

Centre for Research into Environment and Health

Silver: water disinfection and toxicity

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Appendix 1: Disinfectant mode of action

List of abbreviations

Ag	silver
AgCl	silver chloride
AgNO ₃	silver nitrate
AgNP	silver nanoparticles
CICAD	concise international chemical assessment document
DLS	direct light scattering
DNA	deoxyribonucleic acid
EC ₅₀	half maximal effective dose
GSH	glutathione
HRT	hydraulic retention time
ic	initial concentration
IC ₅₀	half maximal inhibitory concentration
LC ₅₀	median lethal dose – dose required to kill half the members of a test population after a specified test duration
LDH	lactate dehydrogenase
MIC	minimum inhibitory concentration
NO	nitric oxide
PCNA	proliferating cell nuclear antigen
POU	point-of-use
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
SOD	superoxide dismutase
TEM	transmission electron microscopy

1. Background

This literature review, on the use of silver (Ag) as a water disinfectant and the toxicity of silver, is designed as a background document (based on a literature review up to the end of July 2013) for the Expert Working Group on Drinking-water Guidelines and to provide the basis for the development of a short factsheet. The report considers both ionic silver, silver nanoparticles (AgNP) and copper/silver applications. The aim is to look at the literature to determine:

- Is silver an effective water disinfectant (does it work)?
- Is silver toxic (does it do you harm)?
- Is there enough evidence to make a judgement on the above?

2. Introduction

2.1 Antimicrobial properties

Silver has been known to have antibacterial properties since Roman times, however, the increased use of nanosilver in a range of (as yet largely) experimental drinking-water treatment systems, its use in conjunction with ceramic filters and its perceived potential to be a water disinfectant that does not result in disinfection by-products (DBP) in the treated water have raised the profile of this chemical.

Silver and AgNP have been shown to have general (i.e. not specifically water disinfection related) anti-bacterial properties against a range of both Gram-negative (e.g. *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Salmonella* and *Vibrio*) and Gram-positive bacteria (e.g. *Bacillus*, *Clostridium*, *Enterococcus*, *Listeria*, *Staphylococcus* and *Streptococcus*) – Wijnhoven *et al.* (2009). Some researchers have also demonstrated that fungi, such as *Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisia*, are sensitive to silver (reviewed by Marambio-Jones and Hoek, 2010). In addition, a number of studies have suggested a biocidal action of AgNP against hepatitis B virus (Lu *et al.*, 2008), HIV-1 (Elechiguerra *et al.*, 2005), syncytial virus (Sun *et al.*, 2008) and murine norovirus (De Gussemme *et al.*, 2010).

2.2 Nanoparticles

According to a review issued by the European Commission (2013) and cited by Bondarenko *et al.* (2013), nanomaterial is defined as “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more of the external dimensions is in the size range 1-100nm.” In the scientific literature, nanoparticles are usually defined as particles having one or more dimensions in the order of 100nm or less (Moore *et al.*, 2006). Although the terminology may be relatively new, the use of AgNP is not (Nowack *et al.*, 2011), with the first report of nanosilver probably being in 1889, when Lea reported the synthesis of a citrate-stabilised silver colloid (which has an average particle size between 7 and 9nm).

The most common method of producing AgNP is the chemical reduction of a silver salt (often AgNO₃) dissolved in water with a reducing compound such as sodium borohydride, citrate, glucose, hydrazine and ascorbate (Marambio-Jones and Hoek, 2010). There is, however, an almost bewildering array of different manufacturing methods (including spark discharging, electrochemical reduction, solution irradiation and cryochemical synthesis) some of which have been outlined by Marambio-Jones and Hoek (2010). In addition to different manufacturing methods, different capping or stabilising agents may be used; these are generally used to prevent the AgNP from aggregating or agglomerating (Ema *et al.*, 2010) and common examples include polyvinylpyrrolidone (PVP) and citrate (Völker *et al.*, 2013). The different methods employed in the manufacturing process result in

AgNP with different sizes (typically <50nm), shapes (e.g. spheres, rods and cubes) and characteristics.

2.3 Water-related applications

In terms of water-disinfection-related applications, silver is most commonly used in domestic water filters (either to reduce the level of biofilm growth within the filter or as an additional level of treatment). It is also quite commonly used in conjunction with copper ionization as a preventative measure against colonization of *Legionella* spp. in hospital hot water systems. AgNP are currently being tested in a number of experimental point-of-use (POU) treatment systems and ionic silver has been investigated for its potential for use as a secondary disinfectant (to reduce levels of chlorine) in drinking-water supplies. Silver ions (in combination with both copper and chlorine) have also been investigated for use in swimming pool disinfection.

3. Water disinfection efficacy

Numerous studies have been conducted on the disinfection efficacy of silver and AgNP applications against a range of microorganisms found in water. Although the majority of these have focused on bacterial disinfection, some have also looked at the impact on bacteriophages and viruses. In addition to the material below, which focuses on water disinfection, there is also a short section (Appendix 1) on the general disinfectant mode of action of Ag and AgNP.

3.1 Ionic silver applications

3.1.1 Efficacy of ionic silver for disinfection of potable water

In the studies outlined below silver ion (Ag^+) efficacy (generated from silver salts [AgNO_3 , AgCl] or produced electrolytically) was tested against a range of bacteria and the inactivation was principally assessed by the log reduction in bacterial numbers. Initial bacterial concentrations ranged from 3.5 cells/ml up to 1.5×10^7 cells/ml.

Hwang *et al.* (2007) looked at the efficacy of silver ions (up to $100\mu\text{g/l}$), derived from silver nitrate, against *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Escherichia coli* (all at 1.5×10^7 cells/ml) in synthetic drinking-water (pH 7, temperature 25°C). After a three hour contact time with the highest concentration of silver the following log reductions were reported:

- 2.4 log reduction - *L. pneumophila*;
- 4 log reduction – *P. aeruginosa*;
- 7 log reduction – *E. coli*.

Similar work was conducted by Huang *et al.* (2008), where the efficacy of silver ions, derived from AgCl , against 3×10^6 cfu/ml of *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* was investigated. A 5 log reduction in *P. aeruginosa* was seen with $80\mu\text{g/l}$ Ag (the highest concentration used) after 12 hours. *S. maltophilia* was more sensitive to Ag, with a 5 log reduction seen after 6 hours when exposed to $80\mu\text{g/l}$. For *A. baumannii*, however, a 5 log reduction was only seen after 72 hours exposure to $80\mu\text{g/l}$ Ag.

Silvestry-Rodriguez *et al.* (2007) investigated the inactivation of *Pseudomonas aeruginosa* and *Aeromonas hydrophila* by silver in tap water, with a view to assessing the possibility for using silver as a secondary disinfectant to replace or reduce the level of chlorine. Dechlorinated municipal water (obtained from a groundwater source) was seeded with 10^6 cfu/ml bacteria and silver nitrate added to a concentration of $100\mu\text{g/l}$. Experiments were performed at pH 7 and pH 9 at 24°C for both bacterial species and at 4°C for *P. aeruginosa*. In addition, 3 mg/l humic acid was added to the dechlorinated tap water (to simulate a surface water source). Inactivation of the bacteria was time

and temperature dependent and after 8 to 9 hours of exposure to 100 µg/l silver at 24 °C, there was more than a 6 log reduction in both bacteria (at 4 °C a 4.5 log reduction in *P. aeruginosa* was seen only after 24 hours). Silver was found to be almost as effective in reducing bacteria in the presence of humic acid (5.5 log reduction in *P. aeruginosa* at pH 7, 24 °C after 8 hours in the presence of 3mg/l humic acid). This group also looked at the potential for exposure to silver (100 µg/l) to reduce biofilm formation in drinking-water distribution systems (Silvestry-Rodriguez *et al.*, 2008). In this role, silver was found to be ineffective, and there was no difference seen between the silver treatment and the control.

Cunningham *et al.* (2008) used flow cytometry to examine the minimum inhibitory concentration (MIC) of AgNO₃ on *E. coli*, with a view to the methodology being used to examine water and wastewater disinfection. They reported a 24 hour MIC of between 60 and 80µg/l for silver. A 4 log reduction (approximately) was seen at 100µg/l after 24 hours of exposure.

Pathak and Gopal (2012) evaluated the efficacy of silver ions against *E. coli*. Bacteria (concentration - 1.75 x 10³ cfu/ml) were exposed to various concentrations of silver ions (1, 2, 5, 10 and 20 µg/l), produced from silver electrodes, for up to 60 minutes. Complete bacterial inactivation was seen at neutral pH and ambient temperature after a 20 minute period for the 20 µg/l concentration. 100% bactericidal activity was also seen for the other silver concentrations (with the exception of 1 µg/l), although a longer contact time was required (10 µg/l – 40 minutes; 5 µg/l – 50 minutes; 2 µg/l – 60 minutes). Disinfection was most efficient at pH values between 8 and 9 and at temperatures greater than 20 °C.

Nawaz *et al.* (2012) looked at the efficacy of silver (AgNO₃) in removing *P. aeruginosa* and *E. coli* in rooftop harvested rainwater supplies. Prior to disinfection, samples were found to contain between 350-440 cfu/100ml *P. aeruginosa* and 740-920 cfu/100ml *E. coli*. The disinfection rate and residual effect of silver was determined using final silver concentrations between 10-100 µg/l over a period of up to 168 hours. Samples were taken for microbial analysis every two hours for 14 hours after the application of silver and then daily for 1 week, to examine regrowth. At higher concentrations (80-100 µg/l) complete inactivation of both microorganisms was seen in 10 hours, with no regrowth of *E. coli* seen after 168 hours. Inactivation was slower at lower concentrations (95-99% inactivation for silver concentrations between 10-40 µg/l after 14 hours) and regrowth was also observed (e.g. 7.5% survival of *P. aeruginosa* exposed to 10µg/l silver for 168 hours compared to approximately 4.5% survival at 14 hours), thus, at the lower concentrations, silver only delayed bacterial reproduction and did not cause permanent damage. Adler *et al.* (2013) also looked at the effectiveness of silver disinfection as part of rainwater harvesting treatment. Ten rainwater harvesting systems in Mexico, equipped with silver electrodes were evaluated for a number of water quality parameters. The silver electrodes were located in line with the filtering system (after a mesh filter, designed to remove large particles, and before an activated carbon filter). On average, the ionisers reduced the level of total coliforms by approximately 1 log and *E. coli* by approximately 0.4 log and resulted in a silver concentration of approximately 0.01mg/l in the final water. The systems, as a whole, delivered water containing zero *E. coli* and less than 10/100ml total coliforms.

In a comparative study of disinfectants, the potency of silver ions, derived from AgNO₃, was examined in a batch disinfection test of ground water using 10⁶ cfu/ml *E. coli* (Patil *et al.*, 2013). It was found that for a 6 log reduction (i.e. complete inactivation), the minimum concentration of silver required was 10mg/l with a contact time 3 hours.

It can be seen from these studies that log reductions varied widely with some bacteria being more sensitive to silver (i.e. more easily killed or inactivated) than others. Generally, relatively long contact times were required to effectively reduce bacterial concentrations (e.g. 3 hours or longer), the exception being the study of Pathak and Gopal (2012) where silver ions were generated electrolytically (rather than from silver salts), and complete inactivation (3 log) was seen after 20

minutes at a relatively low silver concentration (20µg/l). In contrast to the lab spiked samples, where generally good log reductions were reported, relatively poor results (lower log reductions) were seen in harvested rainwater samples (low initial bacterial concentration) used by Nawaz *et al.* (2012) and they suggested that this may result from greater resistance to disinfection in microbes grown in low nutrient systems.

3.1.2 Copper/silver applications

Copper/silver is generally applied to water as an ionization process, with the electrolytic generation of copper and silver ions, sometimes used in combination with a halogen (e.g. chlorine, iodine), although it may also be applied as copper and silver salts. Copper/silver systems are generally used for *Legionella* control (typically in hospital hot water systems) and have been investigated for the treatment of swimming pool water.

Hospital water systems

Copper/silver ionization is often used for *Legionella* control in hot water distribution systems especially in hospital environments. The studies outlined in this sub-section typically relate to systems that are in use and so tend to assess samples for the presence/absence of the organism of interest, rather than using laboratory tests to determine log reduction. It is generally considered that ion levels should remain within the range of 0.2-0.4 mg/l copper and 0.02-0.04 mg/l silver to maximize efficacy (Cachafeiro *et al.*, 2007).

Liu *et al.* (1998) looked at the intermittent use of a single copper/silver ionization system in the hot water systems of two buildings. 20 distal sites in each building were examined for *Legionella* before the start of ionization and then monthly after installation. The elimination of *Legionella* took between 4 and 12 weeks. After cessation of disinfection (16 weeks), re-colonization did not occur for between 6 to 12 weeks (depending on the sampling site) in the first building and 8 to 12 weeks in the second building. The control building (no ionization) remained positive for *Legionella* throughout the study period.

In 2003, Stout and Yu reported on surveys of the first 16 hospitals in the USA to install copper/silver ionization systems for *Legionella* control. Prior to installation, all of the hospitals had reported cases of nosocomial Legionnaires' disease and 75% had attempted other disinfection methods. Colonization of distal water sites with *Legionella* was much less frequent after installation (although it did still occur) and no cases of nosocomial Legionnaires' disease had been reported at any of the hospitals since installation.

In Switzerland, Blanc *et al.* (2005) found that copper/silver ionization was not effective at reducing *Legionella* in their hospital hot water system (90% of water samples were positive for *Legionella* before treatment, 93% positive after the introduction of ionization), although they acknowledged that the low concentration of ions (copper 0.3 mg/l, silver not reported) and the high pH (7.8 – 8.0) of the hot water may have explained the poor results. Ionization in conjunction with increased temperature (65 °C), however, was more effective, with the number of *Legionella* positive samples falling to 39% and the level of *Legionella* in the positive samples also decreasing (mean of 7.6 cfu/ml with ionization alone compared to a mean of 0.23 cfu/ml with ionization and a raised temperature).

Mòdol *et al.* (2007) found that while a copper/silver ionization system was only moderately successful in reducing the number of positive *L. pneumophila* samples isolated from the hospital hot water system (57% of samples were positive before installation compared to 16-21% after installation of the system), the level of hospital-acquired Legionnaires' disease dropped dramatically from 2.45 cases per 1000 patient discharges down to 0.18 cases per 1000 patient discharges.

Pedro-Botet *et al.* (2007) investigated the impact of copper and silver ionization on fungal colonization of a number of health care centre water systems after noticing that the number of

consultations regarding fungal infections in their centre had dropped markedly since the installation of an ionization system (for *Legionella* control). Samples from ionized water distribution systems (nine health care centres) were compared with non-ionized systems (seven health care centres). The prevalence of fungi was significantly lower in the samples of ionized water (29% compared to 77%) in both hot and cold water systems – with the most marked difference seen in the cold water samples (14% compared to 88%). A decrease in fungal colonization following the implementation of copper/silver ionization was also reported by Chen *et al.* (2013). They found a 40% reduction in fungal colonization during ionization treatment, with fungi isolated from only 2% of samples during this period.

Chen *et al.* (2008) looked at the efficacy of a point-of-entry copper/silver ionization system (designed to treat both hot and cold water) against *L. pneumophila* in a hospital water distribution system. Prior to installation, typically between 32% and 50% of samples were positive for *L. pneumophila*. In the first three months, no change was seen in the number of positive samples. When ion concentrations were increased (months 4 to 7), however, the number of positive samples decreased significantly to between 5 and 16%. Rates of *Legionella* positivity dropped further to between 0 and 5% after month 7. Mean positivity remained at 50% in the control (non-treated) sites. The ion concentrations varied between sampling sites and over the course of the monitoring. Mean levels were 0.16 mg/l Cu and 0.014 mg/l Ag, slightly below the target concentrations of 0.2 and 0.02 mg/l respectively. The authors note that, while the system did not completely eradicate *L. pneumophila*, no cases of nosocomial Legionnaires' disease were reported during the year-long study.

In a lab-based study Pianetti *et al.* (2008) examined the efficacy of various combinations and concentrations of copper and silver ions (from CuCl₂ and AgCl) and free chlorine in inactivating *L. pneumophila* in water samples (contact period of up to 24 hours). Three different water supplies were used, tap water, spring water and distilled water and were spiked with 10⁶ cfu/ml *L. pneumophila*. Copper and silver ions (0.4/0.04 mg/l) did not completely eradicate the bacteria even after a 24 hour contact time at 22 °C. Using higher concentrations of copper and silver (up to 0.8/0.08 mg/l) produced varying results depending on the type of water, and the authors speculate that the physical and chemical properties of the water, especially its chloride content (which can combine with Ag⁺ reducing ion availability), may affect the copper/silver disinfection process. The combination of copper and silver with 2 mg/l chlorine was more effective than the chlorine dose on its own, suggesting a synergistic effect.

