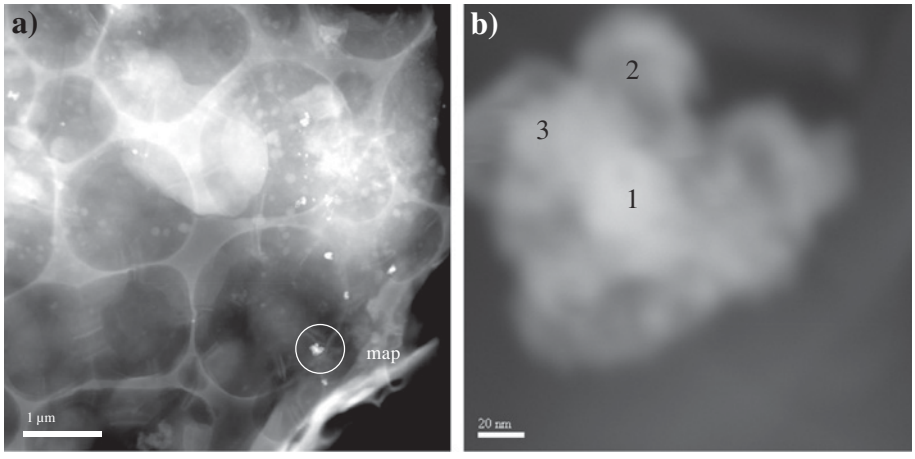

Figure 2 Characterisation of transformed Ag nanoparticles in aerobic sludge samples using STEM-HAADF. (**a**) STEM-HAADF image of a typical NP aggregate containing sulfidised nanoscale Ag particles. The bright aggregates are indicative of high *Z* elements (**b**) STEM-HAADF image of a typical Ag aggregate, particles 1–3 were characterised using energy dispersive X-ray (EDX) analysis (see Figure 3). The sludge sample was collected from the AgNP spiked SBR at the end of the experiment SBR experiment.

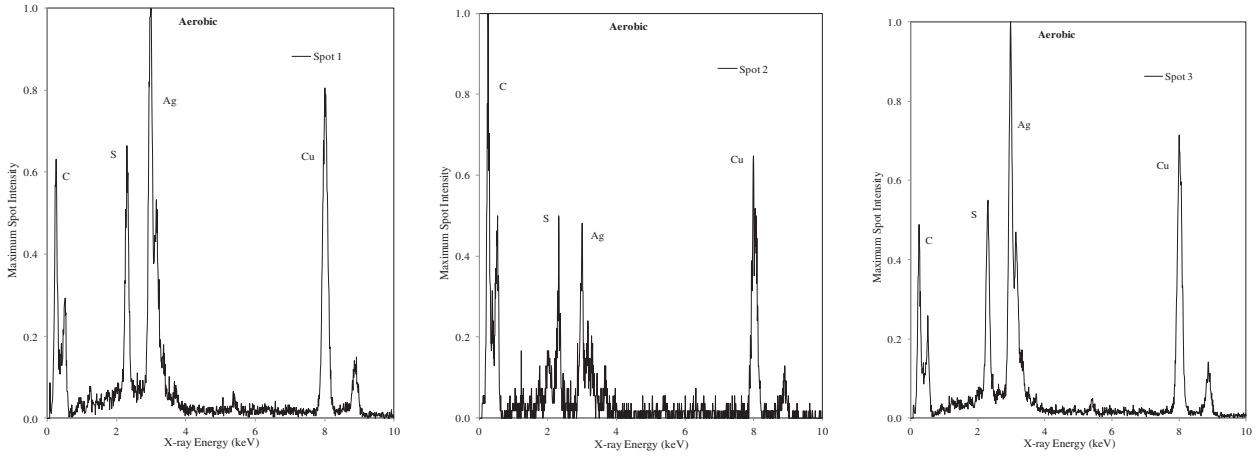

Figure 3 Energy dispersive X-ray (EDX) spectra of sulfidised silver nanoparticles in aerobic sludge. Spectra were collected from the specific spots indicated in Figure 2b.

Ag-glutathione (Ag-GSH), Ag-thiosulfate, Ag-foil (Ag^0) and Ag_2S (Figure 4). Examination of the XANES spectra of the six target compounds (Figure 4) showed that Ag_2S NP and Ag-GSH were very similar (also see XANES difference spectra Additional file 1: Figure SI.2b). Therefore, for these Ag standards, their percentage contributions to the sample model fits were combined (Table 1). The two remaining Ag-S models (Ag-GSH and Ag-thiosulfate) are not easily distinguishable from each other by visual inspection of the spectra; however, examination of the difference spectra does show considerable variation (Additional file 1: Figure SI.2b). Furthermore, the identities of Ag-GSH and Ag-glutathione (Ag-GSH) standards were confirmed by EXAFS analysis (data not shown).

The Ag K-edge XANES spectra of all sludge samples and samples from the wastewater experiment are shown in Figure 4 together with the six references that were used in the linear combination fitting (LCF). The LCF analysis provided good fits to all experimental data (Additional file 1: Figure SI.5). Results show that the dominant Ag species identified in all aerobic and anaerobic sludges was Ag bonded with sulfur (S). The contributions of each standard varied between the different Ag treatments (Ag^+ vs. AgNP) and also between each treatment process (aerobic vs anaerobic) (Table 1). The exception to this was in the wastewater samples.

The two spectra of AgNP dosed sludge shows that AgNPs were completely transformed during the SBR experiment and again during anaerobic digestion (Figure 4, Table 1). To the best of our knowledge this is the first time that sulfidation of AgNPs has been reported in aerated sludge. The aerobic sample (labelled ANP) was dominated (85%) by sulfidised Ag species with minor amounts of elemental Ag (15%). However, in the anaerobic sample (NNP), elemental Ag was not a significant component. The absence of Ag(0) in the anaerobic AgNP sample is supported by analysis of the corresponding EXAFS spectra (Additional file 1: Figure SI.7) where Ag – Ag bonding was not detected (Table 2).

In the anaerobic AgNP treated sludge, bulk Ag_2S was detected (13%) whereas in the aerobic sludge it was not detected by XAS. This suggests that the anaerobic digestion process in WWTPs may be vital for the conversion of nano-sized aggregates to bulk forms. The spectral differences that were observed between bulk Ag_2S and Ag_2S NPs are a common feature when comparing the XANES spectra of NPs and the bulk. Such effects have been reported for numerous NPs, including Au, CdS, ZnO and Fe_2O_3 NPs [28-30]).

Similar sulfidation trends are apparent for the Ag^+ dosed sludge; the major components of the aerobic and anaerobic sludges were Ag-S coordinated species (80% and 86%, respectively).This was supported by EXAFS analysis which identified Ag– S bonding in each sludge sample (Table 2). The major solid phase speciation changes between aerobic and anaerobic AgNP dosed sludge was the decrease in Ag(0) (15% to 0%) and Ag_2S NP (40% to 14%) and the subsequent increase in bulk Ag_2S (8% to 30%). It is most likely that metallic Ag was produced in the Ag^+ dosed aerobic sludge by photocatalytic reduction of $AgNO_3$, or alternatively by reducing agents in the mixed liquor (e.g. hydrogen sulfide, glucose).

Comparison of the XANES spectra for anaerobic Ag^+ spiked sludge and anaerobic AgNP sludge shows only minor differences. However, much greater differences were found between the aerobic AgNP treated sludge and the anaerobic Ag^+ sludge (Additional file 1: Figure SI.6).

Silver acetate was identified as a significant component (as determined by the size of the residual following least squares refinement of the model compounds during LCF) in the XANES fitting of the anaerobic AgNP sludge (8%) but not in the aerobic sample. The structure of this

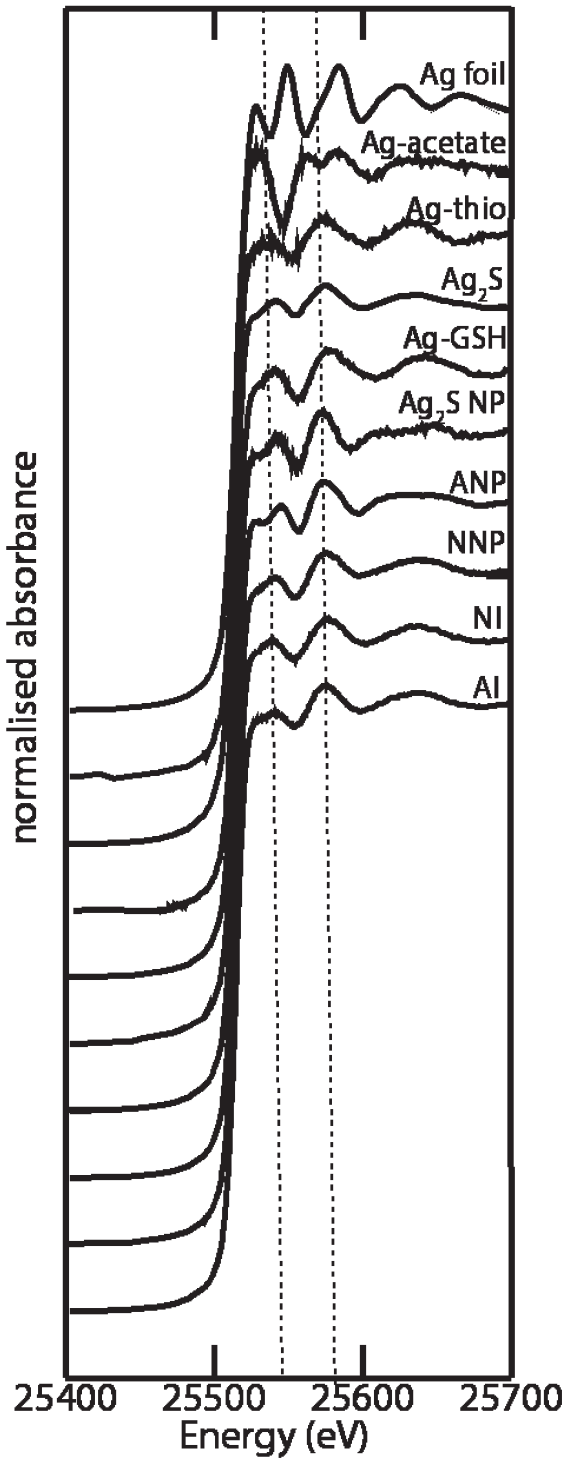

Figure 4 Silver XANES K-Edge spectra of sludges and the 6 Ag references used for the PCA. Where ANP = aerobic sludge from the AgNP dosed SBR; NNP = anaerobic sludge from the AgNP treatment; NI = anaerobic sludge dosed with Ag^+; AI = aerobic sludge from the SBR dosed with Ag^+; Ag-thio = Ag thiosulfate complex; and, Ag-GSH = Ag glutathione complex. For spectra of the control sludge (collected from the SBR that was not spiked with Ag) see Figure SI.4.

standard was not verified by other methods, however, the spectra is significantly different from the Ag-S and Ag(0) standards (Figure 4 & Additional file 1: Figure SI.2b) to be confident that Ag-carboxyl groups are present in the anaerobic sludge sample. In samples where Ag-acetate was detected as a significant component (Table 1), re-fitting the spectra with Ag_2O produced a poorer quality fit with larger R^2 values. Furthermore, when Ag-acetate was excluded from the model, this led to an increase in the fit residuals (see Additional file 1: Table SI. 3 for increased residual values).

Overall the majority of Ag in the AgNP dosed aerobic and anaerobic sludges was sulfidised (85% and 92%, respectively). The results are in agreement with previous studies that have shown sulfidation of AgNPs in sludge [8,12] and highlights the importance of considering Ag speciation in determining the fate and toxicity of AgNPs in terrestrial environments.

The speciation of AgNPs in influent wastewater has not been previously investigated. Based on Ag K-edge XANES results, the results demonstrate that the absence of activated sludge in influent wastewater had a considerable effect on AgNP transformation (Table 1). There was complete transformation of AgNPs to Ag(0) for all wastewater samples; no other species was identified as a significant component in the fit model. Furthermore, there were only subtle differences in the spectra for samples collected initially (4 min after spiking) and after 3.5 h. (Additional file 1: Figure SI.4). The results suggest that when PVP coated AgNPs enter wastewater, their polymer coating will be quickly modified or lost, and aggregation will occur. Additional analysis using the PVP AgNP reference in place of metallic Ag(0) as a target component, produced a poorer fit with a greater residual (3.14 cf. 0.72). It should be noted that the PVP coating of the AgNPs used in the experiments has not been fully characterised (i.e. coating thickness), so this effect may not be observed for all PVP coated NPs. However, it can be concluded that that when AgNPs enter WWTPs, the polymer surface coating may already be modified and AgNPs will no longer be nano in size. The size increase may be caused by a number of factors, including heterocoagulation with natural colloids (e.g. dissolved organic matter and iron and manganese oxyhydroxides) and aggregation due to high ionic strength of the wastewater.

Effect of silver nanoparticles on nitrification

The ammonium (NH_4^+) and nitrate/nitrite (NO_x) profiles of each SBR are illustrated in Figures 5 & 6. There was near complete removal of NH_4^+ (> 99%) observed from each SBR during each cycle. It should be noted that for the cycle analysed on day 2 for the AgNP dosed SBR only 70% of NH_4^+ was removed. In a similar SBR experiment,

Table 1 Linear combination fitting analysis of XANES spectra of sludges collected from the SBRs (aerobic), sludges after anaerobic batch tests (anaerobic) and from the short term wastewater experiment

Sample	Ag_2S	Ag_2S NP + Ag-GSH	Ag(0)	Ag-acetate	Ag-thiosulfate	*Residual*
Aerobic sludges						
Control		40 (5)	20 (3)	19 (3)	23 (4)	0.350
Ag^+	8 (2)	72 (8)	6 (0.9)	14 (0.9)		0.029
AgNP		85 (4)	15 (4)			0.022
Anaerobic sludges						
Control	39 (9)	24 (7)		27 (4)	11 (6)	0.549
Ag^+	30 (1)	56 (2)		13 (0.7)		0.015
AgNP	13 (2)	78 (6)		8 (0.8)		0.030
Wastewater Experiment						
4 min			100 (0.2)			0.776
24 min			100 (0.2)			0.863
210 min			100 (0.2)			0.927

The proportion of species are presented as percentages with the estimated standard deviation (SD) in parentheses. For the Ag_2S NP + Ag-GSH column, the SD is the sum of the individual SD's from each species. The control sludge was collected from the SBR that was not spiked with Ag.

slight inhibition of nitrifying organisms by AgNPs (citrate capped AgNPs, 0.1 mg Ag L^{-1} of mixed liquor) was also observed on the first day of Ag addition [9]. However, the current results are most likely due to unexpected incomplete mixing of mixed liquor. In the cycle immediately following, complete mixing was resumed, and thus complete NH_4^+ removal would be expected as occurred in the other SBRs for day 2. At the beginning of the aeration phase the highest concentrations of NH_4^+ were observed, with low variation between each SBR. The maximum concentrations were recorded on different days for the control (day 4; 24.5 mg NH_4^+ L^{-1}), AgNP (day 6; 20.8 mg NH_4^+ L^{-1}) and Ag^+ (day 3; 20.3 mg NH_4^+ L^{-1}) dosed SBRs. Small amounts of nitrite (NO_2^-) were produced in the reactors, however, even during the cycles that had the highest concentrations, NO_2^- was completely converted to nitrate (NO_3^-) before the end of the phase.

Table 2 Structural parameters of sludges and standards derived from EXAFS analysis

Sample	Shell	CN[a]	R[b]	σ^{2c}
Aerobic sludges				
Ag+	Ag – S	2.0	2.55	0.007
AgNP	Ag – S	1.6	2.51	0.005
	Ag – Ag	2.4	2.89	0.005
	Ag – Ag	3.0	3.09	0.006
	Ag – Ag	2.4	4.98	0.003
Anaerobic sludges				
Ag^+	Ag – S	2.0	2.48	0.008
AgNP	Ag – S	2.0	2.51	0.005
References				
Ag_2S NP	Ag – S	1.5	2.52	0.004
	Ag – Ag	3.0	3.06	0.007
Ag-foil	Ag – Ag	12.0	2.86	0.001
Ag-GSH	Ag – S	2.0	2.49	0.003
	Ag – Ag	1.0	3.03	0.007
Ag-thiosulfate	Ag – Ag	1.0	2.52	0.002

[a] Coordination number, [b] Interatomic bond distance (Å), [c] Debye-Waller factor.

Nitrification rates were calculated using linear regression over time for two complete cycles on days 8 and 9 and normalised for TSS content (Table 3). The rates were calculated from the initial reduction of NH_4^+ at the beginning of the aeration phase (Figure 5). Nitrification occurred rapidly in the first 50 – 60 min of the aerobic phase, and as a result the linear regression is based on 3 time measurements. To support these results, an on-line NHx autoanalyser (YSI, USA) was also used on days 8 and 9 to measure NH_4^+ concentrations in the AgNP and Ag^+ dosed SBRs, respectively (Additional file 1: Figure SI.1). A comparison of the nitrification rates calculated from both analysis methods shows comparable results (Table 3); confirming the accuracy of the chemical data. Probe determined NH_4^+ concentrations are between 4.5 mg L^{-1} and 5.4 mg L^{-1} lower than those obtained from chemical analysis (Table 3). This may be due to the close proximity of the probe to the aeration stone in the SBR. Alternatively, the lower pH of the mixed liquor compared to the calibration solutions may have caused a shift of the NH_4^+ equilibrium ($NH_3 + H^+ \rightleftharpoons NH_4^+$) to the left, decreasing the concentration of NH_4^+. There was very limited variation in the nitrification rates of all SBRs.

Several studies have investigated the effect of AgNPs on nitrification in WWTPs, but results are conflicting

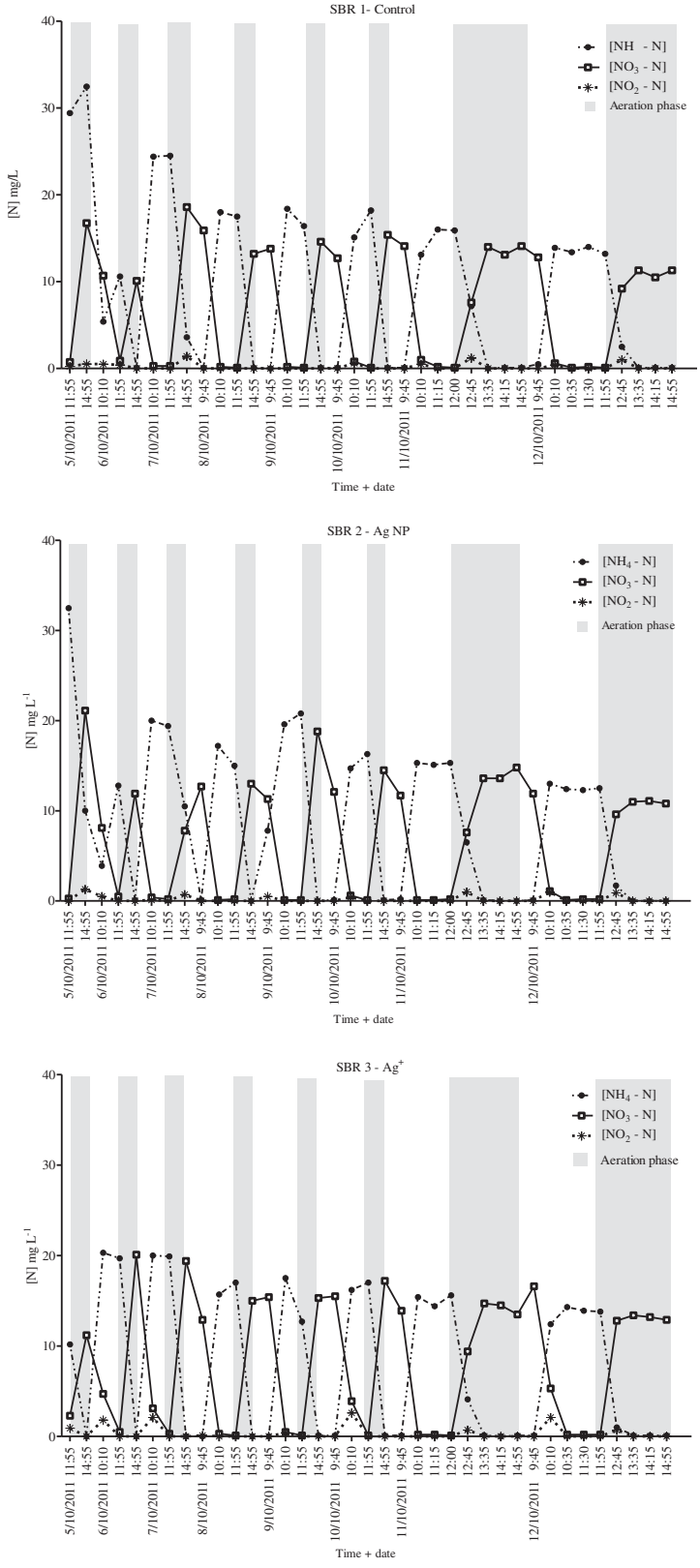

Figure 5 NH_4^+ and NO_x profiles of each SBR. Results are shown from day 2 to day 9 (after spiking). Sample collection commenced on day 1; the second day of SBR operation (results not shown). Samples were collected daily during one cycle (4 cycles in 24 h).

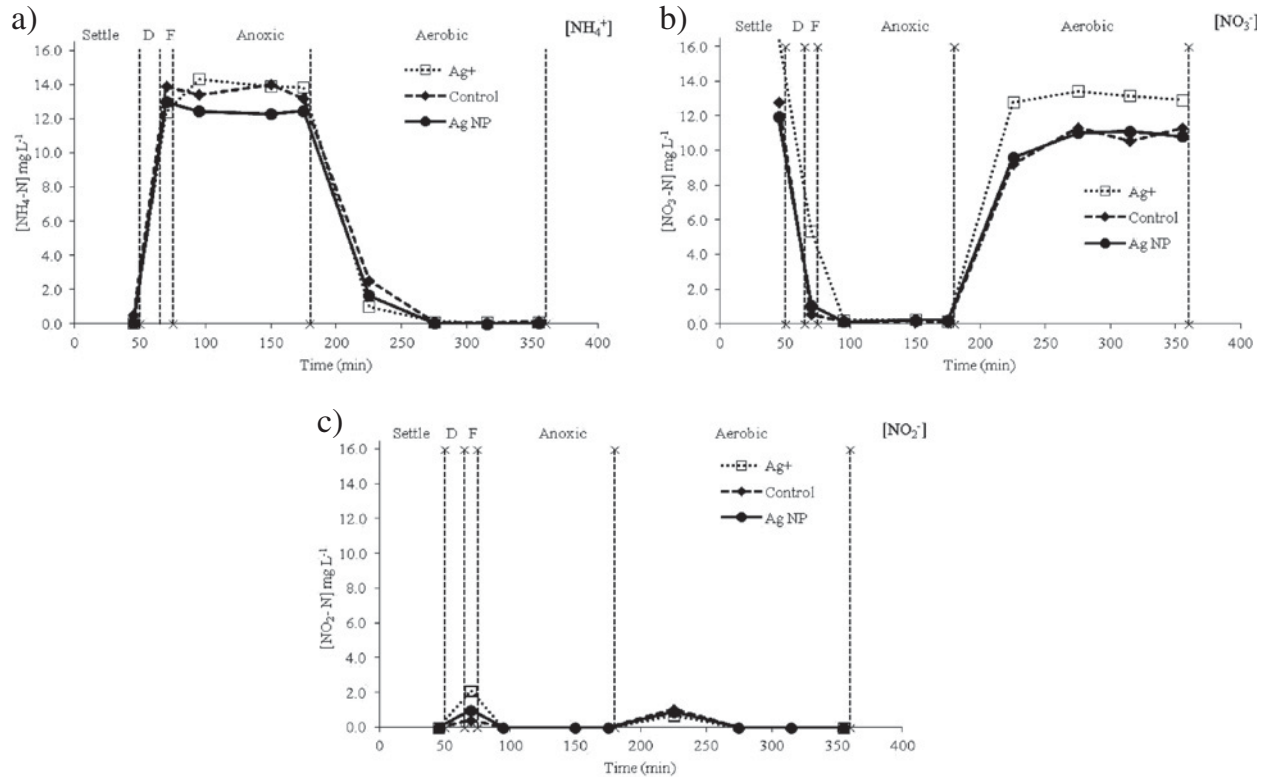

Figure 6 Variations of (a) NH_4^+- N, (b) NO_3 – N and (c) NO_2 - N profiles during one complete 6 h cycle on day 9. Where D and F are the decant and feed phases, respectively.

[9,14,15,18]. As the sludge matrix is likely to have a major influence on the fate of AgNPs in WWTP, the results from the current experiment are most comparable to those experiments that have used WWTP sludge and activated sludge [9,15]; not synthetic wastewater. In a 15 d simulated SBR experiment [9], NH_4^+ removal efficiency was not affected by AgNPs in wastewater (0.5 mg Ag L^{-1}), whereas in a short term (12 h) batch test using a synthetic feed solution [15], a 7% decrease in nitrification rate at 1 mg AgNP L^{-1} was recorded. This inhibition may be due to the relatively high DO concentrations (~ 7.2 – mg L^{-1}) compared to the more realistic concentrations used in our experiment (1.5 – 2.5 mg L^{-1}).

In the current experiment, the complex sludge matrix may have decreased AgNP toxicity for a number of reasons, with two primary factors being the presence of organic matter and the high ionic strength. Organic matter complexes Ag^+ [31,32], which has been linked to AgNP toxicity, whereas high salt concentrations cause NP aggregation which is known to decrease nanoparticle toxicity [33]. In addition, Ag^+ anion binding may produce very stable products such as AgCl ($K_{sp,\ H2O,\ 25°C} = 1.77 \times 10^{-10}$) and Ag_2S ($K_{sp,\ H2O,\ 25°C} = 5.92 \times 10^{-51}$) which will also decrease Ag^+ bioavailability and hence toxicity.

Table 3 Nitrification rate for each SBR on days 8 and 9 of the experiment

	Day 8	Day 9
SBR	mg L^{-1}.h	mg L^{-1}.h
Control	2.4	1.8
Ag^+	1.5	1.3 (1.2)
AgNP	2.6 (2.2)	1.6

Rates calculated from the on-line NH4 probe are shown in parentheses. All rates have been normalised for TSS content.

Effect of silver nanoparticles on methane production

The cumulative production of biogas (methane) during anaerobic digestion of the AgNP and Ag^+ dosed sludges is shown in Figure 7. Based on the calculated anaerobic biodegradability parameters, AgNPs did not have an impact on sludge digestion (Table 4). There was no difference between the methane production of AgNP, Ag^+ and control sludges at Ag concentrations of 184, 77 and 6.3 mg Ag kg^{-1}. The results concur with previous studies that found methanogenesis was not affected by AgNPs at concentrations < 18.9 mg Ag L^{-1} [18] and 40 mg Ag L^{-1} [19]. Similarly, for bulk Ag, the rate and extent of methanogenesis in mixed cultures was not affected by either $AgNO_3$ or Ag_2S at concentrations of 100 mg Ag L^{-1} [34]. To the best of our knowledge, the results are the

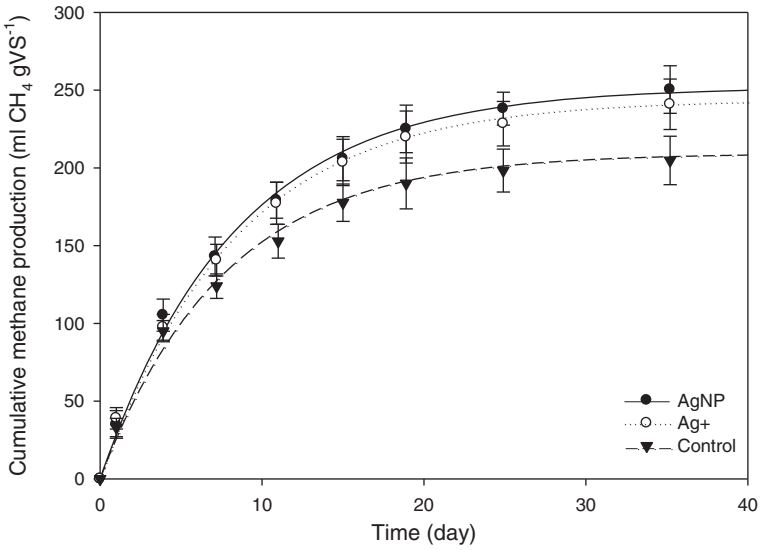

Figure 7 Cumulative methane produced during the 38 d anaerobic digestion. All values are blank corrected and the error bars show 95% confidence intervals calculated from triplicate measurements.

first to demonstrate that transformed AgNPs in sludges (present mainly as Ag bonded to S groups) (Table 1), as opposed to 'pure' AgNPs, have no effect (at 184 mg Ag kg^{-1}) on methanogenic processes which are essential for sludge degradability in WWTPs.

Effect of silver nanoparticles on niche microbial communities

Following mixed liquor digestion in the three SBRs and anaerobic assays, the diversity of bacterial populations was determined and compared to that of influent wastewater, activity sludge mixed liquor (aerobic inoculum) and anaerobic inoculum. The results from a PCA of the individual data sets shows that all samples could be grouped based on their source (Figure 8). In each case, there was a slight shift from control/Ag^+ to AgNP.

One dimension could account for 70% of overall variation. Aerobic samples were heavily dominated by a major dominant γ-Proteobacteria 19%, 22% and 21% for the control, AgNP and Ag^+ aerobic samples, respectively (Figure 8) This is surprising as nitrifiers and phosphate accumulating organisms (PAOs) in activated sludge are usually *β-Proteobacteria* with only a small percentage from the gamma subclass. Removal of organic N is a two-step process where ammonia is initially oxidised to nitrite by ammonia oxidising bacteria (AOB) and then further oxidised to nitrate by nitrite oxidising bacteria (NOB). All AOB belong to two genera each in the *β-Proteobacteria* and γ-*Proteobacteria* phylum, whereas NOB belong to five genera in various classes of the *Proteobacteria*. The primary habitats of *γ-Proteobacteria* are marine environments whereas *β-Proteobacteria* dominate in freshwater systems. The inoculum plant was in a coastal environment (more saline), which could account for this increased dominance.

Table 4 Anaerobic biodegradability of each sludge as indicated by degradation extent (f_d), apparent first order hydrolysis rate coefficient (k_{hyd}) and the estimated methane potential (B_0)

Treatment	k_{hyd} (d^{-1})	f_d	B_0 (mL/gVS)
Control	0.13 ± 0.020	0.31 ± 0.016	195 ± 1
Ag+	0.12 ± 0.014	0.36 ± 0.014	228 ± 9
AgNP	0.12 ± 0.014	0.36 ± 0.014	238 ± 9

Ammonia oxidising bacteria are generally more sensitive to toxicants than NOB [16,35]. There was a very minor response to the AgNP treatment, driven mainly by slight shifts in niche populations. Subdominant microbial structure in the Ag^+ treated sludges was not significantly different to that of the control. What was more surprising was that there was almost no shift in population between the inoculum and Ag^+ and control. This is surprising as the feed is different, the mode of operation is different (continuous in parent vs sequenced in SBR), and at least 1 nominal sludge age occurred through the study. The pyrosequencing data confirms the nitrification results, in that AgNPs at a concentration of 2.5 mg Ag L^{-1} (2.9 g TS kg^{-1}), do not influence the broad microbial population.

Anaerobic samples indicated that control and Ag^+ were very similar, but with a large shift from inoculum to batch, and a small shift from control/Ag^+ to AgNP (Figure 9). This was confirmed through additional PCA analysis on the anaerobic samples only (top 500 OTUs, Hellinger adjusted). This indicated a large shift from inoculum to end

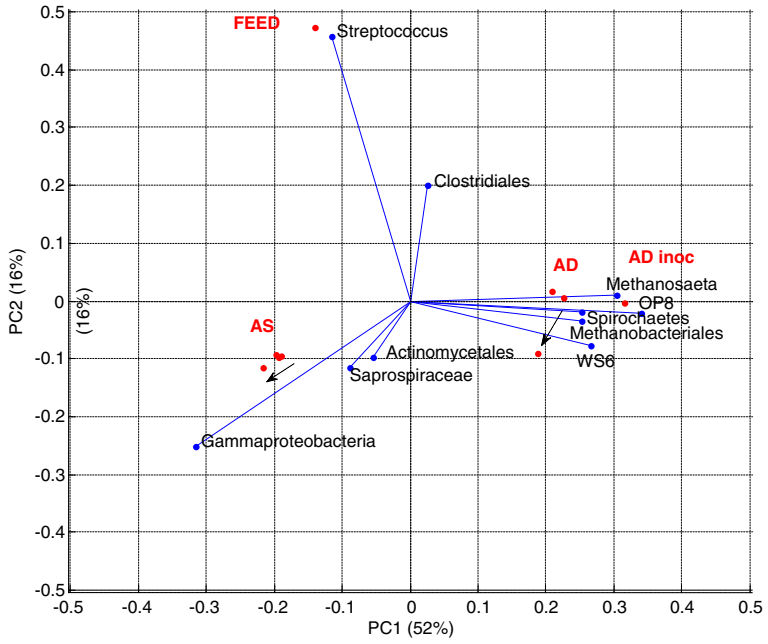

Figure 8 Overall bi-plot of PCA data showing top 10 OTUs. All OTUs were used for analysis. The key clusters of Activated sludge - AS (inoculum Ag^+, and control overlap), Feed, and Anaerobic Digestion (AD) are shown. Arrows show shift from control and Ag^+ to AgNP communities. Note that AD Inoculum is right-shifted on PC1 compared to the control and Ag^+ samples.

BMP, with a dominant WS6 OTU being largely replaced by OP8 (both candidate divisions), and a number of other major OTUs. Silver NPs seemed to cause a subtle shift from Spirochaetes to other organisms. *Archaea* seemed not at all influenced by batch operation, or Ag^+/AgNP treatment. Our data therefore support those in previous studies [19] indicating no impact of AgNPs on *Archaea* compared to controls (at 20 mg AgNP L^{-1}).

What is surprising is the dominance in all anaerobic samples by uncultured division OTUs (Figure 9). The inoculum contained phyla from the uncultured candidate division WS6 (18%), whereas microbial population in anaerobic samples collected after digestion appeared to be dominated (10 – 17%) by organisms from another candidate division (OP8). The cause of this shift in diversity is unclear. The WS6 phyla was first identified in a

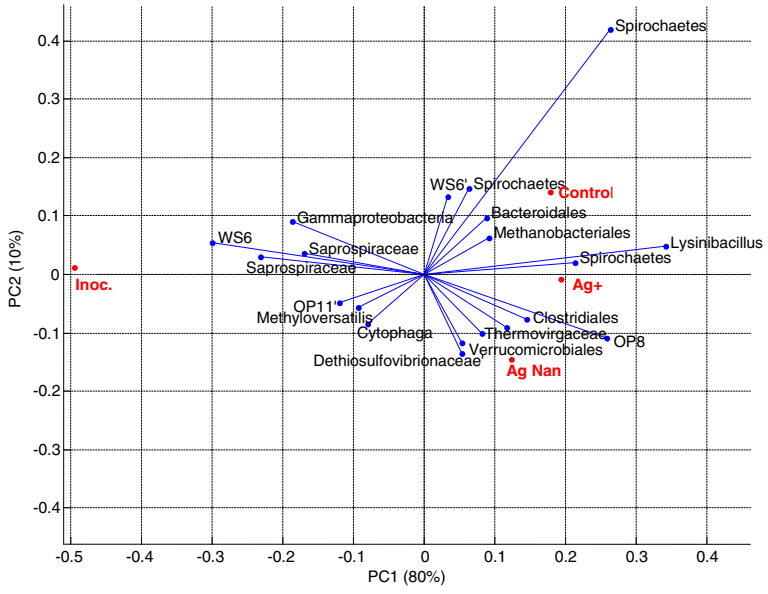

Figure 9 PCA analysis of anaerobic pyrotag results only.

contaminated aquifer and has since been identified in other environments (e.g. anoxic pond sediment [36], sulfur-rich spring sediments [37], eutrophic estuaries [38], hydrothermal vents [39]) but not in sludges or wastewater. Organisms from the OP8 division have been identified in mangrove sediments [40] and an anaerobic sludge digester, where 1% of the operational taxonomic units (OTUs) were represented by organisms from this division [41]. Broadly speaking, environmental bacterial community structure is regulated by local conditions. Hence, in the BMP test, factors including salinity and nutrient conditions [42] may have differed to that in the tank where inoculum was collected causing the population change.

