

Silver as a drinking-water disinfectant

Alternative drinking-water disinfectants: silver

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List of abbreviations and terms used in the document

Ag	elemental silver
Ag ⁺	silver ion
AgAc	silver acetate
AgCl	silver chloride
AgNO ₃	silver nitrate
AgNP	silver nanoparticles
bw	body weight
cfu	colony-forming units
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
EC ₅₀	half maximal effective concentration
GSH	glutathione
HRT	hydraulic retention time
IC ₅₀	half maximal inhibitory concentration
LC ₅₀	median lethal concentration
LD ₅₀	median lethal dose
LDH	lactate dehydrogenase
LOAEL	lowest-observed-adverse-effect level
LRV	log ₁₀ reduction value
MIC	minimum inhibitory concentration
NO	nitric oxide
NOAEL	no-observable-adverse-effect level
NTU	nephelometric turbidity unit
pfu	plaque-forming unit

POU	point-of-use
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
SOD	superoxide dismutase
UK	United Kingdom
USA	United States of America
USEPA	United States Environmental Protection Agency
WHO	World Health Organization

1. Introduction

The emphasis of this literature review is to evaluate available evidence on the efficacy and toxicity of silver as a water disinfectant. The report considers both ionic silver (Ag^+) and silver nanoparticles (AgNP) and also examines the effectiveness of copper-silver disinfection in plumbing systems for the control of *Legionella* spp. The initial review was written in autumn 2013, but some aspects of the report (specifically the efficacy of silver in household water treatment devices, *in vivo* oral toxicity studies, *in vitro* studies on primary mammalian cell lines and genotoxicity) have been updated to account for literature published until September 2015.

1.1 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 in the treated water, have raised the profile of this chemical.

Silver has been shown to have general (i.e. not specifically water disinfection related) antibacterial 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 & Hoek, 2010). In addition, a number of studies have suggested a biocidal action of silver nanoparticles against hepatitis B virus (Lu et al., 2008), HIV-1 (Elechiguerra et al., 2005), syncytial virus (Sun et al., 2008) and murine norovirus (De Gusseme et al., 2010 – see Sections 2.1.1. and 2.2). There is also a suggestion that silver nitrate (AgNO_3) and some silver nanoparticles may reduce the infectivity of *Cryptosporidium* oocysts (Abebe et al., 2015).

1.2 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 1–100 nm.” In the scientific literature, nanoparticles are usually defined as particles having one or more dimensions in the order of 100 nm or less (Moore, 2006). Although the terminology may be relatively new, the use of silver nanoparticles is not (Nowack et al., 2011), with Lea (1889) reporting on the synthesis of a citrate-stabilized silver colloid (which has an average particle size between 7 and 9 nm).

The most common method of producing silver nanoparticles is the chemical reduction of a silver salt (often silver nitrate) dissolved in water with a reducing compound such as sodium borohydride, citrate, glucose, hydrazine and ascorbate (Marambio-Jones & Hoek, 2010). There are, however, numerous different manufacturing methods (including spark discharging, electrochemical reduction, solution irradiation and cryochemical synthesis) some of which have been outlined by Marambio-Jones & Hoek (2010). In addition to different manufacturing methods, different capping or stabilizing agents may be used; these are generally used to prevent the silver nanoparticles 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 silver nanoparticles with different sizes (typically < 50 nm), shapes (e.g. spheres, rods and cubes) and other characteristics.

1.3 Water-related applications

In terms of water disinfection-related applications, silver is most commonly used in domestic water filters (allegedly to reduce the level of biofilm growth within the filter or, hypothetically, as an additional level of treatment); it is used in both granular and powdered activated carbon filters and also domestic ceramic water filters. It is also quite commonly used in conjunction with copper ionization as a preventative measure against colonization by a variety of bacteria (especially *Legionella* spp.) in plumbing hot water systems. Silver nanoparticles are currently being tested in a number of experimental point-of-use (POU) treatment systems and ionic silver has been investigated for its potential use as a secondary disinfectant in drinking-water supplies. Silver ions (in combination with both copper and chlorine) have also been investigated for use in swimming pool disinfection. This report focuses on both established and experimental use of silver for drinking-water disinfection and, briefly, the use of copper-silver ionization for *Legionella* spp. control within plumbing systems.

2. Disinfection efficacy of silver

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

2.1 Ionic silver applications

2.1.1 Efficacy of ionic silver for disinfection of potable water

In the studies outlined below, silver ion (ionic silver) efficacy (generated from silver salts [silver nitrate, silver chloride (AgCl)] or produced electrolytically) was tested against a range of bacteria; the inactivation was principally assessed by the log₁₀ reduction in bacterial numbers. Initial bacterial concentrations ranged from 3.5 cells/mL up to 1.5 x 10⁷ cells/mL. Single studies examined the impact of silver nitrate on bacteriophage (De Gusseme et al., 2010) and *Cryptosporidium* oocysts (Abebe et al., 2015).

Hwang et al. (2007) looked at the efficacy of silver ions (up to 100 µg/L), derived from silver nitrate, against *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Escherichia coli* (all at 1.5 x 10⁷ cells/mL) in synthetic drinking-water (pH 7, temperature 25 °C – defined chemical composition outlined in Hwang et al., 2006). After a three-hour contact time with the highest concentration of silver the following log₁₀ reductions were reported:

- 2.4 log₁₀ reduction – *L. pneumophila*;
- 4 log₁₀ reduction – *P. aeruginosa*;
- 7 log₁₀ reduction – *E. coli*.

Similar work was conducted by Huang et al. (2008), where the efficacy of silver ions, derived from silver chloride, against 3 x 10⁶ colony-forming units (cfu)/mL of *P. aeruginosa*, *Stenotrophomonas*

maltophilia and *Acinetobacter baumannii* was investigated. A 5 log₁₀ reduction in *P. aeruginosa* was seen with 80 µg/L silver (the highest concentration used) after 12 hours. *S. maltophilia* was more sensitive to Ag, with a 5 log₁₀ reduction seen after 6 hours when exposed to 80 µg/L. For *A. baumannii*, however, a 5 log₁₀ reduction was only seen after 72 hours exposure to 80 µg/L Ag.

Silvestry-Rodriguez et al. (2007) investigated the inactivation of *P. aeruginosa* and *Aeromonas hydrophila* by silver in tap water, with a view to assessing the possibility of 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 10⁶ cfu/mL bacteria and silver nitrate added to a concentration of 100 µg/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 of humic acid was added to the dechlorinated tap water (to simulate a surface water source). Inactivation of the bacteria was time and temperature dependent; after 8 to 9 hours of exposure to 100 µg/L silver at 24 °C, there was more than a 6 log₁₀ reduction in both bacteria (at 4 °C a 4.5 log₁₀ 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 log₁₀ reduction in *P. aeruginosa* at pH 7, 24 °C after 8 hours in the presence of 3 mg/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 silver nitrate 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 80 µg/L for silver. A 4 log₁₀ reduction (approximately) was seen at 100 µg/L after 24 hours of exposure.

Pathak & 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. A 3 log₁₀ reduction was seen at neutral pH and ambient temperature after a 20 minute period for the 20 µg/L concentration. A 3 log₁₀ reduction 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 pH 8 and 9 and at temperatures greater than 20 °C.