According to Lin *et al.* (2011) emergence of *L. pneumophila* with resistance to copper/silver ions has been documented in some cases, usually several years after installation of the ionization system, although hospitals where ion concentrations and *Legionella* positivity were monitored were less likely to report resistance problems. Recommended concentrations of ions for *Legionella* eradication are between 0.2 to 0.4 mg/l copper and 0.02 and 0.04 mg/l silver, although Lin *et al.* (2011) recommend slightly different concentrations of 0.2 to 0.8 mg/l copper and 0.01 to 0.08 mg/l silver, suggesting that concentrations towards the lower end of the range may be effective after the initial installation.

In 2012, five confirmed nosocomial cases of Legionnaires' disease and 16 probable nosocomial cases were identified in Pittsburgh, USA at one of the first hospitals to adopt copper/silver ionization for *Legionella* treatment in 1993 (Smeltz, 2012). The disinfection system has been blamed for the outbreak, but it has been speculated that it may have been lack of appropriate monitoring and control that allowed the outbreak to happen, rather than a problem with copper/silver ionization per se (http://www.specialpathogenslab.com/PittsburghVA_Commentary.pdf). Results from the CDC investigation, however, showed that the copper and silver concentrations were generally within the recommended range for *Legionella* control (<http://www.cdc.gov/washington/testimony/2013/t20130205.htm>). All 11 samples assessed for

copper and silver ion concentrations grew *Legionella* (nine of which were positive for the outbreak strain).

Typically, it would seem that copper/silver ionization reduces the number of *Legionella* (and fungal) positive samples in treated systems; it does not completely eradicate the pathogen. This is to be expected, based on the results of the lab study by Pianetti *et al.* (2008) and the point was made by Cachafeiro *et al.* (2007) who, following a review of the literature, noted that eradication cannot be achieved by any method in isolation and that maintaining high temperatures in hot water systems maximises the effectiveness of the ionization approach. Despite the fact that complete eradication of the pathogen is not achieved, a number of studies have suggested that the implementation of copper/silver ionization markedly reduces the number of cases of nosocomial Legionnaires' disease. The observation of resistant *Legionella* and the outbreak in Pittsburgh suggest that ionization as a treatment system may, however, not be a long-term solution.

Swimming pools

Copper/silver ionization is also suggested for treatment of swimming pool water, although peer-reviewed literature on its efficacy is sparse. Although some company websites seem to advocate the use of copper/silver ionization in isolation, it is suggested from the literature that it should be used with, for example, chlorine in order to maximise the disinfection potential and remove organic materials introduced by bathers (skin, hair, urine etc.) and from the environment.

Yayha *et al.* (1990) looked at the disinfection efficacy of copper and silver ions (0.4mg/l and 0.04mg/l respectively) with and without low levels of free chlorine (0.3 mg/l) on simulated swimming pool water, using *Staphylococcus* sp. as a test organism. In combination, the chemicals achieved a 2.4 log removal of the test organism in 2 minutes, compared to only 1.5 and 0.03 log removal for free chlorine alone or copper/silver alone respectively. The combined chemicals were also found to act more quickly on *Staphylococcus* sp. than 1 mg/l free chlorine (the generally recommended level). Similar work was done using *E. coli* and *Streptococcus faecalis* (0.46mg/l copper; 0.075mg/l silver; 0.2 mg/l free chlorine). A combination of chemicals was found to be most effective in reducing bacterial numbers – 3.5 to 4 log reduction in 30 seconds (Yayha *et al.*, 1989). The copper/silver, low chlorine combination was also found to be effective against *L. pneumophila*, *S. aureus* and *P. aeruginosa* (Landeem *et al.*, 1989). Yayha *et al.* (1992) also evaluated the inactivation of coliphage MS2 and poliovirus by copper and silver ions spiked into well water samples. Copper and silver (0.4:0.04mg/l respectively) were found to be far less effective at inactivating both viruses than 0.3 mg/l free chlorine, with inactivation by the copper/silver being at least 100 slower than the free chlorine. However, a combination of copper/silver and free chlorine was found to be more effective when compared with water systems containing either metals or free chlorine alone. Coliphage was found to be approximately ten times more sensitive to the disinfectants than poliovirus.

Abad *et al.* (1994), assessed the efficacy of copper (0.7mg/l) and silver (0.07mg/l) in combination with low levels of chlorine against a number of viruses (hepatitis A virus, human rotavirus, human adenovirus and poliovirus). The metal ion, halogen combination was effective against poliovirus (4 log reduction), but less so against adenovirus. Hepatitis A virus and rotavirus showed little inactivation and the authors concluded that the use of copper and silver ions in water systems may not provide a reliable alternative to high levels of free chlorine for the disinfection of viral pathogens. Viruses are known to show better survival rates when they occur as aggregates, rather than as single particles – divalent and trivalent cations have been reported to induce virus aggregation and hence enhance the survival. Abad *et al.* (1994) suggest that in their experiments, virus aggregation may have been induced by Cu^{2+} ions making the free chlorine less effective.

Beer *et al.* (1999) conducted a field test, using copper/silver ions in combination with low levels of chlorine in a municipal pool in the USA. Measurements of total coliforms and heterotrophic bacteria were made during a baseline period (normal operation of the pool, with a free chlorine level of 1

mg/l) and during operation of the copper/silver ionizer with a free chlorine level of 0.4 mg/l. Apart from a single positive sample during the baseline period, all samples were negative for total coliforms. The average heterotrophic plate count was lower during the test period compared to the baseline measurements (20 cfu/ml compared to 91 cfu/ml) but the difference was not statistically significant. Copper concentrations were, typically, about 0.3mg/l.

3.2 Silver nanoparticle applications

The potential of AgNP for drinking-water disinfection is currently being extensively explored, principally in conjunction with filtration. The medium or matrix utilised for the nanoparticles varies widely and includes coating on polyurethane foams (Jain and Pradeep, 2005), fibreglass (Nangmenyi *et al.*, 2009), copolymer beads (Gangadharan *et al.*, 2010), polystyrene resin beads (Mthombeni *et al.*, 2012), alginate composite beads (Lin *et al.*, 2013), ceramic (Lv *et al.*, 2009), titania (Liu *et al.*, 2012), activated carbon composite incorporating magnetite (Valušová *et al.*, 2012) and bacterial carriers (De Gussemme *et al.*, 2010, 2011). As the focus here is on the efficacy of silver in water disinfection, only studies where this can be distinguished from, say, the filtration effect have been considered below. In addition to considering the log reduction of microorganisms exposed to the test material a number of studies also conducted zone of inhibition tests. (The zone of inhibition is the area on an agar plate where the growth of microorganisms is prevented by the antimicrobial activity of the test material placed on the agar surface).

Jain and Pradeep (2005) coated polyurethane foam with citrate-stabilised AgNP. The antibacterial efficacy was assessed by adding small pieces of Ag-treated or untreated foam to *E. coli* suspensions (10^5 - 10^6 cfu/ml) and assessing bacterial growth after a five or ten minute exposure period. No bacterial growth was seen in the samples exposed to Ag-treated polyurethane, while the untreated polyurethane samples showed “substantial growth”. In addition, no growth of *E. coli* was detected on agar plates beneath pieces of AgNP treated foam in a zone of inhibition test. A prototype filter was created using the treated foam, which was found to be effective at eliminating *E. coli* growth, but equivalent data are not available for untreated foam, making the contribution of the silver treatment difficult to determine.

Nangmenyi *et al.* (2009) looked at the performance of AgNP (<30nm) impregnated fibreglass during immersion and during filtration. For the immersion test, a silver impregnated mat (1% Ag by weight) was added to a 100ml *E. coli* suspension (10^6 cfu/ml). After an hour of immersion, *E. coli* could not be detected in the suspension. Using an *E. coli* concentration of 10^{12} cfu/ml, the AgNP fibreglass mat (1.8% Ag by weight) resulted in a 7 log reduction in concentration in five minutes. Antibacterial filters (5% Ag by weight) were fabricated and a bacterial solution (10^6 cfu/ml *E. coli*) was pumped through the filter at a flow rate of 20 ml/minute. *E. coli* were not found in the treated water (a 6 log reduction, of which the untreated fibreglass accounted for an approximately 1 log reduction).

Lv *et al.* (2009) examined the efficacy of AgNP-coated porous ceramic tiles. The ceramic was modified (using a coupling agent) to ensure that the AgNP were fixed to the material (rather than relying on weak forces of attraction). There was no obvious loss of AgNP when the tiles were exposed to water. Antibacterial action was assessed by exposing a solution of *E. coli* (10^4 – 10^5 cfu/ml) to pieces of the treated and untreated (control) ceramic; by conducting a zone of inhibition test and a flow test. After 24 hours, no bacteria could be grown from the samples exposed to Ag-treated ceramic and, in the zone of inhibition test, there was a clear zone where no bacteria grew on the agar plate after 24 hours of exposure to ceramic. In the flow test using an experimental water filter (flow rate 10 ml/min), no bacteria were detected in the filtered water. Substantial (unquantified) concentrations of bacteria, however, were detected in water filtered through untreated ceramic. The authors suggest two possible antimicrobial mechanisms, namely: (a) the bacteria are killed by Ag^+ released from the ceramic; and/or (b) the bacteria flowing from the

ceramic are contaminated with silver, which prevents their subsequent growth. Silver measurements, however, were not reported from the filtered water.

Gangadharan *et al.* (2010) investigated the antibacterial effectiveness of polymer microspheres containing non-leaching silver nanoparticles by incubating various bacteria (*E. coli*, *P. aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*, with concentrations of between 10×10^6 - 300×10^6 cfu/ml), with the beads for up to 24 hours. The beads were found to be effective against both Gram-negative and Gram positive bacteria, with bacterial counts reduced to zero for all strains tested, with the exception of *B. subtilis* (where a 3 log reduction was seen). Zones of inhibition were seen around agar plated beads for all of the bacteria tested. There was no bacterial adsorption or adhesion to the silver-containing beads.

Heidarpour *et al.* (2011) investigated the ability of AgNP-coated polypropylene filters to remove *E. coli* from water. 15 litres of distilled water containing 10^3 cfu/ml *E. coli* was passed through either uncoated or silver-coated filters at a flow rate of 3 litres/hour. After 7 hours of filtration and re-circulation the *E. coli* level from the silver-treated filter was zero, while the concentration from the untreated filter remained at 10^3 cfu/ml. Scanning electron micrographs demonstrated *E. coli* cells attached to the surface of the AgNP coated filter. No AgNP particles were detected in the treated water. The reported bacterial removal is likely to be a combination of the bacteriostatic/bactericidal impact of the silver and the decreased pore size of the Ag-treated polypropylene in comparison with the untreated material (pore size of 1.3 μm and 9.9 μm respectively).

Dankovich and Gray (2011) investigated the efficacy of nanosilver impregnated paper for reducing bacterial contamination in water. The silver nanoparticles were produced *in situ* by the reduction of silver nitrate in the paper sheet. The bactericidal impact was assessed by passing model bacterial suspensions (*E. coli* and *Enterococcus faecalis*) through the paper and analysing the effluent water for viable bacteria. The average percolation time for 100ml of bacterial solution was 10 minutes. Plate counts showed up to a 7.6 log and a 3.4 log reduction of viable *E. coli* and *E. faecalis* (respectively) in the effluent compared to the initial concentration of bacteria (10^9 cfu/ml) at the highest silver concentration (5.9 mg Ag/dry g paper). Of this reduction, less than 1 log was attributed to the filtration of the paper. The average silver content of the effluent water was 50 $\mu\text{g/l}$.

Mpenyana-Monyatsi *et al.* (2012) compared the bacterial removal by a number of low-cost filter materials coated with AgNP. Various concentrations of AgNP were deposited on zeolite, sand, fibreglass, anion resin and cation resin substrates. In the first phase of analysis, the substrates were tested, using *E. coli* spiked water samples (10^6 cfu/100ml), to determine the optimal silver loading (0.1 mM). In the second phase, each of the substrates (with the optimal silver loading) was tested against *E. coli*, *Salmonella typhimurium*, *Salmonella dysenteriae* and *V. cholerae* in groundwater samples (all bacteria present at 10^3 cfu/100ml). The cation resin/silver filter was found to be the best performing, achieving 100% removal of all the targeted bacteria, with no re-growth over 120 minutes. The silver/zeolite filter was found to have the worst performance, with removal rates between 8 and 67%. The amount of silver ions eluted from the filter material varied according to material type and time, with high concentrations released from zeolite, sand, fibreglass and anion resin substrates within the first 10 minutes (maximum concentration 1.8 mg/l). The cation resin filter released the lowest concentration of silver (less than 100 $\mu\text{g/l}$ silver) in the eluent and, thus was found to be the best performing in terms of bacterial removal and silver loss.

Lin *et al.* (2013) synthesized and studied the efficacy of three types of AgNP-alginate composites for application as a POU technology for water disinfection. Alginate was chosen as the immobilization/delivery material because of both its natural abundance and biocompatibility. The finished beads were used to create porous columns and the bacterial removal abilities of the different beads were compared using *E. coli* (approx 10^5 cfu/ml). Two of the three bead types consistently removed all of the bacteria during filtration, even with a short hydraulic retention time

(HRT) and the third bead type produced a 2 log removal. Silver was also measured in the filtered water, again the three bead types produced different results ranging from 11-98 µg/l to 4-22 mg/l (depending on the type and HRT). The authors speculate that the disinfection efficacy, despite the short retention time, is probably due to released silver ions or AgNP in the effluent, which continue to exert an influence over the test bacteria even after plating, although one bead type produced both low silver concentration in the filtered water and excellent removal/inactivation of *E. coli*. The authors concluded that the results suggest that the beads show promise, but note that long-term breakthrough studies are needed.

Loo *et al.* (2013) explored the use of AgNP in cryogels as a possible POU treatment. The AgNP treated gels were added to water containing 10^8 cfu/ml of *E. coli* or *B. subtilis*. After 15 seconds, to allow swelling, the gel was removed from the bacterially spiked (bulk) water and squeezed to recover the absorbed water. Gels with different silver contents (0 and approximately 20, 90 and 170 mg/g) were assessed. Significantly higher disinfection efficacies (5.4 – 7 log reduction) were seen for the 'squeezed' water compared to the remaining bulk water (maximum 2 log reduction). The highest log removal was seen from the gel with the greatest AgNP content (approximately 6.5 and 7 log removal for *E. coli* and *B. subtilis* respectively). Untreated gel was capable of less than 1 log removal. The silver content of the squeezed water was assessed and found to range between 36.4 to 76.6 µg/l (with the lower concentrations being from the 90 and 170 mg/g gels; 59.6 and 36.4 µg/l respectively). The squeezed water contained both Ag⁺ (45-56%) and AgNP.

De Gussemé *et al.* (2010, 2011) have investigated the possibility of using biogenic silver for water disinfection, where bacteria are used as reducing agents for the production of nanosized Ag⁰ particles. In comparison with chemically produced AgNP, the biogenic particles were found to be far more effective at disinfection. In a spiking experiment using a bacteriophage (10^6 pfu/ml), biogenic silver produced a 4 log reduction after three hours, while the chemically produced Ag⁰ particles showed no inactivation. Using the same concentration of ionic silver resulted in a 4 log reduction, but only after 5 hours. The biogenic particles were also found to be effective against murine norovirus, with a greater than 4 log reduction after only 30 minutes. The capacity of biogenic silver for use in continuous disinfection was assessed following coating of an electropositive cartridge filter. Addition of the biogenic silver increased the removal of virus from 1.5 log, with the filter alone, to 3.8 log. Low concentrations of ionic silver (3 µg/l) were initially detected in the filtrate (up to 5 minutes); thereafter none was detected (De Gussemé *et al.*, 2010). This group have also looked at the immobilization of biogenic silver to microporous membranes (De Gussemé *et al.*, 2011). The system was found to be capable of achieving at least a 3.4 log reduction in bacteriophage concentration (compared to a less than 1 log reduction by the membrane alone). Silver was found to leach out of the system and initially levels of 271 µg/l were recorded, but these soon dropped to below 100 µg/l.

Patil *et al.* (2013) conducted a comparative study of disinfectants for use in household water purifiers and considered both silver ions (outlined in Section 3.1.1) and AgNP, using a batch disinfection test of ground water spiked with *E. coli*. As with Ag ions, AgNP (synthesized from AgNO₃ using citrate as a reducing agent) required a 3 hour contact time, however, a lower minimum concentration of active disinfectant was required (1 mg/l).