Sensitive methanogenic microbes (*Methanosaetaceae*) accounted for ~ 11% of the variation in all anaerobic samples, including the inoculum, and was not impacted by the presence of AgNPs (Figure 9). This organism is most sensitive to possible surface active agents [21]. It is highly important that the bacterial population changed so strongly between inoculum and the end of the batch, while the archaeal population seemed untouched. This means that the mode of operation has a strong impact on acidogenic microbial populations but not methanogenic ones. It will be important to further evaluate the role of organisms in candidate divisions, as almost nothing is known of these microbes.

Whilst previous studies have investigated the effects of pure AgNPs on wastewater microbial populations, this is the first time that the influence of transformed AgNPs (primarily as Ag-S species) has been investigated. Moreover, to the best of our knowledge, this is also the first time that transformed AgNPs have been shown to affect niche populations. The results cannot conclusively be attributed to a nano- effect due to the higher spike concentration of Ag in the AgNP treated sludge compared to the Ag^+ sludge. Yet, XAS analysis of the anaerobic sludges did show a greater percentage of nano sized Ag_2S in the AgNP treated sludge compared to Ag^+ dosed sludge (78% cf. 53%, respectively) and a lower percentage of bulk Ag_2S (13% cf. 30%, respectively). This supports our hypothesis that the observed population changes are attributable to a nano-effect, although further research is required to confirm this hypothesis. Nevertheless, the results still demonstrate that even after their transformation to much less toxic Ag-S species, AgNPs have the potential to impact niche microbial communities but are not likely to impact overall WWTP microbial processes (e.g. nitrification and methanogenesis).

Conclusions

In our experiments, > 99% of PVP-coated AgNPs were removed from wastewater when subjected to activated sludge digestion. During the SBR experiment and subsequent anaerobic digestion stage, nitrogen removal and methane production (respectively) were not affected by transformed AgNPs.

Pyrosequencing analysis of microbial communities showed that AgNPs and Ag^+ did not affect the dominant populations of nitrifiers and methanogenic organisms in aerobic and anaerobic generated sludges, respectively. However, in both sludges a subtle shift in niche populations was observed. In the case of aerobic sludge samples, the shift was extremely minor, whilst for anaerobically digested samples there was a much larger shift. Additional studies are required to confirm if this change in population is exclusively a nano- effect.

Two conclusions were drawn from the XAS analysis of sludge: (i) AgNPs were sulfidised during SBR operation followed by near complete sulfidation during anaerobic digestion; and (ii) AgNP dosed anaerobic sludge contained a higher fraction on nano sized Ag_2S species compared to Ag^+ dosed sludge. The production of stable Ag-S species may have limited the toxicity of AgNPs towards nitrifiers and methanogenic bacteria as Ag^+ is believed to be the main toxicity mechanism of AgNPs.

Based on our results, PVP-coated AgNPs will not affect nitrification and methanogenesis during WWT, even in the future with the increasing use of AgNPs. Further investigations are required to confirm whether sub-dominant microbial sludge populations are at risk from AgNP exposure, as this may have long term consequences for the successful operation of WWTPs.

Methods

Preparation and characterisation of nanoparticle stock solutions

Polyvinylpyrrolidone (PVP) coated (0.1%) Ag NP powders were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX) (10 nm nominal particle size diameter). PVP coated NPs were chosen as they are a very common AgNP capping agent. Thus, their use is realistic of the AgNPs that would be released into wastewater streams. Stock suspensions of AgNPs were prepared by adding the NP powder (0.1 g) to ultrapure deionised water (50 mL, 18.2 Ω) and sonicating (1800 W, 3 min) in an ice bath. The AgNP suspension was then centrifuged (2200 g, 15 min) producing a final stock suspension with an average Ag concentration of 35.7 mg Ag L^{-1} (SD = 5.6 mg Ag L^{-1}, n = 11), 8% of which was dissolved ionic Ag^+ [43]. The AgNP stock suspensions were prepared daily (30 – 60 min before spiking). Total Ag concentrations of the NP spiking solutions were determined by an open-vessel acid digestion (HNO_3, 70%) method as described previously [43].

The AgNP suspensions prepared with this method have been extensively characterized previously using dynamic light scattering (DLS, Malvern Zetasizer),

transmission electron microscopy (TEM, Phillips CM200 at 120 keV) and X-ray diffraction analysis [44]. The particle size distribution has also been investigated using disk centrifuge analysis (CPS Instruments disc centrifuge 24000 UHR). In summary, the average particle diameter was between 40 nm with < 8% of Ag existing as dissolved Ag^+ (Additional file 1: Table SI.1 for complete NP characterisation) [44]. Previous work [44] (using the same method and batch of nanoparticles) has showed that the volumetric diameters of the AgNPs observed using TEM corresponded with the crystallite size determined from X-Ray diffraction analysis, the hydrodynamic diameter (d_h) observed using DLS and the Stokes diameter as found using disk centrifugation.

Set-up and operation of sequencing batch reactors

Three individual SBRs (control, Ag^+ and AgNPs) with a working volume of 5 L and an initial TSS of 6.0 g L^{-1} were prepared with 0.9 L of activity sludge mixed liquor (TS = 35.3 g L^{-1}) and 4.1 L of influent wastewater. Activated biological nutrient removal (BNR) sludge was collected from an activated sludge wastewater treatment plant (Luggage Point), and influent wastewater was collected from a local domestic wastewater pumping station (Indooroopilly), both located in Brisbane, Queensland, Australia.

Each SBR was operated with four 6 h cycles per day with a hydraulic retention time (HRT) of 15 h. Each cycle consisted of a 3 h aerated aerobic phase, followed by 50 min settling, 15 min decanting, 10 min feeding and 105 min anoxic [low dissolved oxygen (DO)] phases. Feeding, decanting and sampling ports were located at different positions on the reactors. During the 3 h aerobic stage, DO levels were maintained between 1.5 – 2.5 mg L^{-1} by intermittent aeration, controlled with an online DO detector. Following the settling phase, 3 L of supernatant was decanted and 3 L of cold influent wastewater (20°C) was pumped into each SBR. The reactors were continuously stirred with a magnetic stirrer except during settling and decant phases.

Silver (as NPs or $AgNO_3$) was added once every 24 h at the beginning of a feed cycle and for the remaining three feed cycles in that 24 hours no Ag was added. Prior to spiking, trace amounts of Ag were recorded in the mixed liquor of each SBR (day 0), (36, 26 and 24 μg Ag L^{-1} for the control, Ag NP and Ag^+ dosed SBRs, respectively).

Following the 10 day aerobic digestion, sludge was allowed to settle for 2 h and the supernatant decanted. The remaining sludge in each SBR was centrifuged (2 min, 3250 g), to increase the TS concentrations (Table 5), and approximately half was used in the subsequent anaerobic digestion experiment.

Table 5 Average characteristics of each sequence batch reactor

Characteristic	Control SBR	Ag NP SBR	Ag^+SBR
Average pH	6.71 (0.23)	6.67 (0.07)	6.62 (0.09)
Final TSS (g L^{-1})	4.4	4.5	6.3
Final VSS (g L^{-1})	4.0	4.3	5.5
TS after centrifugation of SBR sludge (g kg^{-1})	72.8	60.5	65.4
Sludge [Ag] before spiking (mg Ag L^{-1})	0.04	0.03	0.02
Total Ag added (mg)	0.00	12.73	6.14

Mixed liquor suspended and volatile solids (MLSS and MLVSS, respectively) were analysed every 2 d according to APHA Standard Methods (1992). The chemical characteristics and Ag spiking concentrations of each SBR are given in Table 5.

Transmission electron microscopy analysis of silver nanoparticles in sludge

Freeze dried sludge was collected at the conclusion of the 10 d SBR process to determine whether physical or chemical transformation of AgNPs had occurred in the AgNP dosed SBR. STEM analysis in HAADF mode was used to investigate the morphology of AgNPs in the sludge, whereas EDX together with TEM was used for elemental analysis. Samples of aerobic sludge were collected at the end of the SBR experiment. Samples were freeze-dried and analysed using a JEOL 2100 TEM operating at 200 kV. Freeze dried samples were ground in methanol using a mortar and pestle and a single drop pipetted onto a 200-mesh copper (Cu) TEM grid with lacey carbon support film and allowed to evaporate at room temperature.

The elemental composition of "bright" NPs/aggregates was investigated using an EDX spectrometer. The TEM was used in scanning mode (STEM) with a high-angle annular dark-field (HAADF) detector. The high angle detector collects transmitted electrons that are scattered (primarily incoherently) to high angles, whilst excluding Bragg (coherent) scattering. The detector provides an image where the contrast is dependent on the approximate square of the atomic number (Z). Accordingly, bright spots in the image correspond to high Z elements; making the detection of Ag containing particles in the complex sludge matrix more straight forward than that in a bright-field image.

Solid phase speciation of silver in sludge using synchrotron radiation

Solid phase speciation of Ag in aerobic and anaerobic sludges was further examined using X-ray absorption

spectroscopy (XAS); specifically X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) analysis.

Silver K-edge X-ray absorption spectra were recorded on the XAS beamline at the Australian Synchrotron (AS), Melbourne, Australia. The 3 GeV electron beam was maintained at a current of 200 mA in top-up during the sample analysis. The X-ray beam was tuned with a Si (311) monochromator in the energy ranges of 25312–25492 eV for pre-edge (10 eV steps), 25492–25562 eV XANES region (0.5 eV steps) and then 0.035 $Å^{-1}$ steps in k-space for EXAFS. A metallic Ag foil, recorded in transmission mode downstream of the sample, was used as an internal standard to calibrate the energy scale to the first peak of the first derivative of the Ag edge (25515 eV). Spectra of the samples were recorded in fluorescence mode on a 100-pixel Ge detector array at 90° to the incident beam (Canberra/UniSys).

Freeze-dried sludges (aerobic and anaerobic) were finely ground to a homogenous powder and compressed into pellets with a hand press. Samples that had a high Ag concentration were diluted with cellulose material (Sigma-Aldrich). All samples were cooled to ~10 K in a Cryo Industries (Manchester, New Hampshire, USA) cryostat. One to eight scans per sample were collected for XANES spectra and 14 scans per sample were collected for EXAFS spectra. Reference materials measured at the XAS beamline included PVP-coated AgNPs (Nanoamor), $AgNO_3$, Ag_2S, Ag_2O, AgCl, Ag_2CO_3, and Ag_2SO_4 (all purchased from Sigma Aldrich). Additional standards were prepared the day of analysis and stored in the dark until use; Ag_2PO_4, Ag-goethite, Ag-kaolinite, Ag-humic acid complex, Ag-fulvic acid complex, Ag thiosulfate (STS), Ag-acetate, Ag-glutathione (Ag-GSH) and Ag_2S NPs. (See Supporting Information for synthesis and preparation of all Ag standards).

Solid phase speciation of silver nanoparticles in wastewater using synchrotron radiation

A short term exposure experiment (3 h) was undertaken to examine the potential rapid transformation/reactions of AgNPs in wastewater (in the absence of activated sludge) using synchrotron based XAS. PVP coated AgNPs were spiked into wastewater (500 mL) to a final concentration of 5.4 mg Ag L^{-1}. The wastewater was the same as that which was used in the SBR study. The AgNP-wastewater suspension was stirred continuously for 210 min and the DO concentration was measured with an online DO detector (7.4 mg O_2 L^{-1} to 7.1 mg O_2 L^{-1}). Approximately one mL of the suspension was collected at t = 4, 10, 24, 45, 60, 94 and 210 min after the addition of AgNPs. Each sample was collected using a two mL glass syringe and injected directly into a leucite cuvette, secured with Kapton tape, immediately flash frozen in liquid N_2 and stored in dry ice until XAS analysis.

XAS data analysis

The chemical speciation of each sample was determined by fitting a linear combination of model compounds to each XANES spectrum (Additional file 1: Figure SI.3). The number of components in the sample XANES spectra was determined using principal component analysis (PCA) of all sample spectra, followed by target transformation to identify the most likely components of the spectra from a model compound library. The number of components to fit were chosen from the eigenvalues from the PCA and visual inspection of the plot of eigenvectors.

Linear combinations of the six spectra were fitted to each sample spectrum with the best fit to the experimental spectrum achieved by least squares refinement of the model compounds to the experimental spectrum. The best fits were improved by the removal of small components (< 1%) and the adequacy judged by the size of the residual and visual inspection to ensure that all features were accounted for.

Calibration, averaging and background subtraction of all spectra and principle component, target and multiple linear regression analyses of XANES spectra were performed using EXAFSPAK software package (G.N. George, SSRL).

Investigation of nitrification inhibition and silver partioning during aerobic digestion

For nitrification analysis, homogenous mixed liquor samples were collected daily at the end of feed, anoxic, aerobic and settling phases during one 6 h cycle for the first 7 d of SBR operation. On days 9 and 10, more frequent sampling was conducted during the aerobic and anaerobic phases. Samples were filtered (< 0.22 μm) and stored at 4°C until analysis.

The choice of Ag spiking rate was a compromise between realistic environmental exposure concentrations [11], previous partitioning studies of Ag NP in wastewater [8,18] and instrumental detection limits. Taking these factors into account, the aim was to produce sludges with a final concentration of ~100 mg Ag kg^{-1} TS for the Ag^+ and AgNP treatments.

The SBRs were operated for 24 h before spiking to allow for equilibration of the mixed liquor. Reactors 2 and 3 received the AgNP and Ag^+ (as silver nitrate ($AgNO_3$)) treatments, respectively. Reactor 1 was assigned the control SBR and received ultrapure deionised water (Millipore) at each spiking event in order to maintain a consistent volume for all three reactors. Treatments were added once daily, for 8 d,

to each SBR at the beginning of the aeration phase by pipetting the spiking solution directly into the reactor. Each reactor received a total of 330 mL of the assigned spiking solution (nominal concentration for $AgNO_3$ and AgNP suspensions = 20 mg Ag L^{-1}). The Ag concentration of the ultrapure deionised water used in the control SBR was below the limit of detection for ICP-MS analysis (< 0.05 μg L^{-1}). Reactors were operated for a total of 10 d.

For silver analysis, mixed liquor (10 mL) and effluent (35 mL) samples were collected once daily from each SBR; 3 and 5.5 h after spiking, respectively. Samples were acidified and stored at 4°C before subsequent digestion and analysis for total Ag by ICP-MS.

Anaerobic digestion and biomethane potential test

The effect of AgNPs on anaerobic digestion was assessed using a biomethane potential (BMP) test as previously described [45]. Anaerobic biomethane potential tests (BMP) were carried out for AgNPs and Ag^+ using sludge collected from each SBR at the conclusion of that experiment (all assays in triplicate). The inoculum (activity sludge) had a broad trophic microbial composition to ensure the substrate would not be limited. Blank assays (in triplicate) were used to determine the background methane production from the inoculum.

Aerobically digested sludges from the SBRs (substrate) were diluted to 30 g L^{-1} (TSS) with ultrapure deionised water (Millipore). Substrate (40 g wet) and inoculum (60 g wet) (anaerobic digestate from a municipal WWTP, Brisbane, Queensland) were added to glass serum bottles (160 mL working volume), flushed with high purity N_2 gas for 3 min (1 L min^{-1}), sealed with a butyl rubber stopper and aluminium crimp-cap and stored in a temperature controlled incubator (36°C) for 38 d. Blanks (n = 3) contained inoculum (60 g) and ultrapure deionised water (40 mL) (Millipore). Each assay was performed in triplicate. Once daily methane production had ceased (38 d) the batches were terminated, and analysis of the microbial community was conducted. Biogas volume was measured periodically (initially daily) and the quality (CH_4, CO_2, H_2) was analysed using gas chromatography, with a thermal conductivity detector (Perkin Elmer). Confidence intervals (95%) were calculated from triplicate measurements and were ≤ 0.02 g COD d^{-1} for all samples. Excess CH_4 was vented from each serum bottle periodically via syringe and measured by liquid displacement.

Microbial community analysis: DNA extraction and 16 s Pyrotag Analysis

To assess the potential impact of AgNPs on microbial diversity of sludge samples (aerobic and anaerobic), a massive parallel sequencing approach using pyrotag sequencing was used. Microbial diversity analysis was conducted on samples of activated sludge mixed liquor (Luggage Point WWTP), feed (Indooroopilly pumping station), sludge after aerobic digestion (control, Ag^+ and AgNP), anaerobic inoculum (Luggage Point WWTP) and anaerobic digestate (control, Ag^+, and Ag NP).

Community genomic DNA from the anodic biofilms were extracted using FastDNA SPIN for Soil kit (MP Biomedicals, USA) and Fastprep beadbeating machine (Bio101, USA) according to the manufacturer's protocol. The 3' region of the 16S/18S rRNA gene was targeted using universal primers 926 F (5'-AAACTYAAAKGAATT GACGG-3') and 1392R (5'-ACGGGCGGTGTGTRC-3'). Primer sequences were modified by the addition of Roche 454 adaptor 1 or 2 sequences and unique 5 bp barcodes at the 5' end of the primer (sequences not shown) [46,47]. DNA concentration and purity was then determined by gel electrophoresis on 1% agarose gel and spectrophotometrically using the NanoDrop ND-1000 (Thermo Fisher Scientific, USA). DNA was lyophilised using Savant SpeedVac Concentrator SVC100H (Thermo Fisher Scientific, USA) and submitted to the Australian Centre for Ecogenomics (ACE) for 16 s rRNA gene pyrotag sequencing on the Genome Sequencer FLX Titanium platform (Roche, USA). Pyrotag sequences were processed using Pyrotagger [48], and QIIME with correction via ACACIA. Operational taxonomic unit (OTU) tables were normalised, and a square root (Hellinger) transformation was applied to emphasise comparison of niche populations over dominants. A principal components analysis was then performed on the square root (Hellinger adjusted) normalised OTU table using Matlab (princomp command), and results visualised using biplot.

Chemical analysis of silver spiking solutions, mixed liquor, effluent, and sludge

Silver concentrations of the AgNP spiking solutions were determined using an open-vessel acid digestion (HNO_3, 70%) method as previously described [43].

Effluent and mixed liquor samples were analysed for Ag following microwave digestion in *aqua regia* according to the method used for wastewater previously [8]. Sludge samples (aerobic and anaerobic) were first dried at 40°C to constant weight, and then allowed to react with H_2O_2 before using the same *aqua regia* digestion method. Silver concentrations in all digested solutions were determined using ICP-MS (Agilent 7500ce) and $He_{(g)}$ as a collision gas (4 mL min^{-1}) and monitoring Ag at *m/z* 107 and 109.

Filtered mixed liquor was analysed for NH_4^+, NO_2^- and NO_3^- using a Lachat QuikChem8000 Flow Injection Analyser.

Additional file

Additional file 1: The following additional information data are available with the online version of this paper in Additional file 1. Methods for synthesis and preparation of synchrotron standards and sample preparation. **Table SI.1**. Characteristics of the silver nanoparticles (AgNPs) and AgNP stock suspensions. **Figure SI.1**. NH_4 – N profiles of the AgNP and Ag^+ dosed SBRs as measured by the NH_4^+ on-line detector. **Table SI.2**. The concentration of major and trace elements in the influent wastewater **Figure SI.2**. Difference XANES spectra of sludge and various Ag references used in LCF analysis. **Figure SI.3**. Ag K-Edge XANES spectra of all reference materials. **Figure SI.4**. Ag K-Edge XANES spectra of aerobic and anaerobic control sludges and wastewater from the influent experiment. **Figure SI.5**. Bulk silver (Ag) X-ray absorption near-edge spectroscopy (XANES) of sludge collected from the SBRs (a-c) and after the anaerobic batch test (d-f). **Figure SI.6**. Ag K-edge XANES spectra showing the considerable difference between aerobic sludge dosed with AgNP, and anaerobic sludge dosed with Ag^+ or AgNP. **Figure SI.7**. k^3-weighted Ag K-edge EXAFS spectra of sludges and their respective phase-corrected Fourier transforms. **Table SI.3**. The higher residual values that resulted from the exclusion of Ag-acetate from the linear combination fitting analysis of XANES spectra of sludges.

Abbreviations

AgNPs: Silver nanoparticles; TEM: Transmission electron microscopy; EDX: Energy dispersive X-ray analysis; XAS: X-ray absorption spectroscopy; TSS: Total suspended solids; TS: Total solids; MNM: Manufactured nanomaterials; PVP: Polyvinylpyrrolidone; SBR: Sequencing batch reactor; DOC: Dissolved organic carbon; WWTP: Wastewater treatment plant; DLS: Dynamic light scattering; BNR: Biological nutrient removal; DO: Dissolved oxygen; HRT: Hydraulic retention time; OTU: Operational taxonomic units; SD: Standard deviation; STEM: Scanning transmission electron microscopy; HAADF: High angle annular dark field; PCA: Principal component analysis; LCF: Linear combination fitting; XANES: X-ray absorption near edge spectroscopy; EXAFS: Extended X-ray absorption fine structure; AS: Australian synchrotron; Ag^+: Dissolved ionic silver; Ag^0: Elemental silver; NH_4^+: Ammonium; NO_3^-: Nitrate; NO_2^-: Nitrite; $AgNO_3$: Silver nitrate; Ag-GSH: Silver glutathione complex; Ag_2S NP: Silver sulfide nanoparticles; Ag-thio: Silver thiosulfate; Ag-HA: Silver – Humic acid; Ag-FA: Silver – Fulvic acid.

Competing interests

Authors declare that they have no competing interests.

Authors' contributions

CD: designed and conducted the experiments, interpreted results and wrote the manuscript. MJM: designed the experiments, interpreted results and participated in manuscript preparation. JK: designed the experiments, interpreted results and participated in manuscript preparation. DJB: assisted in experimental design, interpreted results and participated in manuscript preparation. HHH: conducted XAS experiments, participated in XAS data analysis, interpreted XAS results and participated in manuscript preparation. HG: Set-up the SBR experiments, conducted the anaerobic batch test and assisted in analysis of results. GC: Participated in experimental design, was involved in useful discussions and participated in manuscript preparation. All authors read and approved the final manuscript.

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Supplementary Information

Synthesis of synchrotron standards and sample preparation

Aqueous Ag^+ (1 mM) was prepared by dissolving $AgNO_3$ in ultrapure deionised water and storing in the dark until use. Silver phosphate was precipitated from a homogenous solution by the volatilisation of ammonia as described by [1]. Briefly, phosphorus oxide (1.3 mmol of H_3PO_4) was combined with ultrapure deionised water (50 mL) in an Erlenmeyer flask, followed by the addition of conc. NH_4OH (0.5 mL) and NH_4NO_3 (25 mmol). A precipitant solution was prepared by adding $AgNO_3$ (15 mmol) to conc. NH_4OH (3 mL) in a beaker and adjusting the volume to 75 mL. The precipitant solution was added to the P_2O_5 solution in one portion resulting in a clear solution. The combined solution (pH = 9.8) was heated on a low temperature hot plate for 3 h until the pH was < 7.5 at room temperature. The resulting precipitate was filtered, washed three times with distilled water and dried overnight at 70°C.

Silver thiosulfate (0.02 mM) was prepared by adding an excess of $(NH_4)_2S_2O_3$ (8 mL, 0.1M) to a stock solution of $AgNO_3$ (2 mL, 0.1M), where the ratio of Ag^+:$S_2O_3^{2-}$ was 1:4. The resulting solution was diluted to 1 mM Ag before XAS analysis

Silver acetate was prepared by adding an excess of acetate (as $NaCH_3COOH$) (2 mL, 0.1M) to aqueous Ag^+ (5 mL, 2 mM) and adjusting the final volume to 10 mL with ultrapure deionised water. The nominal Ag concentration was 1 mM.

Silver glutathione (GSH) (1 mM Ag) was prepared by adding 2 mL of a GSH solution (0.1M) to aqueous Ag^+ (5 mL, 2 mM) and adjusting the volume to 10 mL with ultrapure deionised water. A flocculent white precipitate formed immediately on mixing the solutions and remained suspended in the solution. The spectrum was recorded from the frozen suspension.

To model the possible complexes that Ag may form in the presence of organic compounds or mineral phases in wastewater, Ag was added to fulvic/humic acid and to kaolinite/goethite, respectively. Aqueous Ag^+ (1 mL, 2 mM) was added to solutions of fulvic acid (200 mg L^{-1}) (Suwanee River fulvic acid) or humic acid (200 mg L^{-1}) (Sigma Aldrich) to give a final Ag concentration of 1 mM. For the mineral phases, aqueous Ag^+ (0.5 mL, 7.7 mM) was added to goethite or kaolinite (1 g) to give a concentration of 400 mg Ag kg^{-1}. Both spiked mineral samples were homogenised by grinding in a mortar and pestle. Prior to XAS analysis, the samples were again ground, but with the addition of cellulose, and pressed into a disc.

All sludge samples and the following references were also ground with cellulose material and pressed into a disc prior to analysis: AgCl, Ag_2SO_4, Ag_2PO_4, Ag_2O and Ag_2CO_3. We thank Prof. Enzo Lombi for providing a spectrum of Ag_2S(s) recorded in PVP at beamline 10ID at the APS.

Characterisation of silver nanoparticles

The AgNP suspensions were prepared as previously described [2]. All characteristics determined in our previous study are listed in Table S1.

Table SI.1. Characteristics of the silver nanoparticles (AgNPs) and AgNP stock suspensions [2].

Property	Value
Composition (XRFǂ)	Ag (impurities<0.01%)
Crystal structure (XRD‡)	Ag metal (mineral impurities <2 mass%)
Crystallite size (XRD)	41 nm
Suspended dh# (DLS§)	44 nm
Number-average suspended Stokes diameter (Disc centrifuge analysis)	33 nm
pHIEP¡	3.1
pH in unmodified stock suspensions	4.2
CCCɤ at pH 4 ($NaClO_4$)	22 mM
CCC at pH 8 ($NaClO_4$)	45 mM
Coating (nominal)	0.1% PVP

ǂ X-ray fluorescence analysis
‡ X-ray diffraction
# Apparent *z*-averaged hydrodynamic diameter
§ Dynamic light scattering
¡ pH at which isoelectric point is reached
ɤ Critical coagulation concentration

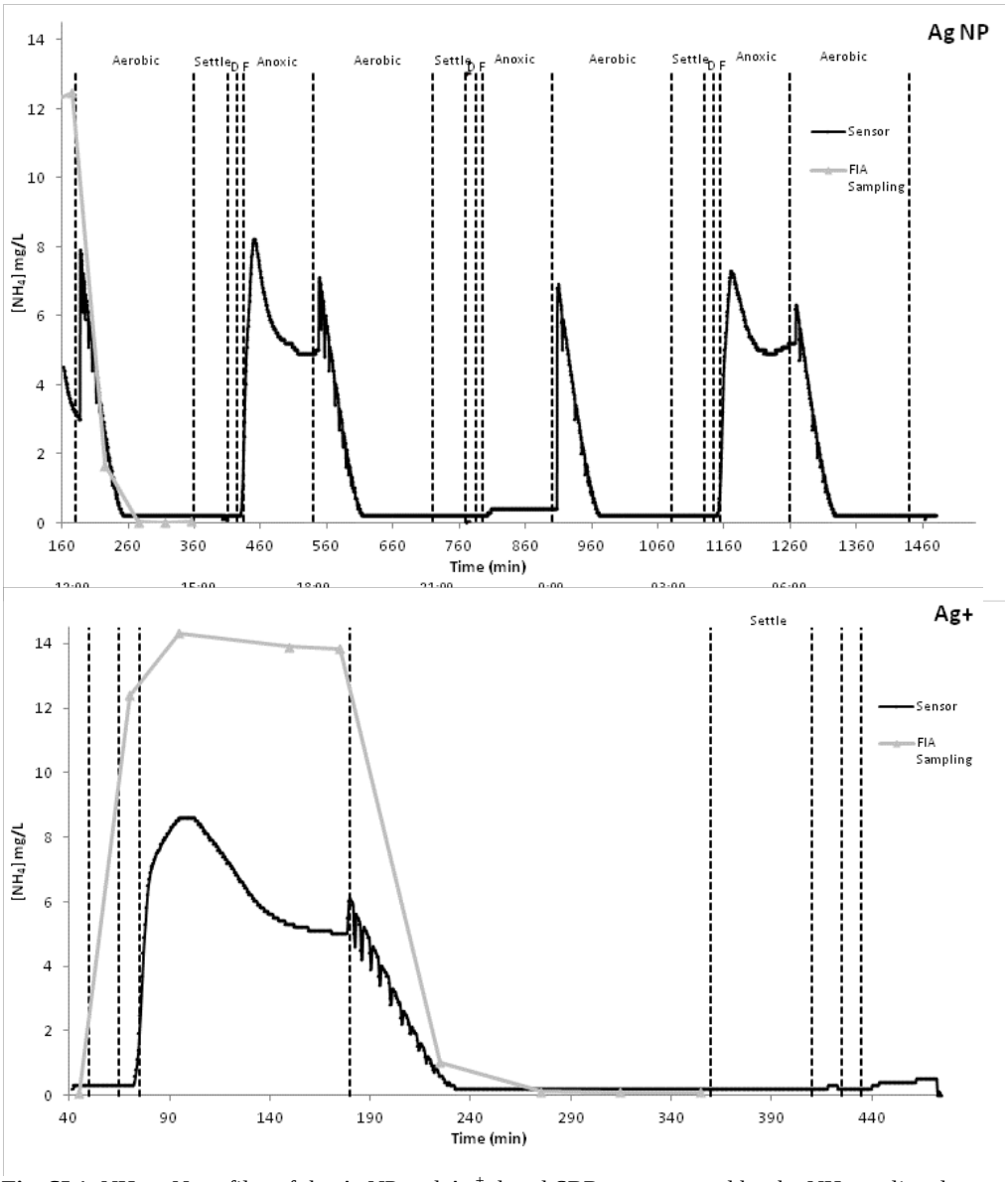

Fig. SI.1. NH_4 – N profiles of the AgNP and Ag^+ dosed SBRs as measured by the NH_4 on-line detector. Data was recorded for 21 h (~3.5 complete cycles) for the AgNP SBR and 6 h (1 cycle) for the Ag^+ dosed SBR. The NH_4 – N concentrations as determined by FIA are also shown.

Table SI.2. The concentration of major and trace elements in the influent wastewater

Element	Ca	K	Mg	Na	P	S	Sr
mg/L	15.3	19.6	12.6	65.8	3.2	12.5	0.1

The concentrations of the following elements were below the ICP-OES detection limit (shown in parentheses in mg/L); Al (0.05), As (0.05), B (0.1), Cd (0.05), Co (0.05), Cr(0.05), Cu (0.05), Fe (0.1), Mn (0.05), Mo (0.05), Ni (0.05), Pb (0.05), Sb (0.1), Se (0.05), Si (0.1) and Zn (0.05).

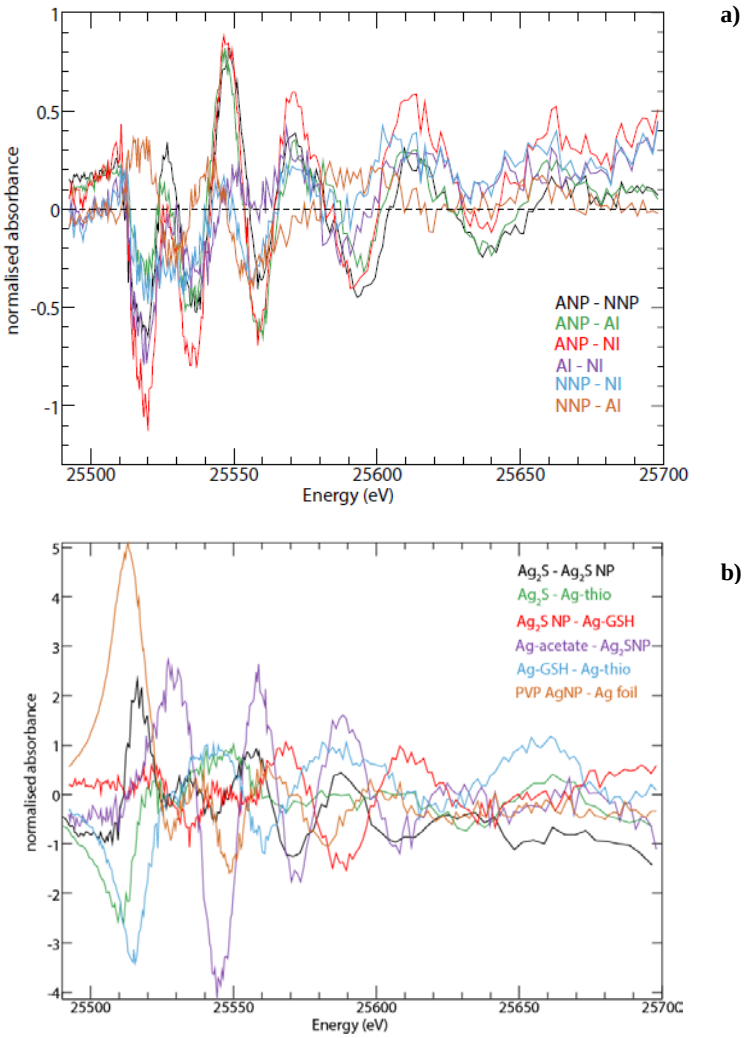

Fig. SI.2. Difference XANES spectra of sludges (a) and various Ag references used in LCF analysis (b). Where ANP = aerobic sludge dosed with AgNPs, NNP = anaerobic sludge dosed with AgNPs, AI = aerobic sludge dosed with Ag^{+}, NI = anaerobic sludge dosed with Ag^{+}, Ag-thio = Ag-thiosulfate complex and Ag-GSH = Ag-glutathione complex.