Nawaz et al. (2012) looked at the efficacy of silver (silver nitrate) in inactivating *P. aeruginosa* and *E. coli* in rooftop harvested rainwater supplies. Prior to disinfection, samples were found to contain between 350–440 cfu/100 mL *P. aeruginosa* and 740–920 cfu/100 mL *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 one week, to examine regrowth. At higher concentrations (80–100 µg/L) complete inactivation (log₁₀ reduction values [LRVs] between 2.5 and 2.9) of both microorganisms was seen in 10 hours, with no regrowth of *E. coli* seen after 168 hours. Inactivation was slower at lower silver concentrations (LRVs between 1.3 and 2 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 10 µg/L silver for 168 hours compared to approximately 4.5% survival at 14 hours). Thus, at the lower concentrations, silver only seemed to delay bacterial reproduction and did not cause permanent damage or loss of ability to increase in number. There were, however, a number of methodological issues with this study, including a lack of follow-up investigation to document regrowth.

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 ionizers reduced the level of total coliforms by approximately 1 log₁₀ and *E. coli* by approximately 0.4 log₁₀ and resulted in a silver concentration of approximately 0.01 mg/L in the final water. The systems, as a whole, delivered water containing 0/100 mL *E. coli* and less than 10/100 mL total coliforms.

In a comparative study of disinfectants, the potency of silver ions, derived from silver nitrate, 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 log₁₀ reduction (i.e. complete inactivation), the minimum concentration of silver required was 10 mg/L with a contact time of 3 hours. The bacterial studies are summarized in Table 1 below.

De Gusseme et al. (2010) in a study of ionic silver and silver nanoparticles (outlined in more detail in Section 2.2) found that silver nitrate, at a concentration of 5.4 mg/L, added to UZ1 bacteriophage-spiked (2 x 10⁶ plaque-forming units (pfu)/100 mL) bottled water, produced a 3.1 log₁₀ reduction after 2 hours and a > 4 log₁₀ reduction after 5 hours.

The infectivity of *Cryptosporidium parvum* to mice following exposure of the oocysts to high levels of silver ions (100 mg/L ionic silver from silver nitrate for 30 minutes) was investigated by Abebe et al. (2015). Infectivity was determined by the effect on animal weight and the number of parasites shed in the stool, relative to those exposed to untreated oocysts. Mice receiving silver nitrate treated oocysts demonstrated 3% weight loss at 3 days post infection, compared with 12% weight loss in those mice exposed to untreated oocysts. Parasite shedding was also significantly lower in the animals receiving treated oocysts.

It can be seen from these studies that LRVs 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 & Gopal (2012), where silver ions were generated electrolytically (rather than from silver salts), and a 3 log₁₀ reduction was seen after 20 minutes at a relatively low silver concentration (20 µg/L). In contrast to the laboratory-spiked samples, where generally higher LRVs were reported, relatively LRVs were seen in harvested rainwater samples (with a low initial bacterial concentration) used by Nawaz et al. (2012). The bacteriophage study of De Gusseme et al. (2010), while suggesting that silver nitrate can result in 3 log₁₀ reduction of UZ1, used a high silver concentration (5 mg/L), which would not be relevant as a drinking-water application. The one study on *C. parvum* (Abebe et al., 2015) indicates that silver may potentially be effective for protozoa reduction, but more research is needed.

Table 1: Summary of ionic silver bacterial disinfection studies by microorganism

Organism	Silver type	Concentration (µg/L)	Medium and conditions	Initial concentration	Duration	Log ₁₀ reduction value	Reference
<i>A. baumannii</i>	AgCl	80	-	3 x 10 ⁶ cfu/mL	72 h	5	B
<i>A. hydrophila</i>	AgNO ₃	100	Dechlorinated tap water, pH 7, 24 °C	1 x 10 ⁶ cfu/mL	9 h	> 6	C
	AgNO ₃	100	Dechlorinated tap water, pH 9, 24 °C	1 x 10 ⁶ cfu/mL	9 h	> 6	C
<i>E. coli</i>	AgNO ₃	100	Synthetic drinking water, pH 7, 25 °C	1.5 x 10 ⁷ cells/mL	3 h	7	A
	AgNO ₃	100	-	2 x 10 ⁴ cells/mL	24 h	4	D
	AgNO ₃	100	Harvested rainwater, pH 7–8, 25–27 °C	740–920 cfu/100 mL	10 h	2.8–2.9	F
	AgNO ₃	40	Harvested rainwater, pH 7–8, 25–27 °C	740–920 cfu/100 mL	14 h	1.3–2 ^a	F
	AgNO ₃	10 000	Ground water	10 ⁶ cfu/mL	3 h	6	G
	Ag ⁺ from electrodes	20	Autoclaved tap water	1.75 x 10 ³ cfu/mL	20 min	3	E
	Ag ⁺ from electrodes	10	Autoclaved tap water	1.75 x 10 ³ cfu/mL	40 min	3	E
	Ag ⁺ from electrodes	5	Autoclaved tap water	1.75 x 10 ³ cfu/mL	50 min	3	E
<i>L. pneumophila</i>	AgNO ₃	100	Synthetic drinking water, pH 7, 25 °C	1.5 x 10 ⁷ cells/mL	3 h	2.4	A
	AgNO ₃	100	Synthetic drinking water, pH 7, 25 °C	1.5 x 10 ⁷ cells/mL	3 h	4	A
<i>P. aeruginosa</i>	AgCl	80	-	3 x 10 ⁶ cfu/mL	12 h	5	B
	AgNO ₃	100	Dechlorinated tap water, pH 7, 24 °C	1 x 10 ⁶ cfu/mL	8 h	> 6	C
	AgNO ₃	100	Dechlorinated tap water, pH 9, 24 °C	1 x 10 ⁶ cfu/mL	8 h	> 6	C
	AgNO ₃	100	Dechlorinated tap water, pH 7, 4 °C	1 x 10 ⁶ cfu/mL	24 h	4.5	C
	AgNO ₃	100	Dechlorinated tap water, pH 7, 4 °C	1 x 10 ⁶ cfu/mL	24 h	5	C
	AgNO ₃	100	Harvested rainwater, pH 7–8, 25–27 °C	350–440 cfu/100 mL	10 h	2.5–2.6	F
	AgNO ₃	40	Harvested rainwater, pH 7–8, 25–27 °C	350–440 cfu/100 mL	14 h	1.3–2 ^a	F
	<i>S. maltophilia</i>	AgCl	80	-	3 x 10 ⁶ cfu/mL	6 h	5

A–Hwang et al., 2007; B–Huang et al., 2008; C–Silvestry-Rodriguez et al., 2007; D–Cunningham et al., 2008; E–Pathak & Gopal, 2012; F–Nawaz et al., 2012; G–Patil et al., 2013; H–Adler et al., 2013; ^aRegrowth observed

2.1.2 Copper/silver ionization

Copper/silver is generally applied to water as an ionization process, with the electrolytic generation of copper and silver ions. Sometimes it is 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), where there are long contact times within the plumbing system. They have been investigated for the treatment of swimming pool water (which is beyond the scope of this report).

2.1.3 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 quantitative tests to determine log₁₀ reduction. It is generally considered that ion levels should be regularly monitored and remain within prescribed concentrations (USEPA, 2015; WHO, 2007); published studies suggest levels of between 0.2 to 0.8 mg/L for copper and 0.01 to 0.08 mg/L silver are needed to maximize efficacy (Cachafeiro et al., 2007; Lin et al., 2011).