The majority of studies considering AgNP for drinking-water treatment applications tested efficacy against bacteria, typically *E. coli*, with the exception being the work of De Gussemé *et al.* (2010, 2011), who considered the effects against bacteriophage and murine norovirus. Typically, good log reduction values were reported, with values up to 7 log reduction (depending upon the spiking concentration) for *E. coli* and, unusually, 4 log reduction for norovirus. Generally the AgNP test materials were effective in both test tube trials (where the silver-treated material is immersed in microbially spiked water) and, where tested, following filtration. Where reported, levels of silver in

the filtered water were usually below 50µg/l. While a wide range of filter materials and different types of AgNP were combined in the studies outlined above the log reduction values, especially in the studies considered virus removal, along with the relatively low silver concentrations in the filtered water, suggest that some of these approaches may have promise for drinking-water treatment, particularly at the household level.

3.3 Silver-coated ceramic filter applications

A number of different types of silver-coated or silver-impregnated ceramic filters (using either AgNP or AgNO₃) have been used as point-of-use (POU) devices, typically in developing countries, for household treatment of drinking-water. Much of the literature on ceramic filter studies, however, has been designed to look at the effectiveness of the filters, rather than the impact of the silver on the effectiveness (e.g. Baumgartner *et al.*, 2007; Brown *et al.*, 2008; Clasen *et al.*, 2004, 2005; du Preez *et al.*, 2008; Salsali *et al.*, 2011). In addition to filters employing silver to improve microbial removal, domestic (and travel) filters may also incorporate silver into the filter to reduce biofilm formation, however, no published literature (in terms of silver efficacy) was found on this application.

Some early work on the efficacy of silver-coated ceramic filters was conducted by Lantagne, 2001 (although this was never formally published), and while some bacterial challenge levels are given (outlined below) units are not. Four untreated filters (i.e. with no silver coating) were tested against water containing unspecified levels of *E. coli*, total coliforms and hydrogen sulphide producing bacteria. Three of the four uncoated filters completely removed *E. coli* but none of the filters completely removed total coliforms or hydrogen sulphide producing bacteria. In contrast, three silver-coated filters were found to completely remove total coliforms (initial concentration [ic] 3108 – no units) and faecal coliforms (ic 1583 no units) and two of the three filters completely removed faecal streptococcus (ic 33 no units). In an assessment of six filters (three with silver, three without), the silver coated filters were found to completely remove total coliforms (ic 3000 no units), faecal coliforms (ic 250 no units), faecal streptococcus (ic 245 no units) and *E. coli* (ic 250 no units). The non-silver coated filters were less efficient and while all three reduced faecal streptococcus levels to zero, total coliform levels in the filtered water ranged between 15 and 300, faecal coliforms ranged between 0 and 45 and *E. coli* ranged between 0 and 45.

Van Halem *et al.* (2007) reported results from filter challenge studies with *E. coli*, *Clostridium* spores and MS2 bacteriophage for six silver-coated and six uncoated Nicaraguan ceramic filters (Table 1). Although the silver coated filter slightly out-performed the uncoated filters for *E. coli* removal, the difference was not statistically significant. There was very little difference in performance for *Clostridium* spore removal and the silver-free filters out-performed the coated filters at both time points for MS2 bacteriophage removal.

Table1: Log removals from coated and uncoated ceramic filters (van Halem *et al.*, 2007)

Microbe	Challenge dose	Log removal	
		Silver coated (n=6)	Silver free (n=6)
<i>E. coli</i>	10 ⁵ to 10 ⁷ cfu/100ml	4.7 to 7.2	2.6 to 5.7
<i>Clostridium</i> spores	10 ³ to 10 ⁵ /100ml	3.6 to 5.3	2.7 to 5.3
MS2 (1)	10 ⁴ to 10 ⁶ pfu/ml	0.5 to 0.7	0.8 to 1.4
MS2 (2)	10 ⁴ to 10 ⁶ pfu/ml	0.8 to 1.4	1.8 to 2.4

The two MS2 experiments were done at different points in a long term study, namely week 5 and week 13. The authors suggest that the improvement in performance between the two time points may be due to biofilm formation.

Wubbels *et al.* (2008) looked at the bacterial removal efficiency of silver impregnated ceramic filters in extensive laboratory-based testing. Ceramic, candle-type filters with and without silver were compared for their ability to remove *E. coli* (10⁶ cfu/l), at two different flow rates, over time. Over

8000 litres of water was passed through each of the filters and samples were periodically spiked with *E. coli* and the log removal efficacy assessed (Table 2). Initially, there was little difference between the filter types with, generally, between 5 and 6 log removal seen in all cases. After passage of almost 5500 litres of water, however, the silver filters started to out-perform the non-silver filters. After over 8000 litres, log removal rates had dropped for both silver (log 2.2-3.2) and non-silver (log 1.1-1.2) filters. The silver concentration in the effluent from the silver filters ranged between 11.95 to 17.68µg/l at the start of the experiment to 1.72 to 3.65µg/l at the end.

Table 2: Log removals from two untreated and two silver-impregnated ceramic filters (Wubbels *et al.*, 2008)

Volume filtered (l)	Reference filter Flow - 6l/h	Silver coated filter Flow - 6l/h	Reference filter Flow - 3l/h	Silver coated filter Flow - 3l/h
Start	5.1	>5.4	>5.4	>5.4
1000	4.4	5.5	5.5	5.5
2067	5.2	>6.0	6.0	>6.0
3452	5.5	>5.8	5.8	>5.8
4487	5.1	>5.6	>5.6	>5.6
5469	3.8	5.5	4.9	>5.8
6411	3.7	>6.4	4.5	>6.4
7390	2.7	4.5	2.9	5.6
8389	1.1	2.2	1.2	3.2

Bielefeldt *et al.* (2009) showed that the disinfection efficacy of ceramic filters could be variable. In tests of untreated (2 filters) or previously heavily used silver-treated filters, log removal varied between <1 log to >4 log for both the untreated and heavily used filters. The initial filter run tended to show the best removal, with removal efficiencies of between 3.7 log and 4 log in the untreated filters and 2.9 log to 4.1 log in the heavily used filters. Re-coating of the previously heavily used filters improved log removal slightly, but filters still showed high variability between filter runs and the improved removal efficiencies were not maintained. As with the initial tests, the initial filter run was generally the one with the best removal (3.5 log to 4.5 log removal). It is not clear from the paper whether the short-term improvement with re-coating was statistically significant. Silver, however, was found to be important in preventing contamination of subsequent batches of clean water passing through the filter (levels of <20 to 41 cfu/ml in recoated filters compared to 10^3 - 10^5 cfu/ml prior to re-recoating).

Bloem *et al.* (2009) looked at *E. coli* and MS2 bacteriophage removal from filters with and without silver. Water was spiked with 10^3 - 10^6 cfu/ml *E. coli* or 10^3 - 10^4 pfu/ml MS2 and passed through the filters. 20 litres of water was treated daily for up to six months. Over the lifetime of the experiment the silver-treated filters outperformed the untreated ones with mean *E. coli* log reductions of 5.9 and 3.1 respectively. No difference was seen between treated and untreated filters in MS2 removal, with removal being uniformly low (0.5 log removal). As reported in some other studies, high variability in removal efficiencies were seen, with *E. coli* log removal in silver-treated filters ranging between 3.82 and 7.65 compared to 2.01 and 4.3 in untreated filters.

Brown and Sobsey (2010) found no significant difference in the removal of *E. coli* (challenge dose 10^4 - 10^7 cfu/ml) or MS2 (challenge dose 10^5 - 10^8 pfu/ml) between silver-treated and untreated filters. *E. coli* removals were between 2.2 and 2.3 log in the silver-treated filters, compared to 2.1 log in the untreated filters. MS2 removal was between 1.3-1.5 log in the silver-treated filters, compared to 1.6 to 1.7 log in the untreated filters.

Kallman *et al.* (2011) looked at *E. coli* removal and compared untreated and silver-treated filters with different pore sizes (according to the percentage of sawdust used in their manufacture). Although

the authors comment that silver improves the log removal values for the filters (Table 3), probably the only significant improvement is that seen for the 17% sawdust filter.

Table 3: *E. coli* log removal values for silver treated and untreated filters with different sawdust content (Kallman *et al.*, 2011)

Percentage sawdust	Log removal	
	Untreated	Silver treated
4%	4.56	4.74
9%	3.52	3.81
17%	2.55	4.91

Zhang and Oyanedel-Craver (2013) compared *E. coli* log removal (challenge dose 10^{10} - 10^{11} cfu/ml) in ceramic disks with or without silver treatment. Silver treatment did not noticeably improve the performance of the disks (4.2-4.3 log removal by the untreated disks compared to 4.4 log removal by the treated disk).

Although some studies have suggested that silver treatment improves the *E. coli* removal performance of filters (e.g. Wubbels *et al.*, 2008; Bloem *et al.*, 2009), others have shown only small benefits, short-term improvements or negligible impact (beyond the filtration effect) as a result of silver incorporation (e.g. van Halem *et al.*, 2007; Bielefeldt *et al.*, 2009; Brown and Sobsey, 2010; Kallman *et al.*, 2011; Zhang and Oyanedel-Craver, 2013). None of the studies has suggested that silver treatment improves the removal of viruses.

4. Silver toxicity

This section outlines the toxicity of silver via a number of possible routes of exposure (i.e. it is not confined to ingestion). The emphasis is on recent papers on toxicity to mammalian systems (*in vivo* and *in vitro*). Much of the recent focus has been on toxicity of silver nanoparticles although, in some cases, ionic silver has been used to provide a comparison.

4.1 Absorption, distribution and excretion

Exposure to silver leads to uptake by the body. This has been reported after:

- inhalation (Dong *et al.*, 2013; Hyun *et al.*, 2008; Ji *et al.*, 2007; Kim *et al.*, 2011; Lee *et al.*, 2010; Lee *et al.*, 2012; Stebounova *et al.*, 2011; Song *et al.*, 2013; Sung *et al.*, 2008, 2009, 2011; Takenaka *et al.*, 2001);
- ingestion (Cha *et al.*, 2008; Chung *et al.*, 2009; Hadrup *et al.*, 2012a; Hosseini *et al.*, 2013; Kim JS *et al.*, 2008, 2009, 2010; Kim WY *et al.* 2009; Loeschner *et al.*, 2011, Maneewattanapinyo *et al.*, 2011; Munger *et al.*, 2013; Park *et al.*, 2010a; Pelkonen *et al.*, 2003; van der Zande *et al.*, 2012);
- injection (De Jong *et al.*, 2013; Dziendzikowska *et al.*, 2011; Gromadzka-Ostrowska *et al.*, 2012; Lankveld *et al.*, 2010; Tiwari *et al.*, 2011; Wang *et al.*, 2013a; Xue *et al.*, 2012); and
- dermal exposure (Korani *et al.*, 2011; Maneewattanapinyo *et al.*, 2011).

4.1.1 Inhalation studies

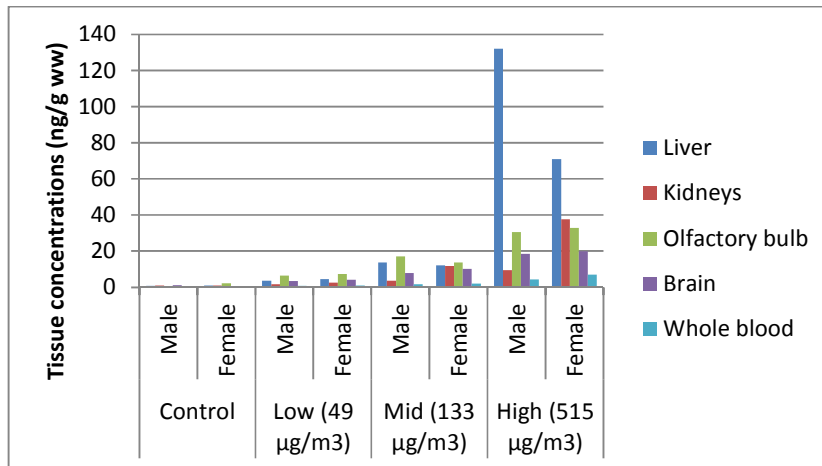
Following AgNP inhalation (in various doses and exposure periods – summarised in Table 4) studies in rats showed that silver could be found in the lungs, liver, kidney, brain, heart, nasal cavity, olfactory bulb, eyes, spleen, ovaries, testes and blood (Ji *et al.*, 2007; Song *et al.*, 2013; Sung *et al.*, 2009; Takenaka 2001). In mice (in a single study) only the lungs were found to exhibit elevated silver concentrations after exposure (Stebounova *et al.*, 2011).

In addition, a study of intra-nasal administration of AgNP in natal rats reported concentrations of almost 20µg/g total silver in the cerebellum (the only tissue investigated) following administration of 1mg/kg AgNP (20-30nm) for 21 consecutive days (Yin *et al.*, 2013).

Table 4: Tissue silver accumulation in inhalation studies

Animal	AgNP/ionic	Max [Ag]	NP size	Duration	Elevated tissue	Ref
Rat	AgNP	133 µg/m ³	15nm	6 h	Lung, Liver, Kidney, Heart, Blood, Nasal cavity, Brain, Lymph nodes	Takenaka <i>et al.</i> , 2001
Rat	AgNP	61 µg/m ³	12-15nm	6 h x 5 days/week x 4 weeks	Lung, Liver, Brain, Olfactory bulb	Ji <i>et al.</i> , 2007
Rat	AgNP	515 µg/m ³	18-19nm	6 h x 5 days/week x 13 weeks	Lung, Liver, Kidney, Olfactory bulb, Brain, Blood	Sung <i>et al.</i> , 2009
Rat	AgNP	381 µg/m ³	14-15nm	6 h x 5 days/week x 12 weeks	Lungs, Liver, Kidneys, Spleen, Ovaries, Testes, Blood, Eyes, Brain	Song <i>et al.</i> , 2013
Mouse	AgNP	3.3 mg/m ³	5nm	4 h x 5 days/week x 2 weeks	Lungs	Stebounova <i>et al.</i> , 2011

Sung *et al.* (2009), for example, exposed rats to low (49 µg/m³), medium (133 µg/m³) or high (515 µg/m³) concentrations of AgNP (18-19nm) via inhalation over a 90 day period. They found a dose-dependent increase in silver in both male and female rats in a number of tissues (Figure 1), with the lungs and liver being the sites with the greatest silver concentrations. A significant gender difference was seen in silver accumulation in the kidney, with reported levels being three times higher in the female kidney, compared to the male, at the high concentration.



Lung concentrations at the high dose: males 14645ng/g wet weight; females 2058 ng/g wet weight

Figure 1: Tissue silver concentrations from a 90 inhalation study in rats (Sung *et al.*, 2009)

Song *et al.* (2013) looked at tissue accumulation immediately post-exposure and also at 4 and 12 weeks post-exposure, following a 90 day inhalation study. They found high initial levels in the lungs (data not shown in Figure 2), liver, kidneys, spleen, ovaries, blood and eyes, with clearance over the recovery period (Figure 2). Although levels in the lungs dropped significantly during the recovery period (concentration immediately post-exposure in females exposed to the high dose – 5587ng/g wet weight to 1475ng/g wet weight after 12 weeks recovery) the post-recovery level was still significantly higher than the unexposed level (0.27ng/g wet weight).

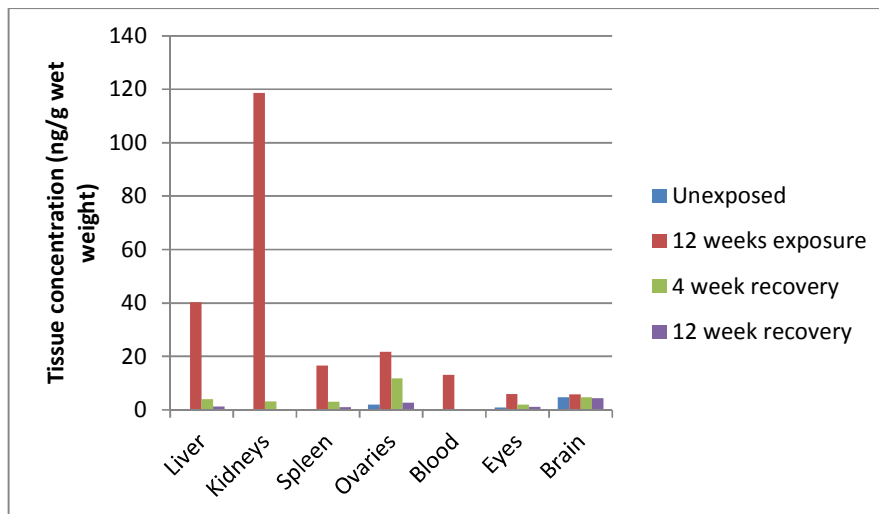


Figure 2: Tissue silver concentrations in female rats exposed to the high AgNP dose (381 µg/m³) in a 90 day inhalation study, followed by a 12 week recovery period (Song *et al.*, 2013)

4.1.2 Ingestion studies

Ingestion studies (typically via gavage) have found silver to be distributed to the bladder, blood, brain, heart, kidney, liver, lungs, muscle, small intestine, stomach and spleen (i.e. generally similar to the distribution seen after inhalation) – as shown in Table 5.