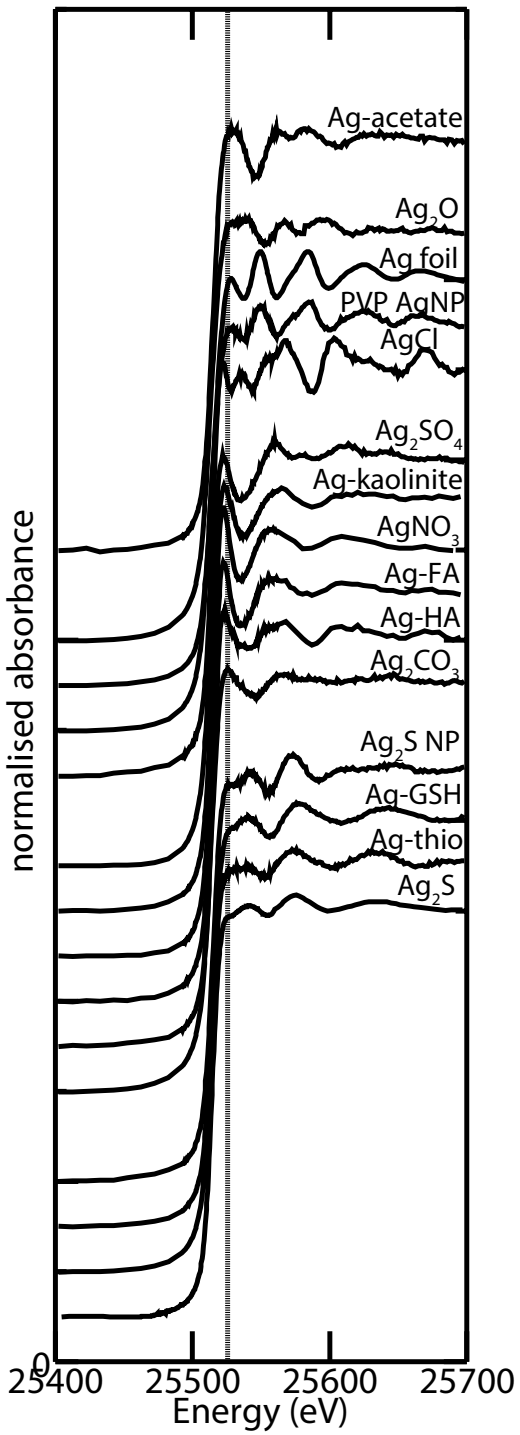

Fig. SI.3. Ag K-Edge XANES spectra of all reference materials. Where Ag-FA = Ag adsorbed to fulvic acid; Ag-HA = Ag adsorbed to humic acid; Ag-GSH = Ag-glutathione complex; and, Ag-thio = Ag-thiosulfate complex. The dashed line is to guide the eye.

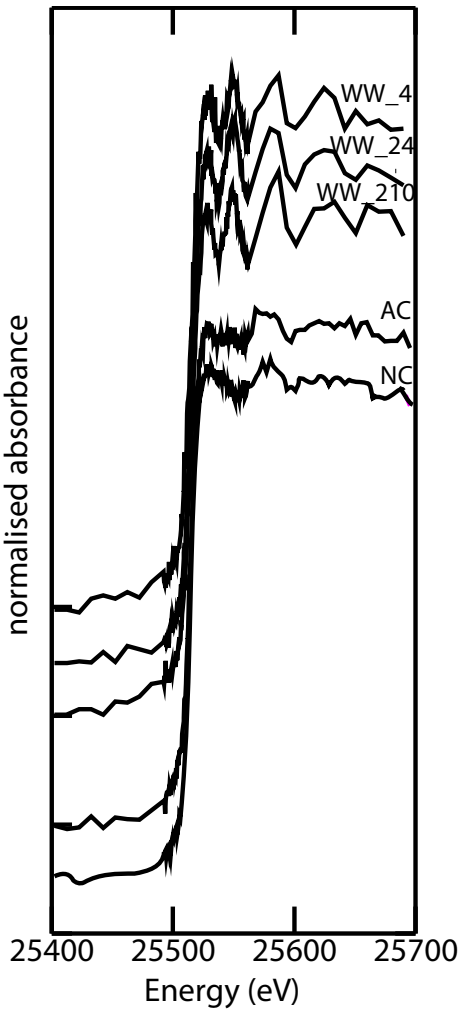

Fig. SI.4. Ag K-Edge XANES spectra of aerobic and anaerobic control sludges (AC and NC, respectively) and wastewater from the influent experiment. Where WW_4, WW_24 and WW_210 are influent samples collected 4 min, 24 min and 210 min after the addition of AgNPs, respectively.

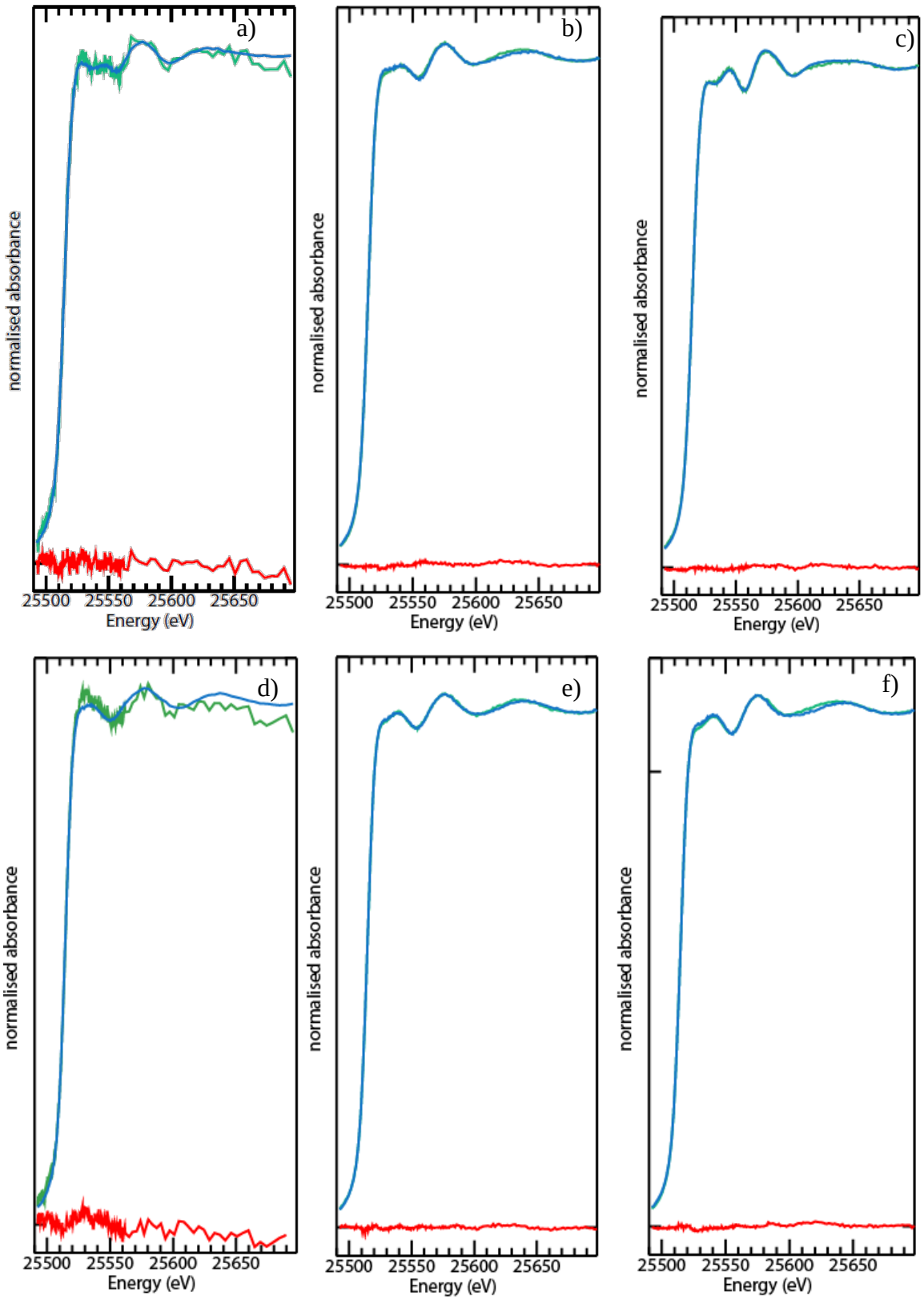

Fig. SI.5. Bulk silver (Ag) X-ray absorption near-edge spectroscopy (XANES) of control, Ag^{+} and AgNP dosed sludges collected from the SBRs (a-c) and after the anaerobic batch test (d-f), respectively. Blue, linear combination fit; green, experimental fit; red, offset residual.

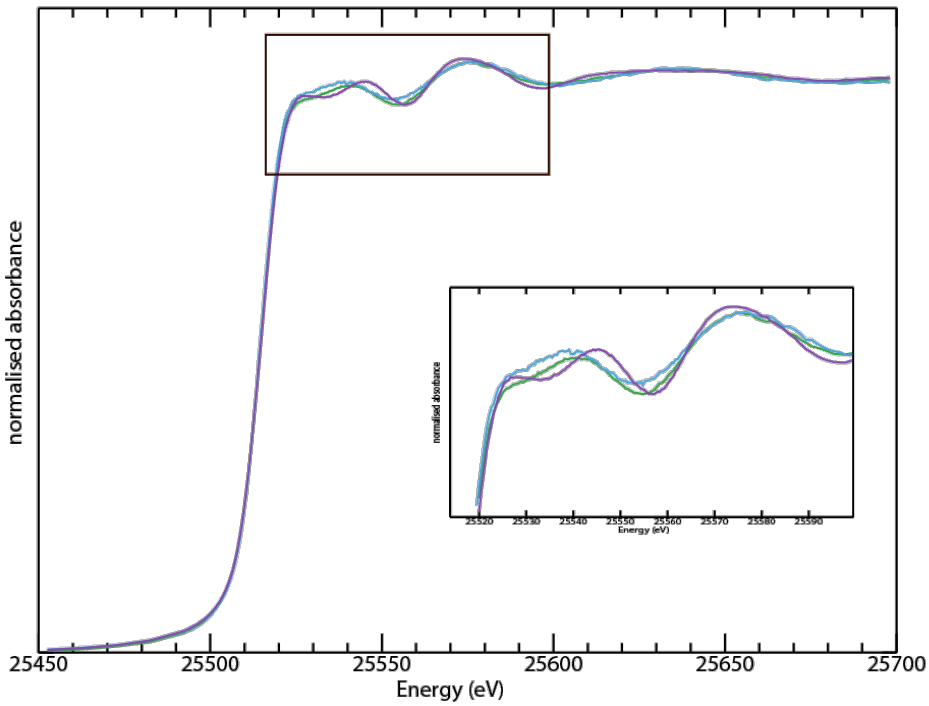

Fig SI.6. Ag K-edge XANES spectra showing the considerable difference between aerobic sludge dosed with AgNP (purple) and anaerobic sludge dosed with Ag^{+}(blue) or AgNP (green).

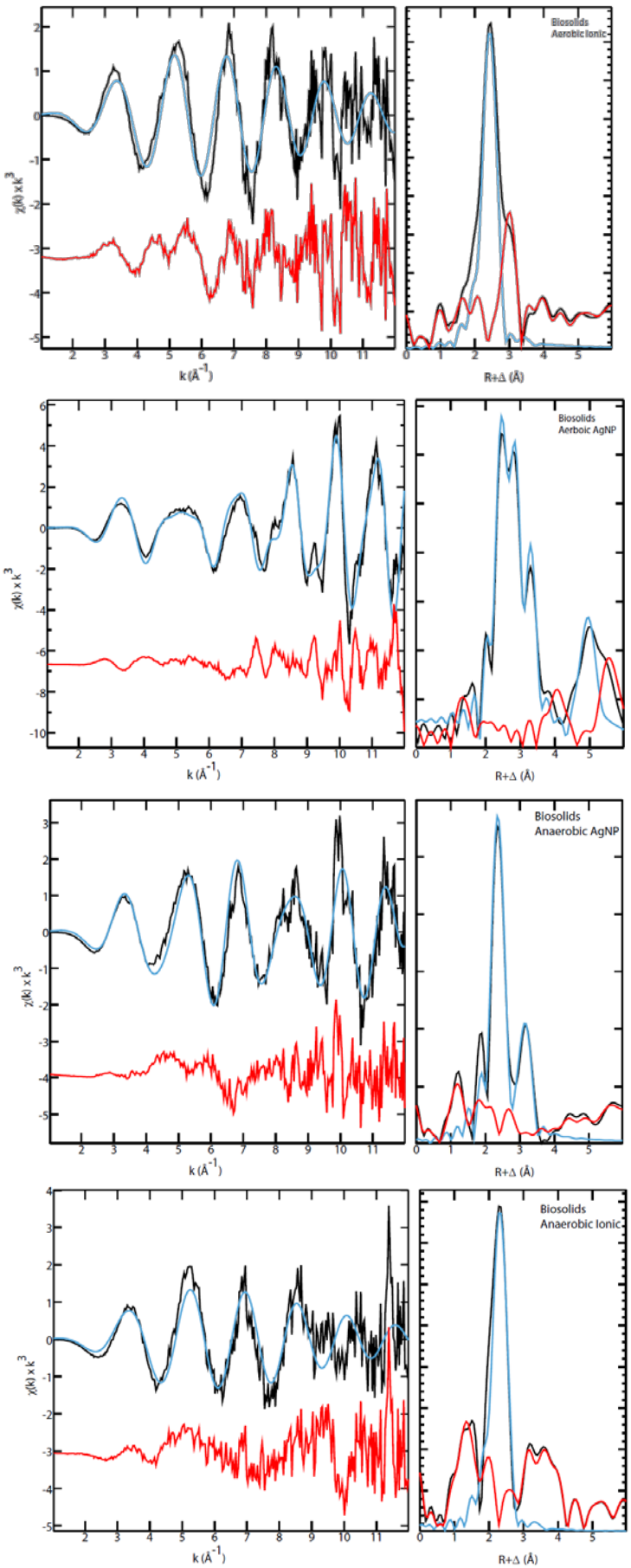

Fig. SI.7. k^3-weighted Ag K-edge EXAFS spectra of sludges (left panels) and their respective phase-corrected Fourier transforms. Data is shown in black, fit in blue and the residual in red.

Table SI.3. The higher residual values that resulted from the exclusion of Ag-acetate from the linear combination fitting analysis of XANES spectra of sludges. Only those sludge samples that were identified as having Ag-acetate as a significant component in the fit are shown. The proportion of species are presented as percentages with the estimated standard deviation **(SD)** in parentheses**.**

Sample	*Residual*	*Residual excluding Ag-acetate*
Sludges		
Aerobic		
Control	0.350	0.414
Ag^+	0.029	0.064
Anaerobic		
Control	0.549	0.703
Ag^+	0.015	0.042
AgNP	0.030	0.041

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[2] G. Cornelis, C. Doolette, M. Thomas, M.J. McLaughlin, J.K. Kirby, D.G. Beak, D. Chittleborough, Retention and Dissolution of Engineered Silver Nanoparticles in Natural Soils, Soil Sci Soc Am J, 76 (2012) 891-902.

CHAPTER 3

Bioavailability of silver and silver sulfide nanoparticles to lettuce (*Lactuca sativa*): Effect of agricultural amendments on plant uptake

Statement of Authorship

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Contribution to the Paper	Experimental design; carried out the experiment; performed all analyses; data interpretation; illustrated all figures; wrote the manuscript		
Overall percentage (%)			
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 3

Bioavailability of silver and silver sulfide nanoparticles to lettuce (*Lactuca sativa*): Effect of agricultural amendments on plant uptake

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ABSTRACT

Silver nanoparticles (AgNPs) can enter terrestrial systems as sulfidised AgNPs (Ag_2S-NPs) through the application of biosolids to soil. However, the bioavailability of Ag_2S-NPs in soils is unknown. The two aims of this study were to investigate 1) the bioavailability of Ag to lettuce (*Lactuca sativa*) using a soil amended with biosolids containing Ag_2S-NPs; and 2) the effect of commonly used agricultural fertilisers/amendments on the bioavailability of Ag, AgNPs and Ag_2S-NPs to lettuce. The study used realistic AgNP exposure pathways and exposure concentrations. The plant uptake of Ag from biosolids-amended soil containing Ag_2S-NPs was very low for all Ag treatments (0.02%). Ammonium thiosulfate and potassium chloride fertilisation significantly increased the Ag concentrations of plant roots and shoots. The extent of the effect varied depending on the type of Ag. Ag_2S-NPs, the realistic form of AgNPs in soil, had the lowest bioavailability. The potential risk of AgNPs in soils is low; even in the plants that had the highest Ag concentrations (Ag^++thiosulfate), only 0.06% of added Ag was found in edible plant parts (shoots). Results from the study suggest that agricultural practises must be considered when carrying out risk assessments of AgNPs in terrestrial systems; such practises can affect AgNP bioavailability.

1. Introduction

The primary exposure pathway for AgNPs into the environment is *via* wastewater streams [1, 2]. Silver NPs and (ionic Ag) may readily enter wastewater streams following their release from AgNP-containing textiles [3, 4], plastics [5], outdoor paints [6] and washing machines [7]. During wastewater treatment (WWT), the majority of AgNPs (> 85%) will be retained by the biosolids [8-10] and most AgNPs will be converted to Ag-sulfides (Ag-S).

In biosolids, the predominant Ag-S species is silver sulfide (Ag_2S) which may exist in the nanometre size range [8, 10] (i.e Ag_2S-NPs). Biosolids that contain Ag_2S-NPs may then be applied to soil as an agricultural amendment to supply nutrients for plant growth [11]. Therefore, the fate and toxicity of AgNPs in soil environments will not be governed by the primary particle released from products but by its transformation products (e.g. Ag_2S-NPs).

Very few studies have investigated the fate and toxicity of AgNPs in terrestrial systems. Recent reviews [12-14] have concluded that there is a need to carry out experiments that use realistic exposure pathways, realistic Ag concentrations and also consider agricultural practices e.g. fertiliser use. It is important to carry out experiments under realistic conditions using soil, otherwise the potential environmental risks of AgNPs may be overestimated if simple solution media are used. For example, soil has been shown to decrease the availability of AgNPs to terrestrial organisms [15] due to the strong interactions of AgNPs with clay and OM [13, 14, 16-18].

Silver NPs are believed to be less toxic and less bioavailable once they are sulfidised [19] due to the low solubility of Ag_2S (K_{sp} = 8 x 10^{-51}) [20]. However, the bioavailability of biosolids-borne Ag_2S-NPs to terrestrial organisms (specifically plants) is unknown. Furthermore, the effect of common agricultural practices on the long term stability of Ag_2S-NPs in soil has not been investigated.

Soil properties can influence the bioavailability of AgNPs to terrestrial organisms. Therefore, any agricultural management practice that affects soil properties, i.e. fertiliser application, has the potential to also impact AgNP bioavailability. Commonly used fertilisers contain a number of different compounds, such as phosphates, chlorides and thiosulfates, which may interact with Ag_2S-NPs. Previous studies have shown that these compounds can affect the bioavailability of Ag or AgNPs to various freshwater and aquatic organisms [21-23]. Therefore, it is important to investigate the effect of these compounds on AgNP bioavailability in terrestrial systems. In addition to nutrient fertilisation, some agricultural amendments are applied to soil for disease control and to improve plant growth (e.g. H_2O_2). Hydrogen peroxide has been shown to interact with Ag [24-26], albeit the effect of H_2O_2 on Ag bioavailability has not been investigated.

The aims of this study were 1) to investigate the plant uptake of Ag from soil that was amended with biosolids containing Ag_2S-NPs and, 2) to evaluate the

effect of commonly used agricultural fertilisers and amendments on plant uptake of Ag from soil treated with $AgNO_3$, AgNPs or Ag_2S-NPs. The study consisted of two pot trial experiments that used the same plant species (lettuce, *Lactuca sativa*) and were designed to be environmentally realistic in terms of 1) soil Ag exposure concentrations 2) biosolids application rate (10 t/ha), and 3) the Ag species that are likely to be present in soils (Ag_2S-NPs) following biosolids amendment.

2. Experimental

2.1. Silver nanoparticle synthesis and preparation of nanoparticle suspensions and silver stock solutions.

Polyvinylpyrrolidone (PVP) coated (0.1%) metallic AgNP powder (10 nm nominal particle size) was purchased from Nanostructured & Amorphous Materials, Inc., Houston, TX. AgNP suspensions, with a concentration of ~ 40 mg Ag/L, were prepared in ultrapure deionised water (Milli-Q, Millipore) as described previously [10].

Ag_2S-NPs were prepared by hydrothermal reaction using L-cysteine as the sulfur source (adapted from Xiang et al. [27]). The Ag_2S-NP suspensions prepared from this powder in ultrapure deionised water (Milli-Q, Millipore) had a concentration of ~ 15 mg Ag/L. See S1.1 for detailed methods of NP synthesis and re-suspension.

The AgNP and Ag_2S-NP suspensions prepared with these methods have been extensively characterized in previous studies using dynamic light scattering (DLS, Malvern Zetasizer), transmission electron microscopy (TEM, Phillips CM200 at 120 keV), X-ray diffraction analysis (XRD, PANanalytical X'Pert Pro) and UV – Vis absorption spectroscopy (200 – 600 nm) (Cary 5000 UV Vis NIR spectrophotometer) [17, 28]. The particle size distribution of AgNPs has also been investigated using disk centrifuge analysis (CPS Instruments disc centrifuge 24000 UHR) [17].

Silver NPs and Ag_2S-NPs were uniformly dispersed and generally spherical with some rod-like particles (for TEM images of AgNPs and Ag_2S-NPs, see [17] and [28], respectively). In summary, the average hydrodynamic particle diameters (d_h) and zeta potentials (ζ) for AgNPs and Ag_2S-NPs were 44 nm and 152 nm, and -50 mV and -43 mV, respectively. The uniform dispersity of NP suspensions was evident from the close correlation between d_h and crystallite size (XRD) for AgNPs [17] and the low polydispersity index (PdI) (0.21) recorded for Ag_2S-NPs [28].

Ionic Ag solutions were prepared to the desired concentrations by dissolving $AgNO_3$ powder (Sigma Aldrich) in ultrapure deionised water (18.2 Ω, Milli-Q, Millipore).

2.2. Overview of Pot Trials 1 and 2

The aim of the first pot trial, herein referred to as PT1, was to examine the bioavailability of Ag in soil that was exposed to biosolids containing Ag_2S-NPs. Biosolids containing Ag_2S-NPs were applied to soil, giving soil Ag concentrations that were between 0.3 to 2.1 mg Ag/kg. To compare the uptake of biosolids-borne AgNPs to that of 'pristine' AgNPs, suspensions of Ag_2S-NPs and polyvinylpyrrolidone (PVP) coated-AgNPs were added directly to soil (1.4 and 4.5 mg Ag/kg soil, respectively). PVP-coated AgNPs will herein be referred to as AgNPs.

The second pot trial (PT2) was the main focus of the study. Commonly used agricultural amendments were applied to soil that had been dosed with Ag_2S-NPs (1.3 mg Ag/kg), AgNPs (1.2 mg Ag/kg) and Ag^+ (as $AgNO_3$, 1.3 mg Ag/kg). The amendments were; ammonium thiosulfate [ATS, $(NH_4)_2S_2O_3$)], potassium chloride (potash, KCl), mono-ammonium phosphate [MAP, $NH_4(H_2PO_4)$] and hydrogen peroxide (H_2O_2). These amendments are used in agriculture to supply crops with the following nutrients: ATS is a common sulfur (S) fluid fertiliser; potash is a common source of K; MAP is applied to soil as a P source; and, finally, H_2O_2 has a variety of uses in agriculture e.g. to control pests/diseases [29], improve fruit quality [30, 31] and oxygenation of the root zone [31, 32]. Fertilisers will be referred to as follows; thiosulfate (for ATS), KCl (for potash); phosphate (for MAP) and H_2O_2.

The bioavailability of Ag was determined by measuring the Ag concentrations of plant parts. In PT1, lettuce shoots were analysed for their total Ag concentration. In PT2, the Ag concentrations in shoots, roots and soil solution were analysed to investigate the effect of agricultural amendments on plant uptake of Ag.

The two key differences between the studies were that 1) in PT1, biosolids were applied to soils (not in PT2), and 2) in PT2, agricultural amendments (KCl, thiosulfate, phosphate, H_2O_2) were applied to soils (not in PT1).

Table 1. Properties of soil used (collected from Mt.Compass, South Australia).

Soil location	EC[a]	pH[a]	Total C	CEC	Particle size analysis			MWHC
	mS/cm		%	(cmol/kg)	Clay (%)	Silt (%)	Sand (%)	(%)
Mt. Compass (South Australia)	0.05	5.8	0.5	2.0	4	1	95	3.5

Acronyms: EC=electrical conductivity; Total C=Total carbon; CEC=cation exchange capacity; MWHC=maximum water holding capacity.

[a]pH and EC measurements were analysed using a 1:5 soil:solution ratio in duplicate. Reported values are the

2.3. Soil properties and pot experiment set-up (Pot Trial 1 and 2)

Lettuce plants were grown in free-draining pots to prevent the accumulation of salts from the added nutrient solutions (see S1.2). Two layers of gravel (large and small sized) were placed in the bottom of each pot followed by 200 g (dry weight) of soil. A sandy soil (from Mt. Compass, South Australia) was oven dried at 60ºC (7 days), sieved (< 2 mm) and thoroughly mixed. The soil was slightly acidic ($pH_{1:5\ soil:water}$ 5.8) and had a high sand content (95%) with a low concentration of total organic carbon (0.5 %) (see Table 1 for soil properties). Soils with these properties are likely to promote AgNP dissolution and thus, potentially increase AgNP bioavailability. See S1.2 for plant growing conditions.

2.4. Pot Trial 1

Soil was amended with biosolids that were collected from a previous experiment that examined the effects of Ag^+ and AgNPs on a simulated WWT plant [10]. Briefly, two Ag treatments –Ag^+ (as $AgNO_3$) and AgNPs – were added to separate sequencing batch reactors (SBRs) that contained activated sludge mixed liquor and influent wastewater. A third SBR was used as a control (no Ag added). Following SBR operation (aerobic stage), sludge was then anaerobically digested. At the end of both the aerobic and anaerobic stages, sludge samples were collected and dried (40°C) for use in PT1. The dominant Ag species in all biosolids treatments was Ag_2S [10].

For PT1, the six biosolids treatments were added to soil at a realistic application rate (for Australia [33]) of 10 t/ha, corresponding to 5.9 g/kg soil. The bioavailability of Ag in biosolids-amended soil was compared to soils that were directly exposed to Ag^+ and 'pristine' AgNPs or Ag_2S-NPs. For each biosolids treatment, two letters, 'A' or 'N', will be used as a prefix to indicate how the biosolids were produced (aerobically or anaerobically, respectively). Overall there were nine soil treatments (Table 2).

The total Ag concentrations of soils in PT1 (Table 2) varied between treatments due to the different Ag concentrations in biosolids (Table 2). This was unavoidable because the biosolids application rate was kept constant for each treatment. To enable comparisons of results between treatments at different Ag exposure concentrations (0.2 – 9.5 mg Ag/kg), Ag^+ (as $AgNO_3$) was also added to 19 pots to establish a dose *vs.* shoot-uptake curve for this soil. Based on the measured shoot Ag concentrations from these treatments, a dose-response curve was developed, enabling the results from all other treatments (i.e. biosolids and NP suspensions) to be compared to similar Ag^+ concentrations.

2.5. Pot Trial 2

In PT2, soil was spiked with the following treatments: 1) Ag_2S-NPs; 2) AgNPs); 3) Ag^+; and, 4) control (no added Ag). All Ag treatments were added over 5 days to prevent waterlogging of the soil. Silver concentrations of the Ag_2S-NP, AgNP and Ag^+ dosed soils were 1.32, 1.20 and 1.32 mg Ag/kg, respectively. Once the spiked soils were homogenised, 200 g (dry weight) of soil was added to each pot (n=4). For each Ag treatment, the agricultural amendments were added, as solutions, to four replicate pots.

The target soil concentrations of S, P and K after 60 days of growth were 100 mg (S/P/K)/kg soil for the thiosulfate, phosphate and KCl fertilisers, respectively. The pots receiving H_2O_2 treatment were watered to weight with a dilute H_2O_2 solution (0.5 %) four times over the course of the experiment. See S1.2 for details of fertiliser addition.

2.6. Plant harvesting

After 60 days of plant growth, shoots from PT1, and shoots and roots from PT2 were harvested. The shoots were cut ~ 0.5 cm above the soil surface and the roots removed from the soil with tweezers. Shoots and roots were oven dried for 5 days (60°C) and shoots then weighed for dry mass. Once dried, soil was removed from PT2 roots using tweezers and a fine brush.

2.7. Measurement of total silver content in plant tissues and soil

Total Ag concentrations of plant tissues (shoots and roots) were determined using a closed vessel microwave-assisted digestion procedure and analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) and ICP-mass spectrometry (ICP-MS, Agilent 7500ce). Further details of analysis methods and Ag recoveries are given in S1.3. The limit of quantification (LOQ) for Ag concentrations in plant tissues and soils was 0.02 mg Ag/kg. Prior to ICP-MS analysis, shoot samples from PT1 required pre-concentration due to low Ag concentrations. This method decreased the LOQ to 0.01 mg Ag/kg (S1.3).

Total Ag concentrations in roots from PT2 were corrected for the presence of Ag bound to soil on root surfaces using a chromium (Cr) correction method. This method assumes that no soil Cr was taken up by the plant (S1.4). On average, 14 ± 3% of the Ag that was detected in root samples was attributable to the presence of soil Ag in the root sample (S1.4).

Pot trial 2 soils were extracted with ammonium nitrate (NH_4NO_3) (ISO 19730) [34] to estimate the potentially bioavailable fraction of Ag, w_{Ag} (µg Ag/kg) (S1.5).

2.8. Statistical analysis

Statistical analysis was performed using GenStat (15th Edition SP2) software with one way ANOVA. Details on all statistical analysis and the data fitting methods used in PT1 are described in S1.6.

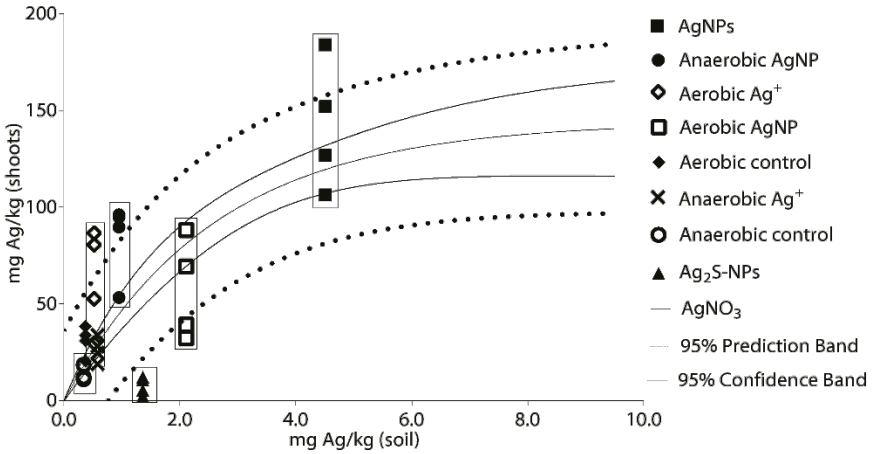

Figure 1. Concentration of silver in lettuce shoots harvested from Pot Trial 1. The Mitscherlich model was used to fit the $AgNO_3$ data (solid line) and calculated using SigmaPlot® (see S1.6 for details). Four replicates per treatment are shown.

3. Results and Discussion

3.1. Pot trial 1

There were no significant differences in the above-ground biomass (dry weight) of plants at harvest ($p \leq 0.05$). Shoot concentrations of Ag were considerably lower than that of the soil (Fig. 1). This demonstrates that both Ag^+ and transformed AgNPs (present in the applied biosolids) had very low bioavailability to lettuce in this soil. The highest Ag shoot concentrations (138 ± 37 µg Ag/kg) were found in plants that were grown in AgNP-dosed soil (no biosolids). This soil also had the highest Ag concentration (4.5 ± 0.3 mg Ag/kg) (Table 2). The results also show that plant uptake of Ag cannot be predicted by soil Ag concentrations alone (Fig. 1). For example, the Ag concentration of the A-nB treated soil was greater than that of the A-iB soil (2.1 *vs.* 0.5 mg/kg, respectively), yet the average Ag shoot concentration was lower (50 and 62 µg Ag/kg, respectively). This suggests that the speciation of Ag played a significant role in the bioavailability of Ag to lettuce.

The shoot concentrations of Ag for all treatments, except for Ag_2S-NP, fit within the 95% prediction intervals for shoot uptake of Ag^+ in this soil (Fig. 1). This suggests that dissolved Ag was the main Ag species taken up by lettuce and not AgNPs or Ag_2S-NPs. Therefore, the dissolution of AgNPs and Ag_2S-NPs to Ag^+ will be an important factor that controls their bioavailability. For Ag_2S-NPs, very little Ag^+ is released in natural soils due to its low solubility [28]. Note that in the soil solution, free Ag^+ may be complexed by dissolved organic carbon or partitioned to solid phases depending on soil properties [35].

Plants that were grown in biosolids-amended soil had higher Ag shoot concentrations than Ag_2S-NP plants, despite the fact that Ag_2S was the dominant species in biosolids. It is unclear why this occurred. One possibility is that Ag speciation differed between the biosolids and the manufactured Ag_2S-NPs. Silver speciation analysis of the biosolids has shown that sulfidation of AgNPs was not complete [10]; carboxylated and thiolated Ag species were also present. Another contributing factor may be that during biosolids storage, a fraction of Ag_2S may have

Table 2. Mean silver concentrations (± 1 standard deviation) of soil and biosolids used in Pot Trial 1.

Treatment	Abbreviation	Soil silver concentration (mg Ag/kg)	Biosolids silver concentration (mg Ag/kg)
Anaerobic control biosolids	N-cB	0.34 ± 0.19	6.3 ± 0.3
Aerobic control biosolids	A-cB	0.37 ± 0.23	5.7 ± 0.1
Anaerobic ionic Ag biosolids	N-iB	0.58 ± 0.44	76.7 ± 0.2
Aerobic ionic Ag biosolids	A-iB	0.52 ± 0.01	167.9 ± 8.5
Anaerobic AgNP biosolids	N-nB	0.95 ± 0.01	183.6 ± 0.3
Aerobic AgNP biosolids	A-nB	2.11 ± 0.22	418.4 ± 70.6
Soil with Ag_2S-NPs	Ag_2S-NPs[a]	1.36 ± 1.30	-
Soil with AgNPs	AgNPs[b]	4.51 ± 0.32	-
	$AgNO_3$[c]	0.2 – 9.5	-

Acronyms: N=anaerobic biosolids; A=aerobic biosolids; cB=control biosolids (no added Ag); iB=ionic Ag ($AgNO_3$) added initially to biosolids; nB=silver nanoparticles added initially to biosolids.

[a]Silver sulfide nanoparticles added directly to soil (no biosolids).
[b]Silver(0) nanoparticles (with PVP coating) added directly to soil (no biosolids).
[c]19 concentrations of $AgNO_3$ in the given range were added directly to soil .

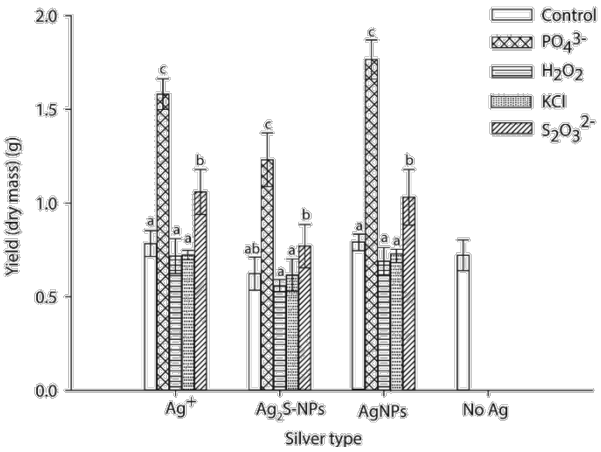

Figure 2. The shoot yield (dry weight) of lettuce from Pot Trial 2 (± 1 standard deviation). There were no significant differences in yield between silver treatments. Significant differences calculated using ANOVA Fischer's Protected LSD Test ($p \leq 0.05$).

been transformed to more available forms (e.g. Ag weakly bound to organic matter). Aging has been shown to affect the speciation and lability of metals in biosolids and biosolids-amended soil [35-37]. Speciation changes will affect bioavailability.