Liu et al. (1998) looked at the intermittent use of a single copper/silver ionization system in the hot water systems of two buildings. Twenty 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 & Yu (2003) 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 hospital-acquired Legionnaires' disease and 75% had attempted other disinfection methods. Two postal surveys (1995 and 2000) gathered information on environmental monitoring of *Legionella*, identification of hospital-acquired legionellosis, and monitoring and maintenance of the copper/silver ionization systems. *Legionella* monitoring was conducted at 15 out of the 16 hospitals at both time points, although the frequency of monitoring was markedly lower at the second survey (9/16 hospitals reported monthly or quarterly monitoring in 1995, compared to only 4/16 hospitals reporting quarterly monitoring in 2000). Regular monitoring (undefined) of copper/silver concentrations was reported by 15/16 hospitals in 1995; no information is presented for the 2000 survey. Colonization of distal water sites with *Legionella* was much less frequent after installation of the copper/silver ionization (with between 7 and 8 of the hospitals reporting zero positivity of monitoring sites, and the remaining hospitals reporting 30% positivity or lower). A single case (shortly after installation) of hospital-acquired Legionnaires' disease was reported from the surveyed hospitals after implementation of copper/silver ionization.

In Switzerland, Blanc et al. (2005) found that at a water temperature of 50 °C, 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% were 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. High pH had previously been shown to have a detrimental effect on the ability of copper ions to kill *Legionella* (Lin et al., 2002).

In addition, trisodium phosphate was used within the hospital hot water system to protect against corrosion; copper ions are known to bind to phosphate (Lin & Vidic, 2006), which will reduce their efficacy against *Legionella*. Blanc et al. (2005) found that ionization in conjunction with increased temperature (65 °C) 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).

In Spain, Mòdol et al. (2007) looked at hospital-acquired Legionnaires' disease following introduction of a copper/silver ionization system. Prior to installation, hospital-acquired Legionnaires' disease was 2.45 cases/1000 patient discharges. After installation, the level dropped to 0.18 cases/1000 patient discharges and, after increasing ion levels, no further cases of Legionnaires' disease were reported up to the end of the study period (19 months). Prior to installation, 57% of water samples were positive for *L. pneumophila* compared to 16% after installation, when the system was running consistently with copper/silver ion levels greater than 0.3 and 0.03 mg/L, respectively.

Pedro-Botet et al. (2007) investigated the impact of copper/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 (when ion levels were well below recommended levels: average copper and silver levels of 0.095 and 0.012 mg/L respectively), no change was seen in the number of positive samples. However, when ion concentrations were reportedly increased (average copper and silver levels of 0.135 and 0.011 mg/L respectively) in months 4 to 7, 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 (between months 4 to 12) were 0.132 mg/L copper and 0.012 mg/L silver, below the target concentrations of 0.2 and 0.02 mg/L respectively. The authors note that, while the system was not operating at the required ion concentrations and did not completely eradicate *L. pneumophila*, no cases of hospital-acquired Legionnaires' disease were reported during the year-long study.

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.

In 2012, five confirmed and 16 probable hospital-acquired cases of Legionnaires' disease were identified in Pittsburgh, USA at one of the first hospitals to adopt copper/silver ionization for *Legionella*

treatment in 1993. The system was successful for several years. Reports suggest that prior to the outbreak and until the outbreak was formally identified the system had not been subject to appropriate monitoring and control and the system had not been properly run (with issues such as an incorrect amperage being used and staff not appreciating the importance of maintaining the correct pH range).¹ A Centre for Disease Control study noted that the outbreak coincided with construction work at the hospital, which may have introduced organic matter to the water system, increasing consumption of chlorine leading to amplification of *Legionella*.²

Typically, it would seem that copper/silver ionization reduces the number of *Legionella* (and fungal) positive samples in treated systems; however, it may not completely eradicate the pathogen. This 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 maximizes 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 hospital-acquired Legionnaires' disease.

2.2 Silver nanoparticle applications

The potential of silver nanoparticles for household POU drinking-water disinfection is currently being extensively explored, principally in conjunction with filtration. The medium or matrix utilized for the nanoparticles varies widely and includes coating on polyurethane foams (Jain & Pradeep, 2005), fibreglass (Nangmenyi et al., 2009), copolymer beads (Gangadharan et al., 2010), paper (Dankovich & Gray, 2011), 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, for example, the filtration effect, have been considered below. In addition to considering the LRVs of microorganisms exposed to the test material, a number of studies also conducted zone of inhibition tests.³

Jain & Pradeep (2005) coated polyurethane foam with citrate-stabilized silver nanoparticles. 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 5- or 10-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 silver nanoparticle-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 silver nanoparticle (< 30 nm) impregnated fibreglass during immersion and during filtration. For the immersion test, a silver-impregnated mat (1% silver by weight) was added to a 100 mL *E. coli* suspension (10^6 cfu/mL). After an hour of immersion,

¹ <http://www.post-gazette.com/local/city/2014/03/11/Studies-blame-VA-outbreak-on-employees-errors/stories/201403110229>

² <http://www.cdc.gov/washington/testimony/2013/t20130205.htm>

³ The zone of inhibition is the area on an agar plate containing a lawn of bacteria where the growth of the microorganisms is prevented by the antimicrobial activity of the test material placed on the agar surface.

E. coli could not be detected in the suspension. Using an *E. coli* concentration of 10^{12} cfu/mL, the silver nanoparticle fibreglass mat (1.8% silver by weight) resulted in a 7 \log_{10} reduction in concentration in five minutes. Antibacterial filters (5% silver 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/min. *E. coli* were not found in the treated water, amounting to a 6 \log_{10} reduction. The untreated fibreglass accounted for only an approximately 1 \log_{10} reduction.

Lv et al. (2009) examined the efficacy of silver nanoparticle-coated porous ceramic tiles. The ceramic was modified (using a coupling agent) to ensure that the silver nanoparticles were fixed to the material (rather than relying on weak forces of attraction). There was no obvious loss of silver nanoparticles 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, followed by conducting a zone of inhibition test and a flow test. After 24 hours, no bacteria could be grown from the samples exposed to silver-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 the 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 ionic silver 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*, *B. 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_{10} 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 silver nanoparticle-coated polypropylene filters to remove *E. coli* from water. Fifteen 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 per 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 silver nanoparticle-coated filter. No silver nanoparticles 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 silver-treated polypropylene in comparison with the untreated material (pore size of 1.3 μm and 9.9 μm , respectively).

Dankovich & 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 100 mL of bacterial solution was 10 minutes. Plate counts showed up to a 7.6 and a 3.4 \log_{10} 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 silver/dry g paper). Of this reduction, less than 1 \log_{10} was attributed to the filtration of the paper. The average silver content of the effluent water was 50 $\mu\text{g/L}$. Dankovich (2014) has done further work, examining the effect of producing the nanosilver paper using a safer technique. Filtration through the paper, produced using glucose as a reducing sugar in combination with a domestic microwave oven, produced similar results to the earlier work, with an 8.1 \log_{10} reduction for *E. coli* (initial concentration 1×10^9 cfu/mL) and a 2.3 \log_{10} reduction for *E. faecalis* (initial concentration 2×10^8 cfu/mL), with a 1 \log_{10} reduction attributed to the paper alone. It was thought, based on filtration time, that there was a greater physical retention of *E. coli* compared to *E. faecalis*. The average silver content in the treated water was $105 \pm 36.3 \mu\text{g/L}$.