Table 5: Tissue silver accumulation in ingestion studies

Animal	AgNP/ionic	Max [Ag]	NP size	Exposure	Duration	Elevated tissue	Ref
Rat	AgNP (CMC)	1000 mg/kg	60 nm	Oral (unstated)	28 days	Stomach, Liver, Kidney, Lungs, Testes, Brain, Blood	Kim <i>et al.</i> , 2008
Rat	AgNP (CMC)	500mg/kg bw	56nm	Gavage	90 days	Liver, Kidney, Lungs, Testes, Brain, Blood	Kim <i>et al.</i> , 2010
Rat	Ag acetate AgNP (PVP)	9 mg/kg bw 12.6 mg/kg bw	NA 14nm	Gavage	2 x /day x 28 days	Small intestine, Stomach, Liver, Kidney, Lungs, Muscle, Brain, Plasma	Loeschner <i>et al.</i> , 2011
Rat	AgNO ₃ AgNP	9 mg/kg bw 90 mg/kg bw	NA <20 <15- PVP	Gavage	28 days	Liver, Spleen, Testis, Kidney, Brain, Lungs, Blood, Bladder, Heart	van der Zande <i>et al.</i> , 2012
Mouse	AgNO ₃	0.03 mg/l	NA	Oral – drinking-water	2 weeks	Musculus soleus, Cerebellum, Spleen, Duodenum, Myocardium, Lungs, Cerebellum, Musculus gastrocnemius, Liver, Kidneys, Blood	Pelkonen <i>et al.</i> , 2003
Mouse	AgNP (bare)	1 mg/kg bw	22nm – 71nm	Gavage	14 days	Kidney, Testis, Liver, Brain, Lung	Park <i>et al.</i> , 2010a

PVP – polyvinylpyrrolidone

CMC – carboxymethylcellulose

NA – not applicable

Organs showing the greatest silver deposition following ingestion tended to vary between studies although the liver and kidney were typically key organs. Generally, where tested, ionic and AgNP showed similar patterns of distribution (although with lower silver tissue concentrations following AgNP exposure, e.g. Loeschner *et al.*, 2011). One study (Park *et al.*, 2010a) demonstrated preferential tissue silver accumulation in mice exposed to smaller AgNP (22nm), as shown in Figure 3.

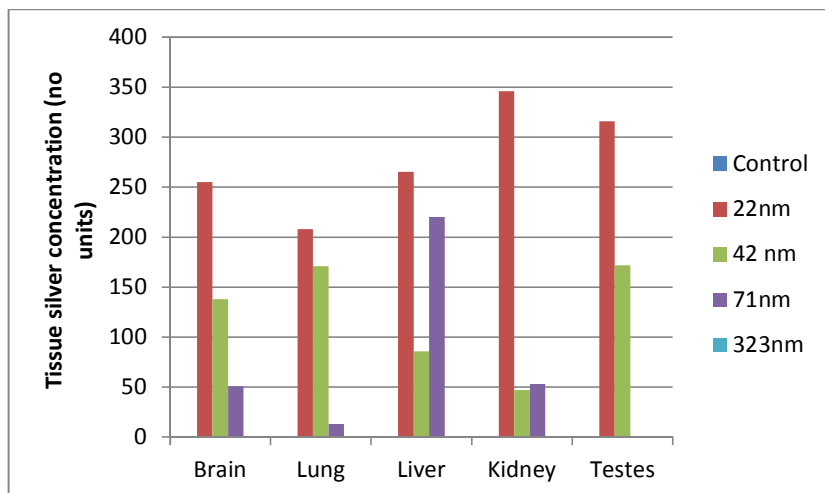


Figure 3: Silver accumulation according to AgNP size (Park *et al.*, 2010a)

Van der Zande *et al.*, (2012) reported a generally rapid reduction in tissue silver concentrations following 28-days of ingestion exposure. In most tissues, silver concentrations were already significantly reduced (to below 50% of the immediate post-exposure levels) just one week after exposure ceased and approached a return to control levels in most samples 12 weeks after exposure. There were, however, four exceptions, namely brain, testis, kidney and spleen, where silver concentrations were still elevated after 12 weeks, with the brain retaining over 90% of the original post-exposure levels.

The faecal and urinary levels of silver, post-exposure, were measured in two of the ingestion studies. Loeschner *et al.* (2011) found very low levels in the urine (<0.1%) and reported slightly different levels in the faeces, depending upon the nature of the original challenge, with higher faecal levels from AgNP compared to Ag acetate (63% and 49% of the daily dose, respectively). The faecal excretion levels reported by Loeschner *et al.* (2011) are notably lower than those reported by van der Zande (2012), who reported that over 99% of the daily dose was excreted in faeces.

4.1.3 Injection studies

Although not relevant to food or environmental exposures, injection may be useful to consider for studying tissue distribution, as absorption is bypassed (and has clearly been demonstrated in the studies outlined above) and this can allow tissue distribution and elimination to be studied more precisely (Lankveld *et al.*, 2010). As with other routes of administration, silver was found to be widely distributed following injection (Lankveld *et al.*, 2010; Dziendzikowska *et al.*, 2012). Lankveld *et al.* (2010) found that following injection of rats with either 20nm, 80nm or 100nm AgNP, the AgNP disappeared rapidly from the blood and was subsequently found in all the organs evaluated (liver, lungs, spleen, brain, heart, kidneys and testes). They found a difference in distribution pattern, according to the size of the particle, with the 20nm particle being found mainly in the liver (followed by kidneys and spleen), while the larger particles distributed mainly to the spleen, followed by the liver and lung. Dziendzikowska *et al.* (2012) demonstrated time-dependent changes in silver levels in

a number of organs, following a single intravenous injection of AgNP (nominally 20nm or 200nm). They found that concentrations of silver were greatest in the liver 24 hours after injection and then decreased, while concentrations in the kidney and brain were at their lowest 24 hours after injection but subsequently increased and reached their maximum levels at the end of the experiment (28 days after injection). Both individual AgNP and/or clusters of nanoparticles were identified in the red and white pulp of the spleen, all regions of the kidney (inner medulla, cortex and outer medulla - but with preferential accumulation in the renal tubules), lung macrophages, brain and liver endothelial and Kupffer cells. Wang *et al.* (2013a) found that in mice injected with AgNP, silver could cross the placental barrier and accumulate in foetuses.

4.1.4 Human exposure

It is clear that silver (largely irrespective of the route of exposure or form) can distribute widely within the body and cross both the blood-brain and placental barriers in experimental animals. Silver, has also been found to be widely distributed throughout the body in exposed humans. Human data relating to silver ingestion is largely limited to a number of case reports where people have ingested varying amounts of colloidal silver, generally over a protracted period (Chung *et al.*, 2010). The most common presenting feature is argyria (Brandt *et al.*, 2005; Wadhwa and Fung, 2005) where tissues become impregnated with silver sulphide, which forms a complex in elastic fibres; large amounts of this complex under the skin give it a bluish, grey-blue or (in extreme cases) a black colour. Generalised argyria results from increased serum silver levels and silver granules can be detected in all body tissues, with the highest concentrations found in the skin, liver, spleen and adrenal glands (Brandt *et al.*, 2005). Silver has also been found to cross the placental barrier in humans. Lyon *et al.* (2002) looked at liver samples, collected at autopsy, and found significant levels of silver (median 15.5ng/g ww) in livers of children under 6 years old. It was speculated that silver (probably from maternal mercury amalgam fillings) is accumulated from the mother during pregnancy and lactation.

4.2 *In vivo* toxicity

The research outlined below includes studies conducted on rats, mice, guinea pigs and a single study on human subjects. It covers inhalation, ingestion and dermal exposure routes. There are indications from this research that high doses of silver and/or repeated administration can result in signs of, generally, mild toxicity in some of the test species.

4.2.1 Inhalation

A number of inhalation studies have been performed, these generally use AgNP (typically 12-18nm in size) produced by evaporation/condensation of source silver using a ceramic heater (Ji *et al.*, 2007) and expose rats or mice to different concentrations of AgNP over different periods of time, via whole body inhalation chambers. Whether toxicity is reported as a result of inhalation seems to depend on the duration of the exposure. With short-term exposure (up to 28 days) resulting in no significant impacts. Ji *et al.* (2007) found no change in body weight, haematology or blood biochemistry in rats exposed to up to 61µg/m³ over a 28 day period. Hyun *et al.* (2008) focussed on the nasal cavity of rats exposed over a 28 day period. While they found an increase in the size and number of goblet cells in the nasal cavity containing neutral mucins in rats exposed to 3.5 µg/m³ and 61 µg/m³ this was not considered to have toxicological significance. Sung *et al.* (2011) exposed rats on a single occasion to between 76 and 750 µg/m³. The rats were monitored for two weeks following exposure and there were no significant body weight or clinical changes and there was no difference in lung function between the groups. Stebounova *et al.* (2011) exposed mice (via aerosol generation) to 5nm AgNP in concentrations up to 3.3 mg/m³ for 10 days over a two week period, followed by a three week recovery period. Although increased numbers of neutrophils were detected in the bronchoalveolar lavage fluid (at both zero and three weeks post-exposure) compared to the control group the change was considered to be "*of little biological significance*" and no pathological changes were seen.

In contrast to the acute and sub-acute studies, some of the longer-term exposure studies (90 days) suggest a toxic effect of AgNP inhalation. Sung *et al.* (2008, 2009) subjected rats to a 90 day whole body inhalation study, where animals were exposed to AgNP (18-19nm) at low ($49 \mu\text{g}/\text{m}^3$), medium ($133 \mu\text{g}/\text{m}^3$) or high ($515 \mu\text{g}/\text{m}^3$) doses for six hours a day, five days a week for 13 weeks. The main targets of toxicity were the lungs and liver. Bile duct hyperplasia in the liver was found to increase dose dependently in both male and female rats. Histopathological examinations indicated dose-dependent increases in lesions related to AgNP exposure, including mixed inflammatory cell infiltrate, chronic alveolar inflammation and small granulomatous lesions. Test animals showed a reduction in lung function indicated by decreases in the tidal volume and minute volume. The authors investigated the possible impact of the increased silver accumulation seen in kidneys of female animals by measuring kidney function (based on N-acetylglutamate and protein concentration in the urine) in both males and females. No significance differences were seen, except for an increase in protein in the urine in males in the high-dose group. This group also looked at recovery from AgNP-exposed lung inflammation and lung function changes, by allowing the animals to recover for up to 12 weeks after a 12 week exposure period (Song *et al.* 2013). It was found that although some recovery was evident, an exposure-related lung function decrease in males (exposed to the highest AgNP dose) persisted during the recovery period. This finding was supported by the histopathology results which showed persistent lung inflammation in this group.

There was no evidence of genetic toxicity in male or female rats, based on an analysis of micronucleus induction from bone marrow, following exposure of rats to up to $515 \mu\text{g}/\text{m}^3$ over a 90 day period (Kim *et al.*, 2011). Dong *et al.* (2013) exposed rats to up to $381 \mu\text{g}/\text{m}^3$ for 12 weeks. Although they found a change in gene expression in the kidneys with, overall, male rat kidneys showing a higher expression of genes involved in xenobiotic metabolism and the female rat kidneys showing a higher expression of genes involved in extracellular signalling, this was not considered to be of toxicological significance.

4.2.2 Ingestion

There is a suggestion from some studies that, especially at high doses, silver may have mild toxic effects via ingestion, with the liver being the target organ. Although, while some studies suggest toxicity (Cha *et al.*, 2008; Kim *et al.*, 2008, 2010; Park *et al.*, 2010a; Hadrup *et al.*, 2012a), others found no evidence (Maneewattanapinyo *et al.*, 2011; Kim *et al.* 2013; van der Zande *et al.*, 2012; Munger *et al.*, 2013) – although the difference may reflect the type of silver administered or the dosing regimen.

Following a large dose (2.5 g) of AgNP (13nm) or silver microparticles (2-3.5 μm) administered by gavage, Cha *et al.* (2008) reported focal lymphocyte infiltration in the mouse liver portal tracts, suggesting the induction of inflammation. They also reported nonspecific focal haemorrhages in the heart, focal lymphocyte infiltration in the intestine and nonspecific medullary congestion in the spleen in the mice exposed to AgNP.

In a 28 day study, Kim *et al.* (2008) investigated the toxicity, genotoxicity and gender-related tissue distribution of AgNP in rats. The animals were treated with 30, 300 or 1000 mg/kg AgNP (60 nm, suspended in carboxymethyl-cellulose). While there was no significant change in body weight as a result of AgNP exposure, some significant dose-dependent changes were found in alkaline phosphatase and cholesterol values in either male or female rats. This led the authors to suggest that exposure to levels of AgNP greater than 300 mg/kg may result in slight liver damage. There was no indication of genetic toxicity. In a sub-chronic oral study (Kim *et al.*, 2010) using 30, 125 and 500 mg/kg doses of AgNP (60nm as above) over a 90 day exposure period, the group found that there were significant differences in the body weights of the males exposed to the mid and high dose of AgNP compared to the control animals. As in the short-term study, the group also found significant dose-dependent changes in alkaline phosphatase and cholesterol for male and female rats. In addition, histopathologic examination revealed a higher incidence of bile duct hyperplasia.

In a 28 day feeding study in mice, Park *et al.* (2010a) found that AgNP (42nm) at the highest dose given (1mg/kg) resulted in some changes in serum biochemistry, with increased levels of alkaline phosphatase and aspartate transaminase in both male and female mice. Levels of alanine transaminase were also increased following high dose administration, but only in female mice. They also found that pro-inflammatory cytokines were increased in a dose-dependent manner. Minor histopathological changes were seen in the kidney (slight cell infiltration in the cortex), but not in the liver or small intestine following high dose administration.

Hadrup *et al.* (2012a) examined the oral sub-acute toxicity of 14 nm AgNP (stabilised with polyvinylpyrrolidone – PVP) and silver acetate in rats. Doses of 2.25, 4.5 or 9mg/kg bw of AgNP or 9mg/kg bw silver acetate were given daily, by gavage, for 28 days. The authors found no toxicological effects following AgNP administration. Following silver acetate administration, however, they found lower body weight gain, increased plasma alkaline phosphatase, decreased plasma urea and lower absolute and relative thymus weight. This group (Hadrup *et al.* 2012b) also conducted a metabolomics investigation (the concomitant measurement of a wide range of low molecular weight molecules in body fluids) of the rat urine on day 18 of the study. The analysis revealed differences in the urine composition of female (but not male) rats when compared to the control group. Differences were found in the levels of uric acid and its degradation product, allantoin. AgNP ingestion led to an increase in both metabolites, while silver acetate only increased allantoin levels. As both AgNP and silver acetate altered urine composition this suggests that female rat physiology was affected by silver ingestion.

Maneewattanapinyo *et al.* (2011) treated mice with a single dose (5000 mg/kg) of AgNP (10-20nm) by gavage and then observed the animals for up to 14 days after exposure. They found no mortality or acute toxic signs throughout the observation period. In addition, there was no difference in the percentage of body weight gain between the treatment and control groups or a significant difference in haematological parameters. Kim *et al.* (2013) found no difference in body weight, gross findings at necropsy or mortality in rats treated with up to 2000 mg/kg bw citrate-coated AgNP (10nm). Van der Zande *et al.* (2012) found no hepatotoxicity or immunotoxicity in a 28 day feeding study in rats exposed to AgNP (< 20nm non-coated; <15nm PVP coated) at 90 mg/kg bw or AgNO₃ (at 9mg/kg). Munger *et al.* (2013) conducted a study looking at human exposure (60 healthy subjects) to commercial nanoscale silver colloid in a single-blind, controlled, cross-over, intent-to-treat design. Two different commercial AgNP (colloid) solutions were used, one with particle sizes between 5 – 10nm (10ppm solution) and one with particles between 25-40 nm (32 ppm solution). With the 10ppm solution subjects were dosed for 3, 7 or 14 days (100µg/day – equivalent to 1.4µg/kg body weight – assuming 70kg adult), while for the 32 ppm solution all subjects were dosed for 14 days (480µg/day – equivalent to 6.8µg/kg bw). Subjects underwent metabolic, blood counts, urinalysis, sputum induction and chest and abdomen magnetic resonance imaging. Silver serum and urine levels were also determined. The authors reported that no morphological changes were detected in the lungs, heart or abdominal organs and that no significant changes were noted in pulmonary reactive oxygen species or pro-inflammatory cytokine generation. They saw no clinically important changes in human metabolic, haematologic, urine, physical findings or imaging morphology.

4.2.3 Dermal

There seem to be relatively few *in vivo* animal studies looking at the potential dermal toxicity of silver application (Samberg *et al.*, 2010; Korani *et al.*, 2011; Maneewattanapinyo *et al.*, 2011; Kim *et al.* 2013), although a number of studies have looked at the impact of silver impregnated wound dressings (e.g. Trop *et al.*, 2006).