The results demonstrate that when biosolids containing transformed AgNPs (primarily Ag_2S NPs) are applied to soil, an increase in total plant Ag concentrations can occur. However, the overall bioavailability of biosolids-borne Ag_2S-NPs to lettuce was still very low. The amount of Ag in lettuce shoots (edible plant parts), accounted for < 0.02% of the total amount of Ag in each pot for all biosolids-amended soil treatments.

3.2. Pot Trial 2

3.2.1. Shoot biomass

The effect of common agricultural fertilisers and amendments on shoot biomass (dry weight) at harvest is shown in Fig. 2. No Ag treatments had a significant effect on shoot biomass ($p > 0.05$); however, some fertiliser treatments (phosphate and thiosulfate) did ($p \leq 0.05$). Soils treated with phosphate had significantly greater lettuce shoot biomass ($p \leq 0.05$) than the controls for all Ag treatments (Fig 2). Thiosulfate had less of an effect than phosphate on shoot biomass, and significant increases were only observed in Ag^+ and AgNP treatments ($p \leq 0.05$). Analysis of shoot phosphorus (P) concentrations revealed that some plants were marginally deficient in this element; which may explain the positive growth response to the additional P in this soil.

3.2.2. Thiosulfate fertiliser treatment

The greatest accumulation of Ag in roots occurred in the thiosulfate treatments (Fig 3). Application of thiosulfate to soil at 100 mg S/kg significantly increased the concentration ($p \leq 0.05$) of Ag in roots compared to the controls for AgNPs and Ag_2S-NPs treatments (Fig 3). For the Ag^+ treatment, there was no significant increase in root Ag concentrations compared to the control ($p > 0.05$), possibly due to variation between replicates. Large variability in metal concentrations is often observed with the analysis of roots in soil pot trials due to the close relationship between soil particles and fine root hairs and complex microorganism interactions on the root surface [38].

In the shoots, thiosulfate addition significantly increased Ag concentrations in all Ag treatments ($p \leq 0.05$) (Fig. 4). For example, in the Ag^+ and Ag_2S-NP treatments, the shoot concentrations of Ag were 152 and 110 µg Ag/kg (respectively), whereas in the controls (no fertiliser) concentrations were approximately five times less (26 and 20 µg Ag/kg, respectively). Despite this increase, the overall bioavailability of Ag was very low for all treatments and very little Ag was translocated to the shoots (see S2.2 and Table S2). For example, in the Ag^++thiosulfate treatment, only 0.06% of the added Ag was found in the shoots.

The high Ag concentrations of plants grown in thiosulfate-treated soil may be due to the complexation of Ag by thiosulfate, which in turn can

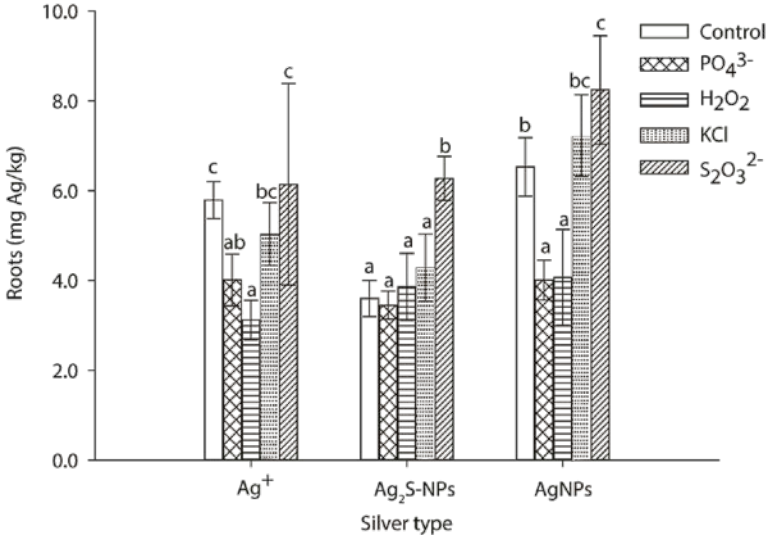

Figure 3. Silver concentrations in lettuce roots (± 1 standard deviation) from Pot Trial 2. Letters indicate significant differences between fertilisers within each silver treatment according to ANOVA Fischer's Protected LSD Test for $p \leq 0.05$.

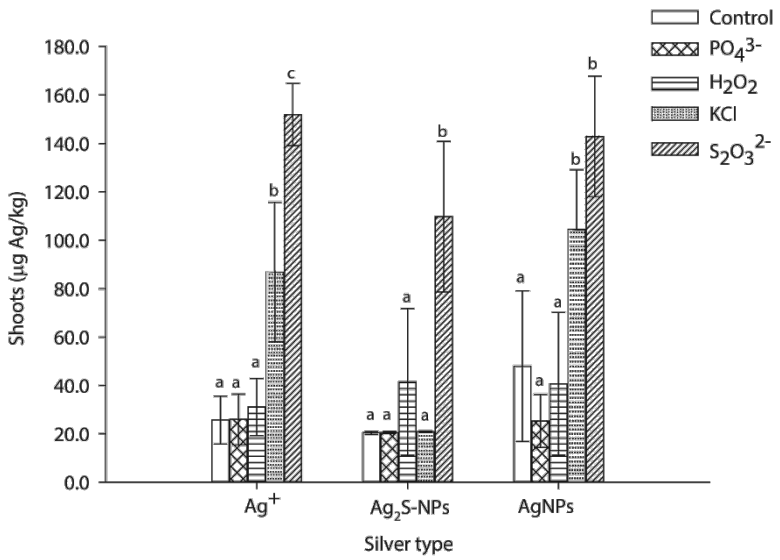

Figure 4. Silver concentrations in lettuce shoots (± 1 standard deviation) from Pot Trial 2. Letters indicate significant differences between fertilisers within each silver treatment according to ANOVA Fischer's Protected LSD Test for $p \leq 0.05$.

increase bioavailability. It is challenging to predict the reaction pathway between Ag^+ and thiosulfate due to the many possible reactions. A number of factors influence the reaction pathway including; ratio of Ag^+:thiosulfate, concentration of thiosulfate, physicochemical properties (e.g. pH) and the oxidation of thiosulfate to simpler molecules (e.g. SO_4^{2-}). In this experiment, one of the most important factors when considering Ag speciation is the ratio of Ag^+:thiosulfate.

When thiosulfate is in excess, Ag can readily form soluble complexes with thiosulfate:

$$Ag^+_{(aq)} \; S_2O_3^{2-}{}_{(aq)} \leftrightarrow Ag(S_2O_3)^-_{(aq)} \quad k_1 = 7.4 \times 10^8 \qquad \text{(Equation 1)}$$

$$Ag(S_2O_3)^-_{(aq)} + S_2O_3^{2-}{}_{(aq)} \leftrightarrow [Ag(S_2O_3)_2]^{3-}_{(aq)} \quad k_2 = 3.9 \times 10^4 \qquad \text{(Equation 2)}$$

[39]

We used the modelling software Visual MINTEQ to predict what Ag and Ag-thiosulfate species would be present in soil solutions using experimentally-determined soil physical and chemical properties [e.g. pH 5.8, total C content, NH_4NO_3 extractable Ag (see S2.1 and Table S1) and thiosulfate fertiliser concentrations in soils]. Modelling predicted that $Ag(S_2O_3)^-$ would be the dominant Ag species (> 90 %) in soil solutions. It is likely that any free Ag^+ in soil solution would have complexed with thiosulfate due to (a) Ag^+ being a soft cation that readily forms stable complexes with soft ligands such as thiosulfate [40] and (b) the strong affinity of Ag^+ for S-containing molecules. A similar reaction mechanism could be envisioned in the AgNP and Ag_2S-NP +thiosulfate treatments; thiosulfate could interact with any dissolved Ag or with Ag exposed at the NP surface. Metal-thiosulfate complexes have been shown to be taken up by plants and algae [22, 41]. Therefore, the complexation of Ag by thiosulfate to form $Ag(S_2O_3)^-$ may have increased the lability and hence, bioavailability of Ag.

3.2.3. Potassium chloride fertiliser treatment

Root concentrations of Ag were not significantly affected by KCl ($p > 0.05$) in any Ag treatments (Fig. 3). However, soil application of KCl (applied at 100 mg K/kg soil) did significantly increase shoot concentrations of Ag for plants grown in Ag^+- and AgNP-treated soils ($p \leq 0.05$) (Fig. 4). For plants grown in Ag_2S-NP-treated soil, although some soluble Ag was present (Table S1), KCl had no significant effect on Ag shoot concentrations compared to the control ($p > 0.05$).

The results suggest that for plants grown in AgNP-treated soil, AgNP dissolution is the dominant process that governs Ag uptake i.e. AgNPs are not directly absorbed from soil. This hypothesis is further supported by the absence of a significant effect in the Ag_2S-NP plants. Silver sulfide NPs are more resistant to dissolution due to their very low solubility and high stability. Since some soluble Ag is present in these soils (Table S1), it is likely that Ag in these extracts were in dispersed Ag_2S-NP form [28]. Under this scenario, uptake of Ag in the Ag_2S-NP+KCl soils would be low.

The increased bioavailability of Ag following KCl fertilisation of Ag^+ and AgNP-spiked soils may be due to changes in Ag speciation in the soil solution and/or solid partitioning via cation exchange. In soils, Ag^+ may exchange with K^+ that is adsorbed onto the permanent negatively-charged sites on soil surfaces; both ions are monovalent cations and have similar hydrated radii (Ag^+ = 3.41 Å, K^+ = 3.31 Å). This competitive ion effect may have increased soil solution concentrations of Ag^+ in the KCl treatment. Note that this exchange mechanism would have been limited in the Ag_2S-NP soils. It has been shown that in clay minerals, Ag^+ may substitute for K^+ under conditions of low acidity [42]. High concentrations of K^+ have also been reported to significantly decrease Ag^+ sorption to a sandy loam (7% clay, pH = 5.3) [43]. In the present study, if Ag^+ was released into the soil solution following exchange with K^+, it is unlikely that it would have remained as a free ion. Instead,

exchanged Ag^+ could have reacted with excess soluble Cl^- present in the soil solution.

Chloride reacts readily with Ag^+ to form a number of soluble (and bioavailable) Ag-chloro complexes e.g. $AgCl^0_{(aq)}$, $AgCl_2^-{}_{(aq)}$, $AgCl_3^{2-}{}_{(aq)}$. However, when over saturation occurs, $AgCl_{(s)}$ can precipitate as insoluble cerargyrite (K_{sp} = 1.7 x 10^{-10}). The concentrations of Ag and Cl^- and the chemical properties of the soil solution (e.g. pH) will determine what Ag-chloro complex dominates in soil solution. We used the modelling software Visual MINTEQ to predict what Ag and Ag-chloro complexes would be present in the soil solution of KCl-amended soils at pH = 5.8. The model predicted that the majority of Ag in solution would exist as the neutral complex $AgCl^0_{(aq)}$ (~ 70%), with smaller amounts of anionic species and free Ag^+ ($AgCl_2^-$ =17%, $AgCl_3^{2-}$ < 1 %, Ag^+=13%). It is possible that free Ag^+ would be complexed by other complexing ligands (e.g. low molecular weight acids from root exudates, organic matter, etc.) [35]. Plant uptake of Ag-Cl species has not previously been investigated. However, experiments with other organisms have shown that the availability of these species is strongly dependent on the type of organism. For example, $AgCl^0$ is bioavailable to aquatic organisms, e.g. Atlantic salmon and rainbow trout [21], and some algae species (*C. reinhardtii* [44] and *T. weissflogii* [45]) but not to *P. subcapitata* [44].

Note that Ag-Cl speciation would vary depending on the concentration of Cl^- in soil i.e. soil salinity. Further modelling showed that in more saline soils, the dominant species would be anionic complexes ($AgCl_2^-$ and $AgCl_3^{2-}$), whereas in less saline soils, insoluble $AgCl_{(s)}$ would dominate. These predictions agree with previous speciation studies [46, 47]. Changes in Ag-Cl speciation would affect the concentration of Ag that is available for plant uptake.

3.2.4. Hydrogen peroxide amendment and mono-ammonium phosphate fertilisation

Root concentrations of Ag for plants grown in H_2O_2 treated soil were significantly less than the controls for Ag^+ and AgNP treatments (Fig. 3). The oxidation pathway of AgNPs in the presence of H_2O_2 is pH dependent. Under alkaline conditions (pH = 9.5), AgNPs can catalyse H_2O_2 degradation and in the process produce Ag^+ [24, 25]. Interestingly, it has been shown that under alkaline conditions, AgNPs rapidly reform in the presence of H_2O_2 decomposition products, specifically superoxide, (O_2^-), this extends the lifetime of AgNPs [25]. At very acidic pH (2.2), AgNPs are also oxidised to produce Ag^+; however, the primary H_2O_2 degradation product (hydroxyl radical, •OH) does not cause continual reformation of AgNPs [26]. In the current experiment, soil pH was slightly acidic (pH = 5.8) so both mechanisms may have been operating. The formation of O_2^- as a result of H_2O_2 degradation may have caused the (re)formation of AgNPs. Reformation of AgNPs would decrease the concentration of plant available Ag.

Soil application of H_2O_2 (as 0.5% solution) did not have a significant effect on Ag shoot concentrations for any Ag treatments ($p > 0.05$) (Fig. 4). Control studies investigating AgNP and Ag_2S-NP dissolution in H_2O_2 (1%) were performed previously (S1.7 and Fig. S1). Results from these experiments (without soil) showed that AgNP dissolution could increase in the presence of H_2O_2 (1%). Therefore, we hypothesised that H_2O_2 (having a high oxidation potential) would oxidise AgNPs and Ag_2S-NPs; the release of free Ag^+ would then lead to greater plant uptake of Ag. This hypothesis was not supported by the results. Although H_2O_2 may have oxidised Ag, shoot Ag concentrations were not significantly greater than the controls ($p > 0.05$). Plant uptake of oxidised Ag may not have occurred due to the absence of suitable complexing agents (e.g thiosulfate or Cl^-) which may keep Ag available in soil solution. It is likely that any released Ag^+ would have strongly adsorbed to soil particles and/or organic matter. The trends that were observed in the H_2O_2 treatments, in terms of shoot and root Ag concentrations, were also observed in plants grown in phosphate-amended soil.

The soil application of phosphate at 100 mg P/kg did not have a significant effect on Ag shoot concentrations ($p > 0.05$) for any Ag treatments (Fig. 4). However, there was a significant decrease in root Ag concentrations compared to the controls for Ag^+ and AgNPs treatments (Fig. 4) ($p \leq 0.05$). Silver ions can react with phosphate [13] to produce insoluble Ag phosphate (Ag_3PO_4). However, under the conditions of the current study (i.e. Ag soil solution concentration, pH etc.), MINTEQ modelling predicted that Ag_3PO_4 would not precipitate. Instead, other aqueous Ag-phosphate complexes with low bioavailability may have formed; decreasing Ag root uptake. Previous studies have shown that in the presence of phosphate, Ag toxicity can decrease [23, 48].

To the best of our knowledge, this experiment is the first to investigate the effect of phosphate on the plant uptake of Ag. However, the effect of phosphate on Ag bioavailability in aquatic and freshwater environments has been investigated. The presence of phosphate has been shown to decrease Ag^+ accumulation in aquatic microalgae (*C. reinhardtii*) [23] and also potentially decrease AgNP toxicity to phytoplankton [49]. As the trends for Ag^+ and AgNP treatments were the same (root Ag concentrations were significantly less than the control), this suggests that Ag^+ was the most likely form of Ag being taken up (not AgNP).

4. Conclusions

This study demonstrates the low plant availability of Ag from soil amended with biosolids containing environmentally relevant Ag_2S-NPs at realistic Ag concentrations. Silver sulfide NPs that were applied directly to soil were less bioavailable than Ag_2S-NPs borne biosolids; possibly due to incomplete sulfidation of AgNPs during WWT or changes to Ag speciation during storage. The results support previous findings that have shown low dissolution of Ag_2S-NPs in soil [28].

Our results demonstrate that commonly used

fertilisers can affect the plant uptake of Ag released from Ag_2S-NPs and AgNPs. Despite the high affinity of AgNPs and Ag_2S-NPs for soil surfaces, bioavailability was found to increase following soil fertilisation with thiosulfate and KCl. This increase may be due to complexation of Ag^+ by thiosulfate and chloride ligands, which act to stabilise Ag^+ in soil solution thereby increasing Ag mobility and bioavailability. Conversely, soil application of H_2O_2 and phosphate fertiliser (MAP) was found to decrease the plant uptake of Ag from soil amended with Ag_2S-NPs, AgNPs or Ag^+.

This study suggests that the potential risk of AgNPs in terrestrial environments is very low. Plant uptake of Ag from soils containing Ag_2S-NPs, the realistic form of AgNPs in biosolids-amended soil, will be limited due to the low solubility of Ag_2S and its resistance to dissolution. Our results demonstrate that agricultural practices, e.g. use of thiosulfate as fertiliser, have the potential to increase Ag_2S-NP bioavailability. However, under the conditions used in this study, the concentration of Ag in edible plant parts was still very low and accounted for <1% of the total Ag added to the soil.

Overall, our study demonstrates that agricultural practises may have implications for the future management of Ag-containing biosolids. The information gained from this study will enable a more accurate risk assessment of AgNPs and Ag_2S NPs in terrestrial systems.

5. Acknowledgments

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Supplementary Information

S1. Experimental methods

S1.1. Silver nanoparticle synthesis and preparation of nanoparticle suspensions

To prepare AgNP suspensions, the PVP coated AgNP powder (0.1 g) was added to ultrapure deionised water (50 mL, Ω 18.2, Milli-Q, Millipore) in a 50 mL centrifuge tube, sonicated (90 W, 3 min) and then centrifuged (2200 g, 15 min) to remove particles with a diameter >100 nm. The supernatant was removed (35 mL) and homogenised (by gently inverting the tube), resulting in a AgNP suspension with a concentration of ~ 40 mg Ag/L.

To synthesise Ag_2S-NPs, L-cysteine (0.165g) was added to ethanol (150 mL) in a beaker. The solution was vigorously stirred for 4 min after which time, $AgNO_3$ (0.233 g) was added and stirred vigorously for a further 15 min**.** As the solution was stirred, a gradual change in colour was observed; from colourless to milky white. The solution was then transferred to a 250 mL stainless steel Teflon-lined hydrothermal autoclave reactor and heated at 180°C for 5 h. Once cool, the resulting suspended black precipitate was filtered (Whatman®, Grade 42), rinsed three times with ethanol followed by ultrapure deionised water (Ω 18.2, Milli-Q, Millipore) and oven-dried at 60°C for 6 h. The dried Ag_2S powder was stored in a desiccator in the dark until preparation of the Ag_2S-NP suspension.

The Ag_2S-NPs suspensions were prepared by weighing Ag_2S-NP powder (0.16 g) into a 50 mL centrifuge tube, adding ultrapure deionised water (50 mL, Ω 18.2, Milli-Q, Millipore) and humic acid (0.5 mL of a 500 mg/L stock solution, Sigma Aldrich) and gently inverting several times. Based on our preliminary experiments, the addition of humic acid to the suspension is necessary to improve the dispersibility of the NPs in water. The suspension was then probe sonicated (90W, 10 min) in an ice bath, centrifuged (2200g, 15 min) and the supernatant removed (30 mL). Ultrapure deionised water (30 mL, Ω 18.2, Milli-Q, Millipore) was added to the remaining suspension/residue (20 mL) which was then re-sonicated in an ice bath (90W, 10 min) and centrifuged (2200 g, 15 min). The supernatant (30 mL) was then removed and combined with the first supernatant (30 mL) to give a final Ag_2S-NP stock suspension (60 mL) with a concentration of ~ 15 mg Ag/L).

S1.2. Plant growing conditions

Lettuce seeds (*Lactuca sativa*, variety Greenway Iceberg) were pre-germinated for 7 days on paper towel with ultrapure deionised water (18.2 Ω, Milli-Q, Millipore,) before adding to pots. Four seedlings of similar size were planted into each pot. Plants were grown in a controlled environment room under UV lights with 14 h day/10 h night photoperiod with mean 20°C/15°C day/night temperatures. The positions of the pots were randomised weekly and water losses through transpiration were replaced by watering to weight with deionised water daily.

On the day of sowing, and twice weekly thereafter, two nutrient solutions were pipetted onto the soil surface of each pot (0.5 mL of each solution). Two solutions were used to prevent the precipitation of iron and phosphorus. The following basal nutrients were added to the surface of each pot: boron as boric acid (H_3BO_4) at 1.8 μg/pot, potassium as potassium sulfate (K_2SO_4) at 1325 μg/pot, molybdenum as molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_20$) at 0.2 μg/pot, phosphorus as monopotassium phosphate (KH_2PO_4) at 187 μg/pot, copper as copper(II) sulfate pentahydrate ($CuSO_4.5H_20$) at 0.1 μg/pot, iron as iron(II) sulfate heptahydrate ($FeSO_4.7H_20$) at 5.0 μg/pot, magnesium as magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) at 218 μg/pot,

manganese as manganese chloride ($MnCl_2$) at 1.9 μg/pot, zinc as zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) at 2.5 μg/pot and nitrogen as ammonium nitrate (NH_4NO_3) at 700 μg/pot. The total S, N and K applied to each pot from these reagents equated to 835, 700 and 1561 μg/pot, respectively. The above listed nutrients were supplied as basal addition across all treatments. However, the total concentrations of some nutrients, specifically K, P, S and N, were greater in pots that were treated with the agricultural amendments (KCl, phosphate and thiosulfate, respectively).

In PT2, the fertiliser treatments, thiosulfate, phosphate and KCl, were added regularly over the duration of the experiment. To achieve the desired soil concentrations of S, P and K after 60 days of growth [100 mg (S/P/K)/kg soil for the thiosulfate, phosphate and KCl fertilisers respectively], 0.2 mL of each fertiliser solution was pipetted onto the soil surface fortnightly. The total volume added to each pot over the duration of the experiment was 1 mL of each solution. H_2O_2 solution (0.5%) was pipetted onto the soil as described in 2.5. In agricultural settings, the concentration of H_2O_2 that is applied to soil varies depending on its reason for use; when used as a pesticide, the recommended concentration is below 1% [1].

S1.3. Analysis methods for total silver concentrations in plant shoot and root tissues

Approximately 0.25 g of plant tissue sample was digested in 50 ml Teflon® vessels with HNO_3 (9 mL,70%) and HCl (2 mL, 37%), using a modified US EPA method 3052 [2]). The temperatures of vessels were ramped using a CEM Mars Express system (1600 W) for 10 min to 175°C and maintained at 175°C for 45 min.

Due to low Ag shoot concentrations in PT1, the microwave digested solutions were pre-concentrated before ICP-MS analysis. In brief, shoot digests (11 mL) were evaporated to dryness in open Teflon® beakers (150°C) on a hot plate and re-suspended in HCl (5 mL, 5%) for analysis.

Total Ag concentrations of soils was determined using a modified US-EPA 3051A microwave-assisted digestion procedure and ICP-OES or ICP-MS analysis [3]. Dried soil samples (0.25 g) were strong acid extracted in Teflon® vessels with HNO_3 (2.5 mL, 70%) and HCl (7.5 mL, 37%). Soils were initially open vessel digested at room temperature for 12 h, then sealed and heated in a microwave oven (CEM, Mars Express, 1600 W) for 45 min at 175 °C (after a 10 min ramp period).

After digestion, all plant tissues and soil samples were filtered through 0.45-μm mixed cellulose ester membrane filters (Millex®). All samples were diluted to 10% acid concentration immediately prior to analysis (except PT1 shoot samples) using ICP-OES (Spectro ARCOS) or ICP-MS Agilent 7500ce). The limit of quantification for Ag concentrations in plant tissues and soils was 0.02 mg Ag/kg. This was applicable for all samples except for PT1 shoots samples which had a lower LOQ (0.01 mg Ag/kg). The following reference materials were analysed with soil and plant samples; NRC-CNRC PACS-2 (1.22 ± 0.14 mg Ag/kg) and NIST-SRM 1573a 'Tomato Leaves' (17.0 μg Ag/kg), respectively. Silver concentrations measured in NRC-CNRC PACS-2 (1.27 ± 0.14 mg Ag/kg) and NIST-SRM 1573a (15.4 ± 0.3) were in close agreement with reference values.

S1.4. Behaviour of chromium in soil

Plants have no specific Cr uptake mechanisms. In soil, Cr exists as either Cr (III) or Cr (VI), with the former oxidation state being more dominant [4]. Chromium (III) species are highly immobile in soils as they adsorb very strongly to soil particles. Accordingly, the majority of plants take up Cr (VI) species over Cr (III) species [5]. The bioavailability of Cr is even less under acidic conditions (as in the current experiment) due to reduction

of Cr (VI) to Cr (III) and the formation of insoluble Cr-hydroxyl precipitates [6, 7]. For these reasons, Cr concentrations were analysed in root samples and then used to correct for any soil that may still be present on the root surface. Silver concentrations in plant roots from PT2 were corrected for soil Ag using Equations 1 – 3:

$$\text{Mass of soil in root digest (g) (MS)} = \frac{\text{Mass of Cr in root digest (µg)}}{\text{Soil concentration of Cr (µg Cr g soil}^{-1}\text{)}}$$

Equation 1.

$$\text{Mass of Ag in root digest from soil (µg) (MA)} = \text{Ag soil concentration (µg Ag g soil}^{-1}\text{)} \times \text{MS (g)}$$

Equation 2.

$$\text{Root concentration of Ag (µg Ag g soil}^{-1}\text{)} = \frac{\text{(Mass of Ag in root digest (µg)} - \text{MA (µg))}}{\text{Mass of root digest (g)}}$$

Equation 3

S1.5. Extraction of the easily soluble Ag fraction in soil (Pot Trial 2)

Ammonium nitrate (NH_4NO_3) extraction was used to estimate the potentially bioavailable fraction of Ag in soils , w_{Ag} (µg Ag/kg) (ISO 19730) [8]. This method determines the easily soluble Ag soil concentration (Ag present in porewater and weakly associated with solid phases) by extracting a soil sample (10 g) with NH_4NO_3 solution (25 mL, 1M). The soil suspension was mixed on an end-over-end shaker for 2 h and allowed to settle. The suspension was centrifuged (2000g, 20 min) and the supernatant removed which was then filtered through a 0.45-µm mixed cellulose ester membrane filter (Millex®), acidified with concentrated HNO_3 (70%) and analysed by ICP-OES or ICP-MS for total Ag concentrations.

S1.6. Statistical Analysis

Significant differences compared to the control were obtained using Fischer's Protected LSD Test ($p \leq 0.05$). Silver concentrations in shoots used to develop the dose-response curve for ionic Ag^+ in PT1, were fitted to the exponential form of the Mitscherlich equation for a single variable. This equation has the form $y = A(1 - e^{-c_1 x})$, where y = shoot concentration of Ag (µg Ag/kg), A = maximum value of y, x = soil concentration of Ag (mg Ag/kg) and c is a constant that represents the effect 'factor' of x on y. Confidence and prediction bands (95%) were also fitted to the data using SigmaPlot (Version 12.3).

S1.7. Effect of hydrogen peroxide on AgNPs and Ag_2S-NPs dissolution

To investigate the effect of H_2O_2 on NP dissolution, a solution experiment was performed without soil. Silver NPs, Ag_2S-NPs and $AgNO_3$ were added to 50 mL of sodium acetate solution (1mM) with hydrogen peroxide (1%). The solutions were maintained at pH 4 over 8 days by drop-wise addition of HNO_3 (70%). The total Ag concentration of each treatment was 5 mg Ag/L. Treatments were in triplicate. The 'dissolved' concentration of Ag was measured after 24 h, 48 h and 8 d by filtering (< 3 kDa) a 2 mL subsample of each treatment and analysing the total Ag concentration with ICP-MS. Hydrogen peroxide had a significant effect ($p \leq 0.05$) on Ag_2S-NP and AgNP dissolution compared to the controls (no H_2O_2) (Figure S1).

S2.Additional results

S2.1 Ammonium nitrate-extractable silver concentrations

Silver was detected in the NH_4NO_3 extracts of Ag-treated soils; however, not in all fertiliser treatments (Table S1). For Ag^+ and Ag_2S-NP dosed soils, Ag was only detected in the thiosulfate and KCl treatments, whereas in AgNP-dosed soil, Ag was only detected in the KCl treatment. For all other treatments, the concentrations of extractable Ag were below the LOQ (2.5 µg Ag/kg). For samples that were > LOQ, all w_{Ag} values were < 5.5 µg/kg. This demonstrates that the easily soluble soil fraction of Ag was very low. Even in the treatment that had the highest w_{Ag} value (Ag^++KCl), only 0.4% of the total soil Ag concentration was in the potentially bioavailable fraction. All NH_4NO_3 extractable Ag concentrations were below the suggested action value to affect soil micro-organisms (5 µg/kg) [9], with the exception of one treatment (Ag^++KCl, 5.4 µg/kg).

Table S1. Soil concentrations of ammonium nitrate extractable silver (± 1 standard deviation) of soils from Pot Trial 2. This represents the easily soluble silver fraction in soil (< 0.45 µm).

Agricultural amendment/ fertiliser	Silver			
	Ag^+ (µg kg^{-1})	Ag_2S-NPs (µg kg^{-1})	AgNPs (µg kg^{-1})	No Ag
-	< LOQ	< LOQ	< LOQ	< LOQ
PO_4^{3-}	< LOQ	< LOQ	< LOQ	
H_2O_2	< LOQ	< LOQ	< LOQ	
KCl	5.4 ± 2.2	3.1 ± 0.5	4.2 ± 1.2	
$S_2O_3^-$	3.6 ± 0.5	3.8 ± 1.7	< LOQ	

Acronyms: < LOQ = below limit of quantification (approximately 2.5 µg Ag/kg).

S2.2 Translocation of silver in lettuce

Root concentrations of Ag were significantly greater than shoot concentrations for all treatments (Fig. 3; Fig. 4). In roots, the concentrations ranged from 3.1 – 8.2 mg Ag/kg (Ag^+-control and AgNP+thiosulfate, respectively) whereas in shoots, the concentrations were much lower (0.026 – 0.151 mg Ag/kg, AgNP+phosphate and Ag^++thiosulfate, respectively).

Silver translocation was not significantly affected by fertilsers/amendments ($p > 0.05$) and was not significantly different between Ag treatments (Table S2). Translocation factors (TFs) for all treatments ranged from 0.005 to 0.08 (Table S2). A translocation factor < 1 indicates that there is low accumulation in roots and high root-to-shoot translocation. The results show that the translocation of Ag from lettuce roots to shoots was minimal in comparison with other metals such as Cd and Cu. In contrast to Ag, Cd has been shown to accumulate in lettuce shoots [10]. For example, Fontes et al. [11] recorded a TF_{Cd} of 2.7 for lettuce that was grown in Cd-spiked soil (1.14 mg Cd/kg). Copper has the opposite behaviour to Cd in lettuce; it does not accumulate in shoots. However, the TF for Cu in lettuce (TF = 0.45 [12]) is still greater than that of Ag in the current study.

There are limited studies on the plant uptake of AgNPs (or Ag) from natural soils [13]; the majority of studies use hydroponic exposure media [14] or artificial soil [15]. Previous studies on the translocation of AgNPs (or Ag) in plants are also conflicting. Thuesombat et al. [14] showed that rice shoots could accumulate Ag when exposed to high concentrations of AgNPs (100 and 1000 mg Ag/L) (20 nm diameter). Conversely, when other crop species (*Phaseolus radiatus* and *Sorghum bicolor)* were grown in a AgNP-spiked soil (> 100 mg AgNP/kg), Ag accumulated in the roots [15]. Bulk Ag has shown similar results, whereby root accumulation is predominant [16].

Table S2. Silver translocation factors calculated for plants grown in Pot Trial 2. The translocation factor (TF) equals the shoot Ag concentration (μg Ag kg^{-1}) divided by the root Ag concentration (μg Ag kg^{-1}). There were no significant differences for any treatments at $p \leq 0.05$.

	Translocation Factor (TF)		
Agricultural amendment/ fertiliser	Ag^+	Ag_2S-NPs	AgNPs
-	0.005 – 0.01	0.01 – 0.04	0.008 – 0.02
PO_4^{3-}	0.01 – 0.02	0.01 – 0.03	0.008 – 0.01
H_2O_2	0.01 – 0.04	0.01 – 0.08	0.008 – 0.06
KCl	0.01 – 0.03	0.01 – 0.02	0.01 – 0.02
$S_2O_3^{2-}$	0.05 – 0.07	0.02 – 0.04	0.02 – 0.02

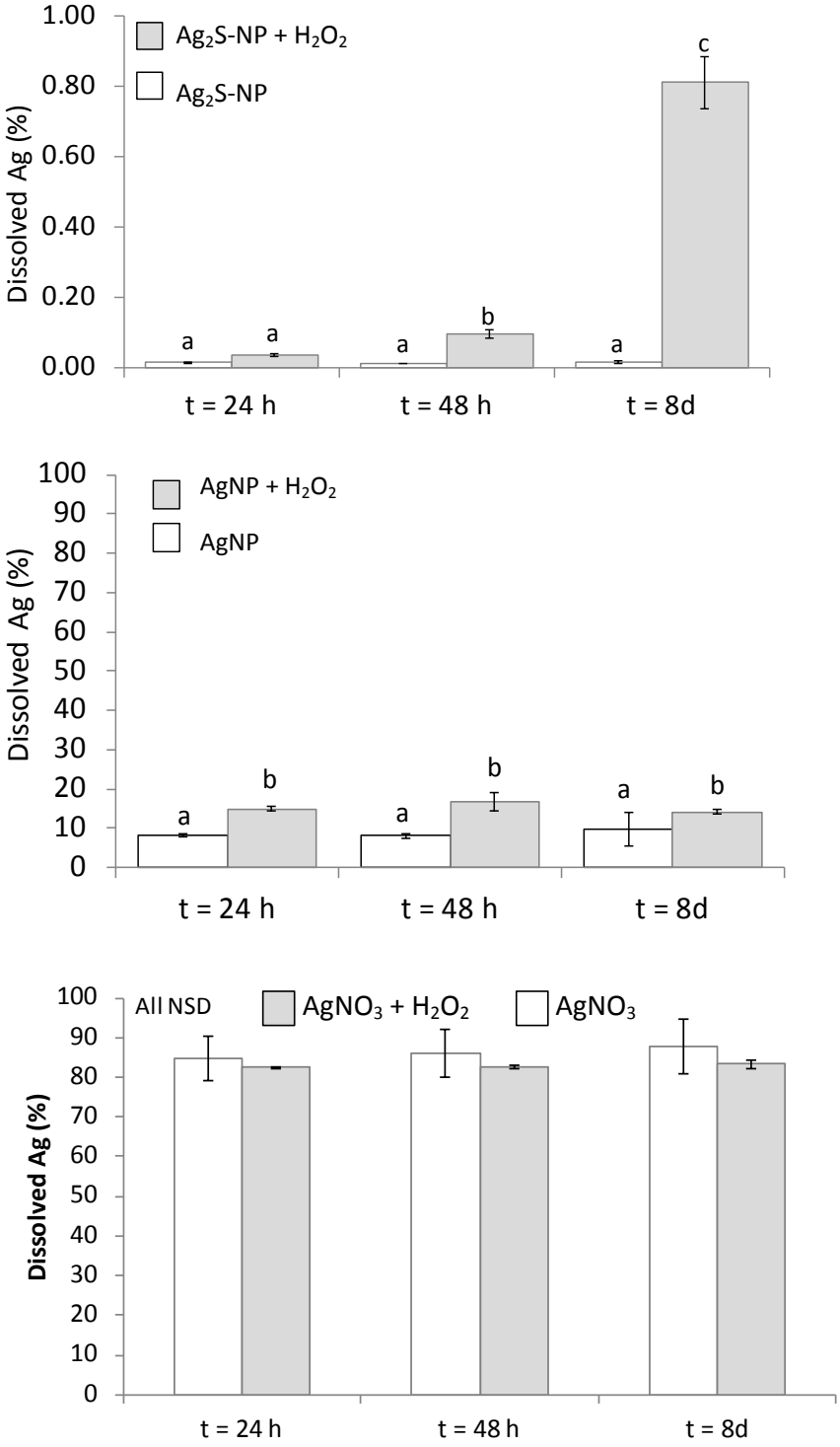

Figure S1. Effect of hydrogen peroxide (1%) on the concentration of dissolved silver in Ag_2S-NP (top), AgNP (middle) and $AgNO_3$ (bottom) solutions at pH 4. The total concentration of silver in each solution was 5 mg Ag/L. Letters indicate significant differences between samples as determined form ANOVA ($p \leq 0.05$), NSD = no significant difference ($p > 0.05$). Note the different y-axis scale for Ag_2S-NP (top).