Mpenyana-Monyatsi et al. (2012) compared the bacterial removal by a number of low-cost filter materials coated with silver nanoparticles. Various concentrations of silver nanoparticles 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/100 mL), 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*, *S. dysenteriae* and *Vibrio cholerae* in groundwater samples (all bacteria present at 10^3 cfu/100 mL). The silver/cation resin filter was found to be the best performing, achieving 3 \log_{10} reduction of all the targeted bacteria, with no regrowth over 2 hours. The silver/zeolite filter was found to have the worst performance, with \log_{10} reduction rates between 0.5 and 2. 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 $\mu\text{g/L}$) in the eluent and, thus was found to be the best performing in terms of bacterial reduction and silver loss.

Lin et al. (2013) synthesized and studied the efficacy of three types of silver nanoparticle-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 produced a 5 \log_{10} reduction during filtration, even with a short hydraulic retention time (HRT) and the third bead type produced a 2 \log_{10} reduction. Silver was also measured in the filtered water, and again the three bead types produced different results ranging from 11–98 $\mu\text{g/L}$ 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 silver nanoparticles in the effluent, which continue to exert an influence over the test bacteria even after plating for culture assay, although one bead type produced both low silver concentration in the filtered water and excellent removal of *E. coli*. The authors concluded that the results suggest that the beads show promise, but note that long-term breakthrough studies are needed. Other researchers have noted that the validity of efficacy studies could be biased because they did not correct for the presence of toxic contaminants. Samberg et al. (2011), for example, investigated the efficacy of silver nanoparticles against a number of bacteria in culture medium and found that washed and unwashed silver nanoparticles had notably different MICs (e.g. for the 20 nm silver nanoparticles against *E. coli* J53, MICs were 64 $\mu\text{g/mL}$ and $< 4 \mu\text{g/mL}$, respectively). The additional toxicity of the unwashed particles was attributed to the presence of formaldehyde.

Loo et al. (2013) explored the use of silver nanoparticles in cryogels as a possible POU treatment. The silver nanoparticle-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 log₁₀ reduction) were seen for the “squeezed” water compared to the remaining bulk water (maximum 2 log₁₀ reduction). The highest log₁₀ reduction was seen from the gel with the greatest silver nanoparticle content (approximately 6.5 and 7 log₁₀ reduction for *E. coli* and *B. subtilis*, respectively). Untreated gel was capable of less than 1 log₁₀ reduction. 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 ionic silver (45–56%) and silver nanoparticles.

De Gusseme 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 elemental silver particles. In comparison with chemically produced silver nanoparticles, the biogenic particles were found to be far more effective at disinfection. In a spiking experiment using a bacteriophage (10⁶ pfu/mL), biogenic silver produced a 4 log₁₀ reduction after three hours, while the chemically produced elemental silver particles showed no inactivation. The biogenic particles were also found to be effective against murine norovirus, with a greater than 4 log₁₀ 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 reduction of virus from 1.5 log₁₀, with the filter alone, to 3.8 log₁₀. Low concentrations of ionic silver (3 µg/L) were initially detected in the filtrate (up to 5 minutes); thereafter, none was detected (De Gusseme et al., 2010). This group has also looked at the immobilization of biogenic silver to microporous membranes (De Gusseme et al., 2011). The system was found to be capable of achieving at least a 3.4 log₁₀ reduction in bacteriophage concentration (compared to a less than 1 log₁₀ reduction by the membrane alone). Silver was found to leach out of the system; 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 treatment systems and considered both silver ions (outlined in Section 2.1.1) and silver nanoparticles, using a batch disinfection test of ground water spiked with *E. coli* (10⁶ cfu/mL). Silver nanoparticles (synthesized from silver nitrate using citrate as a reducing agent) at 1 mg/L required a 3 hour contact time for a 6 log₁₀ reduction.

Mecha & Pillay (2014) investigated the efficacy of silver nanoparticle-impregnated woven fabric microfiltration membranes. Turbidity removal was examined using water with up to 700 nephelometric turbidity units (NTU) and disinfection performance was assessed using three concentrations of *E. coli*, based on river water (2500 and 10 000 cfu/100 mL) and one on synthetic feed water (77 000 cfu/100 mL). The silver nanoparticle impregnated membranes, after an initial priming period, reduced turbidity to below 1 NTU, irrespective of the turbidity of the feed water. *E. coli* removal for the membrane alone was between 84–91%, while the coated membrane completely removed *E. coli* (i.e. up to 5 log₁₀ reduction). Silver eluted from the coated filters was below 0.02 mg/L. A long-term study showed that the silver-coated membrane was effective at removing *E. coli* for at least 2 months of continuous operation (Mecha et al., 2014).

Liu et al. (2014) examined the use of nanosilver textile fixed to a plastic tube as a POU disinfection kit. The kit is immersed in the water to be treated and then used to stir the water (for 1, 5 or 10 minutes). The system produced minimal reduction in bacteria naturally present in rainwater (LRVs between 0.61

and 0.96 depending upon contact time) and also spiked concentrations of *E. coli* (1.65 log₁₀ reduction after 5 minutes).

In mice infectivity tests (outlined in Section 2.1.1), Abebe et al. (2015) found that treatment of *Cryptosporidium* oocysts with proteinate-capped or PVP-capped silver nanoparticles resulted in a minor reduction (statistical significance not stated) in the level of weight loss (12% reduction in body weight in mice receiving untreated oocysts compared with an 8% and 6% loss [respectively] for the mice receiving silver nanoparticle-treated oocysts). Some reduction was also seen in the amount of oocyst shedding in the stool, with the proteinate-capped silver nanoparticles resulting in a marked (but non-statistically significant) reduction in shedding.

The majority of studies considering silver nanoparticles 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 and Abebe et al. (2015) who considered *Cryptosporidium* infectivity. Typically, good bacterial LRVs were reported (as summarized in Table 2), with values up to 7 log₁₀ reduction (depending upon the spiking concentration) for *E. coli*. However, in many cases, contact times were generally long. Generally, the silver nanoparticle 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 50 µg/L.

Table 2: Summary of silver nanoparticle bacterial disinfection studies by microorganism