Samberg *et al.* (2010) looked at the impact of AgNPs (20, 50nm) on porcine back skin. Pigs were dosed topically with solution of AgNP (0.34-34µg/ml) for 14 consecutive days and the skin evaluated for erythema and oedema. Macroscopic observations showed no gross irritation in the porcine skin. Microscopic observation, however, showed a different picture and the skin exhibited a

concentration-dependent response for both the 20nm and 50nm AgNP. Skin treated at the lowest dose (AgNP 20nm) showed slight intracellular and intercellular epidermal oedema, while that exposed to the highest concentration showed severe intracellular and intercellular epidermal oedema with severe focal dermal inflammation. AgNPs could be detected in the upper stratum corneum layers of the skin.

Maneewattanapinyo *et al.* (2011) exposed guinea pigs (via a small shaved area of skin) to either 50 ppm or 100,000 ppm AgNP (10-20nm) for 24 hours and then observed the animals for signs of toxicity for 14 days. Despite the high dose no gross abnormalities were detected nor were any significant microscopic changes observed and there was no evidence of penetration or infiltration through the epidermal and dermal layers.

Korani *et al.* (2011) conducted both an acute and a sub-chronic dermal toxicity study in guinea pigs. In the acute study, 10% of the body surface of the test animals was shaved and exposed to either 1000 or 10,000µg/ml AgNP, with observations after exposure for 14 days. In the sub-chronic study, the shaved skin of experimental animals was rubbed five times a week for 13 weeks with the same AgNP concentrations used in the acute study. In both cases a positive control was used, in the form of 100µg/ml AgNO₃. In the acute test, dermal dose-dependent changes were seen for AgNP, with reduced thickness in the epidermis and papillary layer. Histopathological changes were also seen for AgNO₃, but at a lower level than those seen for AgNP. In the subchronic test, toxic skin responses were dose and time-dependent and the impact of AgNO₃ was similar to that from the same dose of AgNP. Negative impacts from AgNP were also seen on the liver and spleen, including overproduction of Kupffer cells and degeneration of hepatocytes in the liver. The authors comment that, based on their results, exposure to >0.1 mg/kg (100µg/ml dose) of AgNP may result in slight liver, spleen and skin damage.

Kim *et al.* (2013) conducted dermal toxicity/irritation tests on rats, rabbits and guinea pigs using citrate-coated 10nm AgNP. Ten rats were exposed for 24 hours to up to 2000mg/kg bw and then observed for 15 days; no toxicity was observed. Similarly, no skin reaction was seen in three rabbits subjected to the same form of AgNP. In a skin sensitisation test using 20 guinea pigs, a single animal showed some erythema, suggesting that the tested AgNP could be classified as a weak skin sensitiser.

4.3 *In vitro* toxicity

There has been a marked increase in the number of studies looking at the *in vitro* toxic effects of silver (principally AgNP) in recent years, with a wide range of cells investigated, including cells derived from:

- blood (e.g. Zhang *et al.*, 2013),
- brain (e.g. Haase *et al.*, 2012a),
- bone (e.g. Hardes *et al.*, 2007),
- cervix (e.g. Mukherjee *et al.*, 2012),
- immune system (e.g. Pratsinis *et al.*, 2013),
- intestine (e.g. Gopinath *et al.*, 2010),
- kidney (e.g. Kermanizadeh *et al.*, 2013),
- liver (e.g. Gaiser *et al.*, 2013),
- lung (e.g. Suliman *et al.*, 2013),
- skin (e.g. Samberg *et al.*, 2010) and
- testes (e.g. Ema *et al.*, 2010),

along with stem cells from a number of different sources (e.g. Braydich-Stolle *et al.*, 2010; Hackenberg *et al.*, 2011).

In vitro studies covering exposure to cells derived from many of the target organs identified from *in vivo* studies are outlined below.

4.3.1 Liver

In the studies outlined below, researchers tested different AgNP (typically below 20nm in size) against six different types of liver cell. With the exception of the cells used by Arora *et al.* (2009) these were all secondary cells (i.e. cancer-derived or immortalized cell lines). Different tests were used to assess toxicity but, generally, at least one test of cytotoxicity was included. Results were expressed in a variety of ways and include measures of IC₅₀ and LC₅₀.

In 2005, Hussain *et al.* showed that AgNP (15nm and 100nm) were toxic to immortalised rat liver (BRL 3A) cells. AgNP resulted in a concentration-dependent increase in lactate dehydrogenase (LDH) leakage and showed significant cytotoxicity at 10-50µg/ml. The MTT assay (used to assess mitochondrial function) also showed that AgNP caused significant cytotoxicity above 5µg/ml. In addition, the level of reactive oxygen species (ROS) was found to increase in a concentration-dependent manner and a significant depletion of glutathione (GSH) was observed relative to control cells.

Cha *et al.* (2008) exposed Huh-7 (hepatoma) cells to AgNP (13nm) and found little impact on mitochondrial activity or glutathione production. DNA contents in the treated cells, however, decreased by 15% and the expression of genes related to apoptosis and inflammation were altered.

Kim S *et al.* (2009) compared the cytotoxicity of AgNP (5-10nm) and AgNO₃ to human hepatoma (HepG2) cells using three different measures of cell viability. The MTT and Almar Blue tests assess cell metabolic activity, while the LDH tests assesses membrane integrity. The IC₅₀ values for the LDH tests in both AgNP and AgNO₃ were markedly lower than the other tests (Figure 4), suggesting that in HepG2 cell membrane integrity is more readily affected by silver than the tested metabolic activities (which is in contrast to the results of Hussain *et al.*, 2005).

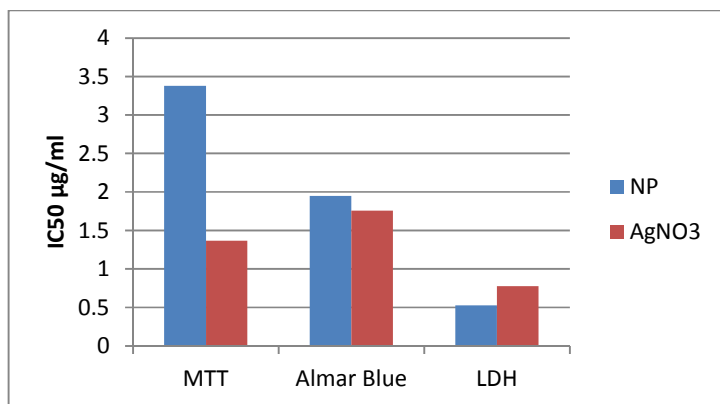


Figure 4: IC₅₀ results for cytotoxicity tests in HepG2 following exposure to AgNP and AgNO₃ (Kim S *et al.*, 2009)

The finding that the cytotoxicity seen in all three tests could be prevented by the addition of N-acetylcysteine (a precursor for the synthesis of glutathione and, thus, an important antioxidant) suggests that the cytotoxicity may be due to oxidative stress. Nowrouzi *et al.* (2010) reported an IC₅₀ value (based on MTT and XTT tests) for HepG2 cells exposed to AgNP (5-10nm) of between 2.75 to 3 mg/l, very similar to that reported by Kim S *et al.*, 2009. They went on to subject HepG2 cells to 0%, 1%, 4% and 8% of the IC₅₀ value, and found significant impacts on indicators of oxidative stress at levels of 4% and above (increases in the activity of LDH, alanine aminotransferase and aspartate aminotransferase activity; increase in nitric oxide concentration; increases in lipid peroxidation and

cytochrome c content; decrease in GSH content and a decrease in SOD activity). Kawata *et al.* (2009) investigated the effects of AgNP (7-10nm – stabilised with polyethylenimine) and Ag₂CO₃ on HepG2 cells at concentrations below those resulting in cytotoxicity. AgNP was found to result in significant toxicity above 1mg/ml (although Ag₂CO₃ still appeared to be non-cytotoxic at that dose), so a concentration of 1mg/ml was used in further experiments. At that concentration AgNP was found to significantly increase the frequency of micronucleus formation, indicating DNA damage and chromosome aberrations (Ag₂CO₃ did not increase levels above those seen in the control). In addition, exposure to AgNP also altered gene expression, including the up-regulation of stress-related genes.

Arora *et al.* (2009) also looked at the toxicity of AgNP (7-20nm) to mouse liver cells but, in contrast to other studies (e.g. Hussain *et al.*, 2005 and Kim S *et al.*, 2009), used primary cells. Exposure of the liver cells to up to 100µg/ml for 24 hours did not alter cell morphology. The onset of apoptosis was seen at 12.5µg/ml, which was much lower than the necrotic concentration (500µg/ml). The primary cells seemed to be more resistant to the cytotoxic effects of AgNP, with an IC₅₀ for the XTT assay of 449µg/ml (although, not strictly comparable, the IC₅₀ for the MTT test [similar to XTT] in human hepatoma HepG2 cells reported by Kim S *et al.*, 2009 was <3.5µg/ml). Exposure of the cells to AgNP at half of the IC₅₀ value resulted in increased levels of superoxide dismutase (SOD) and GSH as compared to unexposed cells suggesting that antioxidant defence mechanisms were triggered by AgNP exposure.

In a study in Chang liver cells, AgNP (5 to 10nm - 28-35nm in the cell culture medium) were found to be more cytotoxic (MTT test) than AgNO₃ (IC₅₀ 4µg/ml and 8µg/ml respectively). AgNP induced ROS generation and suppression of reduced glutathione. The ROS generated resulted in damage to various cellular components, DNA breaks, lipid membrane peroxidation and protein carbonylation (Piao *et al.* 2011).

Gaiser *et al.* (2013) looked at the impact of AgNP (mean 17.5nm) on C3A cells. The AgNP were found to be highly toxic to the cultured cells (LDH LC₅₀ - 2.5µg/cm³; Almar Blue LC₅₀ 20 µg/cm³). It was also shown that hepatocyte homeostasis was affected, with a decrease in albumin release.

4.3.2 Lung

A number of studies have been conducted on the toxicity of various types of AgNP (different sizes and coatings) to lung cells *in vitro*. Typically either A549 cells (a lung carcinoma alveolar epithelial cell) or, less frequently, human lung fibroblasts (e.g. IMR-90) have been used as test systems. Generally, authors have found impacts on cell viability and demonstration of oxidative stress (Carlson *et al.*, 2008; Foldbjerg *et al.*, 2011; Li *et al.*, 2012; Suliman *et al.*, 2013). Other studies have also considered impacts on the cell cycle (AshaRani *et al.*, 2009a; Lee *et al.*, 2011; Chairuangkitti *et al.*, 2013). As with the results from studies on liver cells, there is an indication that size and coating of the AgNP impacts on toxicity (with smaller AgNP typically being more toxic than larger particles – Carlson *et al.*, 2008; Li *et al.*, 2012).

Carlson *et al.* (2008) explored the possible toxicity of inhaled AgNP using rat alveolar macrophages. The toxicity of three AgNP (coated in hydrocarbon) of different sizes (15nm, 30nm, 55nm) was assessed at various doses. In general, the 15nm AgNP showed the greatest toxicity and the 55nm AgNP showed the least toxicity (e.g. the EC₅₀ for increased LDH leakage was 27µg/ml for the 15nm AgNP and > 75µg/ml for the larger particle). The authors also found a significant increase in ROS and a correlated decrease in levels of GSH following exposure to AgNP 15nm and increased secretion of inflammatory cytokines/chemokines.

Foldbjerg *et al.* (2011) compared the toxic effects of AgNP (PVP-coated, 69nm, up to 20µg/ml) and AgNO₃ (up to 10µg/ml) on A549 cells. Both AgNP and AgNO₃ were cytotoxic (as determined by impact on mitochondrial activity), although the cytotoxic impacts of AgNO₃ (EC₅₀ - 6µg/ml) were

seen at lower doses than those following AgNP exposure ($EC_{50} = 12.5\mu\text{g/ml}$). The measured toxicity of both types of Ag could be significantly reduced by pre-treating cells with antioxidant. It was found that cell death was primarily due to a dose-dependent increase in necrosis/late apoptosis, whereas only a minor increase in early apoptosis was detected. The AgNP was found to induce a greater increase in ROS than the AgNO_3 . In comparison to the control, ROS levels were increased almost 16-fold at $10\mu\text{g/ml}$ AgNP, but only approximately 8-fold by the same concentration of Ag from AgNO_3 . This group (Foldbjerg *et al.*, 2012) also looked at the effects of AgNP (16nm) and AgNO_3 at low (non-cytotoxic) doses on gene expression in A549 cells. Exposure to AgNP altered the regulation (2-fold difference or greater) of more than 1000 genes, compared to only 133 genes following exposure to silver ions.

Suliman *et al.* (2013) investigated the toxicity of AgNP (56 nm, 10-100 $\mu\text{g/ml}$) on A549 cells using a wide array of methods. Morphological changes were clearly seen in cells exposed to 25 $\mu\text{g/ml}$ AgNP for 48 hours. The AgNP caused cytotoxicity, as measured by mitochondrial function (MTT assay) and membrane permeability (LDH assay). AgNP induced the generation of ROS and induced oxidative stress (shown by a depletion of GSH and increases in lipid peroxidation, SOD and catalase concentrations). Increased apoptosis following exposure to AgNP was seen, the expression of pro-inflammatory cytokines was up-regulated and a concentration and a time dependent increase in DNA damage was also observed.

In addition to changes in mitochondrial activity, membrane permeability and increases in ROS generation (etc.), a number of authors have shown that AgNP modulates the cell cycle in A549 cells. Lee *et al.* (2011) showed that AgNP (hydrodynamic diameter 480nm) with an IC_{50} of 106 $\mu\text{g/ml}$ for cell viability caused accumulation of cells at G2/M and sub-G1 (apoptosis) following exposure to 50 $\mu\text{g/ml}$ for 4 hours. Chairuangkitti *et al.* (2013) showed that AgNP increased the proportion of cells in the sub-G1 population, increased S phase arrest and caused down regulation of the cell cycle associated proliferating cell nuclear antigen (PCNA) protein. Pre-treatment with an antioxidant, while decreasing some of the effects, did not change the AgNP mediated impact on S phase arrest or down-regulation of PCNA, leading the authors to suggest that the *in vitro* toxic effects on A549 cells are mediated via a ROS-dependent (cytotoxicity) and a ROS-independent (cell cycle arrest) pathway. Asha-Rani *et al.* (2009a) looked at the anti-proliferative activity of AgNP (6-20nm, starch coated) in normal human lung fibroblasts (IMR-90). Electron micrographs showed that AgNPs were taken up by the cells and showed a uniform distribution both in cytoplasm and nucleus. Although the AgNP treated lung fibroblasts exhibited chromosome instability and mitotic arrest, the cells recovered completely from the proliferation arrest.

Sur *et al.* (2010) looked at the impact on toxicity of modifying AgNP with glucose, lactose, oligonucleotides and combinations of these ligands in comparison with bare AgNP on A549 cells. While the modification seemed to increase the uptake of the AgNP into the cells it also acted to decrease the toxicity, with the bare AgNP being cytotoxic at a lower dose than the modified particles.

Li *et al.* (2012) treated human lung fibroblasts (unspecified) with five different sized PVP-coated AgNPs (25, 35, 45, 60 and 70nm) at the same doses (31.75, 62.5, 125, 250 $\mu\text{g/ml}$). Both tests of cell viability (MTT and LDH assay) showed size-dependent cytotoxicity which decreased with increasing AgNP size.

4.3.3 Brain and the blood-brain-barrier

The brain is essentially made up of two key cell types – neurons and glial cells (including microglia, astrocytes/astroglia and oligodendrocytes). A number of recent toxicity studies focusing on brain cell cultures, a model system for neuronal differentiation (PC12 cells) and cells involved in the blood-brain-barrier (BBB) have been identified in the literature.

Astrocytes

The ability of astrocytes to withstand Ag seems to depend upon the form of the Ag and the AgNP coating. Luther *et al.* (2011) for example exposed primary cultures of rat astrocytes to PVP-coated AgNP (70nm) for up to 24 hours (approximately 10µg Ag/ml) and found that while incubation led to a time- and concentration-dependent accumulation of silver in the cells, it did not affect the cell viability or lead to a reduction in cellular glutathione level. In contrast, exposure to a similar concentration of AgNO₃, was found to severely compromise cell viability. This group found that the AgNP taken up by the astrocytes remained sequestered in the cells following 7 days of incubation in AgNP free medium (Luther *et al.*, 2012). The same robustness to AgNP toxicity was not seen when rat astrocytes were exposed to smaller, peptide coated AgNP (20 and 40nm), where the AgNP were seen to induce a strong size-dependent cytotoxicity and an increase in ROS formation (Haase *et al.*, 2012a). In secondary astrocyte cells lines, derived from human glioblastomas, AgNP (starch coated, 6-20nm) were found to result in cytotoxicity and genotoxicity in U251 cells (AshaRani *et al.*, 2009a,b) and AgCl was found to cause oxidative stress in A172 cells (Simmons *et al.*, 2011).