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CHAPTER 4

Sensitivity of whole soil microbial communities to silver sulfide and silver nanoparticles

Statement of Authorship

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Overall percentage (%)			
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Assisted with experimental design; prepared the supplied DNA samples for pyrosequencing analysis; performed qPCR analysis; processed raw data from pyrosequencing and qPCR analysis; manuscript review.		
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Chapter 4

Sensitivity of whole soil microbial communities to silver sulfide and silver nanoparticles

ABSTRACT

Agricultural soils are a sink for manufactured silver nanoparticles (AgNPs), yet there is limited research on the impact of AgNPs on terrestrial organisms. Soils microorganisms are particularly susceptible to the deleterious effects of AgNPs due to the broad spectrum anti-microbial properties of Ag. However, in soils, pristine AgNPs (Ag^0) are unlikely to exist; instead, sulfidised AgNPs or nano- sized aggregates of Ag-sulfide (Ag_2S-NPs) will be the dominant form of Ag. The effect of Ag_2S-NPs on soil microorganisms in a natural soil has not been investigated. A common approach to investigate the ecotoxicity of a stressor on soil microbial communities is to measure the toxicity to cultured microbial species. However, considering that a large percentage of soil microorganisms are uncultured, this method is inadequate for predicting the risk of AgNPs to a whole soil microbial community. Therefore, a new method was developed to calculate toxicity values for individual soil microbial populations following their exposure to ionic Ag (Ag^+), AgNPs and Ag_2S-NPs. A combination of quantitative PCR (qPCR) and 16S rRNA amplicon sequencing was used to develop dose-response curves for sensitive microbial populations. Toxicity values (EC_{20}) for sensitive soil operational taxonomic units (OTUs) were then plotted on a species sensitivity distribution (SSD) in order to calculate the Ag concentration that would theoretically protect a specified percentage of soil microorganism gene sequences (HC_x values). For the HC5 and HC10 values (95% and 90% of soil OTUs protected), there were no significant differences between Ag treatments but at the HC20 (80% of OTUs of protected), Ag_2S-NPs were significantly less toxic than AgNPs and Ag^+. The most sensitive OTUs (EC_{20} < HC5) were predominantly from the *Bacillaceae* family, with lower abundances of other families including *Frankiaceae*, *Comamonadaceae* and *Pseudonocardiaceae*. A new method was developed to investigate the effect of Ag_2S-NPs on a whole microbial community. Overall, the risk of Ag_2S-NPs to soil microorganisms was found to be less than that of Ag^+.

1. Introduction

Metallic silver nanoparticles (AgNPs) are the most commonly used manufactured nanomaterial (MNM) in consumer products. When AgNP-containing products are washed, a portion of AgNPs or soluble Ag (Ag^+) can be released into wastewater streams [1]. During wastewater treatment, the majority of AgNPs or Ag^+ will be transformed to silver sulfide NPs (Ag_2S-NPs) which then adsorb to, or are incorporated in, the biosolids [2]. Depending on location, biosolids are often applied to land as an agricultural amendment to improve soil fertility. Therefore, soils may act as a sink for Ag_2S-NPs. Despite this, only a very limited number of studies have investigated the impact of Ag_2S-NPs on terrestrial organisms. In a recent plant hydroponic study [3], Ag_2S-NPs were shown to decrease the biomass of wheat (*Triticum aesticum* L.) and cowpea (*Vigna unguiculata* L. Walp) following a 2 week exposure to Ag_2S-NPs at 6 mg Ag L^{-1}. The effects of sulfidised AgNPs on the model soil organism *Caenorhabditis elegans* have also been investigated in solution exposure media [4]. Mortality was found to decrease by a maximum of 20% when the nematode was exposed to sulfidised AgNPs at a concentration of 10 mg Ag L^{-1}. In both of these previous experiments it should be noted that Ag_2S-NPs were less toxic than pristine AgNPs and Ag^+ and exposures were in hydroponic media.

It is currently unclear how transformed AgNPs (Ag_2S-NPs) will affect soil microorganisms. Soil microorganisms and, therefore, soil processes that are mediated by microorganisms may be affected by Ag_2S-NPs due to the broad-spectrum antimicrobial properties of Ag: Ag_2S-NPs may act as a slow-release form of toxic Ag^+ [3]. It is essential to address this lack of information given that soil microorganisms play a vital role in many soil processes (e.g. nutrient cycling, stabilisation of soil aggregates and transformation of organic matter).

Previous studies on the effects of AgNPs on soil microorganisms have primarily focused on three main areas: 1) the composition of the microbial community [5]; 2) the changes in soil microbial biomass [6]; and 3) the toxicity to individual cultured microbial species [7]. Perhaps more importantly, the effects of Ag_2S-NPs – the relevant form of AgNPs in the environment – on soil microbial communities remains unknown. The effects of a toxicant on soil microorganisms are usually assessed using a single class of organisms or a single function. Therefore, the impacts of a toxicant at the whole community level in soil systems are largely unknown; this is particularly true for AgNPs and Ag_2S-NPs. This lack of information prevents the development of threshold values to quantify the risks of AgNPs or Ag_2S-NPs to soil microorganisms. Threshold values for a contaminant are required in order to perform accurate risk assessments. Such values are incorporated into regulatory soil quality guidelines (SQGs) which can then be used as a framework to protect soil ecosystems [8].

Advances in molecular-based techniques may provide a powerful tool to determine the impacts of Ag_2S-NPs on whole soil microbial communities. Genomic studies are one approach that can be used to investigate the ecotoxicity of a particular stressor or toxicant. Such studies analyse the stress-induced

changes in gene expression and can be applied to individual organisms or a whole community. For example, a genomics study investigating the effect of AgNPs on *C. elegans*, found that the measured endpoints (e.g. reproduction) could be related to the expression of specific genes [9]. At a community level, genomics tools can be used to investigate the changes in community dynamics when it is exposed to a stressor (e.g. change in the composition of a soil microbial community when exposed to AgNPs [5]). In the current study, a community-based approach was applied; however, instead of focusing on changes in community composition, toxicity values were determined for individual populations within a community. By focusing on affected populations instead of the response at a community level, more accurate toxicity thresholds can be estimated as the influence of non-sensitive organisms is reduced.

To assess the ecological risk of toxicants, a common approach is to apply the species sensitivity distribution (SSD) methodology. A SSD is a cumulative statistical distribution that describes the sensitivity of a group of species to a particular toxicant [10]. The purpose of a SSD is to predict the concentration of a toxicant that will protect a specific percentage of species in an ecosystem. This point estimate, known as the hazardous concentration, is commonly set at a concentration that will protect between 50% and 99% of species (HC50 and HC1, respectively) [11]. When using SSDs to derive environmental quality guidelines, the chosen level of protection varies between countries and is dependent on the specific land use. The specific species and ecological functions that are being protected will also influence the chosen protection level. While SSDs are frequently used for aquatic systems, the lack of toxicity data for a range of soil organisms at different trophic levels has prevented their larger application to terrestrial systems [12].

The goal of the current study was to develop a new technique that could be used to compare the sensitivity of soil microorganisms to Ag^+, AgNPs and Ag_2S-NPs. This study aimed to take advantage of the recent advancements in molecular techniques in order to develop dose response curves and SSDs for whole microbial communities in soils, rather than cultured species only. The study was divided into two experiments. Firstly, a soil nitrification experiment was carried out to determine the concentration range over which Ag^+, AgNPs and Ag_2S-NPs would affect a soil microbial process (nitrification). The second experiment used pyrosequencing-based analysis of the 16S rRNA gene region to construct dose-response curves for individual soil microbial populations exposed to Ag^+, AgNPs and Ag_2S-NPs. Species sensitivity distributions were then constructed from these results, enabling comparisons to be made between the sensitivities of soil microorganisms to different Ag treatments. We believe that this is the first study to use DNA sequencing to develop sensitivity distributions for whole soil microbial communities exposed to a toxicant in soil. The method provides a benchmark for future studies and the development of Soil Quality Guidelines for AgNPs.

2. Materials and methods

2.1. Soil properties

Two soil incubation experiments were carried out: 1) a soil nitrification toxicity test and 2) a microbial DNA sequencing experiment. The same soil was used for both experiments. Soil was collected from Charleston (South Australia, Australia) and has been characterised previously [13]. The soil was a Chernozem with a slightly acidic pH (pH_{CaCl_2} = 5.1) and high organic carbon content (6.9 %) (Table 1). This soil was chosen due to its high concentration of organic carbon; an essential energy source for soil microorganisms. Prior to incubation, soil was oven dried at 40ºC (7 days), sieved (< 2 mm) and homogenised prior to experimentation.

2.2. Rates of silver addition

For both experiments, three Ag treatments were applied; $AgNO_3$ (Ag^+), AgNPs and Ag_2S-NPs. The number of spiking rates differed between experiments. In the nitrification experiment there were eight rates of Ag_2S-NP application and seven rates of Ag^+ and AgNPs, while in the sequencing experiment, 14 Ag rates were applied for each Ag treatment.

In the nitrification experiment, soil spiking concentrations ranged from 0.1 to 72 mg Ag kg^{-1}, 0.1 to 456 mg Ag kg^{-1} and 0.1 to 2285 mg Ag kg^{-1} for Ag^+, AgNP and Ag_2S-NP treatments, respectively. In the sequencing experiment, spiking concentrations ranged from 0.1 to 93 mg Ag kg^{-1}, 0.1 to 404 mg Ag kg^{-1} and 0.1 to 5590 mg Ag kg^{-1} for the Ag^+, AgNP and Ag_2S-NP treatments, respectively. An untreated control (soil with no added Ag), was included in both experiments. The Ag concentration of the control soil was 0.1 mg Ag kg^{-1}.

For the nitrification experiment, Ag treatments were added to 30 g of soil which was then separated into three 8 g replicates. In the sequencing experiment, Ag treatments were added to either 30 g or 5 g of soil

Table 1. Physicochemical properties of the soil that was used in both experiments (collected from Charleston, South

						Particle size analysis		
$pH_{(CaCl_2)}$	$pH_{(H_2O)}$	Total Ag (mg kg^{-1})	Organic C (%)	MWHC (%)	CEC ($cmol^+ kg^{-1}$)	Clay %	Silt %	Sand %
5.1	6.6	< 0.04	6.9	51	12.0	14	12	63

CEC = cation exchange capacity; organic C = organic carbon; MWHC = maximum water holding capacity

depending on the target soil concentration. Silver treatments were added to soil either as a solution (Ag^+) or in suspension form (AgNP and Ag_2S-NP).

2.3. Silver addition to soil

Silver NPs were added to soil as a suspension. For soils with spiking concentrations between 1 and 200 mg Ag kg^{-1}, a AgNP suspension was used that has been described previously [14]. Briefly, 0.1 g of PVP-coated AgNP powder (Nanoamor) was added to 50 mL of ultrapure Milli-Q water, sonicated (90 W, 3 min) and then centrifuged (2200 *g*, 15 min). For the highest AgNP spiking rate, a more concentrated AgNP suspension was used. This concentrated suspension was prepared by weighing AgNP (0.019 g) into a 10 mL centrifuge tube, adding ultrapure Milli-Q water (7.65 mL) and probe sonicating (90 W) for 20 sec before adding to soil (30 g).

For Ag_2S-NP treatments that were between 1 and 100 mg Ag kg^{-1}, a previously described Ag_2S-NP suspension was used [15]. Silver sulfide NP treatments that were greater than 500 mg Ag kg^{-1} were spiked with a more concentrated Ag_2S-NP suspension that was prepared separately for each treatment. These concentrated suspensions were prepared by weighing increasing amounts of Ag_2S-NP powder into 10 mL centrifuge tubes, adding ultrapure Milli-Q water (7.65 mL) and probe sonicating (90 W) for 45 sec.

Solutions of Ag^+ were prepared to the desired concentrations by dissolving $AgNO_3$ powder (Sigma Aldrich) in ultrapure Milli-Q water.

2.4. Characterisation of silver nanoparticles

The AgNP and Ag_2S-NP suspensions that were used in this experiment have been extensively characterised in previous studies using dynamic light scattering (DLS, Malvern Zetasizer), transmission electron microscopy (TEM, Phillips CM200 at 120 keV), X-ray diffraction analysis (XRD, PANanalytical X'Pert Pro) and UV – Vis absorption spectroscopy (200 – 600 nm) (Cary 5000 UV Vis NIR spectrophotometer) [15, 16]. The particle size distribution of AgNPs has also been investigated using disk centrifuge analysis (CPS Instruments disc centrifuge 24000 UHR) [16]. Silver NPs and Ag_2S-NPs were uniformly dispersed and generally spherical with some rod-like particles (for TEM images of AgNPs and Ag_2S-NPs, see [16] and [15], respectively). The average hydrodynamic particle diameters (d_h) and zeta potentials (ζ) for AgNPs and Ag_2S-NPs were 44 nm and 152 nm, and -50 mV and -43 mV, respectively. The uniform dispersity of NP suspensions was evident from the close correlation between d_h and crystallite size (XRD, 41 nm) for AgNPs [16] and the relatively low polydispersity index (PdI, 0.21) recorded for Ag_2S-NPs [15]. These are the characteristics for 'pristine' AgNP and Ag_2S-NP suspensions; it is expected that in a real soil environment these properties may change.

2.5. Chemical analysis of silver concentrations in soil

Total Ag concentrations of soils were determined using a closed vessel microwave-assisted digestion procedure and analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 7000 DV) and ICP-mass spectrometry (ICP-MS, Agilent 7500ce) [16]. Approximately 0.25 g of soil was digested in 50 ml Teflon® vessels with HCl (7.5 mL,37%) and HNO_3 (2.5 mL, 70%), using a modified US EPA method 3051A [17]. Prior to microwave digestion, soils were open vessel digested at room temperature for 12 h. The temperature of vessels was then ramped using a CEM Mars Express system (1600 W) for 10 min to 175°C and maintained at 175°C for 45 min. The vessels were then cooled at room temperature and the digest solutions were diluted 2.5 times with HCl (10%), filtered (0.45 μm) and stored at 4°C until analysis. For quality control and quality assurance, blank samples and a certified reference material (CRM [PACS-2]) were included in each digestion run. The Ag concentration of the digested CRM (1.24 ± 0.19 mg kg^{-1}) was in good agreement with the certified value (1.22 ± 0.14 mg kg^{-1}).

2.6. Soil nitrification toxicity test – impact of silver treatments on soil nitrification processes

Nitrification is a two-step process that is primarily controlled by two distinct prokaryotic groups. In the first step, ammonia is oxidised to nitrite by ammonia oxidising bacteria or archaea (AOB or AOA, respectively). This is followed by the oxidation of nitrite to nitrate by nitrite-oxidising bacteria (NOB). The first step is the rate limiting step and is catalysed by ammonia monooxygenase (AMO), and can be monitored by analysing the abundance of the functional marker gene *amoA*. Often, the abundance of the *amoA* gene is used as a basis for determining the toxicity of metal contaminants to nitrification processes in soils.

2.6.1. Experimental set-up

The effect of Ag treatments on soil nitrogen transformation processes, was investigated using OECD Method No. 216 for substrate induced nitrification [18]. Spiked soil treatments were adjusted to 50% of their maximum water holding (MWHC) capacity using ultrapure Milli-Q water and pre-incubated for 7 d. During pre-incubation, soils were maintained at 50% MWHC and stored in the dark at a constant temperature (22°C) with daily aeration. After 7 d, each soil was divided into three replicates (10 g rep^{-1}) and amended with powdered lucerne (C:N ratio 13.6:1) at a rate of 5 mg g $soil^{-1}$ (dry weight). Soils were maintained at 50% MWHC for 28 d and stored in the dark with daily aeration. One subsample was removed from each of the three replicates immediately after lucerne addition (t=0) and 28 d later (t=28 d). Subsamples were extracted with KCl as described below.

A 1 M solution of KCl was added to each subsample at a ratio 5:1 (soil to solution) and mixed (end-over-end) for 1 h to extract nitrate from soils. The samples were centrifuged (800 *g*, 5 min) and the supernatants filtered through a 0.45 μm mixed

cellulose ester membrane filter (Millex®) and stored at -18°C until analysis. The nitrate (NO_3^-) concentrations in the liquid extracts were determined using flow-injection analysis (FIA) (Lachat QuikChem 8500 Series 2 FIA automated ion analyser system). The total production of NO_3^- after 28 d was calculated for each sample by subtracting the NO_3^- soil concentration at t=0 from that at t=28 d; this corrects for the NO_3^- that was present in the control soil and for the NO_3^- that was added with the soluble salt in the $AgNO_3$ treatment.

2.6.2. Determination of ECx values

For each Ag treatment, dose-response curves were constructed in order to calculate EC_{10}, EC_{20} and EC_{50} concentrations, representing a decrease of 10%, 20% and 50% in nitrate production compared to the control. All data were fitted to a four parameter sigmoidal function [13]; the most commonly used dose-response model [19] (Equation 1):

$$y = c + \frac{d-c}{1+\left(\frac{x}{e}\right)^b} \quad \text{(Equation 1)}$$

where y = NO_3^- produced as a percentage of the control at Ag concentration x; d = response in the control (upper asymptote); c = minimum effect (lower asymptote), e = point of inflection, or the dose when $d - c$ is reduced by 50% (EC_{50}); and, b = slope of the curve around e [20]. EC_{10}, EC_{20} and EC_{50} values were then interpolated from the fitted curve with a 95% confidence interval.

2.7. Sequencing experiment – impact of silver treatments on the whole soil microbial community

Spiked soils were adjusted to 50% of their MWHC using ultrapure Milli-Q water and stored in the dark at a constant temperature (22°C) for 28 d. The samples were aerated daily and ultrapure Milli-Q water was added every alternate day to maintain the soils at 50% of their MWHC. After 28 d, soils were removed and stored at -20°C for 7 d until DNA extraction. Each soil sample was extracted in duplicate.

2.7.1. DNA extraction and 16SrRNA pyrosequencing

DNA was extracted from soils using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions with minor modifications. Specifically, 0.24 ± 0.02 g of soil (dry soil equivalent) was placed in a bead tube for extraction and homogenised using a FastPrep machine (2 x 30 sec at 5 m sec^{-1}). Fifty µL of extract from each sample replicate was combined to give one DNA extract for each treatment.

DNA concentrations were determined spectrophotometrically with a NanoDrop ND-1000 (ThermoScientific, USA). DNA (200 ng) from each sample was submitted to the Australian Centre for Ecogenomics (ACE) for 16S Amplicon sequencing by Illumina Miseq Platform using the 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392wR (5'-ACGGGCGGTGWGTRC-3') primer sets [21].

2.7.2. Quantitative polymerase chain reaction (qPCR) analysis

Two qPCR reactions were performed; one to determine the total bacterial and archaeal biomass load and a second to analyse the total copy number of the bacterial *amoA* gene. Total bacterial and archaeal biomass load was estimated by qPCR according to Vanwonterghem et al. [22] by ACE with primer sets 1406F (5'-GYACWCACCGCCCGT-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3').

The DNA from the ammonia oxidizing bacterial community was quantified using quantitative PCR (qPCR) using the group specific primers *amoA* 1F (GGGGHTTYTACTGGTGGT) and *amoA* 2R (CCCCTCKGSAAAGCCTTCTTC) [23] and was based on methods described previously [24]. Briefly, the PCR reaction contained 1x Biotaq SYBR green master mix (Biorad, Australia), 0.2 µM of forward and reverse primers, 5 µl of template DNA (1:10 dilution) in a total of 25 µL reaction. The PCR conditions were as follows; initial denaturation occurred at 95°C for 15 min, followed by 40 cycles of 95°C for 45 sec, 57°C for 60 sec, 72°C for 45 sec and a final extension of 72°C for 5 min. To confirm the specificity of amplified PCR products, all PCR reactions were followed by melting curve analysis and agarose gel electrophoresis. The melt curve conditions were 55°C to 95°C at a ramp rate of 0.5°C per 5 sec. Standard curves containing known copy numbers of the gene were generated using serial dilutions of linearised plasmids containing the *amoA* gene (from pure cultures of *Nitrosomonas* sp.); data were linear for 10^1–10^6 gene copies. Quantitative PCR was performed using a Maxpro 3000 qPCR machine (Stratagene, Australia) and data analysis was carried out using the software supplied. The abundance of *amoA* gene copies per g of soil or per ng of DNA was calculated using data from the standard curve and the DNA yield from the soil extraction.

2.7.3. Data analysis

Raw paired reads from pyrosequencing were first trimmed to remove short reads (less than 190bp) and low quality reads (lower than Phred-33 of 20) using Trimmomatic [25]. The trimmed paired reads were then assembled by Pandaseq [26] with default parameters. The adapter sequences were removed by FASTQ Clipper of FASTX-Toolkit [27]. The joined high quality sequences were then analysed by QIIME v1.8.0 [28] using open-reference OTU picking strategy by uclust [29] at 1% phylogenetic distance and assigned taxonomy by uclust against the Greengenes database (13_05 release, [30, 31]. OTUs with only one read were filtered from the OTU table using the command filter_otus_from_otu_table.py in QIIME. Both the qPCR results and filtered OTU table were imported into Galaxy [32] for gene copy number correction and to generate the final absolute abundance of each OTU in each sample using CopyRighter [33].

2.7.4. Dose-response curve fitting of OTUs

Automated curve fitting of each OTU was performed using the statistical software R (version 3.1.3), with the goal of calculating EC_{20} values from the fitted model. The R extension package *drc* (version 2.3-96 [34]) was used to fit all data to the log-logistic form of Equation 1. All parameters had the same definition as given previously except for *y*, which was equivalent to the total count of single copy 16S gene per µL of sample. Fits were deemed acceptable only when the following criteria were met: 1) $b > 0$, this denotes a negative slope and hence inhibition; 2) e (EC_{50}) < the maximum spiking concentration; and 3) $R^2 > 0.65$. Otherwise, that OTU was excluded from further EC_{20} calculations.

All data were then fitted to a second model (Equation 2); the Brain-Cousens model [35]. This model takes into account the potential stimulation of OTUs at low Ag concentrations (hormesis) and was fitted to the data using the *BC.5* function in the *drc* package.

$$y = c + \frac{d - c + fx}{1 + \left(\frac{x}{e}\right)^b} \qquad \text{(Equation 2)}$$

where parameters *d*, *c*, *x* and *y* retain the same definition as in Equation 1 and parameters *e* and *b* have no clear biological meaning [35] as they lose their definition as the point of inflection and slope, respectively. The hormesis model includes an additional parameter, *f*, which relates to the initial rate of increase at low doses. Hormesis was deemed statistically significant at the 0.05 probability level if the 95% confidence intervals for *f* did not intercept zero [36]. Confidence intervals for *f*, and other parameters, were calculated as follows:

$$f_{CI(95\%)} = f_{est} \pm \left(f_{SE} \cdot t_{\alpha/2}\right) \qquad \text{(Equation 3)}$$

where $f_{CI(95\%)}$ = upper and lower 95% confidence intervals for parameter *f*; f_{est} = estimated value for *f* (calculated in R); f_{SE} = standard error of *f* (calculated in R); $t_{\alpha/2}$ = the critical value of the Student's *t* distribution at a 5% significance threshold (95% confidence interval) with nine degrees of freedom (2.262), where $\alpha = 0.025$.

Based on the results from curve fitting, the OTUs that could not be described by either model (using the criteria outlines above) were excluded from SSD calculations. For the OTUs that were successfully fitted, EC_{20} values were calculated using R (ED.drc function). Note, in this case, EC_{20} refers to the Ag concentration that reduces the absolute abundance of an OTU by 20%. This percentage decrease is calculated from the upper asymptote of the fitted model and is not based on the absolute abundance of the control. If an OTU could be described by both models, the hormesis model was selected; this ensures a conservative EC_{20} estimate.

2.7.5. Species sensitivity distribution

Calculated EC_{20} values were then used to construct a species sensitivity distribution [37]; here, it is more correctly termed an 'OTU sensitivity distribution' (OSD) as each OTU was not assigned to the species level. This type of distribution plots the cumulative percentage of OTUs affected against the soil concentration of Ag. The primary aim of producing an OSD was to calculate the Ag concentration that would theoretically protect a specified percentage of soil microbial populations. . By constructing an OSD for each Ag type, the potential toxicity of AgNPs and Ag_2S-NPs to soil microorganisms was compared to that of Ag^+. It is recommended that the distribution of species sensitivity (i.e. OTU sensitivity) be applied to the Burr Type III family of distributions [38]. Accordingly, EC_{20} values were fit to a Burr Type III function using the software package Burrlioz [39] (https://research.csiro.au/software/burrlioz/).

The OSDs were then used to calculate the Ag concentrations that are protective of a specific percentage of OTUs (HC_x values). The Burrlioz software was used to calculate HC5, HC10 and HC20

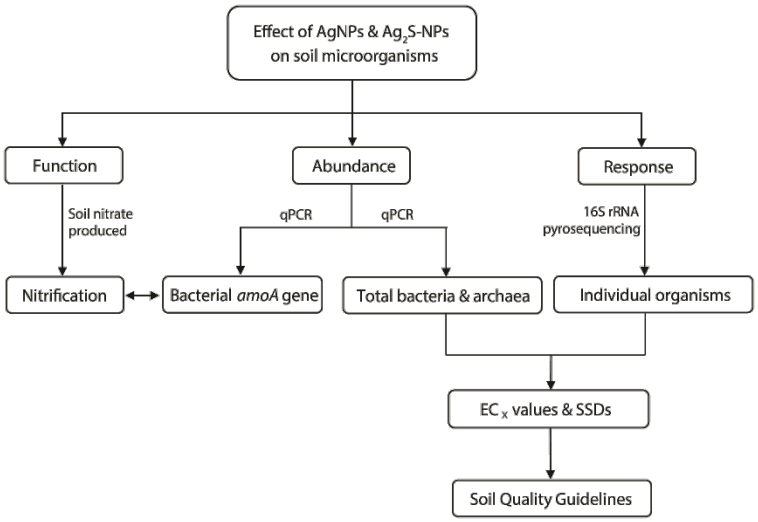

Figure 1. Schematic overview of the study. The techniques that were used to investigate each parameter are shown on the arrows.

Table 2. Silver concentrations (mg Ag kg soil^{-1}) that correspond to a 10%, 20% and 50% reduction in soil nitrate production compared to the control (EC_{10}, EC_{20} and EC_{50}, respectively). Mean values are shown with 95% confidence intervals in parentheses. Significant differences between Ag treatments for each EC_x value are indicated by the asterisks.

EC (mg Ag kg^{-1})	Ag^+	AgNP	Ag_2S-NP
EC_{10}	8 (6 – 9)	7 (4 – 12)	9 (3 – 21)
EC_{20}	11 (9 – 12)	13 (8 –20)	44 (24 – 72)**
EC_{50}	19 (17 – 21)*	42 (30 – 57)**	619 (411 – 899)***
r^2	0.98	0.97	0.94

values for each Ag type i.e. Ag concentrations that would affect 5%, 10% and 20% of OTUs, respectively. For each HC value, 95% confidence intervals were calculated by the Burrlioz software using a bootstrap technique [39]. To classify the OTUs that were less sensitive to Ag treatments, HC80 values were also calculated.

2.7.6. Responses of OTUs that did not fit the models

For OTUs where the response to Ag dose could not be fitted to either dose response model, additional curve fitting was attempted. Operational taxonomic units were again fitted to the log-logistic form of Equation 1; however, *b* was constrained to < 0. This equates to a positive slop and thus, OTU stimulation by Ag, not inhibition. Fits were further analysed if R^2 was > 0.65 and *e* was < the maximum spiking concentration. Linear regression was then performed on the remaining OTUs and they were classified based on their slope.

An overview of the study is shown in Figure 1.

3. Results

3.1. Nitrification experiment

3.1.1. Silver decreased soil nitrate production

After 28 d, nitrate concentrations in the control soil increased from 8.2 mg kg^{-1} to 153.7 mg kg^{-1}. Dose-response relationships were observed across all Ag treatments (Figure 2). EC_{10} concentrations for the three forms of Ag were similar ($p > 0.05$) (Table 2). However, EC_{20} and EC_{50} values were significantly different between Ag treatments ($p \leq 0.05$). The EC_{20} value for Ag_2S-NP treated soil was significantly greater ($p \leq 0.05$) than that of AgNP and Ag^+ treatments. At the EC_{50}, all Ag treatments were significantly different from each other ($p \leq 0.05$) and toxicity increased in the order Ag_2S-NPs < AgNPs < Ag^+ (Table 2).Therefore, it can be concluded that in a Chernozem soil, Ag_2S-NPs – the most realistic form of Ag in the environment – are significantly less toxic ($p \leq 0.05$) to soil nitrification processes than AgNPs or Ag^+

3.1.2. Abundance of the bacterial amoA gene

The abundance of the bacterial *amoA* gene ranged from 1476 copies g^{-1} to 1.77 × 10^5 copies g^{-1} (dry soil basis). For each Ag treatment, the abundance of the bacterial *amoA* gene increased at low Ag concentrations. All data were fitted to the hormesis dose-response equation (Equation 2) and hormesis was found to be significant for all Ag treatments (Figure 3). The calculated EC_{10}, EC_{20} and EC_{50} values were significantly different between Ag treatments, where values increased in the order Ag^+ < AgNP < Ag_2S-NP (Table 3). Therefore, the bacterial *amoA* gene was most sensitive to Ag^+.

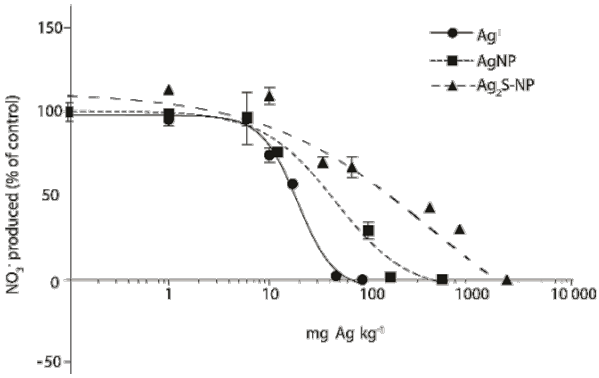

Figure 2. Dose-response curves for NO3 production in soil over 28 d for ionic Ag (Ag+ – dashed line); Ag nanoparticles (AgNP – short dashed line); and Ag sulfide nanoparticles (Ag2S-NP – long dashed line). Mean values (*n* = 3) ± 1 standard deviation are shown. Silver concentrations are on a log_{10} scale.

3.2. Sequencing experiment

3.2.1. Microbial community distribution

Bacterial and archaeal community composition of the control sample was determined from the pyrosequencing data (Figure 4). A total of 139,862 OTUs were identified. Following the removal of singletons and doubletons, 51,025 OTUs remained. The remaining OTUs could be assigned to 27 different phyla; 26 of which were bacterial and one archaeal. A proportion of OTUs (6.9%) could not be assigned to any archaeal/bacterial phyla. The most dominant phyla in the microbial community were; Proteobacteria (29.2%), Actinobacteria (27.4%) and Firmicutes (21.3%). Crenarchaeota, the only archaeal phylum, had a relative abundance of 0.2%. Gemmatimonadetes (4.5%), Bacteroidetes (3.0%) and Planctomycetes (2.5%) were also present in the sample (Figure 4).

Table 3. Silver concentrations (mg Ag kg soil^{-1}) that correspond to a 10%, 20% and 50% reduction in total copy number of the bacterial *amoA* gene. Mean values are shown (n = 4) with 95% confidence intervals in parentheses. Significant differences between Ag treatments for each ECx value are indicated by the asterisks.

EC (mg Ag kg^{-1})	Ag^+	AgNP	Ag_2S-NP
EC_{10}	26 (21 – 35)*	73 (47 – 128)**	1636 (1235 – 2530)***
EC_{20}	28 (22 – 38)*	77 (48 – 138)**	1788 (1328 – 2624)***
EC_{50}	35 (26 – 51)*	98 (52 – 186)**	2503 (1687 – 4125)***
r^2	0.64	0.50	0.58

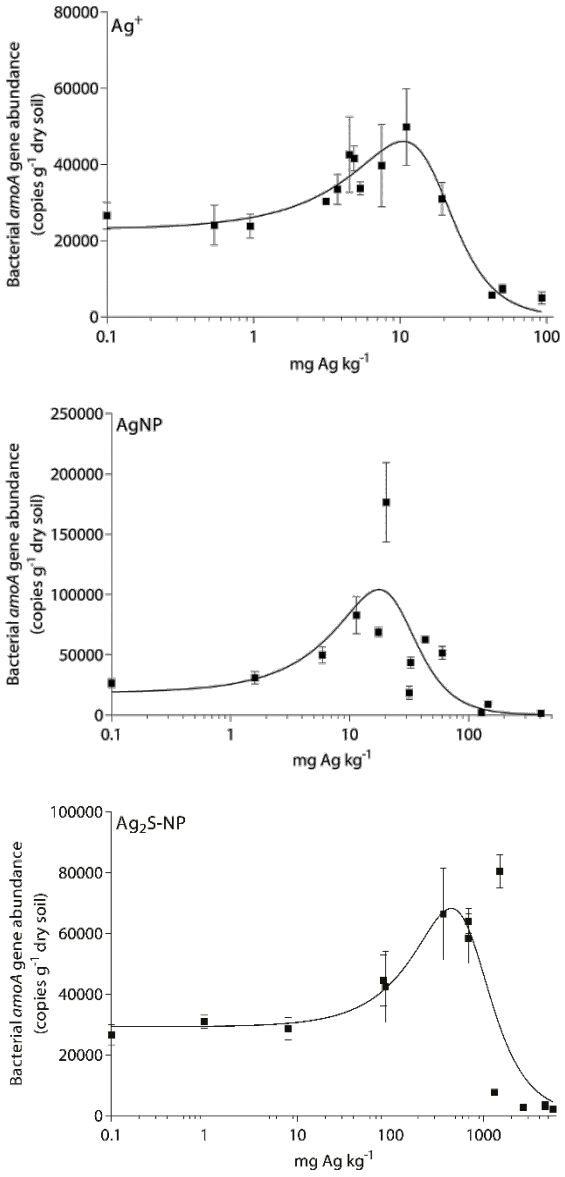

Figure 3. Dose-response curves showing the decrease in total abundance of bacterial *amo*A gene over 28 d in soil spiked with ionic Ag (Ag^+ – top), Ag nanoparticles (AgNP – middle) and Ag sulfide nanoparticles (Ag_2S-NP – bottom). Mean values (n = 4) ± 1 standard deviation are shown. Silver concentrations are on a log scale. All Ag treatments were fit to the five parameter Brain-Cousens hormesis model as hormesis was significant ($p \leq 0.05$).