Organism	Medium/matrix	Conditions	Initial concentration	Duration or volume	Log ₁₀ reduction value	Reference
<i>B. subtilis</i>	Copolymer microspheres	Immersion (200 mg AgNP)	24 x 10 ⁶ cfu/mL	4 h	3.3	D
	AgNP-decorated cryogels	Gel (170 mg Ag/g) added to spiked water for 15 s and then removed and squeezed	10 ⁸ cfu/mL	15 s	7	I
<i>E. coli</i>	Coated polyurethane foam	Immersion of foam into bacterial suspension	10 ⁵ cfu/mL	5–10 min	5	A
	Impregnated fibreglass	Immersion of fibreglass (1% Ag by weight) in bacterial suspension	10 ⁶ cfu/mL	1 h	6	B
	Impregnated fibreglass	Filter created with fibreglass (5% Ag by weight), flowrate 20 mL/min	10 ⁶ cfu/mL	3 L	6	B
	Porous ceramic tiles	Immersion of tiles in bacterial suspension	10 ⁴ –10 ⁵ cfu/mL	24 h	4-5	C
	Porous ceramic tiles	Filtration of <i>E. coli</i> solution, flow rate of 10 mL/min	10 ⁵ cfu/mL	500 mL	5	C
	Copolymer microspheres	Immersion (200 mg AgNP)	7 x 10 ⁶ cfu/mL	4 h	6	D
	Polypropylene filters	Filtration of 15 L (at 3 L/h)	10 ³ cfu/mL	7 h	3	E
	Paper	Filtration (5.9 mg Ag/dry g of paper), flow rate 10 mL/min	10 ⁹ cfu/mL	10 min	7.6	F
	Cation resin filter substrate	Filtration of bacterially spiked groundwater, flow rate 0.12 L/h	10 ³ cfu/100 mL	10 min	3	G
	AgNP-alginate composites, created using different methodologies	Filtration through adsorption reduction beads	10 ⁵ cfu/mL	HRT of 1 min	5	H
AgNP-alginate composites, created using different methodologies	Filtration through AgNP incorporation beads	10 ⁵ cfu/mL	HRT of 1 min	2	H	
AgNP-alginate composites, created using different methodologies	Filtration through simultaneous gelation-reduction beads	10 ⁵ cfu/mL	HRT of 1 min	5	H	
AgNP-decorated cryogels	Gel (170 mg Ag/g) added to spiked water for 15 s and then removed and squeezed	10 ⁸ cfu/mL	15 s	6.4	I	

Organism	Medium/matrix	Conditions	Initial concentration	Duration or volume	Log ₁₀ reduction value	Reference
<i>E. coli</i> contd.	AgNP	AgNP solution (varying concentrations) added to bacterial suspension	10 ⁶ cfu/mL	1 mg/L at 3 h	6	J
	AgNP impregnated woven fabric microfiltration membranes	Filtration through membrane	Up to 7 x 10 ⁴ cfu/100 mL	5 min	4	K
	AgNP immobilized onto cotton textile	Immersion and stirring of bacterial suspension	1.9 x 10 ⁵ cfu/mL	5 min	1.65	L
<i>E. faecalis</i>	Paper	Filtration (5.9 mg Ag/dry g of paper), flow rate 10mL/min	10 ⁹ cfu/mL	10 min	3.4	F
<i>P. aeruginosa</i>	Copolymer microspheres	Immersion (200 mg AgNP)	22 x 10 ⁶ cfu/mL	4 h	6	D
<i>S. aureus</i>	Copolymer microspheres	Immersion (200 mg AgNP)	46 x 10 ⁶ cfu/mL	4 h	6	D
<i>S. dysenteriae</i>	Cation resin filter substrate	Filtration of bacterially spiked groundwater. Flow rate 0.12 L/h	10 ³ cfu/100mL	10 min	3	G
<i>S. typhimurium</i>	Cation resin filter substrate	Filtration of bacterially spiked groundwater. Flow rate 0.12 L/h	10 ³ cfu/100mL	10 min	3	G
<i>V. cholerae</i>	Cation resin filter substrate	Filtration of bacterially spiked groundwater. Flow rate 0.12 L/h	10 ³ cfu/100mL	10 min	3	G

A–Jain & Pradeep, 2005; B–Nangmenyi et al., 2009; C–Lv et al., 2009; D–Gangadharan et al., 2010; E–Heidarpour et al., 2011; F–Dankovitch & Gray, 2011; G–Mpenyana-Monyatsi et al., 2012; H–Lin et al., 2013; I–Loo et al., 2013; J–Patil et al., 2013; K–Mecha & Pillay, 2014; L–Liu et al., 2014

2.3 Silver-coated ceramic filter applications

A number of different types of silver-coated or silver-impregnated ceramic filters (using either silver nanoparticles or silver nitrate) have been used as 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; Abebe et al., 2014). 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.

Van Halem et al. (2007) reported results from filter challenge studies with *E. coli* (K12), *Clostridium* spores and MS2 bacteriophage for six silver nanoparticle-coated and six uncoated Nicaraguan ceramic filters (Table 3). Although the silver-coated filters slightly outperformed 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 outperformed the coated filters at both time points for MS2 bacteriophage removal.

Table 3: Log₁₀ reduction values from coated and uncoated ceramic filters (van Halem et al., 2007)

Microbe	Challenge doses	Log ₁₀ reduction values	
		Silver-coated (n=6)	Silver-free (n=6)
<i>E. coli</i>	10 ⁵ to 10 ⁷ cfu/100 mL	4.7 to 7.2	2.6 to 5.7
<i>Clostridium</i> spores	10 ³ to 10 ⁵ n/100 mL	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 (unspecified) 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* WR1 (10⁶ cfu/L) spiked into drinking water (no further details available), at two different flow rates, over time. Over 8000 litres of drinking water were passed through each of the filters and samples were periodically spiked with *E. coli* and the log₁₀ reduction efficacy assessed (Table 4). Initially, there was little difference between the filter types with, generally, between 5 and 6 log₁₀ reduction 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, log₁₀ reduction rates had dropped for both silver (log₁₀ reduction 2.2–3.2) and non-silver (log₁₀ reduction 1.1–1.2) filters. The silver concentration in the effluent from the silver filters ranged between 11.95 to 17.68 µg/L at the start of the experiment to 1.72 to 3.65 µg/L at the end.

Table 4: Log₁₀ removal values for *E. coli* from two untreated and two silver-impregnated ceramic filters (Wubbels et al., 2008)

Volume filtered (L)	Reference filter Flow – 6 L/h	Silver-coated filter Flow – 6 L/h	Reference filter Flow – 3 L/h	Silver-coated filter Flow – 3 L/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 nanoparticle-treated filters, *E. coli* (K12) LRVs varied between < 1 to > 4 for both the untreated and heavily used filters. The initial filter run tended to show the best reduction, with LRVs of between 3.7 and 4 in the untreated filters, and 2.9 to 4.1 in the heavily used filters. Re-coating of the previously heavily used filters improved LRVs slightly, but filters still showed high variability between filter runs and the improved removal efficiencies were not maintained. As with the initial tests, the first filter run was generally the one with the best reduction (3.5 log₁₀ to 4.5 log₁₀). 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 un-spiked dechlorinated water passing through the filter (levels of < 20 to 41 cfu/mL in recoated filters compared to 10³–10⁵ cfu/mL prior to re-coating).

Bloem et al. (2009) looked at *E. coli* and MS2 bacteriophage removal from filters with and without silver (silver nitrate). Water was spiked with 10³–10⁶ cfu/mL *E. coli* or 10³–10⁴ pfu/mL MS2 and passed through the filters. Twenty 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* LRVs 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 log₁₀). As reported in some other studies, high variability in removal efficiencies were seen, with *E. coli* LRVs in silver-treated filters ranging between 3.82 and 7.65 compared to 2.01 and 4.3 in untreated filters.

Brown & Sobsey (2010) found no significant difference in the removal of *E. coli* – CN13 (challenge dose 10⁴–10⁷ cfu/mL) or MS2 (challenge dose 10⁵–10⁸ pfu/mL) between silver-treated (silver nitrate) and untreated filters. *E. coli* LRVs were between 2.2 and 2.3 in the silver-treated filters, compared to 2.1 in the untreated filters. MS2 removal was between 1.3–1.5 log₁₀ in the silver-treated filters, compared to 1.6 to 1.7 log₁₀ in the untreated filters.