Neurons

Some studies show that AgNP seem to be particularly toxic to neurons (Yin *et al.*, 2013; Xu *et al.*, 2013), although Haase *et al.* (2012a) found that in their study astrocytes were more sensitive to peptide coated AgNP than neurons. In rat cerebellum granule cells, commercial AgNP (sized between 20-30nm) were found to cause cytotoxicity, based on an AB staining assay, at very low doses – with a reported IC₅₀ of 0.96µg/ml. Cell-body shrinkage was seen after 24 hour exposure to 1µg/ml AgNP and the AgNP were seen to cause oxidative stress (Yin *et al.*, 2013). Xu *et al.* (2013) found that 20nm AgNP caused cytotoxicity in rat cortical cell cultures at the lowest concentration examined (1µg/ml) in developing cells and at 5µg/ml in more mature cultures. The AgNP were found to not only inhibit the sprouting of neuronal branches and elongation of neurites, but they also caused fragmentation and degeneration of mature neurons. In contrast, Haase *et al.* (2012a) found that a significant cytotoxic effect of peptide stabilised 20nm AgNP was not seen until 50µg/ml and above on their rat neuronal-enriched cultures.

Neurodevelopment

The possible impacts of Ag on neurodevelopment have been examined using PC12 cells. PC12 cells, which are derived from rat adrenal medulla, stop dividing and terminally differentiate when treated with nerve growth factor and they are used as a model for neuronal differentiation. Powers *et al.* (2010, 2011) have looked at the impact of AgNO₃ and AgNP on these cells. A one hour exposure of undifferentiated PC12 cells to 10µM Ag⁺ was found to inhibit DNA synthesis and protein synthesis. Longer exposure resulted in oxidative stress and loss of viability. Ag⁺ directly inhibited mitotic activity. The same concentration of Ag⁺ was found to elicit even stronger effects with the onset of cell differentiation, with greater DNA synthesis inhibition and greater levels of oxidative stress. In addition selectively impaired neurite formation was seen and there was suppressed development of the acetylcholine phenotype in favour of the dopamine phenotype (Powers *et al.*, 2010). This group have also looked at the effects of AgNP (citrate- and PVP-coated) in PC12 cells. In undifferentiated cells citrate-coated AgNP (10nm) impaired DNA and protein synthesis, but did not result in significant oxidative stress or loss of cell viability. In differentiating cells, however, the citrate coated AgNP caused oxidative stress and impaired differentiation into the acetylcholine phenotype. In undifferentiated cells PVP-AgNP (10nm and 50nm) reduced DNA synthesis; with the 50nm particle size have a greater effect. All three AgNP significantly suppressed the acetylcholine phenotype, but the small PVP-AgNP enhanced differentiation into the dopamine phenotype (Powers *et al.*, 2011). The authors suggest that their results point to the likelihood that Ag and AgNP are developmental neurotoxicants.

Brain endothelial cells

Two recent studies have examined the impact of AgNP on rat brain endothelial cells (Trickler *et al.*, 2010; Grosse *et al.*, 2013). Trickler *et al.* (2010) used cultured rat brain microvessel endothelial cells as a model to examine the cellular accumulation, changes in pro-inflammatory mediators and changes in morphology and permeability following exposure to PVP-coated AgNP (25, 40 and 80nm in size). AgNP were found to accumulate in the cells in a size-dependent manner (with less accumulation seen for the 80nm AgNP). The cellular association of AgNP led to significant cytotoxicity and caused the release of cytokines and other inflammatory mediators from the cell monolayers. The changes in the pro-inflammatory mediators correlated with morphological changes and increased cell permeability.

Grosse *et al.* (2013) investigated the impact of citrate-coated AgNP (10, 50 and 100nm) on rat brain endothelial cells (RBE4). Based on the neutral red uptake assay (membrane permeability as an indicator of cytotoxicity), toxicity was seen for all of the AgNP examined, with the smaller particles being more toxic (effects seen at lower concentrations and after a shorter period of time). Exposure of the cells to AgNO₃, suggested that the ionic form was less toxic to the endothelial cells than AgNP.

4.3.4 Gut

A number of studies have looked at the impact of AgNP on intestinal cells, some of which have attempted to account for the likely effects of digestion or have used synthetic drinking-water as a medium for AgNP, rather than cell culture medium, to try and more closely simulated *in vivo* conditions.

Bouwmeester *et al.* (2011) used an *in vitro* model of the human intestinal epithelium (consisting of Caco-2 and M-cells) to study the passage of four different preparations of AgNP (nominal sizes 20, 34, 61 and 113nm) and silver ions (from AgNO₃). Concentrations of AgNP of up to 50µg/ml (irrespective of size) reduced metabolic activity in the Caco-2 cells by less than 20%, while a concentration of 5µg/ml AgNO₃ resulted in a 70% reduction in metabolic activity. Translocation of silver derived from either AgNP suspensions or AgNO₃ was clearly shown and the authors speculate that the translocation of silver is likely to be in the ionic and not the particulate form.

Walczak *et al.* (2013) studied the likely impact of digestion on 60nm AgNP (citrate) and silver ions (AgNO₃). The model comprised artificial saliva, gastric, duodenal and bile juice, simulating digestion in the oral, gastric and intestinal compartments with salt and protein composition, pH differences and transit times similar to human *in vivo* digestion. The AgNP, in the presence of proteins, were found to survive gastric digestion and reach the intestine where they were present in large clusters and co-localised with chlorine. The chlorine was thought to be involved in connecting separate AgNP inside clusters with 'chlorine inter-particle bridges'. Following intestinal digestion, the AgNP were found to be present in, essentially, their original form. Silver ions were also found to reach the intestine, but they were generally present as complexes of silver, sulphur and chlorine (20-30nm in size). The authors suggest that ingestion of AgNP and silver ions results in intestinal exposure to nanoparticles, albeit with different chemical compositions. Böhmert *et al.* (2014) conducted some similar work, subjecting AgNP to simulated digestion (both Böhmert *et al.*, 2014 and Walczak *et al.*, 2013 based their digestion model on the method described by Versantvoorlet *et al.*, 2005) but then examining their toxicity to Caco-2 cells. Cells were exposed to primary and digested particles as well as a digestion fluids mixture without AgNP to act as a control. It was found that AgNP seem to overcome gastrointestinal juices in their particulate form, without forming large quantities of aggregates, and there seems to be only a slight reduction in their cytotoxic potential following digestion.

Hsin *et al.* (2008) looked at the impact of two different commercially available preparations of AgNP (1-100nm) on human colon cells (HCT116). One preparation (Ching-Tai) was found to result in significant decreases in cell viability after 24 hours at 50µg/ml, while the other (at the same

concentration) did not result in significant cytotoxicity even after 72 hours. Compared to the other cells examined (mouse fibroblasts - NIH3T3 and rat vascular smooth muscle cells – A10), HCT116 cells were relatively insensitive to AgNP.

Gaiser *et al.* (2009) looked at the potential human exposure to AgNP via ingestion of contaminated food sources. They looked at both bare AgNP (35nm) and 'bulk' Ag (0.6 – 1.6 μm) on secondary intestinal epithelial cells (Caco-2) and human hepatocytes (C3A). Cytotoxicity was only assessed on the hepatocytes, with AgNP being more cytotoxic (LDH assay) than bulk Ag. Both AgNP and Ag were, however, shown to be taken up by Caco-2 cells.

The impact of AgNP (18nm) on gene expression in HT29 cells (and human kidney cells – see below) was explored by Gopinath *et al.* (2010). A concentration of 11 $\mu\text{g}/\text{ml}$ (less than half of the concentration required to inhibit cell growth by 50% - Gopinath *et al.* 2008) resulted in changes in cell morphology and caused an 11% increase in early apoptotic population, 21% increase in late apoptotic population and a 7% increase in necrotic population. Exposure to AgNP resulted in an up-regulation of apoptotic genes and a down-regulation of anti-apoptotic genes.

Kruszewski *et al.* (2013) looked at the impact of bare 20nm and 200nm AgNP on liver (HepG2), lung (A549) and gut (HT29) cells in terms of DNA damage and colony forming ability. They found a substantial difference in the cell uptake of AgNP, with uptake by the gut cells being markedly lower than the other cell lines. The authors suggest that this might be due to the production of mucin by HT29 cells which prevents NP uptake. The cellular uptake of AgNP was found to correspond to the formation of ROS and the subsequent pattern of DNA breakage and base damage induction was found to correspond to intracellular ROS formation.

Abbott Chalew and Schwab (2013) looked at the cytotoxic effects of uncoated AgNP (20-30nm) on Caco-2 and SW480 intestinal cells. The AgNP was not found to be particularly toxic to the intestinal cells when dispersed in cell culture medium (with LC₅₀ values for the two cell lines greater than 100 mg/l). Far greater cytotoxicity was seen for SW480 when the cells were exposed to AgNP in buffered synthetic water, with a significant drop in viability seen after exposure to 1 mg/l. The authors suggest that the lower toxicity in AgNP in cell culture media may be due to the stabilising effect of foetal bovine serum in the cell culture medium.

4.3.5 Kidney

A number of different kidney cell types have been subjected to silver, these include embryo kidney cells, which are a heterogeneous mix of almost all the types of cells present in the body (although most are endothelial, epithelial or fibroblasts), proximal tubule cells (HK 2) and renal epithelial cells (A498).

Gopinath *et al.* (2010) used baby hamster kidney cells (BHK21) to investigate the impact of 18nm AgNP on primary cells. Cells exposed to 11 $\mu\text{g}/\text{ml}$ (a concentration below the IC₅₀ value) showed altered morphology and a 9% increase in the early apoptotic population compared to control cells. An examination of gene expression showed that AgNP induced p53-mediated apoptotic pathway. Hudecová *et al.* (2012) exposed human embryo kidney cells (HEK293) to 20nm AgNP. Although there was clear agglomeration of the particles, the AgNP were still taken up by the cells and could be identified in vacuoles and cytoplasm. No cytotoxicity was reported after exposure of the cells to 100 $\mu\text{g}/\text{ml}$ for 30 minutes (based on Trypan blue exclusion), although there was a 48% reduction in proliferation activity and a 21% reduction in colony number at that concentration. No cytotoxicity (in any of the employed tests) was seen at concentrations up to 25 $\mu\text{g}/\text{ml}$, although DNA damage could be detected even after exposure to 1 $\mu\text{g}/\text{ml}$ AgNP. Singh and Ramarao (2012) found that renal epithelial cells (A498) were sensitive to 44nm AgNP, with a significant reduction in viability (MTT and Coomassie Blue assay) at 1 $\mu\text{g}/\text{ml}$. This group looked at five different cell lines and the kidney cells were the most sensitive. Kermanizadeh *et al.* (2013) looked at the impacts of a variety of

nanomaterials on renal proximal tubule epithelial cells. The AgNP (<20nm, capped with polyoxylaurat Tween) was one of the more toxic nanomaterials examined, with an LC₅₀ of between 4.5-10µg/cm² (depending on the cell culture medium used). AgNP exposure resulted in a significant increase in ROS, interleukins 6 and 8 and evidence of DNA damage. Ionic silver has also been found to be toxic; with Simmons *et al.* (2011) reporting that AgCl caused an increase in the oxidative stress response in four out of five cell lines examined, including kidney cells - HEK293T.

4.3.6 Blood

The toxic effects of silver on blood have been studied by a number of groups, using a variety of different methodologies. Foldbjerg *et al.* (2009) looked at the toxicity of PVP-coated AgNP (69nm) and silver ions (from AgNO₃) on the human monocytic leukaemia cell line (THP-1). Cells were exposed for up to 24 hours and it was found that both AgNP and Ag⁺ induced apoptosis and necrosis (depending upon the dose and exposure time) and caused increased ROS levels after six hours. In the cytotoxicity test (Annexin V/PI) silver ions were found to be four times more toxic than AgNP (EC₅₀ 0.62µg/ml Ag⁺ compared to 2.44µg/ml AgNP). Haase *et al.* (2012b) also looked at the toxicity of AgNP on THP-1 cells. They used two peptide coated AgNP (20nm and 40nm) and found that while both AgNP were toxic to the monocytes, AgNP20 was more toxic. The toxic effect was found to increase with time, thus the IC₅₀ for AgNP20 at 24 hours was 110µg/ml compared to 18µg/ml at 48 hours.

Wang *et al.* (2013) used mouse erythroleukemia cells to study the impact of a range of PVP-coated AgNP (10, 25, 40, 45 and 110 nm) on mRNA transcription. At 1µg/ml (a non-cytotoxic dose) a large reduction in alpha- and beta-globin was seen. The shape of the AgNP seemed to be important as the spherical AgNP showed a greater impact on globin expression compared to the plate form and it was speculated that spherical AgNP may have a greater capability to cross the plasma membrane. Small spherical AgNP (10, 25nm) showed a greater inhibition of globin expression than the larger particles. The group demonstrated that AgNP caused a significant suppression of RNA polymerase activity and overall RNA transcription through direct Ag binding to RNA polymerase.

Jun *et al.* (2011) looked at the effect of AgNP on platelet aggregation. The group used human washed platelets as an *in vitro* test and rats as an *in vivo* test. In platelets, the AgNP (<100nm) were found to induce platelet aggregation:

- control - 5.4% aggregation;
- 100µg/ml AgNP - 28% aggregation;
- 250µg/ml AgNP - 54% aggregation.

The aggregation was potentiated by co-treatment with a sub-threshold concentration of thrombin. Consistent with the human platelet studies, *in vivo* exposure of rats to AgNP (0.05 – 0.1 mg/kg by intravenous administration or 5 – 10 mg/kg by intratracheal instillation) enhanced venous thrombus formation and platelet aggregation. The authors suggest that AgNP may increase the prothrombotic risk in susceptible patients with compounding cardiovascular diseases.

Choi *et al.* (2011) used heparinized human blood to look at the impact of silver on haemolysis. They used four different silver preparations (two nano and two micron sized). Both AgNP preparations (citrate stabilised and bare particles) were significantly more haemolytic than the micron sized particles (equivalent mass concentration). The haemolysis was related to the release of silver ions (with the AgNP releasing considerably more than the micron preparations).

Silver nitrate, at various concentrations (up to 33µM) was added to human whole blood and levels of GSH measured at time intervals (Khan *et al.* 2011). The GSH level was found to decrease in a concentration- and time-dependent manner in both the plasma and cytosolic fraction, with the

depletion suggesting that the AgNO₃ penetrated the blood cells and resulted in oxidation of the reduced glutathione or the formation of a silver-glutathione complex.

4.3.7 Skin

Most researchers have used cell lines (keratinocytes, dermal fibroblasts and skin epithelial cells) to look at the potential toxicity of skin application of silver, but the potential for skin penetration of AgNP has also been investigated in an *in vitro* system.

Skin penetration

Larese *et al.* (2009) looked at the penetration of AgNP through human skin using an *in vitro* test system that utilised abdominal full thickness skin obtained as surgical waste. Skin was essentially bathed in AgNP (25nm in size, dispersed in ethanol and diluted with synthetic sweat) for 24 hours. The experiments were conducted using both intact and abraded skin. Low, but detectable, AgNP absorption through intact skin was seen. As might be expected, penetration through damaged skin was five times greater than that through intact skin. AgNP could be seen (using transmission electron microscopy) in the stratum corneum and upper layers of the epidermis.

Skin cells

Arora *et al.* (2008) used secondary human skin epithelial cells (A431) to study cellular responses induced by spherical AgNP (7-20nm). As the IC₅₀ (XTT assay) was 11.6µg/ml, cells were subsequently exposed to a dose roughly half of that value. At 6.25µg/ml, cellular morphology was unchanged, but there were clear signs of oxidative stress, namely decreased GSH (~ 2 fold), decreased SOD (~ 3 fold) and increased lipid peroxidation (~ 2 fold). Comfort *et al.* (2011) also found indicators of oxidative stress in A431 cells after exposure to low levels of AgNP (10nm). In addition was inducing high quantities of ROS, AgNPs caused a disruption in the epidermal growth factor (EGF) signalling response.

Cortese-Krott *et al.* (2009) treated primary human skin fibroblasts with low levels of AgNO₃ (below that impacting on proliferation, mitochondrial activity or cell viability) and found that subtoxic concentrations (5-10µM) strongly increased the intracellular production of ROS (including superoxide anion radicles) and impacted on intracellular zinc homeostasis.