3.2.2. Total bacterial and archaeal abundance

The abundance of bacteria and archaea across the Ag concentration range for each Ag treatment was estimated using qPCR (Figure 5). There was an observed decrease in abundance of bacteria and archaea with increasing Ag concentrations for the Ag^+ (0 – 93 mg kg^{-1}) and Ag_2S-NP (0 – 5590 mg kg^{-1}) treatments (Figure 5). The abundance of bacteria and archaea in AgNP treated soil remained fairly constant with increasing Ag concentrations (0 – 404 mg kg^{-1}).

3.2.3. Curve-fitting of dose-response models and non-linear regression analysis of OTUs

For the remaining 51,025 OTUs, 47,426 were classified as bacteria, 102 as archaea and 3,497 could not be classified. An OTU needed to appear in at least six samples per Ag type to satisfy degrees of freedom requirements from contestable parameters in Equations 1 and 2. Operational taxonomic units that did not satisfy these requirements were not assessed further (Table SI.1). As a consequence, each Ag treatment had a different number of OTUs that were analysed using non-linear regression (Ag^+ = 5,444, AgNP = 4,272, Ag_2S-NPs = 4,259 OTUs).

Multiple dose-response curves were successfully constructed for each Ag treatment (examples are shown in Figures 6 and 7); however, the number of curves differed between each Ag type (Table SI.2). For the Ag_2S-NP treatment, 498 OTUs were successfully fitted to the dose-response models (Equations 1 and 2), whereas fewer curves were generated for the Ag^+ and AgNP treatments (390 and 146, respectively). For the dose-response curves that are shown in Figures 6 and 7, curve-fitting results are given in Tables SI.4 & SI.5 (estimated values for the fitted parameters are listed with their associated error).

When comparing the taxonomy of the fitted OTUs between Ag treatments, a similar distribution of phyla was observed (Table SI.3). In all Ag treatments, the microbial communities were dominated by a group of phyla including Actinobacteria, Proteobacteria and Firmicutes (78-85%). Other phyla including Bacteroidetes, Acidobacteria, Planctomycetes, and Chloroflexi were present in all treatments and accounted for less than 10% of the microbial community. Nitrospirae and Elusimicrobia were only present in the Ag^+ treatment (0.3%).

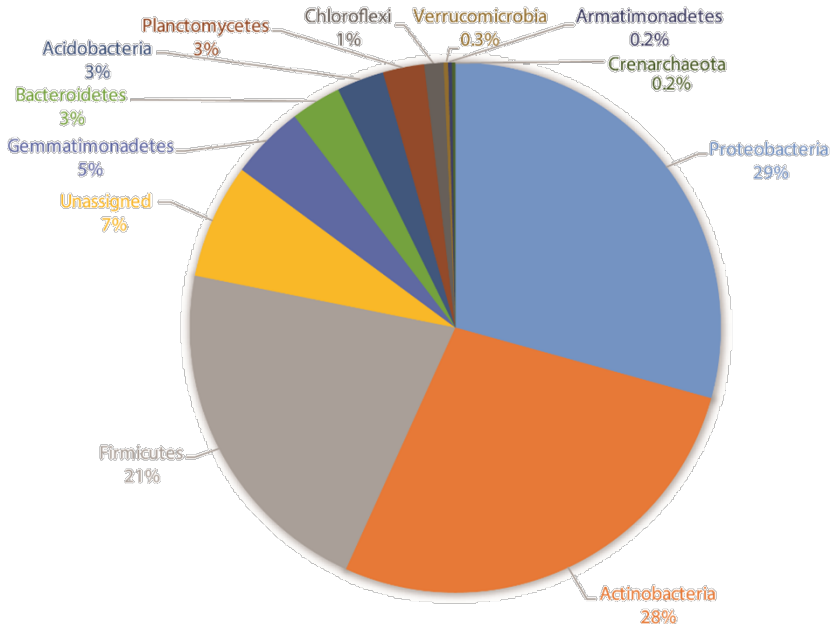

Figure 4. Distribution of bacterial and archaeal phyla in Chernozem soil collected from Charleston, South Australia. Phyla accounting for > 0.1% of sequences are shown. Sequences were clustered into OTUs at 99% similarity

When OTUs were assigned to the family level, minor differences between Ag treatments were observed (Figure 8). For example, in the AgNP treatment, no OTUs were affiliated with *Chitinophagacea*e, while in the Ag_2S-NP and Ag^+ treatments, this family accounted for approximately 5% of the microbial community. *Bacillaceae* was also reduced in the AgNP treatment (8% compared to 14% and 19% in Ag^+ and Ag_2S-NP treatments). In the Ag^+ treatment, *Comamonadaceae* was the third most dominant family comprising 7% of sequences. However, it was less dominant in the Ag_2S-NP treatment (3%) and was absent in AgNP treatment.

3.2.4. Calculation of toxicity values (EC_{20}) and hazardous concentrations

For OTUs that are termed 'fitted OTUs' in the preceding sections, their response to Ag treatments can be described by either the sigmoidal dose-response function (Equation 1) or the hormesis function (Equation 2) – or both (Table SI.2). Toxicity values (EC_{20}) were determined for these OTUs and plotted on separate OSDs for each Ag type (Figure 9). The distribution of EC_{20} values followed a sigmoidal shape and was fitted to a Burr Type III function.

Hazardous concentrations (HC_x) for each Ag treatment were derived from the OSDs. For HC5 or HC10 values, there were no significant differences between Ag treatments ($p > 0.05$) (Table 4). However, at the least protective HC value (HC20, 80% protection), Ag_2S-NPs were significantly less toxic than Ag^+ and AgNPs ($p \leq 0.05$).

3.2.5. Taxonomy of silver-sensitive and silver-tolerant OTUs

Across all Ag treatments, the most sensitive OTUs (EC_{20} < HC5) were predominantly from the *Bacillaceae* family. Four of the 20 most sensitive OTUs in the Ag^+ treatment were assigned *Bacillacaeae*. In the AgNP and Ag_2S-NP treatments, *Bacillacaeae* were again the most dominant of the sensitive OTUs. In addition to *Bacillaceae*, the most abundant sensitive OTUs were affiliated to family *Frankiaceae* and *Comamonadaceae* for Ag^+ (6 OTUs of 20); *Planococcaceae*, *Thermomonosporaceae* and *Micromonosporaceae* for AgNP (3 OTUs of 5); and *Pseudonocardiaceae* and *Micromonosporaceae* for Ag_2S-NPs (8 OTUs of 34)

Less sensitive OTUs, those in the upper part of the OSD (EC_{20} > HC80) (Table 4), were assigned to consistent families. Again, *Bacillacaeae* was the most dominant family for Ag_2S-NP OTUs (26%), followed by *Chitinophagaceae* (13%), *Solirubrobacteraceae* (16%) and OTUs that were not classified at the family level (8%). In the Ag^+ treatment, unassigned OTUs (family level) were the most abundant (22%), followed by *Solirubrobacteraceae* (10%), *Frankiaceae* (9%) and *Ellin5301* (7%). Similarly, for AgNPs, dominant OTUs were either unassigned or classified as *Bacillaceae*, *Geodermatophilaceae* or *Streptomycetaceae* (22%, 15%, 11% and 7%, respectively).

3.3. Responses to silver by OTUs that could not be modelled

The response of many OTUs to Ag could not be modelled by either dose-response function (Equations 1 and 2). This was the case for 5054, 4126 and 3761 OTUs (Ag^+, AgNPs, and Ag_2S-NPs, respectively). However, for each Ag treatment, a small number of OTUs (< 25) were successfully fitted to an increasing dose-response function, signifying stimulation by Ag (Table SI.2). For Ag^+ and Ag_2S-NP treatments, these stimulated OTUs were predominantly assigned to the family *Xanthomonadaceae* (37% and 53%, respectively). In the AgNP treatment, *Bacillaceae* (27%) and *Planococcaceae* (20%) comprised the majority of OTUs stimulated by Ag. *Oxalobacteraceae* were present in the Ag^+ and AgNP

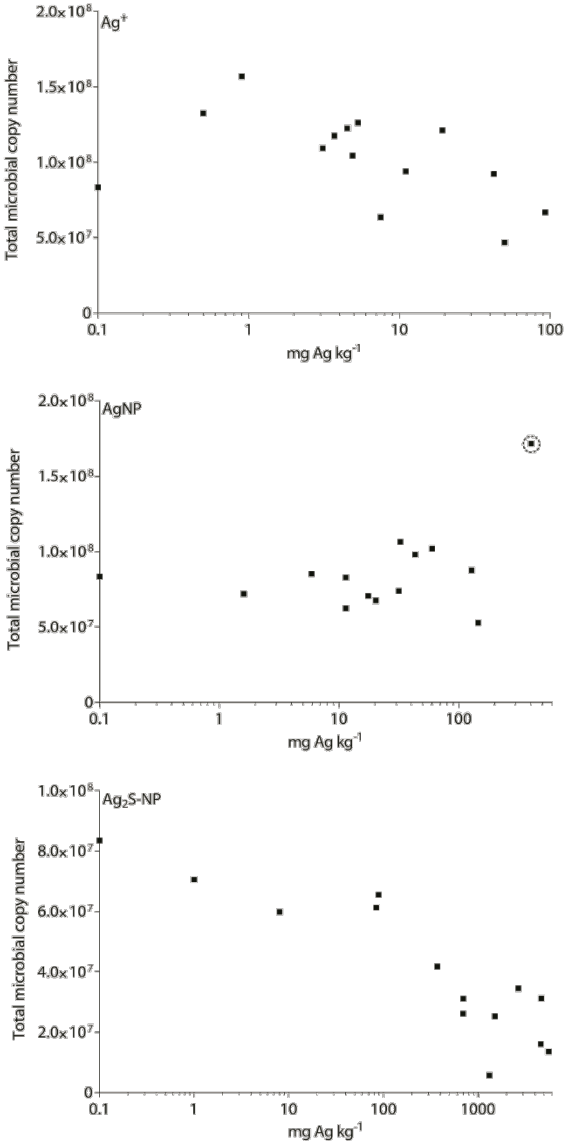

Figure 5. The abundance of bacteria and archaea, as indicated by the number of 16S ribosomal DNA (rDNA) copies measured using quantitative PCR (qPCR). Results are shown for each Ag treatment: ionic Ag (Ag^+ – top), Ag nanoaparticles (AgNP – middle) and Ag sulfide nanoparticles (Ag_2S-NP – bottom). A likely outlier is circled in the AgNP treatment.

treatments (13%). The remaining OTUs were analysed using linear regression and classified according to their slope (Table SI.2). The distribution of the most abundant families (contributing to 80% of the total) are given in Supplementary Information (Excel file).

4. Discussion

4.1. Silver treatments decrease soil nitrate production

Nitrification is particularly sensitive to metal contamination and as a result is often used to assess the potential risk of metals in soils [40]. Results from the nitrification experiment support previous studies that have shown that following sulfidation, AgNPs are less bioavailable and as a result less toxic to organisms [41-43]. This has been attributed to the high stability of Ag_2S in environmental matrices; a consequence of its very low solubility (K_{sp}= 1.6 x 10^{-49} [44]). At the EC_{50}, AgNPs were less toxic to soil nitrification processes than Ag^+. This may be due to heterocoagulation of AgNPs with natural soil colloids (e.g. clay particles) [45]. Such mechanisms have been shown to reduce dissolution and, thus, limit the release of toxic Ag^+ [46].

In a similar soil nitrification experiment, Langdon et al. [13] calculated the EC_{50} values for Ag^+ in six Australian soils, including the Charleston soil that was used in the current study. The EC_{50} value was higher in the previous study (43 – 53 mg Ag kg^{-1}, 95% confidence interval) compared to the current results (17 – 21 mg Ag kg^{-1}, 95% confidence interval). It is unclear why this discrepancy occurred. One explanation may lie in the different models that were used to fit the data. The previous study used a hormetic model, whereas in the current experiment, a regular sigmoidal model was used as hormesis was not significant. Differences in sample preparation and changes in the microbial community composition over time (during soil storage) may have also contributed.

Soluble Ag in some soils has been shown to have a have a stimulatory effect on soil nitrification at low Ag concentrations (< 10 mg Ag kg^{-1}) [13]. Upregulation of nitrifying genes in the nitrifier *N. europaea* has also been reported at low Ag^+ and AgNP concentrations (2.5 µg L^{-1}) [47]. However, in the current study, no significant hormesis effects due to the addition of Ag were found (Figure 2). Therefore, the sigmoidal dose-response model (Equation 1) was used.

There are limited data on the effect of AgNPs on nitrification processes in soils; the majority of research has focused on wastewater and sludge. For example, when sludge nitrifying bacteria were exposed to AgNPs at 40 mg Ag L^{-1}, bacterial abundance was shown to significantly decrease [48]. Similarly, when the model ammonium oxidising bacteria (AOB) *Nitrosomonas europaea*, was exposed to PVP coated AgNPs at 20 mg Ag L^{-1} [49], nitrate production decreased by 90% compared to the control. However, in other experiments, AgNPs have had no significant effect on wastewater nitrification at 2.5 mg Ag L^{-1} [14] and were found to be up to 48-times less toxic than Ag^+ to various nitrogen-cycling bacteria [47]. Only one previous study has used a natural soil to investigate the impacts of AgNPs on nitrification. The authors concluded that over 24 h, AgNPs were more toxic to nitrification than Ag^+ when added to a soil slurry at 1 mg Ag L^{-1} [50].

As discussed above, it is still debatable whether AgNPs are more toxic than Ag^+ to nitrification processes. Given the limited amount of research that has been carried out using natural soils, this is particularly true for terrestrial systems. Furthermore, the effect of sulfidised AgNPs on soil nitrification processes has not been investigated. The findings from the current study suggest that the risk of Ag-based NPs (especially Ag_2S-NPs) to soil nitrification is overestimated (and conservatively covered) by the risk of ionic Ag^+ in soil environments. The results also

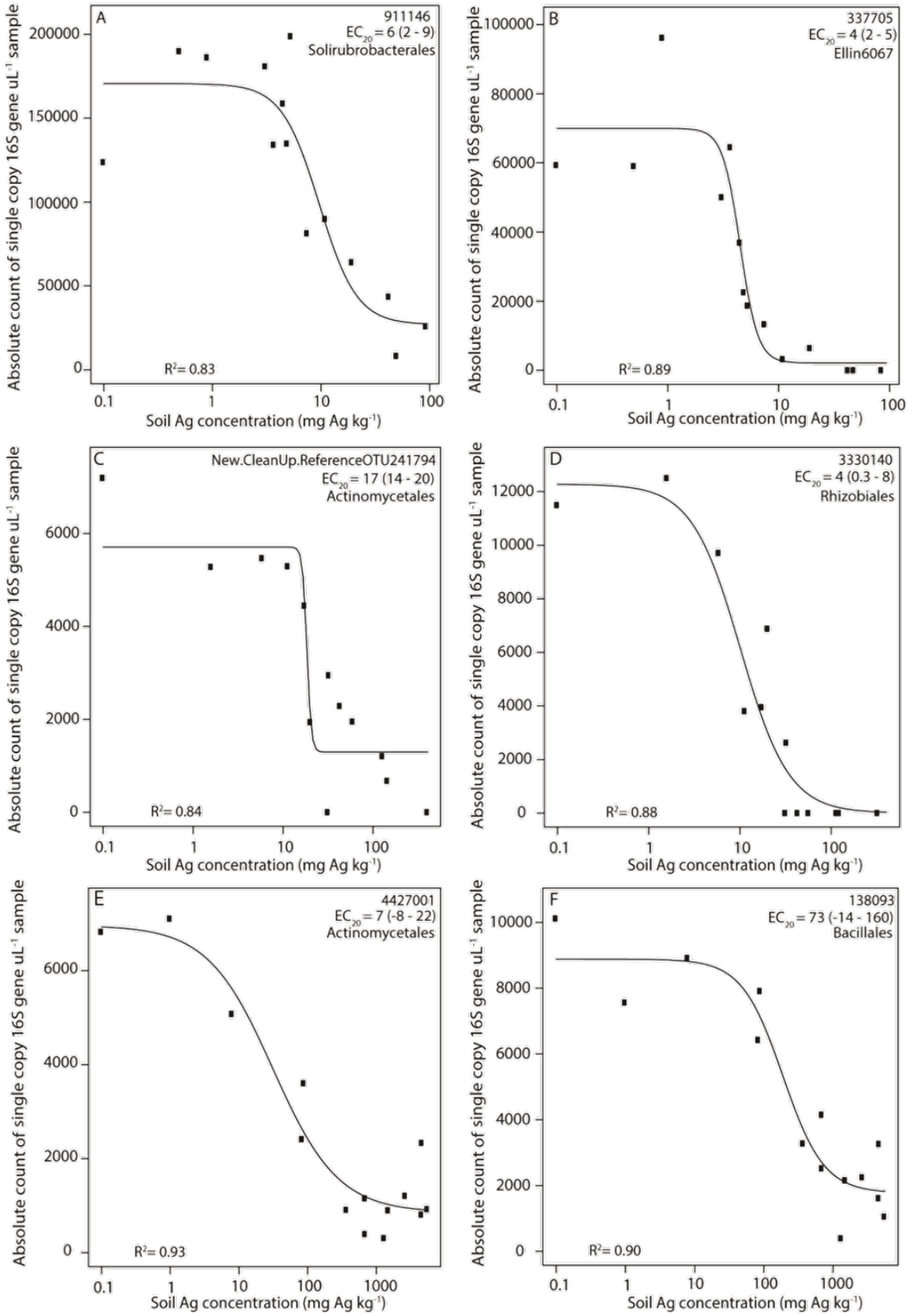

Figure 6. Dose-response plots of the absolute abundance of selected OTUs following 28 d soil incubation. Selected plots are shown for ionic Ag (Ag^+ – A,B); Ag nanoparticles (AgNP – C,D); and Ag sulfide nanoparticles (Ag_2S-NP – E,F) . The OTU identity, assigned order and the calculated EC_{20} values (with 95% confidence intervals) are shown at the top right of each plot. See Table SI.4 for estimates of the fitting parameters and associated errors.

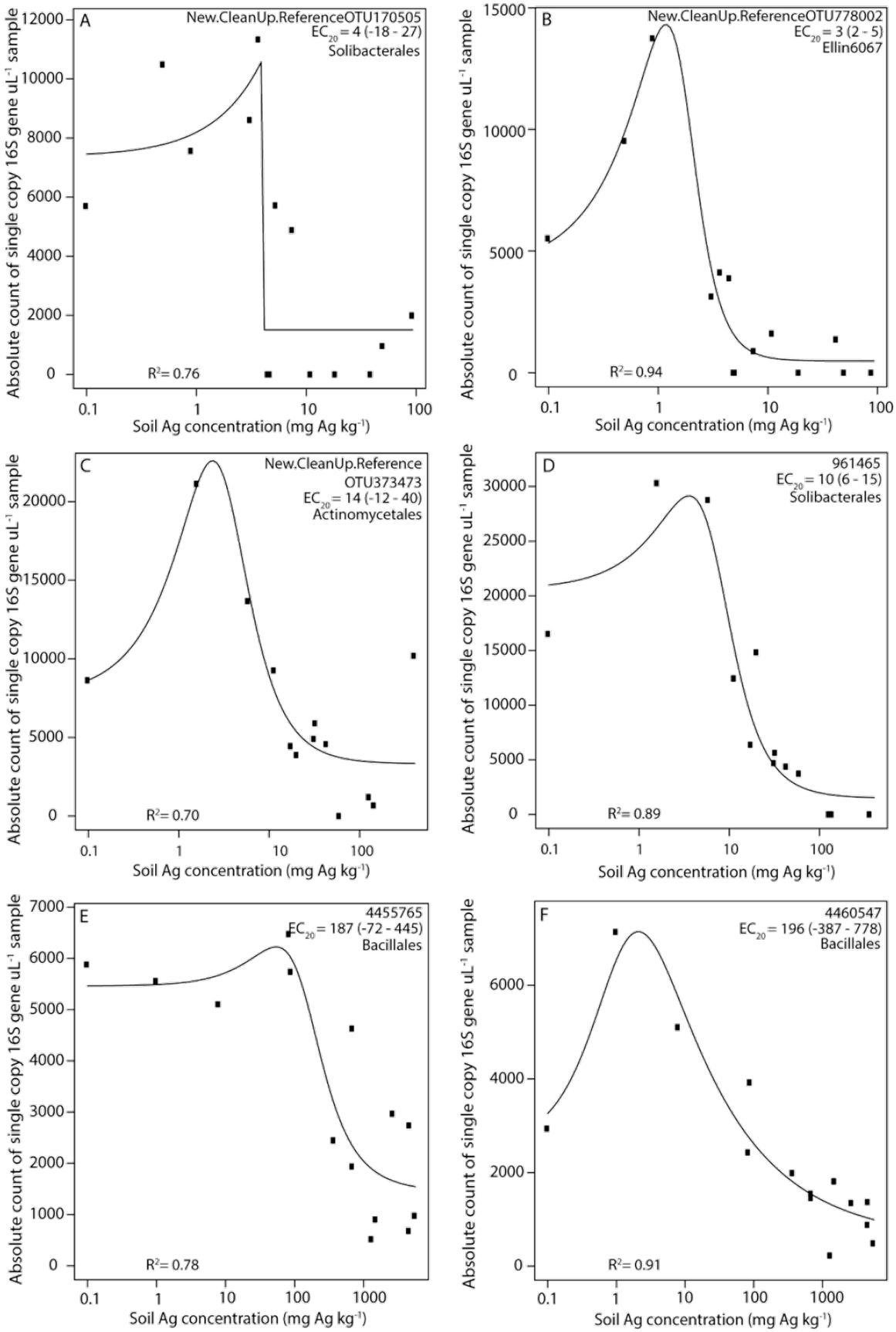

Figure 7. Dose-response plots of the absolute abundance of selected OTUs that demonstrated significant hormesis ($p \leq 0.05$) following 28 d soil incubation. Selected plots are shown for ionic Ag (Ag^+ – A,B); Ag nanoparticles (AgNP – C,D); and Ag sulfide nanoparticles (Ag_2S-NP – E, F) . The OTU identity, assigned order and the calculated EC_{20} values (with 95% confidence intervals) are shown at the top right of each plot. See Table SI.5 for estimates of the fitting parameters and associated errors

Table 4. Hazardous concentrations (HC) for ionic Ag (Ag^+), Ag nanoparticles (AgNP) and Ag sulfide nanoparticles (Ag_2S-NP) at which 95%, 90% and 80% of soil OTUs are protected (HC5, HC10 and HC20, respectively). Upper and lower 95% confidence intervals are shown in parentheses. Hazardous concentrations at which only 20% of soil OTUs are protected (HC80) were calculated to define the less sensitive OTUs. For a given HC, significant differences ($p \leq 0.05$) between Ag treatments are indicated by the asterisk.

Hazardous concentration (mg Ag/kg)	Silver type		
	Ag^+	AgNP	Ag_2S-NP
HC5	0.49 (0.32 – 0.73)	0.14 (0.056 – 0.35)	0.25 (0.13 – 0.47)
HC10	0.83 (0.61 – 1.1)	0.44 (0.22 – 0.86)	1.2 (0.76 – 2.0)
HC20	1.4 (1.2 – 1.7)	1.4 (0.89 – 2.2)	5.9* (4.4 – 8.1)
HC80	5.1* (4.7 – 5.6)	17.0** (14 – 22)	171.0*** (144 – 203)
Number of data points in SSD curve	390	146	498

demonstrate the concentration range over which this soil microbial process will be affected by Ag.

4.1.1. The toxicity of silver nanoparticles to soil microbial processes is controlled by multiple factors

A number of factors will affect the toxicity of AgNPs to soil nitrification; namely, the abundance of the total microbial population, the community composition and the functional capabilities of individual organisms (and their inter-relationships). This complex interaction can be observed when comparing the dose-response curves for the effect of Ag treatments on soil nitrification and bacterial *amoA* gene abundance (Tables 2 and 3). For example, the abundance of the bacterial *amoA* gene increased at low Ag concentrations (hormesis), whereas the production of soil nitrate was not stimulated at low Ag concentrations for any Ag treatment. Therefore, although *amoA* gene abundances increased, the composition of the whole community also changed, which may have counteracted this increase and resulted in no observable stimulation of nitrification. This highlights the need to use a variety of approaches when investigating the effect of contaminants, including Ag-based NPs on soil microbial communities. Analyses should include: qPCR, for analysing total community abundance; pyrosequencing, for determining community composition; measurement of the abundance of specific genes, e.g. *amoA*; and, measurement of the effects on specific functions (e.g. nitrate production).

4.2. Microbial community distribution

Overall, the community composition of the soil is

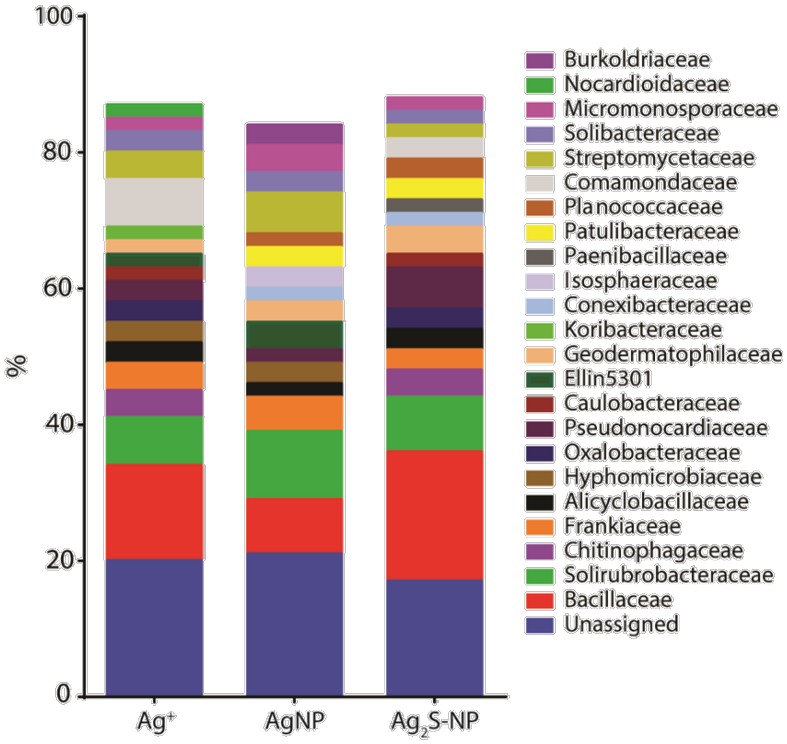

Figure 8. Taxonomy of the OTUs that were successfully fitted to the dose-response models. For clarity, only the bacterial families that contributed ≥ 2% to the overall distribution are shown.

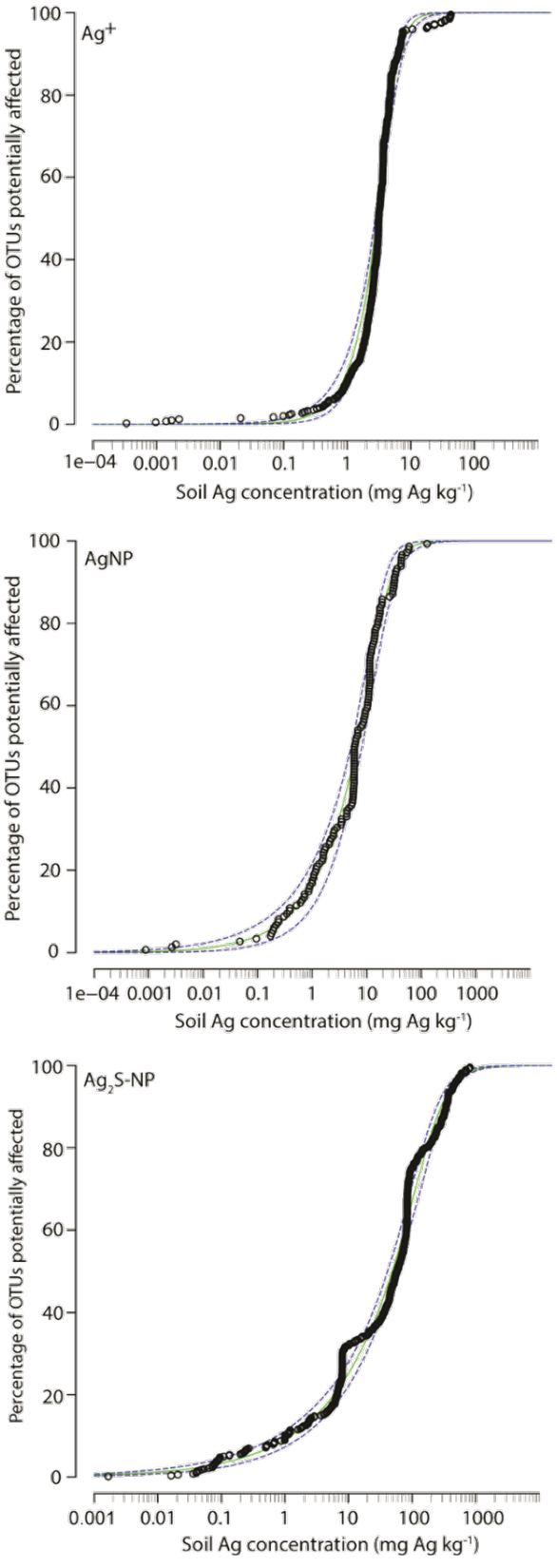

Figure 9. Operational taxonomic unit (OTU) sensitivity distributions (OSD) comparing the sensitivity of OTUs to ionic Ag (Ag^+ – top), Ag nanoparticles (AgNP – middle) and Ag sulfide NPs (Ag_2S-NP – bottom). Each data point corresponds to the Ag concentration that decreased the absolute abundance of a specific OTU by 20% (EC_{20}). Data were fitted to a Burr Type III function, where the fitted function is shown in green and 95% confidence intervals are indicated by the blue dashed line.

consistent with previous observations, where Actinobacteria and Proteobacteria are the most dominant phyla in soil. However, the abundance of Firmicutes (21.3%) was relatively high compared to other studies (e.g. 0.9% [51]) and Acidobacteria (2.9%) was slightly lower than expected (e.g. 13.8% [51]). Although Firmicutes are usually considered a low-abundance phylum [52], they have been found to dominate the total bacterial soil community in a loamy-sand agricultural soil (27.3%) [53]. The abundance of Firmicutes has been shown to increase in the presence of chitin in a Chernozem soil [54].

4.3. Estimated hazardous concentrations of silver treatments

Overall, the hazardous concentrations of AgNPs and Ag_2S-NPs to soil OTUs were less than or equal to that of Ag^+. When considering a protection level of 80%, Ag_2S-NPs were significantly less toxic than Ag^+ or AgNPs. In biosolids-treated soils, the concentration of AgNPs has been reported to be between 0.1 – 1 μg kg^{-1}, with a yearly increase of 110 ng Ag kg^{-1} [55]. All HC_x values that were calculated (HC5 to HC20) exceeded this concentration range. Therefore, based on the findings in this study and current and predicted near future soil concentrations, AgNPs and Ag_2S-NPs pose a low risk to whole soil microbial communities.

Currently, geogenic concentrations of bulk Ag in soils (0.01 – 1 mg kg^{-1} [56]) are much greater than that for predicted loadings of Ag-based NPs; this lower concentration will be exceeded in approximately 90 years according to current predictions. Therefore, based on the calculated HC1, HC5, and HC10 values in this study, soil microorganism are potentially at risk in some of these soils. However, it is important not to overestimate this risk based on these soil concentrations alone as the bioavailability of Ag (and other metals) in soils is affected by many factors, including speciation, effects of aging on speciation and, the physical and chemical properties of the soil [57, 58].

4.4. Implications for risk assessment of transformed silver nanoparticles in soils

Compared to aquatic systems, very few studies have applied the SSD methodology to terrestrial systems [59, 60]. This is due to a lack of toxicity data for soil species. The construction of SSDs for soils requires multiple data points and for many toxicants, including AgNPs, these data are not available. Furthermore, as toxicity studies are usually not designed with the aim of developing a SSD, toxicity thresholds (EC_x values) are often not reported. The SSDs that have been constructed for soil organisms have only considered soil invertebrates (e.g. nematodes and annelids) and plants (monocots and dicots); microorganisms have not been modelled except when included in multi-trophic level SSDs. Therefore, in this study, we have developed a new approach for constructing a SSD (OSD) for soil microorganisms based on the analysis of the whole soil microbial community (and not just culturable species).

To construct a reliable SSD, the proposed number of data points (species geometric mean values) is between five and thirty [8]. In the current study, the minimum number of data points for each OSD was 146 which provides more reliable predications of the potential risks of AgNPs and/or Ag_2S-NPs. On the other hand, given the complexities of microbial communities and the fact that ~ 4000 OTUs were in the original dataset, it may seem unsatisfactory to be using a reduced dataset. However, this was done to avoid using fitted models that do not adequately describe the observed data. In the instances where OTUs did not respond to Ag in a manner that was consistent with commonly described dose response models, we assumed no response. Including these OTUs in an OSD would be similar to the erroneous inclusion of unbounded no observed effect concentrations (NOECs) in SSDs [61]. If poor model fitting data is included in an SSD, incorrect estimates will be generated and the problems of interpreting 'natural' variance will be magnified [11].

4.5. Limitations of the study and future recommendations for the risk assessment of transformed silver nanoparticles

The results and findings from this experiment are only applicable to the soil type that was tested (Chernozem). Soil properties are likely to influence the sensitivity of soil microorganisms to AgNPs and Ag_2S-NPs. It has been shown that soil pH, the concentration of organic carbon and clay content are key determinants of toxicity of Ag^+ and Ag-based NP [13, 62]. Therefore, future investigations should focus on the role of these soil properties and their influence on Ag_2S-NP toxicity to soil microorganisms. Soil SSDs are usually constructed from "normalised" toxicity data (EC_x values) where the effect of soil properties is used to modify the EC_x values before inclusion in the SSD to produce HC_x values [63]. This approach could also be applied to the toxicity data generated for OTUs identified using molecular methods, so that the OSD is comprised of normalized EC_x data from many soils. This would significantly increase the wider applicability of the HCx values derived.

To investigate the long-term effects of Ag_2S-NPs on soil microbial communities, longer incubation times are required. Whilst 28 d is the recommended period of time for soil nitrification tests [18], it may not be adequate for predicting the chronic effects of Ag_2S-NPs on soil microbial communities. Therefore, further studies that use longer incubation times (e.g. 6 months) are required.