Kallman et al. (2011) looked at *E. coli* (wild strain) removal and compared untreated and silver nanoparticle-treated filters made with different percentages of sawdust. Although the authors comment that silver improves the LRVs for the filters (Table 5), probably the only significant improvement is that seen for the 17% sawdust filter.

Table 5: *E. coli* log₁₀ reduction values for silver-treated and untreated filters with different sawdust content (Kallman et al., 2011)

Percentage sawdust	Log ₁₀ reduction value	
	Untreated	Silver-treated
4%	4.56	4.74
9%	3.52	3.81
17%	2.55	4.91

Zhang & Oyanedel-Craver (2013) compared *E. coli* (wild strain) log₁₀ reduction values (challenge dose 10¹⁰–10¹¹ cfu/mL) in ceramic disks with or without silver nanoparticle treatment. Silver treatment did not noticeably improve the performance of the disks (4.2–4.3 log₁₀ reduction by the untreated disks compared to 4.4 log₁₀ reduction by the treated disk).

Rayner et al. (2013) investigated the impact of the type of silver (ionic or silver nanoparticles) on bacterial removal efficacy using ceramic disks. Using different silver concentrations (0.003, 0.03 and 0.3 mg of silver/g of disk), bacterial removal performance was assessed using 10⁶ cfu/mL *E. coli* continuously fed to the disks at a flow rate of 0.5 mL/minute. Samples were taken daily for 10 days, with the concentration of bacteria measured in both the influent and effluent. In addition, viable bacterial retention on and within the disks was also examined. A sharp decrease in LRVs was seen from day 1 to day 4, with a levelling off seen thereafter, so the LRVs are based on samples taken from days 5 to 10. The results varied according to the source of the clay used for the disks. For the Indonesian and Tanzanian clays, 0.3 mg/g silver nanoparticles was found to be the most effective, with > 4 log₁₀ reduction seen on day 10 (1 to 1.7 log₁₀ reduction improvement over the control disks without silver). Disks coated with other concentrations of silver either produced no improvement or a less than 1 log₁₀ reduction improvement over the control. Silver desorption was greater for the disks coated with silver nitrate compared to silver nanoparticles. Effluent silver concentration, bacterial removal and viable bacteria retention were dose-dependent on the amount of silver applied. The authors recommend that, based on the results, ceramic filter factories should use silver nanoparticles rather than silver nitrate, to improve silver-filter retention and that silver nanoparticle application should be increased to 0.3 mg/g to maximise microbiological performance without compromising the effluent quality.

Although Ren & Smith (2013) did not measure microbial reduction, they compared the retention of silver nanoparticles in a ceramic porous medium following different application methods (paint-on, dipping and fire-in methods). The fire-in method appeared to significantly improve silver nanoparticle retention and even where the amount of silver applied to the disk was increased by a factor of 10 (27.3 mg silver per disk), the effluent silver concentrations did not exceed 0.02 mg/L.

Bielefeldt et al. (2013) looked at the impact of various water quality parameters on the detachment of silver nanoparticles from a solid silica surface (representative of ceramic pot filters). Over typical ranges of pH, ionic strength, turbidity and dissolved organic matter, minimal impact was seen on the rate of release and dissolution of silver nanoparticles. Free chlorine (added as sodium hypochlorite), however, rapidly removed silver (whether applied as ionic silver or silver nanoparticles) even at drinking-water levels, leading to the suggestion that contact between ceramic pot filters and pre-chlorinated water and cleaning with bleach should be avoided. Mittelman et al. (2015) also examined the impact of different water chemistries on silver dissolution and release from ceramic water filters (coated with casein-coated

silver nanoparticles or silver nitrate) and concluded that saline, hard or acidic waters should be avoided to minimize eluent silver concentrations and preserve silver treatment integrity.

Van der Laan et al. (2014) looked at the role of silver (silver nitrate) during both filtration and storage, comparing different silver applications (non-silver-treated, silver-treated either on the outside or on both sides of the filter) in long-term loading experiments using *E. coli* (K12 and WR1 strains) and MS2 bacteriophage. Comparison of samples taken within 5 minutes of filtration and after 11 hours of storage showed that, for silver nitrate coated filters, there is very little inactivation during the filtration phase, with no significant difference found immediately after filtration between the filters, with or without silver treatment (median LRVs between ~ 0.7 and 1.1 for *E. coli* and an average of 0.6 for MS2). Storage time, post filtration, was found to be the dominant parameter in *E. coli* inactivation (no post-storage data presented for MS2); after 11 hours of storage the median LRV was approximately 4 in the silver treated filters.

Simonis et al. (2014) tested silver-treated ceramic filters (with silver applied either by sputter coating or through dipping [silver impregnation] and drying in different atmospheres to coat the filters with ionic silver or silver oxide) against F-specific and somatic phages. Initial spiking levels were 2.5×10^5 pfu/mL for F-specific phage and 2.1×10^3 pfu/mL for the somatic phage. The LRVs varied according to both the coating method and the phage type. For the F-specific phage, LRVs were between < 0.1 and 0.56 . Greater inactivation was seen for the somatic phage (LRVs between 1.2 and 1.84). In both cases the sputter coated filter was the most effective.

Matthies et al. (2015) looked at bacterial and bacteriophage removal using silver-coated (silver nitrate) Indonesian filters. Bacterial LRVs from spiked samples were between 3.4 and 5 (*E. coli* 5.1 – 5.2 \log_{10} reduction; *Enterococcus faecium* 3.4 – 4.5 \log_{10} reduction; *P. aeruginosa* 3.4 – 5.0 \log_{10} reduction). \log_{10} reduction values for bacteriophages MS2 and ϕ X174 were considerably lower and, typically, were between 0.5 and 0.6 .

Although some studies have suggested that silver treatment improves the *E. coli* removal performance of filters, others have shown only small benefits, short-term improvements or negligible impact (beyond the filtration effect) as a result of silver, with no clear pattern of removal in relation to the type of silver used (i.e. ionic or nanoparticles) – see the summary of LRVs in Table 6.

Work directly comparing filters using ionic silver versus silver nanoparticles, however, suggest that silver nanoparticles may be as effective at bacterial reduction but shows better retention within the filter (Rayner et al., 2013). The silver concentration used to treat the filter is likely to be important reducing bacterial numbers. Rayner et al., (2013) suggest that this can be increased to 0.3 mg/g silver nanoparticles without high silver leaching. The use of differing concentrations and application methods may go some way to explaining the contradictory results. Studies which looked at the impact on bacteriophage concentrations, typically found that silver treatment has a limited effect, with LRVs often below 1 .