Samberg *et al.* (2010) looked at the cytotoxicity of bare AgNP (20, 50 and 80nm) and carbon coated AgNP (50 and 80nm) to primary neonatal human epidermal keratinocytes. If AgNP were applied to the keratinocytes unwashed, a 24-hour exposure resulted in a significant dose-dependent decrease in viability. However, application of the carbon-coated AgNP or washed AgNP did not cause a decrease in cell viability, suggesting that the toxicity seen in the unwashed AgNP is a result of residual contamination from the AgNP synthesis (in this case formaldehyde). Although washed AgNP did not result a decrease in viability, they were taken up and were found to be internalized into the membrane-bound vacuoles in the keratinocytes.

Zanette *et al.* (2011) found that PVP coated AgNP (25-50nm) caused a concentration- and time-dependent decrease in cell viability (based on mitochondrial function) in HaCaT cells at concentrations of 11µg/ml and greater. A long-lasting inhibition in cell proliferation was seen as cell proliferation was still showing a concentration-dependent decrease 6 days after the AgNPs had been washed out of the system.

Comparative AgNP (~ 65nm) cytotoxicity tests using HaCaT and cervical cancer cells (HeLa) were conducted by Mukherjee *et al.* (2012). They used a wide range of cytotoxicity tests and found that, in both cell lines, a measure of mitochondrial function (MTT) was the most sensitive test (HaCaT LD₅₀ at 24 hours 51.8mg/l). After 24 hours, the LD₅₀ values for the MTT test for both cell types were similar. After 48 and 72 hours, however, HeLa cells were found to be much more sensitive (LD₅₀ 72 hours: HaCaT 30.4mg/l compared to HeLa 0.04mg/l). The authors note that a major difference

between the two cell types is their natural antioxidant levels, with HaCaT having over 30 times more glutathione than HeLa and this could be an important factor in the different sensitivity to AgNP.

Srivastava *et al.* (2012) investigated the impact of AgNP (size unstated) and silver ions (AgSO_4) on selenium metabolism in keratinocytes (HaCaT). They found that while there was no clear cytotoxic effect of AgNP (up to $10\mu\text{M}$) or AgSO_4 (up to 1000nM) exposure on the keratinocytes, AgNP and Ag^+ led to a dose-dependent inhibition of selenium metabolism. The authors commented that the decrease in selenoprotein synthesis could have significant implications in the defence against oxidative stress in the event of long-term exposures.

4.3.8 Macrophages

Macrophages constitute the first line of defence upon uptake of AgNP by humans and other mammals (Pratsinis *et al.* 2013). Macrophages function in both non-specific defence (innate immunity) as well as helping to initiate specific defence mechanisms (adaptive immunity).

Shavandi *et al.* (2011) looked at the cytotoxicity of AgNP (18-34nm) to murine peritoneal macrophages using an assessment of mitochondrial activity (MTT assay). A significant decrease in viability was seen at concentrations of 1 ppm and above after 24 hours of exposure. Significant reductions in nitric oxide production were seen at 0.4 ppm AgNP. Park *et al.* (2010b) also used murine peritoneal macrophages (RAW 264.7) to examine the impact of AgNP. AgNP with an average size of ~ 70 nm were dispersed in foetal bovine serum and cells were exposed for up to 96 hours to concentrations between 0.2 to 1.6 ppm. Cell viability (MTT assay) decreased in a concentration and time-dependent manner, with the lowest concentration causing significant cytotoxicity after 96 hours. The AgNP also significantly reduced levels of intracellular GSH at concentrations of 0.4 ppm and above. In contrast to Shavandi *et al.* (2011), Park and colleagues found that NO was significantly increased. Park *et al.* (2010b) reported that AgNP were ingested by phagocytosis, but that they weren't observed in the dead cells, suggesting that the particles were released back into the culture medium by the damaged cells where they were available for further biological responses.

Four different AgNP with a similar size ($<10\text{nm}$) and shape (spherical), but different coatings and surface charge were tested against two cell lines: mouse macrophage (RAW 264.7) and lung epithelial cells (C-10). The same pattern of toxicity was seen in both cell lines with, essentially, the AgNPs with the greater positive surface charge being more toxic. The macrophage cells were more sensitive to the AgNP than the lung epithelial cells (Suresh *et al.* 2012). Singh and Ramarao (2012) also found that RAW 264.7 macrophages were highly sensitive to AgNP (44nm) toxicity, with a significant reduction in cell viability (MTT assay) seen after 72 hours exposure to $3\mu\text{g}/\text{ml}$. Of the six cell lines examined only renal epithelial cells (A498) were more sensitive. Interestingly, J774.1 macrophages were one of the more resistant cells line (significant cytotoxicity was seen at $30\mu\text{g}/\text{ml}$).

Pratsinis *et al.* (2013) synthesised uncoated AgNP (6 to 20nm) supported on inert nanostructured silica and looked at the impact of silver ion release on the viability of murine macrophages (RAW 264.7). Small AgNP ($<10\text{nm}$) released or leached larger fractions of their mass as Ag^+ upon dispersion in water and this strongly influenced the cytotoxicity.

5. Quality of evidence

In order to increase the transparency and improve confidence in the conclusions, it is becoming increasingly common to assess the quality of the body of evidence as part of the literature review process. A framework, which has been used by WHO in other areas, is the GRADE approach, which has been derived for use in a clinical/healthcare context (Guyatt *et al.*, 2008a). GRADE is an acronym for Grading of Recommendations Assessment, Development and Evaluation. The GRADE system specifies four categories for the quality of evidence – high, moderate, low and very low – with the definitions shown in Table 6 (Balshem *et al.*, 2011).

The starting point is that randomized control trials constitute high quality evidence, while observational studies are low quality. A number of factors, shown below, are then considered that can increase or decrease the rating (Guyatt *et al.*, 2008b).

Reduce the rating:

- Study limitations (risk of bias)
- Unexplained inconsistencies
- Indirectness of evidence
- Imprecision
- Publication bias

Increase the rating:

- Large magnitude of effect
- Plausible confounding (which would reduce a demonstrated effect)
- Dose response gradient

Table 6: Current and previous definitions of the four levels of evidence (Balslem *et al.*, 2011)

Quality level	Current definition	Previous definition
High	We are very confident that the true effect lies close to that of the estimate of effect	Further research is very unlikely to change our confidence in the estimate of effect
Moderate	We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of effect, but there is a possibility that it is substantially different	Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate
Low	Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect	Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate
Very low	We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect	Any estimate of effect is very uncertain

In addition, Klimisch *et al.* (1997) has outlined a systematic approach for evaluating the quality of experimental toxicological data in hazard and risk assessment processes. This is based on an assessment of reliability, relevance and adequacy, as outlined below (taken directly from the paper).

- Reliability – evaluating the inherent quality of a test report or publication relating to preferably standardized methodology and the way that the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings.
- Relevance – covering the extent to which data and/or tests are appropriate for a particular hazard identification or risk characterization.
- Adequacy – defining the usefulness of data for risk assessment purposes.

It is felt, however, that these aspects can be captured in a modified version of GRADE which can be used to assess the quality of all of the evidence presented (rather than using Klimisch *et al.* (1997) for the toxicology and a different method for the non-toxicological data).

In a USA, the National Toxicology Program have recently published draft approach for the systematic review and evidence integration for literature-based health assessments that is based on the GRADE approach (NTP, 2013).

The starting point in the modified version of GRADE is that experimental studies are high quality evidence (i.e. they are equivalent to randomized control trials). The quality rating may be reduced or increased according to similar criteria to those outlined in the GRADE process. These are outlined below, in the context of the current review (and the *in vitro* testing of AgNP).

Downgrading the quality rating

- Study limitations – this is taken from the GRADE assessment, but also captures the ‘reliability’ element described by Klimisch *et al.* (1997). The study limitations will depend on the area under assessment but, as an example, in assessing *in vitro* toxicological studies of AgNP study limitations would include lack of assessment of AgNP agglomeration, lack of consideration of AgNP purity, use of one or only a small number of tests and lack of a positive control.
- Unexplained inconsistencies (GRADE) – there may be good reasons for inconsistent results, such as the use of different cell types (primary or secondary cell lines, cells from different animals or tissue types) or using AgNP manufactured using different methodologies or using different capping agents. Where there are inconsistencies in study results and no clear explanation is provided by the authors, then the quality rating is decreased.
- Indirectness (GRADE) – direct evidence comes from research that specifically sets out to answer the question in which we are interested. In terms of the current review and the question of whether silver is toxic to humans, any toxicological studies using animals or animal cells are clearly indirect.
- Imprecision (GRADE/Klimisch *et al.*, 2007) – Imprecision might occur where there are studies with small sample sizes and wide confidence intervals in the measured outcome. It might also result from a lack of published studies (capturing the adequacy component outlined by Klimisch *et al.*, 1997).
- Publication bias (GRADE) – this can be difficult to assess but should be considered when available evidence comes from a small number of studies, most of which have been commercially funded (Guyatt *et al.*, 2011a).

Upgrading the quality score

- Large reported effect (GRADE) – this may principally apply to epidemiological studies, where it has been suggested that methodologically rigorous observation studies (initial quality rating of poor) may be upgraded where they show at least a two-fold reduction or increase in risk of effect (Guyatt *et al.*, 2011b).
- Dose-response gradient (GRADE) – the presence of a dose-response relationship has long been an important component in showing a causal relationship. Many of the *in vitro* studies show that as the dose of AgNP increases the level of cytotoxicity increases (usually as measured by more than one analytical test).
- Cross species/population/study type consistency (NTP) – although GRADE does not recommend upgrading the quality rating if studies show consistency (Guyatt *et al.*, 2011c), because the current evaluation also needs to apply to efficacy and toxicity studies (rather than solely epidemiological studies) it was felt appropriate to include this category and

upgrade the rating where consistent results are reported in multiple experimental animal models or cell types, different populations or study types (NTP, 2013).

The literature outlined in the principal areas relating to the efficacy of silver in drinking-water and the toxicity of silver have been subjected to this modified GRADE assessment and the scores considered as a whole in order to come to an overall assessment of MODERATE to LOW for the complete body of evidence.

6. Discussion

A review of the recent literature reveals that there is a great deal of interest in silver and AgNP in particular, both in terms of potential applications and toxicity.

6.1 Efficacy

In many of the studies reported in Section 3, it is often difficult to determine the efficacy of the silver component (especially in the studies outlined in Sections 3.2 and 3.3) as the impact of filtration alone is often not reported. In a number of cases, silver measurements in the treated water are not reported (meaning that it is not possible to assess human exposure to silver via this route).

Although an initial glance at the results suggests that silver may be a promising drinking-water treatment, there are a number of factors that need to be considered, including:

- the strong emphasis on testing against bacteria;
- the use of predominantly lab grown bacteria;
- the lack of consideration that silver may be acting as a bacteriostat; and
- no clear accounting for the presence of potentially toxic contaminants in applications using AgNP.

The majority of studies have used bacteria and, in particular, *E. coli* as a measure of silver efficacy. Where viruses were considered, the removals were generally poor or non-existent (with one notable exception – De Gussemé *et al.*, 2010 – which requires further confirmation). Although silver was generally effective at reducing bacterial levels (to some degree) the log reductions varied widely with some bacteria being more sensitive to silver than others. *E. coli*, an important indicator of water quality, seems to be particularly sensitive (with Hwang *et al.*, 2007, reporting a 7 log reduction).

Nawaz *et al.* (2012), showed relatively poor log removals of environmental bacteria from silver-treated harvested rainwater. The use of lab grown bacteria, which tend to be “*less virulent and hearty than wild microbial consortiums*” (Madrigan *et al.*, 2000) may overstate the effectiveness of treatment and it is suggested that further work should use more realistic testing regimens.

Very few studies considered the possibility of bacterial regrowth in the silver treated drinking-water (i.e. that silver is acting as a bacteriostatic, rather than bacteriocidal, agent). Where silver ions are present in the finished water then regrowth is unlikely, but this often was not analysed or reported.

AgNP can be synthesised in a variety of ways, some of which use toxic reagents. It is often not clear from the studies on AgNP applications whether adequate steps were taken to remove these contaminants before efficacy testing. Samberg *et al.* (2011), for example, investigated the efficacy of AgNP against a number of bacteria in culture medium and found that washed and unwashed AgNP had notably different minimum inhibitory concentrations (e.g. for the 20nm AgNP against *E. coli* J53, MICs were 64µg/ml and <4µg/ml, respectively). The additional toxicity of the unwashed particles was attributed to the presence of formaldehyde.

Given these factors, it is suggested that the efficacy of silver as a drinking-water treatment is, currently, far from convincing.

Copper/silver ionization is often used to treated hospital hot water systems as a defence against *Legionella* spp. and outbreaks of Legionnaire's disease. Although a number of studies have shown that where ion levels are carefully controlled copper/silver ionization can be effective in reducing *Legionella* colonization it does not eradicate the bacteria completely and some water characteristics (e.g. high pH) may decrease the effectiveness of the method. While studies generally suggest that implementation of copper/silver ionization markedly reduces the number of cases of nosocomial Legionnaires' disease, the outbreak of illness seen in Pittsburgh in 2012 is of concern and may suggest that ionization as a treatment system may not be a stand-alone long-term solution.

While the peer-reviewed literature on the use of copper/silver ionization for the treatment of swimming pool water is sparse, it does suggest that it is not generally effective against viruses and is only effective against bacteria in combination with chlorine.

6.2 Toxicity

It is clear than silver (largely irrespective of route of exposure or form) can distribute widely within the mammalian body and is capable of crossing the blood-brain and placental barriers. Tissue distribution varies between studies but the liver and kidneys seem to be target organs following silver ingestion. Drawing clear conclusions about the toxicity of silver and AgNP, however, is more difficult as the following sections illustrate.

6.2.1 AgNP

There are numerous different methodologies for the synthesis of AgNP, they can be produced in a wide range of sizes and shapes and stabilised with a variety of capping agents, and these factors alone make generalisations difficult.

Synthesis and capping

Chernousova and Eppe (2013) have noted that the reproducible laboratory synthesis of AgNP is "*more difficult than expected*". They relate this to the initial formation of the nuclei of metallic silver, which develop different morphologies and crystal sizes when reaction conditions (such as concentrations, reduction agent, temperature or presence of additives) change.

A number of studies have shown that the choice of capping or stabilising agent can change the toxicity of AgNP. Stevanovic *et al.* (2011), for example, found significantly less cytotoxicity (MTT assay) and induction of ROS in HepG2 cells exposed to 10% AgNP capped with poly- α,γ -L-glutamic acid in comparison to uncapped (bare) AgNP. Lin *et al.* (2012) showed similar results for a range of different polymer stabilising agents, i.e. the capped AgNP showed less toxicity to mammalian cell lines than bare AgNP (interestingly, in this case, while maintaining a high level of growth inhibition against *E. coli*).

Size

There are a number of techniques for determining AgNP size, those most commonly used are transmission electron microscopy (TEM) and dynamic light scattering (DLS). TEM is useful to capture the size of the individual (or primary) particle, but it is limited as it can only be used to measure particles after they have been suspended and then dried (it may also be affected by the solvent used for AgNP dispersion prior to drying). DLS captures the hydrodynamic size and is performed in solution, but may be affected by the suspension media and how the sample was mixed, for example sonication intensity and duration (Choi *et al.*, 2011). The size of the AgNP also depends on the medium in which they are suspended, with Bouwmeester *et al.* (2011), for example, finding larger hydrodynamic sizes for AgNP when they were suspended in cell culture medium, compared to

water. In the review sections and below, usually only the primary size of the AgNP has been reported to avoid over complicating the text.

Some studies have suggested that smaller AgNP are more toxic to mammalian cells than larger nanoparticles and microparticles. Carlson *et al.* (2008), for example, found that 15nm carbon-coated AgNP caused more toxicity than 50nm carbon-coated AgNP in rat alveolar macrophages and Li *et al.* (2012) reported similar results for PVP-coated AgNP (25, 35, 45, 60 and 70nm) in human lung fibroblasts. Liu *et al.* (2010) found that small PVP-coated AgNP (5nm) were more toxic to four different cell lines than both ionic silver (AgNO₃) and larger particles, as shown in Table 7.