The results in this study suggest that AgNP toxicity was primarily due to dissolution; similar toxicity was observed between Ag^+ and AgNP treatments and lower toxicity values were found for the highly insoluble Ag_2S-NPs. Based on calculated HC values, soil OTUs were significantly less sensitive to Ag_2S-NPs compared to AgNPs or Ag^+.

A limitation of the curve-fitting procedure that was used in this study is that the EC_{20} value is estimated from the upper asymptote of the curve and not from the control value. As a result, the EC_{20} values of a small number of OTUs were less than the control concentration, i.e. between 0 – 0.1 mg Ag kg^{-1}. This is a numerical artefact that can be attributed to the increased sensitivity of these OTUs: they are possibly affected immediately by Ag addition and no dose can be considered 'safe'. Consequently, the response of these OTUs may be better described by another dose-response model. Less than 10 fitted OTUs had this response in each Ag treatment. Indeed, it is likely that a number of OTUs would follow this response due to random effects; however, further analysis of these OTUs was not performed as it is out of the scope of this study. Operational taxonomic units that were affected in this way were still included in the OSDs, as excluding them would lead to an overestimation of the HC_x values. To better describe the response of all OTUs, the method could be further developed in future studies. However, it is unclear if classifying all OTUs would have a significant effect on the overall calculated hazardous concentrations – which is the primary aim of this study.

An ongoing challenge is to relate the function of soil microbial communities to its structure. In this experiment, the effects of Ag_2S-NPs on individual microbial populations were investigated, yet the functional role of many of these populations is unknown. When more information is known about the role of the affected soil microorganisms, the HC_x values may be refined. Furthermore, our understanding of soil microbial communities will also advance with the development of -omic techniques, specifically genomics, proteomics and metabolomics.

This study provides a unique molecular-based framework for quantifying the effect of a toxicant on a whole soil microbial community. However, the development of SSDs is only the first step in the ecological risk assessment of AgNPs and Ag_2S-NPs. Further studies are needed to ascertain if the approach is applicable across a range of soil types and to determine the functional role of sensitive microorganisms. It is recommended that future revisions of soil guideline values consider the results presented in this study.

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Supplementary Information

Table SI.1. The number of OTUs that were removed from the dataset prior to non-linear regression analysis. OTUs were removed if they were only counted between 0 and 5 times in each silver treatment. The remaining OTUs were used in non-linear regression analysis.

Count of OTUs	Number of OTUs		
	Ag^+	AgNP	Ag_2S-NP
Total OTUs initially	51025	51025	51025
0	5882	8827	10214
1 (singletons)	14401	16411	15078
2 (doubletons)	12970	11887	11315
3 (tripletons)	7033	5433	5720
4	3218	2663	2742
5	2077	1532	1697
Remaining OTUs*	5444	4272	4259

Table SI.2. The number of OTUs that were fit to each model type for each silver treatment. Operational taxonomic units (OTUs) that could be fit to the four parameter log-logistic sigmoidal model (LL.4) and the Brain-Cousens hormesis model (BC.5), were plotted on the OTU-sensitivity curve. OTUs that were fit to the models 'LL.4 (inhibition)', 'BC.5' and 'LL.4 + BC.5' were plotted on the OTU sensitivity distribution.

Fitting model	Ag^+	AgNP	Ag_2S-NP
LL.4 (inhibition)	319	68	273
BC.5	44	46	106
LL.4 + BC.5	27	32	119
LL.4 (stimulation)	22	15	15
Linear regression (slope > 0)	1129	1464	819
Linear regression (slope < 0)	3903	2647	2927
Total	5444	4727	4259

Table SI.3. Distribution of phyla that were successfully fit to the dose-response models and subsequently used in the OTU sensitivity distribution. The abundance of each phylum is displayed as a percentage of the total count of all phyla for each Ag treatment.

Phylum	Ag^+	AgNP	Ag_2S-NP
Actinobacteria	31.5%	48.3%	41.4%
Proteobacteria	28.5%	19.0%	15.5%
Firmicutes	18.7%	13.6%	28.9%
Acidobacteria	5.4%	4.8%	4.2%
Bacteroidetes	4.1%	0.7%	4.2%
Planctomycetes	4.1%	4.1%	2.4%
Gemmatimonadetes	4.1%	6.1%	1.4%
Chloroflexi	1.0%	1.4%	0.6%
Armatimonadetes	0.8%	0.7%	0.2%
Unassigned	0.8%	0.7%	0.8%
Verrucomicrobia	0.5%		0.2%
Elusimicrobia	0.3%		
Nitrospirae	0.3%		
Acidobacteria			
Crenarchaeota		0.7%	0.2%

Table SI.4. Results of dose-response curve fitting for OTUs presented in Figures 5 (main text). The estimated values of the fitting parameters *b*, *c*, *d* and *e* are shown with their 95% confidence intervals.

	Ag^+			AgNP		
OTU	911146	337705	New.CleanUp. Reference OTU241794	3330140	4427001	138093
b	3	5	18	2	1	1
Lower CI	-1	-3	-18	0.4	0.2	0.1
UpperCI	6	14	54	3	2	3
c	27004	2585	1296	0	854	1775
Lower CI	-23016	-9153	394	-2345	134	412
Upper CI	77024	14323	2197	2345	1575	3138
d	170627	70417	5708	12280	6962	8881
Lower CI	136449	54683	4641	9193	5495	7381
Upper CI	204806	86151	6776	15368	8428	10381
e	10	4	18	10	31	188
Lower CI	2	4	16	4	-12	19
Upper CI	17	5	21	16	75	357
EC_{20}	6	3	17	4	7	73
Lower CI	2	2	14	0	-9	-14
Upper CI	9	5	20	8	22	160
R^2	0.83	0.89	0.84	0.88	0.93	0.90

Table SI.5. Results of dose-response curve fitting for OTUs presented in Figure 6 (main text). The estimated values of the fitting parameters *b*, *c*, *d*, *e* and *f* are shown with their 95% confidence intervals.

	Ag+		AgNP			
OTU	New.CleanUp. Reference OTU170505	New.CleanUp. Reference OTU778002	New.CleanUp. Reference OTU373473	961465	4455765	4460547
b	5640	4	2	2	2	1
Lower CI	5618	1	0	1	0	1
UpperCI	5663	1	0	1	0	1
c	1506	487	3327	1488	1445	557
Lower CI	1483	-737	-1018	-3974	-569	-1012
UpperCI	1529	1711	7672	6949	3460	2127
d	7371	4157	7582	20594	5461	2544
Lower CI	7349	911	-2348	12037	3722	529
UpperCI	7394	7404	17512	29151	7200	4560
e	4	2	3	7	135	1
Lower CI	-19	1	-1	2	-301	0
UpperCI	27	3	7	11	571	3
f	823	11774	10642	3986	28	7902
LowerCI	801	3887	-5449	-2609	-141	146
UpperCI	846	19661	26732	10580	198	15658
EC_{20}	4	3	14	10	187	196
Lower CI	-19	2	-12	5	-72	-387
Upper CI	27	5	40	15	445	778
R^2	0.76	0.94	0.70	0.89	0.78	0.91

Dominant families that were fit to each model type.
Values are presented as a % of the total of each column. Families that contriubuted to 80% of the total are presented

Ag Type	Ag+				
		Fitted model			
Family	Used in OSD	Dose-response (inc)	Linear dec	Linear inc	Grand Total
Unassigned	19.7%	9.1%	24.9%	17.9%	23.0%
Bacillaceae	13.6%	9.1%	12.0%	14.2%	12.6%
Xanthomonadaceae	1.3%	36.4%	7.2%	29.0%	11.4%
Solirubrobacteraceae	6.7%	0.0%	3.8%	0.7%	3.3%
Streptomycetaceae	3.6%	0.0%	3.7%	1.9%	3.3%
Planococcaceae	1.3%	4.5%	3.0%	5.0%	3.3%
Oxalobacteraceae	3.1%	13.6%	2.5%	6.0%	3.3%
Chitinophagaceae	3.6%	0.0%	3.0%	2.2%	2.9%
Caulobacteraceae	1.5%	0.0%	2.9%	2.5%	2.7%
Pseudonocardiaceae	3.1%	0.0%	3.0%	1.3%	2.6%
Geodermatophilaceae	1.8%	0.0%	2.2%	3.2%	2.4%
Micromonosporaceae	2.3%	0.0%	2.7%	0.8%	2.2%
Comamonadaceae	7.4%	0.0%	1.8%	0.6%	2.0%
Frankiaceae	3.6%	0.0%	2.2%	0.4%	1.9%
Ellin5301	1.5%	0.0%	1.9%	0.2%	1.5%
Patulibacteraceae	1.3%	0.0%	1.7%	0.4%	1.4%
					79.9%

Ag Type	AgNP				
		Fitted model			
Family	Used in OSD	Dose-response (inc)	Linear dec	Linear inc	Grand Total
Unassigned	21.2%	13.3%	23.5%	15.2%	20.5%
Xanthomonadaceae	0.7%	6.7%	14.5%	16.3%	14.6%
Bacillaceae	8.2%	26.7%	8.9%	22.5%	13.6%
Solirubrobacteraceae	9.6%	0.0%	4.7%	1.8%	3.8%
Oxalobacteraceae	1.4%	13.3%	1.6%	7.1%	3.5%
Planococcaceae	2.1%	20.0%	2.1%	5.7%	3.4%
Streptomycetaceae	6.2%	0.0%	3.4%	2.3%	3.1%
Geodermatophilaceae	3.4%	0.0%	3.2%	2.0%	2.8%
Caulobacteraceae	1.4%	0.0%	4.1%	0.5%	2.8%
Pseudonocardiaceae	2.1%	0.0%	2.6%	1.9%	2.4%
Micromonosporaceae	4.1%	0.0%	1.9%	1.6%	1.9%
Chitinophagaceae	0.0%	0.0%	2.2%	1.5%	1.9%
Frankiaceae	4.8%	0.0%	1.7%	1.4%	1.7%
Sphingomonadaceae	0.0%	0.0%	2.5%	0.2%	1.6%
Ellin5301	4.1%	0.0%	2.2%	0.3%	1.6%
					79.2%

Ag Type	Ag2S-NP				
		Fitted model			
Family	Used in OSD	Dose-response (inc)	Linear dec	Linear inc	Grand Total
Unassigned	17.1%	0.0%	22.5%	15.1%	20.4%
Bacillaceae	19.3%	6.7%	19.5%	14.5%	18.5%
Xanthomonadaceae	0.6%	53.3%	5.7%	33.8%	10.7%
Streptomycetaceae	2.4%	6.7%	3.7%	9.6%	4.7%
Solirubrobacteraceae	8.2%	0.0%	3.6%	2.8%	4.0%
Planococcaceae	2.6%	0.0%	4.3%	2.9%	3.9%
Geodermatophilaceae	4.0%	0.0%	2.5%	2.8%	2.7%
Pseudonocardiaceae	5.8%	0.0%	2.0%	1.3%	2.3%
Caulobacteraceae	2.4%	0.0%	2.8%	0.4%	2.3%
Sphingomonadaceae	0.0%	13.3%	2.5%	0.1%	1.8%
Chitinophagaceae	3.8%	0.0%	1.9%	0.0%	1.8%
Micromonosporaceae	1.8%	6.7%	1.9%	1.0%	1.7%
Frankiaceae	3.2%	0.0%	1.6%	1.1%	1.7%
Paenibacillaceae	2.4%	0.0%	1.8%	1.1%	1.7%
Alicyclobacillaceae	2.6%	0.0%	1.6%	0.2%	1.4%
					79.5%

CHAPTER 5

Summary of major outcomes and future research directions

Summary of major outcomes and future research directions

The objective of this thesis was to better understand the life cycle of manufactured silver nanoparticles (AgNPs), the results of which will enable a more accurate risk assessment of AgNPs in the environment. In the previous chapters, the fate and effects of AgNPs on wastewater treatment (WWT) processes and terrestrial organisms were investigated under realistic exposure scenarios to address the six aims presented in the introduction. The following section outlines the main conclusions and outcomes from this thesis relating to these aims and provides recommendations for future research in this area.

1. Investigate the fate of silver nanoparticles during wastewater treatment and the changes in silver speciation during this process.

Silver nanoparticles are removed from wastewater during the wastewater treatment process and adsorb to the soil phase.

A potential route of entry for AgNPs into the environment is *via* the discharge of effluent containing AgNPs into aquatic systems. A number of studies have investigated to what extent AgNPs are removed from wastewater during WWT; removal efficiencies ranged from 88% – 100% [1, 2]. The results from this thesis (**Chapter 2**) showed that under realistic conditions (i.e. non-synthetic influent wastewater, realistic Ag spiking concentrations and realistic wastewater treatment processes), the majority of Ag (AgNPs and Ag^+) will adsorb to, or be incorporated into, the biosolids. For wastewater that was spiked with Ag^+ or AgNPs only very small amounts of Ag were found in the effluent (0.8 ± 0.1% and 0.4 ± 0.4%, respectively).

Silver nanoparticles are transformed to sulfidised silver species during wastewater treatment.

The two main phases of WWT that were simulated in this study were the aerobic stage (activated sludge process) and anaerobic digestion. Previous studies have shown that AgNPs are sulfidised to form Ag-sulfide (Ag_2S) following anaerobic WWT. In this thesis, results showed that sulfidation can also occur during the aerobic stage – even prior to anaerobic treatment (**Chapter 2**).

After 10 days of aerobic WWT in sequencing batch reactors, AgNP spiked sludge was dominated by nano-sized sulfidised species (85%) with minor amounts of metallic Ag (Ag^0) present. In the anaerobic stage that followed, it was found that > 90% of added Ag was sulfidised and metallic Ag was not detectable. Bulk Ag_2S (aggregates > 150 nm) was detected in anaerobic sludge but not in aerobic sludge which suggests that anaerobic treatment may be important for the conversion of nano-sized aggregates of Ag_2S to bulk Ag_2S. These results support previous studies, and studies published during the conduct of research reported in this thesis, which have shown that the majority of AgNPs will be sulfidised during WWT [2-4]. Therefore, the fate and behaviour of AgNPs in soils will be determined by these transformed Ag species.

2. Determine the effect of silver nanoparticles on wastewater treatment processes using realistic experimental conditions.

The toxicity of silver nanoparticles and ionic silver to wastewater microorganisms will decrease following sulfidation.

As is the case for most metals, the bioavailability and therefore toxicity of AgNPs will be controlled by two factors: mobility and speciation. During wastewater treatment, where microorganisms are already in direct contact with the media containing AgNPs, the toxicity of AgNPs to microorganisms is influenced strongly by speciation rather than mobility.

It is widely accepted that one of the main mechanisms of AgNP toxicity involves the release of Ag^+ from AgNPs. Therefore, the transformation of AgNPs to Ag-S species during WWT would influence the release of Ag^+. Since Ag_2S is much less soluble than other Ag compounds, it is therefore expected that sulfidation would decrease AgNP toxicity. This assumption was supported by results from this thesis. The effect of AgNPs on the microbial diversity of anaerobic and aerobic sludges was investigated using pyrotag sequencing of the 16S rRNA gene (**Chapter 2**). The microbial diversity of sludge samples that were spiked with either Ag^+ or AgNPs was compared to that of influent wastewater, activated sludge mixed liquor, and anaerobic inoculum. The structure of major microbial communities in all samples could be grouped together based on their source but not on Ag type; this suggests that the community structure of activated sludge and anaerobic sludge samples were the same, regardless of Ag treatment. Following further transformation of the data (Hellinger transformation), it was found that AgNPs caused a shift in the community structure of sub-dominant populations. This shift was only minor and only observed in the niche communities.

In this thesis, the effect of transformed AgNPs on wastewater microbial communities was investigated. The majority of previous studies have used *in vitro* conditions and focused on the effects of 'pristine' AgNPs and not the environmentally realistic form (Ag_2S). This distinction is important because as the results show, sulfidation can decrease the effect of AgNPs on wastewater microbial communities. Given that virtually all wastewater treatment processes are mediated by microorganisms, changes in microbial structure have the potential to affect the efficiency of wastewater treatment.

At the predicted wastewater concentrations of silver nanoparticles, wastewater treatment processes are unlikely to be affected.

The effect of Ag on wastewater nitrification was investigated by analysing mixed liquor samples that were collected from sequencing batch reactors (SBRs) spiked with either Ag^+ or AgNPs. The SBRs were operated for 10 days in 6 h cycles and contained influent wastewater and activity sludge mixed liquor. Each cycle consisted of four phases; feed, anoxic, aerobic and a settling phase. Samples were collected daily at the end of each cycle and analysed for NH_4^+, NO_2^- and NO_3^- using Flow Injection Analysis (FIA). For all Ag treatments, there was near complete removal of NH_4^+ during each cycle. In addition, the rates of nitrification for both Ag treatments were comparable to that of the control. Therefore, results from this thesis show that at realistic wastewater Ag concentrations (2.5 mg Ag L^{-1}), AgNPs are unlikely to affect wastewater nitrification processes.

Results from previous studies on the effect of AgNPs on wastewater nitrification are conflicting [1, 5-7]. Some studies have suggested that AgNPs are highly toxic to nitrification, while others have reported limited effects. This is mainly due to the fact that each study used different Ag spiking concentrations and different sludge matrices (the latter is particularly important). Organic matter that is present in 'real' mixed liquor, as opposed to synthetic wastewater, can complex released Ag^+ [8]. This complexation, together with anion binding (e.g. chloride ions), can decrease AgNP toxicity. The advantage of the study carried out in this thesis is that the mixed liquor and influent were collected from a local wastewater treatment plant. Therefore, this study is a realistic simulation of the conditions that AgNPs will be exposed to in WWT plants.

In addition to nitrification processes, wastewater microorganisms are also responsible for methanogenesis. The affect of AgNPs and Ag^+ on methane production during anaerobic sludge digestion was investigated using a biomethane potential test (BMP). Sludge was collected from SBRs after 10 d, added to serum bottles and incubated at 36°C for 38 d. A biogas sample was collected daily and analysed using gas chromatography. Transformed AgNPs did not affect methanogenesis. This is in agreement with previous studies that have shown that methane production is not affected by pure AgNPs at concentrations of 40 mg Ag L^{-1} [7] or < 18.9 mg Ag L^{-1} [9].

Overall, although a slight shift in the sub-dominant microbial communities was detected in AgNP spiked anaerobic sludge, this did not impact nitrification or methanogenesis; two key processes that are essential for efficient wastewater treatment.

3. Compare the bioavailability of transformed silver nanoparticles to that of silver nanoparticles and ionic silver.

Transformed silver nanoparticles are less bioavailable to lettuce than pristine silver nanoparticles and ionic silver.

The end use of biosolids is dependent on location. In Australia, approximately two thirds of biosolids are applied to soil as an agricultural amendment to improve soil fertility and as a soil conditioner. The state of South Australia uses the greatest proportion of biosolids (92%) for agriculture [10], specifically for broadacre pasture and cereal cropping. Therefore, the plant uptake of Ag from soils that are amended with biosolids containing Ag_2S-NP is a possibility.

Results from the pot experiment (**Chapter 3**) demonstrate that the bioavailability of biosolids-borne transformed AgNPs to lettuce (*Lactuca sativa*) is very low. Of the total amount of Ag added to each pot (as Ag_2S in biosolids), < 0.02% was present in the shoots. Therefore, the potential for humans to ingest large amounts of transformed AgNPs from crops grown in biosolids amended soil is negligible under the conditions used in this experiment.

Uptake of Ag by lettuce would have been strikingly different under hydroponic conditions. In soil, AgNPs can adsorb to clay particles and soil organic matter which may decrease the bioavailability of AgNPs [11, 12]. In contrast to the majority of previous studies, one of the primary goals of the experiments discussed in **Chapter 3** was to ensure that environmentally realistic conditions were simulated as far as possible; particularly in terms of biosolids application rate (10 t ha^{-1}) and the growth media (i.e. natural soil cf. hydroponic conditions). While hydroponic studies are essential for understanding uptake mechanisms and toxicity thresholds, they do not present an accurate risk assessment of AgNPs in terrestrial systems.

4. Determine the effects of agricultural amendments on the bioavailability of transformed silver nanoparticles and silver nanoparticles.

Agricultural amendments can increase the plant uptake of silver from soils treated with silver and silver sulfide nanoparticles.

The results presented in this thesis show that in a sandy soil, < 0.02% of Ag_2S-NPs added to soil will be translocated to the edible tissue of lettuce. A slightly higher proportion of Ag can be translocated to the shoots (0.06%) following fertilisation with ammonium thiosulfate or potassium chloride (applied at 100 mg S and K kg^{-1}, respectively). The mechanisms that caused this increase are not fully understood. However, it is hypothesised that Ag^+ ions released from AgNPs are complexed by thiosulfate or chloride and transformed into Ag species that are more bioavailable to lettuce. Whether this minor increase in Ag concentration affects plant physiology remains unknown and requires further investigation.

In contrast to the thiosulfate and chloride treatments, soil application of hydrogen peroxide (H_2O_2) and mono-ammonium phosphate (applied as a 0.5% v/v solution and 100 mg P kg^{-1} soil, respectively) decreased the plant uptake of Ag in some Ag treatments. Root concentrations of Ag for plants grown in AgNP and Ag^+ treated soils were significantly less ($p \leq 0.05$) than that of the control (no fertiliser). The mechanisms that caused this decrease remain unclear.

In both plant uptake experiments (**Chapters 3**), the uptake of Ag from AgNP and/or Ag_2S-NP spiked soil was less than, or equal to, that of Ag^+ spiked soil. This demonstrates that in sandy soils the risk posed by AgNPs is conservatively covered by the risk of Ag^+. The application of thiosulfate and potassium chloride fertiliers has the potential to increase Ag uptake however, this increase is minimal as < 1% of Ag added to the soil will be translocated to edible tissue in lettuce.

5. Investigate the effects of transformed silver nanoparticles on soil nitrification processes.

Silver sulfide nanoparticles are less toxic to soil nitrification processes than silver nanoparticles and ionic silver.

To investigate the effects of toxicity on soil microorganisms, a number of biochemical transformation processes (e.g. nitrogen, carbon, sulfur transformations) can be investigated. Among these processes, nitrification is commonly studied because a) it is considered the most sensitive process to toxicants, and b) it is a good indicator of soil fertility as nitrate (the final product of nitrification) is essential for plant growth. In investigating the effects of AgNPs on soil nitrification, two approaches were used: 1) analysis of the functional response (i.e. nitrate produced) and b) genomic techniques. The functional response was used to estimate Ag toxicity, whereas genomic analysis (i.e. bacterial *amoA*) was used to understand the effects on functional genes.

As presented in **Chapter 4**, Ag^+, AgNPs and Ag_2S-NPs decreased the amount of nitrate produced in soil over 28 d compared to the control (no Ag added). The magnitude of the effect varied between Ag treatments. At the lowest effect concentration, where nitrate production decreased by 10% (EC_{10}), there were no significant differences between Ag treatments. However, the EC_{20} and EC_{50} values for Ag_2S-NP treated soil were significantly greater than that of Ag $^+$– demonstrating that Ag_2S-NPs were less toxic. At low Ag concentrations, nitrate production was not stimulated as has been observed in previous studies (hormesis) **[**13]. At the gene level however, significant stimulation did occur: in all Ag treatments, abundance of the ammonia monooxygenase structural gene, bacterial *amoA*, did significantly increase at low Ag concentrations. Therefore, although the abundances of the *amoA* gene increased, the composition of the whole microbial community also changed. This may have counteracted the increase in *amoA* gene abundance and resulted in no observable stimulation of nitrification.

The potential risk of AgNPs and Ag_2S-NP to soil nitrification processes was shown to be lower than that posed by Ag^+. However, this is only applicable to the soil type that was used in studies described in this thesis (Chernozem); further studies are needed to determine the effects of Ag_2S-NPs on soil nitrification processes in other soil types.

6. Use genomic tools to develop a new method to quantify the ecotoxicity, and thus potential risk, of transformed silver nanoparticles to whole soil microbial communities.

A new method was developed to test the sensitivity of a whole soil microbial community to silver nanoparticles and silver sulfide nanoparticles.

Previous studies on the effects of AgNPs on soil microorganisms have, in general, investigated the toxicity to individual cultured organisms, or, the changes in gene expression and community composition following exposure to AgNPs. However, the effect of AgNPs could very well go beyond the target soil organisms that are traditionally tested and, therefore, may not be captured by methods currently available. Soil quality guidelines for AgNPs, which are currently lacking, could benefit from toxicity data collected from multiple species. In **Chapter 4**, a methodology that is commonly used for ecological risk assessment, specifically, the construction of species sensitivity distributions (SSDs) was applied to soil microorganisms. This approach, using genomic information from soils, has not been attempted previously and is advantageous as it considers the whole soil microbial community and not just cultured species (which are only a small fraction of the total microbial diversity).

This study used a combination of genomic techniques (16S rRNA amplicon sequencing and quantitative PCR) to fit dose-response curves to soil operational taxonomic units (OTUs). Taxonomy was assigned to each OTU based on the reference sequence that defines that OTU. Therefore, the response of specific microbial populations could be classified according to their response to Ag. OTUs were classified into four different response groups depending on what model could best describe their response. The models were 1) a common decreasing dose-response function (sigmoidal or hormetic), 2) an increasing dose-response function, 3) an increasing linear model and 4) a decreasing linear model.

Soil quality guideline values for silver nanoparticles and silver sulfide nanoparticles were derived from sensitivity distributions.

Toxicity values were calculated for OTUs that were successfully fitted to a common dose-response function. For each Ag type, OTU sensitivity distributions (OSDs) were constructed using the calculated toxicity values. The Ag concentrations that would protect a specified proportion of soil microorganism gene sequences (known as hazardous concentrations, HC_x values) were predicted from the OSDs.

Results from **Chapter 4** show that in order to protect 95% of soil OTUs (HC5) Ag concentrations in soil should remain below 0.25 mg Ag kg^{-1}. Given that soils amended with biosolids are predicted to reach this concentration within the next 100 years, the results presented here could hold important implications for the development of soil quality guidelines. For the less conservative HC value, HC10 (90% of OTUs protected), the type of Ag did not affect the sensitivity of soil OTUs. At the HC20, Ag_2S-NPs were significantly less toxic to the soil microbial community than AgNPs or Ag^+. Results from **Chapter 4**, together with those from **Chapters 2** and **3,** demonstrate that the effects of Ag_2S-NPs – and therefore potential risks – on terrestrial organisms are less than or equal to that of Ag^+ and AgNPs.

This is the first time that soil quality guideline values for AgNPs have been developed for a whole soil microbial community. This new approach has the potential to be applied to different toxicants, soil types and also to different microbial communities (e.g. aquatic microorganisms). However, one of the limitations of this study is that it does not directly relate the microbial community structure to its function; this remains a major challenge in microbiology. This knowledge gap is beginning to close as new –omic techniques are developed (e.g. genomics, proteomics and metabolomics) and as more soil microorganisms are classified. The new method described in this thesis for testing the sensitivity of whole soil microbial communities to a toxicant may be refined as the relationship between structure and function is better understood.

Future research directions

To further investigate the results acquired from this study, and to advance our understanding of the potential risk of manufactured AgNPs in the environment, it is recommended that the following five areas be investigated.

1. Separation of nano-specific effects from the effects caused by released silver ions.

In this study, Ag concentrations in plant parts and soils were measured following sample digestion and ICP-MS analysis. These methods were, however, only useful in determining the fate of Ag (e.g. soil, plant, roots, or shoots) based on the total Ag concentration. For the fundamental understanding of Ag (ionic and NPs) behaviour (mechanisms of uptake and toxicity) in soil, Ag speciation and characterisation are necessary. For example, the results that were observed in this study could not be attributed to either released Ag^+ or a specific NP effect. Indeed, a combination of both mechanisms may have occurred. In the literature, the reported mechanisms of AgNP toxicity are conflicting. Some studies have concluded that AgNPs exert their toxicity primarily *via* release of Ag^+ [14, 15], while others have suggested that direct NP effects are more important (i.e. due to intrinsic AgNP properties) [16-18]. Whether or not the same toxicity mechanisms still apply in soils requires investigation. To understand AgNP toxicity mechanisms, the Ag speciation must be known; however, determining Ag speciation in soils or organisms at realistic environmental exposure concentrations is problematic.

2. Development of methods for the analysis of nanoparticles in environmental matrices.

For all experiments in this study, no attempt was made to directly detect AgNPs in either plant tissues or soil. Whilst AgNPs have been detected in plant parts, this is a considerable challenge that has not yet been overcome for soils. The detection and separation of AgNPs from soil matrices is made difficult by the presence of natural soil colloids. Detection of manufactured AgNPs in soils or plants is further complicated by the possibility of AgNP dissolution and the subsequent re-precipitation of AgNPs *in vivo* [19].

Characterisation methods could be improved if techniques were developed that could separate intact NPs from dissolved forms (i.e. ions). Such methods have been developed for aqueous samples but not for solid samples. For example, a new technique to separate AgNPs from Ag^+ in solution using a tangential flow filtration system has recently been described [20]. A similar technique that separates AgNPs into size fractions using single particle ICP-MS is also under development [21-23]. These techniques have not been used to determine the composition of Ag in an environmental sample. For soil samples, an extraction technique will be necessary.

The development of a method that could directly analyse NPs in environmental matrices would provide great insight into the mechanisms by which AgNPs exert their effects on terrestrial organisms.

3. Investigation of the effects of silver sulfide nanoparticles on a variety of terrestrial organisms in different soils.

This thesis studied the uptake of Ag_2S-NPs by one plant species, lettuce (*Lactuca sativa*). It is necessary to consider other plant species; specifically, crops that are commonly grown in biosolids-amended soils (e.g. cereals, olives and vines [24]). Furthermore, the ability of other terrestrial organisms to ingest or accumulate Ag_2S-NPs should also be examined. Such organisms include nematodes, mycorrhizae [25], and other fungi. Whilst some studies have examined the ingestion of AgNPs by earthworms [26, 27], the uptake of Ag_2S-NPs by such species has not been investigated.

Future studies would also benefit from including a variety of different soils, given that soil type is a major determinant of AgNP fate. In this study, only two soil types were used: a very sandy slightly acidic soil in the pot trials, and a Chernozem soil characterised by its high organic carbon content was used for microbial experiments. Experiments in other soil types would help in increasing the validity of Ag_2S-NP risk assessments and in determining whether NP behaviour is broadly similar across soils or is site specific. A range of soils should be tested that have a variety of different properties (e.g. pH, clay content).

From such experiments, the specific soil properties that have the most influence on Ag_2S-NP toxicity to soil organisms could be determined. This would enable a more accurate risk assessment of Ag_2S-NPs in a range of different soils.

4. Chronic effects of silver sulfide nanoparticles in terrestrial environments.

Silver NPs can act as a continual source of bioavailable Ag [28] – this characteristic is exploited in AgNP-containing products. Therefore, given the possibility of long-term Ag^+ release, further research is needed to investigate the chronic effects of AgNPs and Ag_2S-NPs. Both pot trials presented in this thesis (**Chapter 3**) exposed plants to Ag_2S-NPs for what is effectively one growing season. The effects of aged Ag_2S-NPs on subsequent crops are unknown. Results from a recent soil incubation study showed that Ag_2S-NPs were very stable in soil; Ag speciation did not change over a 7 month incubation period [29]. However, plants may affect Ag_2S-NP speciation over time; for example, prolonged exposure to root exudates may promote Ag_2S-NP dissolution.

To investigate the effects of aged Ag_2S-NPs on plant uptake of Ag, long term field and/or pot trials carried out over multiple growing seasons is recommended. In a field situation, there are many variables that could affect Ag_2S-NP bioavailability. For example, heterogeneity of soil properties across the site, wetting/drying cycles, other climatic conditions, crop rotation and regular fertiliser application – which has been shown, in this study, to increase plant uptake of Ag from Ag_2S-NP treated soils (**Chapter 3**). The effect of applying fertiliser mixtures also remains unclear. For example, to meet the nutritional demand of plants, farmers are likely to apply blended fertilisers that contain a mixture of fertilisers in one product. Further investigations are required to understand the effect that this may have on the bioavailability of Ag_2S-NPs.

5. Improvement of statistical techniques to understand the response of soil microbes following their exposure to silver sulfide nanoparticles.

Dose-response models can be classified into groups depending on the statistical distribution that is used for describing the shape of the curve (e.g. Poisson, binomial, Weibull [30]). In this thesis, when investigating the response of soil OTUs to Ag, only two dose-response models were considered. Although they are the most commonly used functions, it is likely that a different model could better describe the response of some OTUs. Further improvement of the automated curve-fitting procedure is needed in order to simultaneously fit multiple dose-response models to each OTU. Ideally, each OTU would be fitted to numerous dose-response models and the best fit chosen automatically by the software program. The development of such a technique would improve the accuracy of toxicity values and would adequately explain the response of more soil OTUs. With the continual improvement of molecular techniques and as the 16S rRNA gene database is expanded, eventually each soil OTU could be assigned to the species level. Ultimately this would allow microbial species sensitivity distributions (SSDs) to be constructed for soil bacteria and archaea; such SSDs could then be used to develop more accurate soil quality guidelines.

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Appendix 1

Optimisation of silver sulfide nanoparticle synthesis and re-suspension methods

1. Introduction

Manufactured nanoparticles (NPs) can be used in either powder form or as a NP suspension for *in vitro* and *in vivo* toxicity testing. The form used would depend on the type of exposure being simulated (i.e. inhalation, ingestion, etc.). For environmental ecotoxicology studies, NPs are recommended to be introduced as stable suspensions. [1]. This is because a) it allows for homogenous distribution of NPs throughout the test media (e.g. soil) and b) it separates large NP aggregates, thereby ensuring that NP behaviour is simulated and not the behaviour of aggregates. To disperse NPs and prevent aggregation, sonication and stabilising/capping agents are commonly used [3]. However, producing a stable NP suspension can still be challenging; the optimal conditions (e.g. sonication time) will vary greatly between different types of NPs.

The first aim of this experiment was to establish a synthesis method for Ag_2S-NPs that was time efficient yet still produced Ag_2S-NPs that had a narrow size distribution, a hydrodynamic diameter < 150 nm, a high negative zeta potential and a relatively high Ag concentration upon re-suspension. The method followed in this thesis was based on that described by Xiang et al. [2]. Their procedure used a hydrothermal reaction to produce Ag_2S-NPs whereby L-cysteine was used as the sulfur source. Briefly, 0.271 mmol of $AgNO_3$ (0.046 g) was added to 0.271 mmol of L-cysteine (0.032 g) in ethanol (40 mL) while stirring (1:1 molar ratio). After stirring for 15 min, the mixture was transferred to a stainless steel Teflon® lined autoclave (also known as a calorimetry bomb) and heated at 180°C in an oven for 10 h. The autoclave was then cooled to room temperature naturally and the black precipitate produced was filtered through filter paper (Whatman No. 42), and washed with ethanol and water several times. The recovered precipitates were then dried for 6 h at 60°C.

The first aim of the current experiment was to decrease heating time and increase yield, whilst at the same time maintaining the desired Ag_2S-NP properties. This optimisation was necessary because our subsequent experiments required high spiking concentrations of Ag_2S-NPs and it was not practical to make > 20 'batches' of Ag_2S-NPs for each experiment.

The second aim of the experiment was to optimise the re-suspension of synthesised Ag_2S-NPs (the published method did not use re-suspended NPs, instead they used 'dry' Ag_2S-NPs). In all of our subsequent experiments, Ag_2S-NPs were used in suspension form, i.e. soils were spiked with Ag_2S-NP suspensions and not with dry Ag_2S-NP powder. By optimising the re-suspension process, the aim was to produce a spiking solution which was stable, mono-disperse and had a reasonably high Ag concentration.