Table 6: Summary of ceramic filter log₁₀ reduction values

Organism	Comments	Log ₁₀ reduction values				Reference
		AgNP coated	AgNO ₃ coated	Ag other	Uncoated	
<i>E. coli</i>	-	4.7–7.2	-	-	2.6–5.7	A
	Ag unspecified; flow 6 L/h; initial measurements	-	-	> 5.4	5.1	B
	Ag unspecified; flow 6 L/h after > 8000 L throughput	-	-	2.2	1.1	B
	Ag unspecified; flow 3 L/h; initial measurements	-	-	> 5.4	> 5.4	B
	Ag unspecified; flow 3 L/h after > 8000 L throughput	-	-	3.2	1.2	B
	LRV in freshly coated filters	3.2–4.2	-	-	< 1–> 4	C
	-	-	Mean 5.9 (3.8–7.6)	-	Mean 3.1 (2.0–4.3)	D
	-	-	2.2–2.3	-	2.1	E
	4% sawdust	4.74	-	-	4.56	F
	9% sawdust	3.81	-	-	3.52	F
	17% sawdust	4.91	-	-	2.55	F
	-	4.4	-	-	4.2–4.3	G
	Tanzanian clay; day 10; 0.003 mg/g Ag	~ 3	~ 3	-	2.5	H
	Tanzanian clay; day 10; 0.03 mg/g Ag	~ 3.5	~ 3.5	-	2.5	H
	Tanzanian clay; day 10; 0.3 mg/g Ag	~ 4.4	~ 5	-	2.5	H
	Ag coating inside and outside	-	1.1	-	0.7	I
	Ag coating outside only	-	1.1	-	0.7	I
	-	-	5.1–5.2	-	-	K
<i>E. faecium</i>	-	-	3.4–> 4.5	-	-	K

Organism	Comments	Log ₁₀ reduction values				Reference
		AgNP coated	AgNO ₃ coated	Ag other	Uncoated	
<i>P. aeruginosa</i>	-	-	3.4–5.0	-	-	K
Clostridium spores	-	3.6–5.3	-	-	2.7–5.3	A
MS2	-	0.5–1.4	-	-	0.8–2.4	A
MS2	-	-	0.5	-	0.5	D
MS2	-	-	1.3–1.5	-	1.6–1.7	E
MS2	Ag coating inside & outside	-	0.59	-	0.4	I
MS2	Ag coating outside only	-	0.65	-	0.4	I
MS2	-	-	0.5–0.6	-	-	K
øX174	-	-	0.5–0.6	-	-	K
F-specific phage	Sputter coated	-	-	0.56	0.03	J
F-specific phage	Ag ⁺	-	-	0.25	0.03	J
F-specific phage	Ag ₂ O	-	-	0.09	0.03	J
Somatic phage	Sputter coated	-	-	1.84	0.02	J
Somatic phage	Ag ⁺	-	-	1.32	0.02	J
Somatic phage	Ag ₂ O	-	-	1.2	0.02	J

A–van Halem et al., 2007; B–Wubbels et al., 2008; C–Bielefeldt et al., 2009; D–Bloem et al., 2009; E–Brown & Sobsey, 2010; F– Kallman et al., 2011; G–Zhang & Oyandel-Craver, 2013; H–Rayner et al., 2013; I–van der Laan et al., 2014; J–Simonis et al., 2014; K–Matthies et al., 2015; Ag₂O–silver oxide

2.4 World Health Organization International Scheme to Evaluate Household Water Treatment Technologies: Performance evaluations of silver

Assessment of the microbial effectiveness of silver as a household-level water treatment option should, as far as possible, model actual use conditions in the field, for example, water of varying quality, realistic contact times and testing of all three classes of pathogens which cause diarrhoeal disease. In order to comprehensively assess effectiveness, the World Health Organization (WHO) has set health-based performance targets for household water treatment products based on the removal of bacteria, viruses and protozoa (WHO, 2011). These targets are based on microbial risk models using assumed levels of reference pathogens in untreated water. Since 2014, WHO has been testing products against those targets through the WHO International Scheme to Evaluate Household Water Treatment Technologies.⁴ Box 1 gives further information on the Scheme and its three tiers of log₁₀ performance targets for bacteria, viruses and protozoans. Two silver products were evaluated in the first round of testing (WHO, 2016); a silver coated ceramic filter and a liquid colloidal silver suspension designed to be added in the form of drops to drinking-water to be treated. Testing of the filter had to be discontinued because of unacceptably low filtration rates and the colloidal silver product demonstrated no reduction against viruses and only a mean LRV of 2 against bacteria (the efficacy against protozoa was not evaluated). Therefore, the two products containing silver failed to meet the WHO performance criteria.

⁴ http://www.who.int/water_sanitation_health/water-quality/household/scheme-household-water-treatment/en/

Box 1. WHO International Scheme to Evaluate Household Water Treatment Technologies

The objective of the Scheme is to independently and consistently evaluate the microbiological performance of household and POU water treatment technologies. The evaluation considers both turbid and non-turbid water, and is carried out to manufacturers' instructions for daily household use.⁴ The results of the evaluation are intended to assist and inform Member States and procuring UN agencies in the selection of these technologies.

The performance targets define treatment requirements in relation to source water quality for each pathogen class as detailed below.

Performance target	Bacteria (log ₁₀ reduction required)	Viruses (log ₁₀ reduction required)	Protozoa (log ₁₀ reduction required)	Classification (assuming correct and consistent use)
★★★	≥ 4	≥ 5	≥ 4	Comprehensive protection (very high pathogen removal)
★★	≥ 2	≥ 3	≥ 2	Comprehensive protection (high pathogen removal)
★	Meets at least 2-star (★★) criteria for two classes of pathogens			Targeted protection
–	Fails to meet WHO performance criteria			Little or no protection

The performance of HWT products is classified as 3-star (★★★); 2-star (★★); and 1-star (★), denoting descending order of performance, based on log₁₀ reductions of bacteria, viruses and protozoa from drinking-water. Performance that does not meet the minimum target is given no stars. Products that meet 3-star (★★★) or 2-star (★★) performance targets are classified as providing “Comprehensive protection” against the three main classes of pathogens which cause diarrhoeal disease in humans. The use of these products is encouraged where there is no information on the specific pathogens in drinking-water (and a prudent approach is to protect against all three classes), or where piped supplies exist but are not safely managed. Products that meet the performance targets for at least 2-star (★★) for only *two* of the three classes of pathogen are given one star (★) and are classified as providing “Targeted protection”. In general, the use of these products may be appropriate in situations where the burden of diarrhoeal disease is high due to known classes of pathogens, such as a cholera outbreak.

3. Safety and toxicity of silver

This section outlines the potential for human exposure to silver and silver nanoparticles through all routes (i.e. it is not confined to ingestion) and outlines opinions from various expert bodies on intake. In addition, an assessment of recent (up to autumn 2015) toxicological literature was undertaken and relevant findings are included here. It should be noted that as the study of silver nanoparticles toxicity is relatively new, it is likely that methods of assessment will continue to develop as different challenges posed by nanoparticles are identified (Doak et al., 2012).

3.1 Human exposure

Silver is not considered an essential metal and therefore any exposure is unwanted (Lansdown, 2010). Since ancient times, silver has been used in a variety of products such as jewellery, utensils and coins. More recently, however, largely due to its antimicrobial properties, its uses (and hence potential for human exposure) have expanded rapidly and it can now be found in food packaging materials, babies' bottles and pacifiers, cleaning products, food supplements, cosmetics, medical devices and products, electronics, odour-resistant textiles (e.g. socks and shirts) and household appliances, such as washing machines and refrigerators (Wijnhoven et al., 2009). Dietary intake of silver is estimated to be in the range of 7–90 µg/day (Wijnhoven et al., 2009; Lansdown, 2010), although this does not take into account some of the more recent forms of exposure, or non-dietary ingestion of silver (e.g. Tulve et al., 2015). Inhalation of silver dust and fumes may occur in some occupational settings and skin contact can occur in occupational settings, from contact with jewellery and application of topical creams for burns (Wijnhoven et al., 2009).