Table 7: Median effective concentration (EC₅₀) for cell mortality in four different cell lines (Liu *et al.* 2010)

Cell line	AgNO ₃ (µg/ml)	AgNP – 5nm (µg/ml)	AgNP – 20nm (µg/ml)	AgNP – 50nm (µg/ml)
A549	3.62	1.02	9.96	14.31
HepG2	1.11	0.59	25.35	33.57
MCF-7	1.81	0.51	14.33	47.64
SGC-7901	3.23	0.92	50.94	112.03

However, this does not seem to be universally the case as Powers *et al.* (2011), for example, found that larger PVP-coated AgNP (50nm) had greater effects on DNA synthesis and caused a higher degree of oxidative stress in PC12 cells than the smaller PVP-coated particle (10nm). Park *et al.* (2010b) reported greater cytotoxicity of 70nm AgNP in mouse macrophages than Shavandi *et al.* (2011), although it has been suggested that this may have been an artefact of the preparation method, which could have led to high Ag⁺, but reduced AgNP concentration (Pratsisnis *et al.* 2013). In a review of toxicity data on mammalian cell lines, Bondarenko *et al.* (2013) found that when plotting L(E)C₅₀ data for PVP-coated AgNP (to avoid coated versus non-coated toxicity issues) against the primary size of the AgNP, no correlation was seen (R²=0.1), plotting the data from Liu *et al.* (2010) resulted in a correlation of R²=0.4, while plotting data from just one study on A549 cells (Liu *et al.*, 2010) revealed a correlation of R²=0.81. This demonstrates how difficult it is to make generalisations about the toxicity of AgNP to mammalian cells.

Experimental quality

There are numerous pitfalls that await the unwary AgNP researcher. These include lack of characterisation of the AgNP, gradual release of silver ions from the dissolved AgNP following preparation, toxicity of the capping agent or suspending solvent, presence of biological contaminants, failure to account for possible contaminants remaining after the manufacture of the AgNP and interference of AgNP with the toxicity tests.

In order to improve comparability between studies it is important that the AgNP used are adequately characterised and it has been suggested that complete characterization of AgNP may include measurements of size distribution, shape and other morphological features, solubility, surface area, state of dispersion, surface chemistry and other physico-chemical properties (Park *et al.*, 2010b). Studies which go to those lengths are rare, but AgNP characterization is increasingly being reported and it is clear that where commercial AgNP are utilised it is not always adequate to rely on the manufacturers claims (Choi *et al.*, 2011)

Kittler *et al.* (2010) examined the toxicity of freshly prepared AgNP and previously stored AgNP on human mesenchymal cells. The aged AgNP were found to be considerably more toxic than those that were freshly prepared, with the AgNP that had been made up for 1 or 6 months causing 100% loss of cell viability compared with a 70% loss of viability seen in the cells treated with freshly prepared AgNP. The difference in toxicity was attributed to differing amount of released Ag ions. The authors

comment that some of the published discrepancies in reported toxicity studies may be explained by this observation.

Oostingh *et al.* (2011) investigated a number of possible issues relating to toxicity testing of nanoparticles. Some of the agents used to stabilise AgNP may have a toxic effect in their own right, with citrate, for example (a common capping agent) exerting a dose-dependent cytotoxic effect on BEAS-2B human primary lung cells. They also looked at biological contamination which may be important when studying immunomodulating/immunotoxic effects. Although the nanoparticles they used were sterile (i.e. devoid of live bacterial contamination), they found that both the nanoparticles and their solvents contained variable levels of endotoxin (to which many immune cells are especially sensitive).

A number of traditional measures of cytotoxicity rely on optically based tests, but it has been shown that nanoparticles can interfere with these tests. Small nanoparticles (4-15nm) have been shown to absorb at the wavelengths typically used in most biological assay readouts (this could suggest improved viability), while some nanoparticles can inhibit colour formation – which would mimic a cytotoxic effect (Oostingh *et al.*, 2011).

6.2.2 *In vitro* toxicity

Primary cells are more representative of tissue and they can be expected to reproduce the normal response of normal individuals (Oostingh *et al.*, 2011) and so are ideal for *in vitro* toxicity studies. The use of primary cells, however, is not always feasible as they may be difficult to obtain (e.g. human lung epithelial cells) and they have limited cellular life spans, which means that fresh cells (probably obtained for different donors) are required for each assay, making standardisation difficult (Oostingh *et al.*, 2011). Thus secondary cell lines (transformed or tumour cells with unrestrained proliferative capacity), which are easier to maintain and produce reproducible results are preferred in many toxicity studies (Arora *et al.*, 2008). There may, however, be a number of issues related to the widespread use of secondary cell lines in *in vitro* toxicity testing. Oostingh *et al.* (2011) make the point that particular caution should be used when testing the cytotoxic and anti-proliferative effects of nanoparticles on secondary cells as they have different cell cycle regulation and cell survival compared to primary cells. Indeed, it has been reported (e.g. Arora *et al.*, 2009) that secondary cells are more susceptible to the impacts of AgNP than primary cells and this has led to the exploration of AgNP as a possible cancer treatment (e.g. Sriram *et al.*, 2010).

As noted by Samberg *et al.* (2012), there is currently no consensus on the cytotoxicity of AgNP; however the majority of publications do show reduced cell viability and increased ROS generation following AgNP exposure. Some however, clearly show that ROS are not always produced (e.g. Xiu *et al.*, 2011). Zanette *et al.* (2011) point out that while many studies consider evidence for the induction of oxidative stress and apoptosis in cells exposed to AgNP, less investigate the intracellular pathways involved in the processes. While such details are beyond the scope of this review, Zanette *et al.* (2011) suggest that AgNP may act on different cellular targets and may differentially affect specific intracellular pathways depending on the cell types used. Chernousova and Epple (2013) in their review of silver as an antimicrobial agent comment that, given the different possibilities for silver to disturb biological processes, a general statement about the origin of the toxic action of silver is not possible.

6.2.3 *In vitro* to *in vivo* extrapolations

Monteiro-Riviere *et al.* (2013) looked at the impact that pre-incubation of AgNP with a number of different proteins (albumin, IgG and transferrin - to form protein-complexed nanoparticles) had on the uptake of AgNP by human epidermal keratinocytes. AgNP association with serum proteins significantly modulated Ag uptake compared to native AgNP uptake. This suggests the need for caution in extrapolating *in vitro* uptake data to predict behaviour *in vivo*, where the nature of the protein corona may determine patterns of cellular uptake.

The results of Abbott Chalew and Schwab, 2013 (who found that the cytotoxicity of AgNP to intestinal cells was greater when the NP were suspended in buffered water than culture medium) also suggest the need for caution in making *in vivo* suppositions from *in vitro* data as AgNP exposure, especially via ingestion, are likely to be complex.

6.2.4 *In vivo* toxicity

The studies outlined in Section 4.2, largely focus on the effects of AgNP (with some comparisons with salts) and seem, generally, reassuring in that no toxicity or fairly mild toxicity is reported. Older studies are, perhaps, not as reassuring, although it is clear that in many cases extremely high doses were given. The following is taken from the Concise International Chemical Assessment Document (CICAD) on the environmental aspects of silver and silver compounds (WHO, 2002).

“Ionic Ag given as AgNO₃ is lethal to lab mice and rabbits at 13.9 and 20 mg/kg bw, respectively, by intraperitoneal injection, to dogs at 50 mg/kg bw by intravenous injection and to rats at 1586 mg/l drinking-water for 37 weeks (ATSDR, 1990). Sublethal effects are reported in rabbits given AgNO₃ at concentrations of 250µg/l drinking-water (brain histopathology) (Smith and Carlson, 1977), in rats given 400µg/l drinking-water for 100 days (kidney damage) (USEPA, 1980) in mice given 95 mg/l drinking-water for 125 days (sluggishness), in guinea pigs given 81 mg/cm² skin applied daily for 8 weeks (reduced growth) (ATSDR, 1990) and in rats given diets containing 6mg/kg for 3 months (high accumulations in kidneys and liver) or 130-1110 mg/kg (liver necrosis) (Smith and Carlson, 1977)”.

Of concern are the studies showing toxicity resulting from drinking-water ingestion in rabbits (250µg/l) and rats (400µg/l).

6.3 Guidelines and regulations

There is currently no WHO health-based guideline value for silver in the drinking-water guidelines (WHO, 2011). Silver was last reviewed by the WHO for the drinking-water guidelines in 1993, when it was concluded that on the basis of epidemiological and pharmacokinetic knowledge at the time a total lifetime oral intake of about 10g of silver could be considered the human no observable adverse effect level (NOAEL). As it was felt that the contribution of drinking-water to this NOAEL would normally be negligible it was not deemed necessary to establish a health-based guideline value. However, it was suggested that where silver salts are used for drinking-water treatment that a concentration of 0.1mg/l could be tolerated without risk to health (a concentration that would give a total dose over a 70 year period of half of the NOAEL outlined above). The 0.1 mg/l level is thus a health advisory rather than a guideline value, a distinction that is rarely appreciated by researchers (e.g. Pelkonen *et al.*, 2003) who often refer to 0.1 mg/l as a guideline or allowable amount.

In Germany the drinking-water regulations (Trinkwasserverordnung) sets an allowable maximum of 0.01mg silver/litre (Chernousova and Epple, 2013).

In the 2012 edition of the USEPA drinking-water standards and health advisories document (USEPA, 2012) silver has the following health advisory values:

- | | |
|---|--------|
| • 10 kg child one-day (mg/l) | 0.2 |
| • 10 kg child ten-day (mg/l) | 0.2 |
| • Reference dose (mg/kg/day) | 0.005* |
| • Drinking-water equivalent level - DWEL (mg/l) | 0.2 |
| • Life time health advisory (mg/l) | 0.1* |

* based on a cosmetic effect

A health advisory is an estimate of acceptable drinking-water levels for a chemical substance based on health effects information; it is not a legally enforceable Federal standard, but serves as technical guidance.

In the UPEPA secondary drinking-water regulations (non-enforceable Federal guidelines), silver has a value of 0.1 mg/l (USEPA, 2012).

6.4 Environmental considerations

Environmental considerations are largely beyond the scope of this report, however, it has been noted that release of silver and AgNP (from whatever source) into the environment may pose a threat to 'non-target' organisms (such as natural microbes and aquatic organisms). Bondarenko *et al.* (2013) reviewed the toxicity of Ag and AgNP to selected environmentally relevant test organisms as well as target organisms. Table 8 shows the median L(E)C₅₀ or MIC data for AgNP and Ag salts.

Table 8: Median L(E)C₅₀ for all organisms except bacteria and median MIC for bacteria for AgNP and Ag salts (Bondarenko *et al.*, 2013)

Group of organisms	Median L(E)C ₅₀ /MIC			
	AgNP (mg compound/litre)	N	Ag salt (mg metal/litre)	N
Target				
Algae	0.36	17	0.0076	10
Bacteria	7.10	46	3.3	27
Protozoa	38	7	1.5	3
Non-target				
Crustaceans	0.01	17	0.00085	8
Fish	1.36	17	0.058	4
Nematodes	3.34	21	4.8	4
Mammalian cells in vitro	11.3	25	2	18

The most sensitive organisms to both Ag salts and AgNP are crustaceans (non-target organisms). Based on the lowest median L(E)C₅₀ value of the key environmental organisms both Ag salt and AgNP would be classified as 'very toxic to aquatic organisms' under EU Directive 93/67/EEC CEC, 1996).

7. Conclusions

The lack of consistency in terms of what has been examined (Ag salts versus AgNP; capped AgNP versus bare AgNP; differently sized AgNP; AgNP created using different synthesis methods) and how it has been examined (use of different methodologies, different cells and microorganisms, different concentrations of test organisms and exposure for different time periods) means that it is difficult to compare data from different studies. There does seem, however, to be increasing recognition of some of the problems and the need for a more standardised approach.

The efficacy of silver, especially in terms of drinking-water disinfection, is currently a long way from convincing and, overall, there are far too many studies just looking at bacteria in general and *E. coli* in particular. Clearly, for any silver product to prove its worth as a drinking-water treatment it needs to show disinfectant efficacy against a range of microorganisms, including viruses. Any product that selectively removes the indicator bacteria is presenting a false picture of the water quality.

The body of evidence seems to suggest that silver (in ionic form or as AgNP) is toxic to mammalian cells, although the sensitivity of the cells varies according to the cell type and the type of silver to which it is exposed. There are a number of reasons why this finding of *in vitro* toxicity does not extrapolate easily to *in vivo* situations.

The mammalian *in vivo* data seem to suggest that generally exposure to silver results in little or no toxic signs, although in some cases high doses and/or repeated exposure to silver can cause mild toxicity and it has been noted that “*that the current application of silver (as metal, ion or AgNP) does not constitute a major risk for humans*” (Chernousova and Epple, 2013). The use of silver, especially in a drinking-water context does, however, present the potential for environmental problems as certain aquatic organisms (crustaceans) are far more sensitive to the effects of silver than bacteria (between 700 and over 3500 times more sensitive, based on the figures of Bondarenko *et al.*, 2013).

Finally, returning to the questions asked in Section 1, while the overall quality of evidence has only been rated as between moderate and low it is suggested that the evidence is sufficient to indicate that:

- while silver is toxic to mammalian cells *in vitro*, *in vivo* studies show minimal toxicity at realistic exposure scenarios and as such it seems unlikely to cause harm to humans from its use as a drinking-water treatment; and
- in its current applications, silver is not an effective drinking-water disinfectant.

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Appendix 1: Disinfectant mode of action

This short section outlines the disinfectant mode of action of silver ions and AgNP.

Silver ions are believed to impact on bacteria in a number of ways, including:

- extracellular binding or precipitation of silver to bacterial cell walls (Bellatone *et al.*, 2002);
- the inhibition of essential enzymatic functions via interaction of the ions with the thiol-group (sulfhydryl group) of L-cysteine (Liau *et al.*, 1997);
- the production of reactive oxygen species (Park *et al.*, 2009); and
- interaction with DNA (Thurman and Gerba, 1989).

Feng *et al.* (2000) conducted a mechanistic study of the antibacterial effect of silver ions on *E. coli* and *S. aureus*. Following treatment with silver nitrate, silver ions were detected inside the cells and both types of bacteria showed similar morphological changes, with the cytoplasmic membrane detaching from the cell wall. In addition, an electron-light region appeared in cells, with condensed DNA molecules within the centre of this region. DNA in a condensed form is unable to replicate.

Thurman and Gerba (1989) showed that silver binds to DNA, with the metal displacing the hydrogen bonds between adjacent nitrogens of purine and pyrimidine bases.

Dibrov *et al.* (2002) investigated the antimicrobial activity of silver ions on *Vibrio cholerae* and found that, at low concentrations of Ag⁺, massive proton leakage through the cell membrane could be observed, which resulted in complete de-energization and, probably, cell death.

In their study, Park *et al.* (2009) found that almost half of the log reduction, caused by the silver ion disinfection in the bacteria they studied (*Escherichia coli* and *Staphylococcus aureus*), could be attributed to reactive oxygen species (ROS) -mediated activity, with the major form of ROS generated being the superoxide radical. The authors comment that the silver ions are likely to generate superoxide radicals by impairing enzymes in the respiratory chain and that this impairment may be caused by the thiol-interaction mechanism (as mentioned above).

The antimicrobial mode of action of AgNP is not fully understood (Wijnhoven *et al.*, 2009), although some of the mechanisms may be the same as those for ionic silver or, as increasingly seems likely (e.g. Xiu *et al.*, 2012), may result from the release of Ag⁺ from the AgNP (Li *et al.*, 2008). A number of authors have shown that AgNP can anchor to and then penetrate the cell walls of Gram-negative bacteria (Sondi and Salopek-Sondi, 2004; Morones *et al.*, 2005). Such damaged cell walls enhance cell permeability and inhibit appropriate regulation of transport through the plasma membrane.

Sondi and Salopek-Sondi (2004) looked at the biocidal effect of AgNP on *E. coli* using both scanning and transmission electron microscopy. The bacteria were cultured in a liquid medium supplemented with AgNP (50 µg/cm³) for four hours before electron microscopy. The silver-treated cells were significantly changed in comparison with untreated *E. coli* and showed major damage, which was characterized by the formation of pits in the cell walls. The analysis showed that the AgNP were incorporated into the cell walls and accumulated in the membrane, with some penetrating the cells. As a result, intracellular substances were found to be leaking from the affected bacteria.

As with ionic silver, it has been suggested that AgNP may cause free-radical generation, leading to subsequent cell damage. Kim *et al.* (2007) looked at the free-radical generation effect of AgNP on microbial growth inhibition using electron spin spectroscopy. The group showed that free-radicals were generated by the AgNP and that addition of an antioxidant reduced the bactericidal efficacy of the AgNP; they suggested that the free-radicals may be derived from the surface of the AgNP.

Shrivastava *et al.* (2007) studied the impact of AgNP on *E. coli*, *S. aureus* and *Salmonella typhus*. The AgNP were found to be more effective against the Gram-negative bacteria. The group found that the principal antimicrobial mechanisms were AgNP anchoring and penetration of the cell wall, along with modulation of cellular signalling (leading to growth inhibition).

Hwang *et al.* (2008) performed a study on stress-specific bioluminescent bacteria, based on which they proposed a synergistic toxic effect between the AgNP and the silver ions that they produce. The stress-specific bacterial strains used were designed to respond to protein/membrane, oxidative stress and DNA damage. They found that the AgNP caused toxicity via protein/membrane and oxidative damage. In their study the AgNP released silver ions and subsequently superoxide radicals.

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