Figure 1. The stainless steel autoclave (left) that was used for silver sulfide nanoparticles synthesis and the Teflon® vessel insert (right).

Table 1. Effect of sonication time on silver sulfide nanoparticle size (hydrodynamic diameter, d_h), homogeneity (PdI = polydispersity index), zeta potential (ζ) and silver concentration (n = 3). Nanoparticles were re-suspended in ultrapure Milli-Q water.

Sonication time	d_h	PdI	Zeta potential (ζ)	Silver concentration[#]
(min)	(nm)		(mV)	(mg/L)
2	149 ± 3	0.16	-28	16.0 ± 0.4
10	138 ± 2	0.11	-30	20.3 ± 0.2
20	163 ± 2	0.12	-39	19.7 ± 0.1
45	134 ± 2	0.14	-30	19.0 ± 0.9

2. Materials and methods

2.1. Optimisation of silver sulfide nanoparticle re-suspension

Initially, Ag_2S-NP synthesis was carried out using the method described by Xiang et al. [2]; all experimental parameters were followed as described in the method i.e. heating time, masses, volume of ethanol etc. The resulting black precipitate was then re-suspended: it is this re-suspension process that was optimised first. Once the best conditions for NP re-suspension were determined, the synthesis procedure itself was optimised (see *2.2*).

After heating, the black precipitate was collected from the Teflon® autoclave (Figure 1), filtered (0.45 µm), washed and then dried as described in the introduction. The resulting NP powder was gently ground with a mortar and pestle before re-suspension. Three different masses of Ag_2S-NP powder (0.165g, 0.200g, 0.226g) were weighed into 50 mL centrifuge tubes. In previous experiments using AgNPs we found that 0.1 g of AgNP powder was the optimal mass to use (unpublished).

Silver sulfide nanoparticle powder was weighed into a 50 mL centrifuge tube containing 50 mL of ultrapure Milli-Q water. The tube was gently inverted several times and then probe sonicated and centrifuged (NB: bath sonication was tested but was unsuccessful at re-suspending the Ag_2S-NPs). To determine the optimal re-suspension conditions, sonication time and the composition of the suspension solution were varied. Probe sonication (90W) was carried out in an ice bath using four different times of sonication; 2 min, 10 min, 20 min and 45 min. Four re-suspension solutions were tested: 1) ultrapure Milli-Q water; 2) ultrapure Milli-Q water + citrate (3 mM); 3) ultrapure Milli-Q water + humic acid (5 mg L^{-1}) (Sigma Aldrich); 4) ultrapure Milli-Q water adjusted to pH 9.8 with NaOH (0.01 M).

After sonication, the suspension was immediately centrifuged at 2300 *g* for 15 min to settle particles/aggregates that had a diameter of ~ 0.2 µm. However, this size is only approximate because the density of Ag_2S-NPs was unknown; therefore, the density of AgNPs (10.5 g cm^{-3}) was substituted into Stokes' equation in order to calculate the equivalent Stokes diameter.

2.2. Optimisation of silver sulfide nanoparticle synthesis

The synthesis procedure was optimised in order to increase the yield of Ag_2S-NPs per batch and to decrease the time required for synthesis. In the original method [2], 0.271 mmol of both L-cysteine and $AgNO_3$ were used (32 mg and 46 mg respectively. We increased the amount of both constituents and varied other parameters including; addition of a stabilising agent (citrate) to the solution before heating and changing the heating time and temperature. The success of a procedure was determined by re-suspending the NPs (according to *2.1*) and measuring d_h and polydispersity (PdI) using dynamic light scattering (DLS). Acceptable d_h and PdI were < 200 nm and < 0.5, respectively. Silver concentrations of the suspensions were analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES) following open vessel acid digestion.

3. Results and Discussion

3.1. Results and Discussion

3.2. Optimisation of silver sulfide nanoparticle re-suspension

Silver sulfide NPs were synthesised according to the method described previously [2] and successfully re-suspended. The effect of sonication time was investigated first, specifically, the effect on Ag concentration in the resultant Ag_2S-NP suspension.

3.2.1. Effect of sonication time on silver concentration

The results demonstrate that either 10 min or 20 min of sonication produces the highest Ag concentrations in suspension (Figure 2). Given that the two concentrations are not significantly different (p > 0.05), the shorter sonication time of 10 min was used in subsequent experiments. The purpose of sonication is to separate NP aggregates and form a stable suspension. The 10 minute sonication time was preferred since excess sonication can cause NPs to overheat and dissolve – even if an ice bath is used – and increase particle aggregation. The Ag concentrations of the NP suspension were further increased by applying two sonication and centrifuge steps. Following the first sonication (90 W, 10 min) and centrifugation (2300 *g*, 15 min), 30 mL of

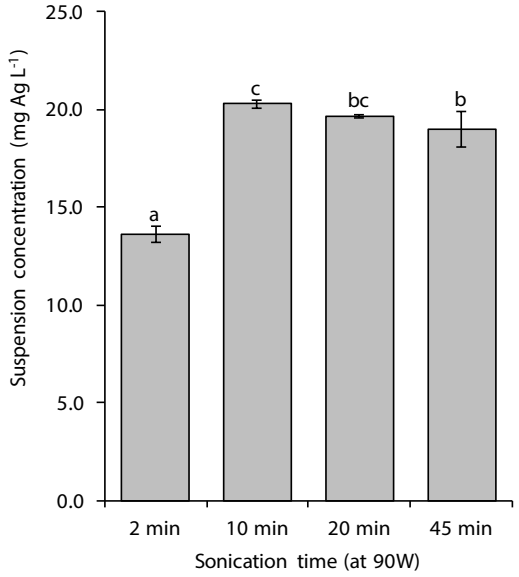

Figure 2. Impact of increasing sonication times on the average silver concentration in silver sulfide nanoparticle suspensions. Average values are presented ± standard deviation (n = 3). Significant differences were calculated using ANOVA Fischer's Protected LSD Test ($p \leq 0.05$) and are indicated by letters *a – c*.

supernatant was removed (R1). The remaining 20 mL of suspension was subjected to another sonication – after addition of ultrapure Milli-Q water (30 mL). Following the second sonication and re-centrifugation, only 20 mL of supernatant removed (R2). Humic acid was added to each suspension prior to sonication (5 mg L^{-1}). Both supernatants (R1 and R2) were then combined and gently shaken to give 50 mL of Ag_2S-NP suspension. In addition to Ag concentration, the 'quality' (d_h, PdI, and ζ-potential) of the NP suspension was also analysed (Table 1).

3.2.2. Effect of sonication time on nanoparticle size and polydispersity

Sonication times of 10 min and 45 min produced the smallest NPs (138 and 134 nm, respectively). Given that the average diameters were not significantly different from each other a 10 min sonication time was again chosen for future experiments. All Ag_2S-NP suspensions were well dispersed (as indicated by low PdI values) and their particle size distribution was monomodal. High PdI values indicate that particle size distribution is either very broad or multimodal. The zeta potential (ζ) of -30 mV for the sample sonicated for 10 min shows that the suspensions were stable: for charged particles, if zeta potential is less than -30 mV and greater than +30mV it is considered stable and unlikely to aggregate.

3.2.3. Effects of the suspension solution on nanoparticle size and polydispersity

To decrease NP aggregation, either humic acid or citrate was added to the NP suspension immediately prior to sonication. Analysis of the resulting NP suspension showed that citrate increased particle size considerably (d_h from ~ 140 nm to ~ 360 nm). Furthermore, DLS analysis showed that the suspension was very polydisperse (PdI > 0.5); large aggregates > 1000 nm were detected. In contrast, the addition of humic acid (5 mg L^{-1}) only slightly increased particle size (d_h = 152 nm) compared to the non-citrate suspension (d_h = 138 nm) and, the particle size distribution was unimodal. Therefore, humic acid (5 mg L^{-1}) was added to each Ag_2S-NP re-suspension prior to sonication. Humic acid may provide electrostatic or steric stabilisation of NPs. For example, it has been shown that at low ionic strength, clay colloids are stabilised electrostatically, whereas at high ionic strength (> 0.1 M) steric stabilisation is dominant [4].

The effect of pH on AgNP stability has been shown previously [5]. For example, at higher pH values there is greater electrostatic stabilisation of AgNPs because the isoelectric point (IEP) of coated AgNPs is usually in the acidic region. Therefore, in the current experiment, pH was increased with the aim of improving stability by decreasing particle aggregation. At pH 9.8, the average d_h was 134 nm which suggests that aggregation was limited by the increased pH. However, at the optimised sonication time, the Ag concentration in the solution was only 5 mg L^{-1}. Therefore, this method was not used.

3.3. Optimisation of silver sulfide nanoparticle synthesis

The influence of heating temperature and heating time were investigated. The starting materials were heated at a lower temperature (110°C vs. 180°C) and for a shorter length of time (5 h vs. 10 h). After 5 h and 10 h of heating at 110°C, the reaction appeared incomplete as L-cysteine was still visible and the amount of precipitate was less than that collected from heating at 180°C. In addition, the average particle diameter for both time regimes was > 1 µm (e.g. $d_{h(5h,\ 110°C)}$ = 1863 ± 739 nm). Therefore, in subsequent syntheses, the temperature was maintained at the

Table 2. Mass of starting materials used in the current study compared to those used in the original study by Xiang et al. [2]. Two different masses were tested in the current study (A and B = 5x and 10x greater than the original method).

Method	Ethanol	L-cysteine		$AgNO_3$		Ratio
	(mL)	(mmol)	(mg)	(mmol)	(mg)	(Cys:Ag)
Original study [2]	40	0.27	33	0.27	46	1:1
Current study (A)	150	1.36	165	1.37	233	1:1
Current study (B)	150	2.71	328	2.74	465	1:1

recommended 180°C.

Shortening the duration of heating also increased average particle size; however, this effect was minimal ($d_{h,\ (5h,\ 180°C)}$ = 143 ± 2 nm). Therefore, for practical reasons this slight increase was deemed acceptable and for future syntheses a heating time of 5 h was used.

To increase Ag_2S-NP yield, the amount of starting materials were increased by approximately five times (A) and ten times (B) (Table 2). When the masses of starting materials were increased by 10x (B), the resulting NP suspension was unsuccessful; d_h was > 1000 nm and the majority of Ag_2S was not re-suspended (solution was clear and colourless). This may be due to unsuccessful sonication: the quality of the particles may have changed with the use of higher masses of starting materials which, in turn, may have promoted the precipitation of larger Ag_2S aggregates.

When starting masses were increased by 5x (A), d_h remained below 150 nm (~140 nm) and the suspension was a clear yellow colour which is evidence for successful NP re-suspension.

The final variable that was investigated was the addition of citrate (3 mM) to the solution before heating, with the aim to electrostatically stabilise the NPs which in turn would decrease average particle size. However, DLS analysis showed that this method actually increased particle size (264 ±10 nm). Therefore this method was rejected.

4. Conclusion

In order to disperse Ag_2S-NPs in a stable homogenous suspension, the following parameters for synthesis and re-suspension were successful: 1.36 mmol $AgNO_3$ and 1.36 mmol of L-cysteine; heating at 180°C for 5 h; addition of humic acid (5 mg L^{-1}) prior to sonication; sonication for 10 min at 90W and centrifugation at 2300 *g*; and, a second sonication and re-centrifugation step using the same conditions to increase Ag concentration. This method produces a Ag_2S-NPs suspension that has an average hydrodynamic diameter of 143 ± 2 nm PdI of 0.11, zeta potential of -30 mV and Ag concentration of ~ 20 mg L^{-1}.

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Appendix 2

Methods for growing lettuce in sandy soil

1. Introduction

For the pot trials described in **Chapter 3**, lettuce was grown in a slightly acidic sandy soil (pH = 5.8, sand = 95% sand). This soil was collected from Mt. Compass, South Australia and represented a worse-case scenario in terms of potential plant uptake of AgNPs for two reasons: 1) acidic conditions are conducive to AgNP dissolution and 2) the low soil concentrations of organic matter and clay may increase the bioavailability of AgNPs and Ag^{+} [1]. Given the very low concentration of soil organic matter and nutrients in Mt. Compass soil, a nutrient solution that provided the required nutrients without being toxic to lettuce had to be found.

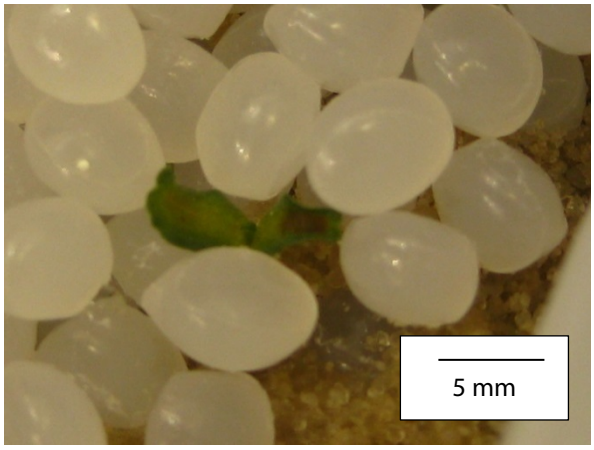

Figure 1. Lettuce shoots in Mt. Compass soil after three weeks of growth using Nitrophoska® Blue granular fertiliser. Only one shoot emerged (four were planted per pot) and shoots did not grow any more than shown.

2. Materials and methods

The efficacy of two nutrient solutions and one fertiliser granular application were tested. The two nutrient solutions (A and B) had been used previously in our laboratory to grow wheat and barley in a sandy soil. The chosen granular fertiliser, Nitrophoska® Blue Special, is recommended by the Western Australian Department of Agriculture and Food for growing lettuce in sandy soils [2].

Lettuce was grown in free-draining pots to prevent the accumulation of salts. Two layers of gravel (large and small sized) were placed in the bottom of each pot followed by 200g (dry weight) of Mt. Compass soil. Five pre-germinated lettuce seeds were added to each pot. Soils were maintained at 150% of their maximum water holding capacity (MWHC) by adding de-ionised water to pots daily. This value is higher than usual due to the low MWHC of Mt. Compass soil (0.035 g g^{-1}).

The composition of each nutrient solution is given in Table 1. To avoid precipitation of some elements (e.g. phosphorus and iron), a minimum of two solutions were prepared for each nutrient solution. Solutions were prepared and added to the pots in order to achieve the target nutrient soil concentrations after 8 weeks of plant growth; 0.5 mL of each nutrient solution per week. After 4 weeks of growth, a visual inspection of plants was used to determine the best nutrient solution for future experiments.

3. Results

Lettuce did not grow in soil that was amended with nutrient solution A or B. The fertiliser granule was slightly more successful; however, after 4 weeks of growth, shoots were < 1 cm and did not continue to grow after this (Figures 1 & 2). Therefore, a new nutrient solution was developed.

Figure 2. Lettuce shoots in Mt. Compass soil. Left - growth after three weeks using nutrient solution A; middle – growth after three weeks using the new nutrient solution; right – growth after 7 weeks using the new nutrient solution.

Table 1. Composition of the three nutrient solutions and the granular fertiliser that were tested. Target soil concentrations of each nutrient are shown soil (mg kg $^{-1}$). Two fertiliser granules (Nitrophoska® Blue) were added to each pot. Concentrations of certain micro-nutrients in the granular fertilise were not specified by the manufacturer ('Trace').

		Nutrient solution (mg nutrient kg soil^{-1})			
Compound	Nutrient	A	B	New	Granule ^ (%)
KH_2PO_4	P	100	34.1	75.0	5.2
NH_4NO_3	N	50	122.4	280.0	12.0
$(NH_4)_2SO_4$	N		0.03		
KCl	K	100			14.1
K_2SO_4	K		53.2	530.0	
$MnSO_4.H_20$	Mn	5	1.1		Trace
$MnCl_2$	Mn			0.75	
$CuSO_4.5H_20$	Cu	3	0.5	0.05	Trace
$ZnSO_4.7H_20$	Zn	10		1.0	
$MgSO_4.7H_2O$	Mg	25	8.8	87.5	0.01
$CoSO_4.7H_2O$	Co		0.2		
$FeSO_4.7H_2O$	Fe		0.3	2.0	Trace
H_3BO_3	B		0.2	0.75	0.02
$(NH_4)_2SO_4$	N		0.03		
$(NH_4)_6Mo7O_{24}.4H_20$	Mo			0.1	Trace
$CaCO_3$	Ca			187.5	Trace
	S				8.0

Table 2. Elemental concentrations of internal standard (lettuce). The heads of 20 mature lettuce plants were analysed to produce this standard.

	Ca	K	Mg	Na	P	S	B	Cu	Fe	Mn	Mo	Zn
Average	9527	30127	5287	21648	7711	2383	35.4	8.00	94.8	59.8	1.52	50.1
Standard deviation	376	2059	234	1424	355	121	5.2	0.38	9.9	2.3	0.10	4.4

Concentrations of nutrients in the new solution were calculated from an internal lettuce standard that is used in our laboratory. The internal standard was previously prepared from 20 lettuces (Iceberg variety) that were purchased from a supermarket. Lettuce was oven dried (40°C), homogenised, acid-digested and analysed using inductively couple plasma mass spectroscopy (ICP-MS) or ICP-optical emission spectroscopy. Elemental concentrations of the internals standard are shown in Table 2. Based on these concentrations, the nutrient requirements for each lettuce plant in the pot trial were calculated. To convert these concentrations to target soil concentrations, a number of factors were taken into consideration: 1) four plants were planted per pot; 2) plants would be harvested after 8 weeks of growth; 3) at harvest, each plant would weigh 8.2 g (fresh weight); 4) 90% of lettuce weight is water, therefore, at harvest the dry weight of each plant would be approximately 0.82 g. The nutrient solution was prepared so that 1 mL would be added to each pot once a week for eight weeks. Theoretically, after 8 weeks, the amount of each nutrient added to each pot would equal the amount required by four lettuce plants. This method assumes that 100% of the added nutrients are available to the plant, which is unlikely to occur.

After 4 weeks of growth, shoots that were grown in the new nutrient solution were significantly larger than those that were treated with nutrient solutions A and B and/or the granular fertiliser (Figure 2). After 7 weeks, the plants continued to grow and appeared healthy (Figure 2) and, therefore, this nutrient solution was used for the pot trials that are described in **Chapter 3.**

4. References

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Appendix 3

Effect of agricultural amendments on silver speciation in soil

Results from **Chapter 3** showed that the application of soil amendments, specifically H_2O_2 and mono-ammonium phosphate (MAP, $NH_4H_2PO_4$), significantly decreased ($p < 0.05$) Ag concentrations in plant roots compared to the control (no fertiliser). A number of mechanisms may have caused this unexpected result, including the formation of insoluble Ag complexes. Therefore, the Ag speciation in soil was investigated using synchrotron based X-Ray absorption spectroscopy (XAS).

1. Materials and methods

1.1. Soil preparation and silver addition to soil

The same soil that was used in **Chapter 3** (collected from Mt. Compass, South Australia), was dried at 60°C for 5 d and sieved (2 mm). Silver was spiked at a nominal concentration of 100 mg Ag kg^{-1} as $AgNO_3$, AgNPs or Ag_2S-NPs. For each Ag treatment, 100 g of soil was spiked with either a NP suspension or Ag^+ stock solution. Soil was weighed into a zip-lock plastic bag and the spiking solution pipetted directly onto the soil which was then thoroughly homogenised. Soil was diluted to 10 mg Ag kg^{-1} by gradually adding 90 g (equivalent dry mass) of unspiked soil to 10 g (equivalent dry mass) of spiked soil.

To investigate the effect of agricultural amendments on Ag speciation, the same fertiliser treatments that were used in **Chapter 3** were added to 2 g of each Ag spiked soil (No.6 vial). The agricultural amendments – ammonium thiosulfate [ATS, $(NH_4)_2S_2O_3$)], potassium chloride (potash, KCl) and mono-ammonium phosphate [MAP, $NH_4(H_2PO_4)$] – were prepared as 250 mL stock solutions at a concentration of 2 g L^{-1} of S, K and P, respectively. Hydrogen peroxide (0.5%) was pipetted directly on to soil to achieve 150% maximum water holding capacity (MWHC), this MWHC was used in **Chapter 3**. Concentrations of S, P and K and H_2O_2 were the same as that used in the pot experiments (100 mg nutrient kg^{-1}), as was the concentration of H_2O_2 (0.5% solution). All samples were prepared in duplicate. Soil samples were stored in the dark for 10 d in a sealed box at 20°C. Samples were aerated daily and maintained at 150% MWHC using ultrapure Milli-Q water, except for H_2O_2 treated soil which was maintained at the desired MWHC using H_2O_2 solution. After 10 d, soils were dried for 48 h (40°C) and analysed within 4 d as described below.

1.2. Preparation of silver spiking solutions

Silver nanoparticle and Ag_2S-NP suspensions that were added to soils were prepared as described previously (**Chapters 2 – 4**). See **Chapters 2 – 4** for NP characterisation. In summary, the average hydrodynamic particle diameters (d_h) and zeta potentials (ζ) for AgNPs and Ag_2S-NPs were 44 nm and 152 nm, and -50 mV and -43 mV, respectively. The uniform dispersity of NP suspensions was evident from the close correlation between d_h and crystallite size (XRD) for AgNPs [1] and the low polydispersity index (PdI) (0.21) recorded for Ag_2S-NPs [2].

Ionic Ag solutions were prepared to the desired concentrations by dissolving $AgNO_3$ powder (Sigma Aldrich) in ultrapure Milli-Q water.

1.3. Solid phase speciation of silver in soils using synchrotron radiation

Solid phase speciation of Ag in soils treated with agricultural amendments was examined using X-ray absorption spectroscopy (XAS), specifically, X-ray absorption near edge structure (XANES) analysis. Silver K-edge X-ray absorption spectra were recorded on beamline 5 BM-D at the Advanced Photon Source (APS) at Argonne National Laboratory, Argonne, IL. The 7 GeV storage ring was maintained at a current of 100 mA with a top-up fill status during sample analysis. The 5-BM-D beamline of DND-CAT has an energy range of 3.6 eV – 75 eV. The X-ray beam was tuned with a Si (111) double-crystal monochromator which was detuned 65% to minimise the harmonic contamination in the incident beam. To calibrate the X-ray energy, the spectrum of a metallic Ag foil was recorded in parallel in transmission mode. Three spectroscopic grade ionisation chambers (Oxford-Danfysik), placed in series and filled with gas mixtures appropriate for the Ag K-edge (25514 eV) measurements, were used to monitor the incident X-ray intensity and to conduct the XANES measurement in transmission mode for the Ag standards.

XANES spectra of soil samples were collected in fluorescence mode by collecting the Ag K-edge fluorescence emission. The incident X-ray beam impinged on the sample at 45° and fluorescence emissions from the sample were collected by a Canberra 13-element Ge solid stage detector system at 90° to the incident beam using XIA electronics (DXP2C, X-ray Instrument Associate). Silver standards that were measured at the APS beamline included PVP-coated AgNPs (Nanoamor), Ag_2S-NPs, silver nitrate ($AgNO_3$), Ag_2S (bulk) , silver oxide (Ag_2O), silver chloride (AgCl), silver carbonate (Ag_2CO_3), silver acetate and silver sulfate (Ag_2SO_4) (all silver salts purchased from Sigma Aldrich). Additional standards were prepared on the day of analysis and stored in the dark until use, including silver phosphate (Ag_3PO_4) and Ag bound to thiosulfate, glutathione, goethite and kaolinite. Standards were prepared according to the methods described in **Chapter 2**.

The chemical speciation of each sample was determined by fitting a linear combination of standard compounds to each XANES spectrum. Principal component analysis and target transformation were

used to determine the number of components in each sample XANES spectrum. The number of significant components that were used to fit the sample spectra was chosen based on the minimum indicator value (IND). Linear combination fitting of the identified standards by PCA was performed using Athena over the XANES region (−20 eV to + 50 eV). The adequacy of the fits was judged based on the R factor and reduced chi square values. The XANES spectra were calibrated, averaged and normalised (over the range of 12 to 95 eV) using Athena.

2. Results and discussion

Nine standards were used in the linear combination fitting (Figure 1). The R-factor for all samples was < 0.001, indicating good quality of fits. XANES spectra of control soils (no fertiliser) that were spiked with Ag^+, AgNPs, and Ag_2S-NPs differed to that of the standards, meaning that Ag soil speciation changed over the 14 d incubation period (Figure 2). The Ag^+ control soil was dominated by AgCl (51%), Ag_2CO_3 (33%) and AgNP (17%) (Figure 3). In AgNP treated soil, although AgNP was the predominant species (71%), a proportion of Ag was present at Ag_2S-NP (30%). Previous studies have also observed Ag_2S in AgNP spiked soil (pH = 6.9, 42 mg Ag kg^{-1} [3]). In that experiment, XAS analysis was carried out immediately after AgNPs were added to the soil, and it was found that 73% of Ag was present at AgNP and 27% as Ag-S species. In the current study, Ag_2S-NP treated soil was dominated by Ag_2S-NP (63%) and contained approximately equal amounts of other Ag-S bound species; specifically Ag_2S-bulk, Ag-glutathione and Ag-thiosulfate.

The XANES spectra of Ag_2S-NP treated soils were relatively unchanged following the addition of fertilisers. However, in all fertiliser amended soils, the proportion of Ag_2S-NP did increase compared to the control (> 89% vs. 63%); this increase was consistent for all fertiliser treatments. Given that in the pot trial, plants that were grown in Ag_2S-NP treated soil had the

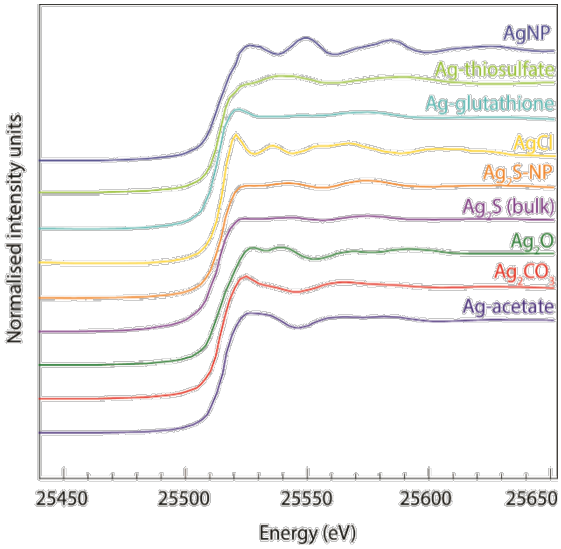

Figure 1. Silver (Ag) XANES K-Edge spectra for the Ag standards that were used in linear combination fitting of sample spectra.

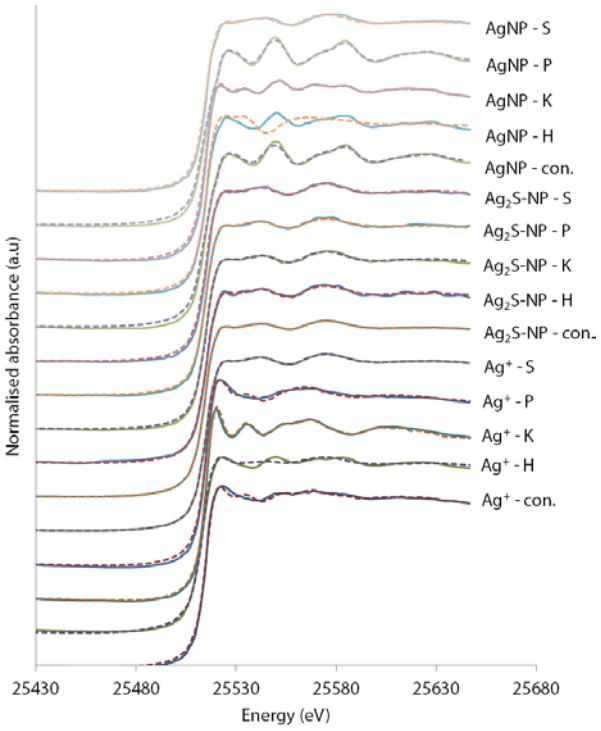

Figure 2. Silver XANES K-Edge spectra of soils spiked with ionic Ag (Ag^+), silver nanoparticles (AgNP) and silver sulfide nanoparticles (Ag_2S-NP) amended with ammonium thiosulfate (S), mono-ammonium phosphate (P), potassium chloride (K) or hydrogen peroxide (H) or unamended (cont.).

lowest Ag concentrations, the speciation results suggests that Ag_2S-NPs are relatively stable and resistant to dissolution. The speciation results for Ag_2S-NP treated soil are somewhat surprising because in **Chapter 3** the application of thiosulfate was shown to significantly increase the plant uptake of Ag. In fact for all Ag treatments in the current study, when thiosulfate was applied, Ag_2S-NP was the dominant Ag species. The synchrotron results do not adequately explain the differences in Ag uptake that were observed in **Chapter 3**. However, the XANES profiles are very similar for Ag-thiosulfate, Ag_2S-NP and Ag_2S-bulk (Figure). Therefore, as Ag_2S-NPs cannot be distinguished from other Ag-S bound species, the synchrotron results are inconclusive in regards to the specific Ag species being taken up by plants. In addition, the results may due to a concentration effect as the Ag soil concentration in this experiment (100 mg Ag kg^{-1}) was greater than in the pot trials.

Results from **Chapter 3** showed that KCl could significantly increase the plant uptake of Ag in Ag^+ and AgNP treated soils but not in Ag_2S-NP treated soil. XAS analysis showed that AgCl was the dominant Ag species in Ag^+ and AgNP treated soils that were amended with KCl (98% and 56%, respectively). In Ag_2S-NP–KCl treated soil, AgCl was not detected. This suggests that AgCl is more bioavailable than Ag-S species and that Ag_2S-NPs are more resistant to dissolution that AgNPs.

The main focus of this experiment was to understand why PO_4^{3-} and H_2O_2 significantly decreased the plant uptake of Ag from Ag^+ and AgNP treated soil compared to the controls. XANES spectra

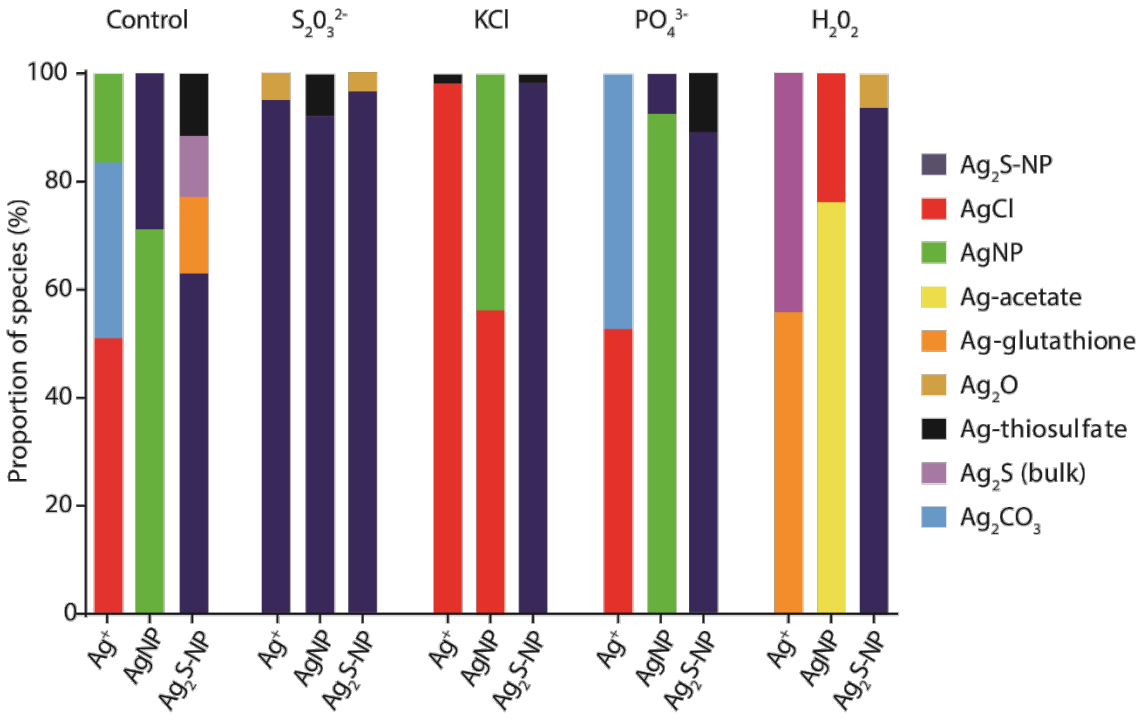

Figure 3. Silver speciation of soil samples. The percentage of each silver species was determined from linear combination fitting analysis of the XANES spectra of soils amended with fertilisers: ammonium thiosulfate ($S_2O_3^{2-}$); potassium chloride (KCl); mono-ammonium phosphate (PO_4^{3-}); and hydrogen peroxide (H_2O_2).

of Ag^+–$PO_4^{3-}$and AgNP–PO_4^{3-} treated soils showed only minor differences compared to the controls (no fertiliser amendment). For example, in Ag^+ treated soil, the main differences between the control and PO_4^{3-} treated soil was the slightly greater proportions of AgCl (51% cf. 53%) and Ag_2CO_3 (33% cf. 47%) and the absence of AgNP species in PO_4^{3-} treated soil. If speciation alone was responsible for the decreased uptake of Ag in PO_4^{3-} treated soil, then these results would suggest that AgNP is the main species being taken up by plants and that its absence in PO_4^{3-} treated soil was the reason for low Ag uptake observed in **Chapter 3**. However, this hypothesis is in direct conflict with the results obtained for AgNP–PO_4^{3-} treated soil. In that soil, AgNP was the dominant Ag species (92%), yet the plant uptake of Ag was significantly less than the control. Therefore, results from speciation analysis alone are unable to explain the decrease in plant Ag uptake following PO_4^{3-} addition.

Following H_2O_2 treatment, XANES spectra of Ag^+ and AgNP treated soils changed considerably. In Ag^+ treated soil, Ag-glutathione and Ag_2S-bulk were dominant (56% and 44%, respectively), whereas they were not significant components in the control. Similarly, in AgNP treated soil, Ag-acetate and AgCl were predominant (76% and 24%, respectively), yet these species were not detected in the AgNP control soil. The structure of the Ag-acetate standard was not verified by other methods; however, its XANES spectrum does appear significantly different to that of AgNP and Ag-S. Hydrogen peroxide may have promoted AgNP dissolution and any released Ag^+ may have adsorbed to carboxyl groups on the surface of soil organic matter [4]. These results suggest that Ag-carboxyl species are less bioavailable than AgNP and Ag–S species. This is surprising given the very low bioavailability and high stability of Ag_2S [5].

Results from XAS analysis are inconclusive and do not adequately explain the results that were obtained in the pot trail (**Chapter 3**). A number of factors may have caused this including; quality of the standards, inadequate incubation time and the fact that soils were not collected from pots that contained the plants. The latter may have had a significant impact as root exudates can alter the metal speciation around the root zone [6-8].

In the current experiment, differences in Ag speciation were observed between the control soils and H_2O_2 and PO_4^{3-} treated soil; however, due to similarities between standard spectra, the exact Ag species could not be confirmed. The results suggest that the formation of Ag-carboxyl species may decrease AgNP bioavailability. However, further XAS experiments are required to confirm the speciation of Ag in soils amended with fertiliser in the presence of growing plants.

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