3.2 Guideline values

3.2.1. WHO drinking-water quality guidelines

There is currently no health-based guideline value for silver in the WHO Guidelines for Drinking-water Quality (WHO, 2017). Silver was last reviewed by WHO in 1993 (WHO, 1993), when it was concluded that, on the basis of epidemiological and pharmacokinetic knowledge at the time, a total lifetime oral intake of about 10 g of silver could be considered as the human no-observable-adverse-effect level (NOAEL). It was felt that the contribution of drinking-water to this NOAEL would normally be negligible and so 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, a concentration of 0.1 mg/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.

3.2.2 Other values

In Germany, the drinking-water regulations (Trinkwasserverordnung) set an allowable maximum for silver of 0.08 mg/L.

In the 2012 edition of the USEPA drinking-water standards and health advisories document (USEPA, 2012) silver has the following health advisory⁵ values:

⁵ A USEPA health advisory is defined as 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.

- 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 (based on a cosmetic effect)
- Drinking-water equivalent level (mg/L): 0.2
- Life time health advisory (mg/L): 0.1 (based on a cosmetic effect)

3.3 Human toxicity data

3.3.1 Toxicokinetics

3.3.1.1 Absorption

Absorption following oral administration in humans has been described qualitatively in several case studies (Chang et al., 2006; Mirsattari et al., 2004; Ohbo et al., 1996). The occurrence of generalized argyria⁶ in a woman who repeatedly applied silver nitrate solution to her gums has been reported as evidence to show that absorption of silver can occur across oral mucosa (Marshall & Schneider 1977).

Quantitative data has been reported by East et al. (1980) who estimated an absorption of 18% of the administered dose in a 47-year-old woman who already suffered from argyria. Transit time in the gastrointestinal tract has been shown to affect absorption, with a faster transit time leading to lower absorption (Furchner et al. 1968).

Evidence of the absorption of silver in humans following inhalation exposure is limited to occupational studies. Inhalation of silver nitrate and silver oxide in the range 0.039 to 0.378 mg silver/m³ was associated with detectable blood silver levels in workers (Rosenman et al., 1979). Di Vincenzo et al. (1985) reported detectable silver levels in the blood and faeces of workers exposed to time weighted average levels of 0.001 to 0.1 mg/m³ insoluble silver in a photographic materials manufacturing facility.

Several silver compounds appear to be absorbed through the intact skin of humans, although the degree of absorption is thought to be low.

3.3.1.2 Distribution

Silver and silver nanoparticles have been reported to be distributed to a wide range of organs in the human body following oral administration, however some organs are suggested to be more prone to silver deposition than others (Hadrup & Lam, 2014). 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 argyria⁶ (Brandt et al., 2005; Wadhera & Fung, 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 15.5 ng/g wet weight) in livers of children under 6 years old. It was speculated that silver is accumulated from the mother (probably from maternal mercury amalgam fillings) during pregnancy and lactation.

Very limited information was identified concerning the distribution of silver in humans following inhalation of elemental silver or silver nanoparticles. Newton & Holmes (1966) estimated that 25% of

⁶ Argyria is where tissues become impregnated with silver sulfide, 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. Generalized 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).

a dose of radioactive silver (dose unknown) was distributed to the liver between 2 and 6 days after exposure.

Following the topical application of silver nitrate for the treatment of burns in two humans, silver was distributed to the muscles (0.03–2.3 ppm), liver (0.44 ppm), spleen (0.23 ppm), kidney (0.14 ppm), heart (0.032–0.04 ppm), and bones (0.025 ppm) (Bader 1966).

3.3.1.3 Metabolism

Metallic silver is inert and absorption through any route is determined by ionization (under oxidizing conditions) to release the biologically active ionic silver. Ionic silver subsequently binds to sulfhydryl groups and other anionic ligands of proteins, cell membranes, and tissue debris (Hadrup & Lam, 2014). Controversies exist on the predominant routes of silver metabolism in the human body (Wan et al., 1991; East et al., 1980).

3.3.1.4 Excretion

Following absorption via any route, ionic silver can be excreted in bile, urine, hair and nail, with the biliary route predominating over the urinary route. However, urinary silver measurement provides a convenient index of silver absorption by all routes. At higher concentrations, patterns of urinary excretion are irregular (Lansdown, 2010).

Following oral exposure to silver acetate (AgAc) in humans, silver is eliminated primarily in the faeces, with only minor amounts eliminated in the urine. The rate of excretion is most rapid within the first week, after a single oral exposure (East et al., 1980).

Accidental inhalation of silver isotope (^{110m}) resulted in rapid removal from the lungs (primarily by ciliary action), with subsequent ingestion and ultimate elimination in the faeces, with biological half-lives of 1 and 52 days (Newton & Holmes 1966).

No studies were identified concerning the excretion of silver or silver nanoparticles by humans following dermal exposure. Once absorption through the skin and distribution to bodily tissues occurs, it can be expected that elimination would be similar to that of silver ions absorbed via oral or inhalation exposure, that is, primarily via the faeces, with minimal amounts excreted in the urine.

3.3.2 Acute toxicity

No information concerning toxicity in humans following acute exposure to silver or silver nanoparticles through oral or dermal routes could be identified. Acute exposure through inhalation during work with molten silver has been linked to acute respiratory failure in one worker. However, quantitative measurements of exposure and history of pre-exposure are unavailable (ATSDR, 1990).

3.3.3 Repeat dose toxicity

3.3.3.1 Systemic effects

Munger et al. (2013) conducted a study looking at human exposure (60 healthy subjects) to commercial nanoscale silver colloid in a single dose, blinded, cross-over, intent-to-treat design. Two commercial silver nanoparticle (colloid) solutions were used, one with particle sizes between 5–10 nm (10 ppm solution) and one with particles of sizes between 25–40 nm (32 ppm solution). With the 10 ppm solution, subjects were dosed for 3, 7 or 14 days (150 µg/day – equivalent to 2.1 µg/kg body weight [bw] – assuming 70 kg adult), while for the 32 ppm solution all subjects were dosed for 14 days (480

µg/day – equivalent to 6.8 µg/kg bw). No clinically important changes in human metabolic, haematologic and urinalysis were noted. Physical findings and imaging morphology of organs was also unchanged.

Occupational inhalation exposure to silver nitrate and/or silver oxide at estimated exposure levels of between 0.039 and 0.378 mg silver/m³ for less than 1 to greater than 10 years, has been linked to upper and lower respiratory tract irritation. The same exposure levels can also cause gastric discomfort in humans. Occupational exposure to silver compounds has not been observed to affect blood counts or the cardiovascular system (ATSDR, 1990).

No studies could be identified to assess the potential systemic toxicity of silver or silver nanoparticles in humans following dermal exposure.

3.3.3.2 Neurotoxicity

No studies could be identified to assess the potential neurotoxicity of silver or silver nanoparticles in humans by any route of exposure.

3.3.3.3 Reproductive and developmental toxicity

No studies could be identified to assess the potential reproductive and developmental toxicity of silver or silver nanoparticles in humans by any route of exposure.

3.3.3.4 Immunotoxicity

No studies could be identified to assess the potential immunotoxicity of silver or silver nanoparticles in humans by any route of exposure.

3.3.3.5 Genotoxicity (*in vivo*)

A single cross-sectional study in Turkey (Aktepe et al., 2015) has examined DNA (deoxyribonucleic acid) damage in peripheral mononuclear leucocytes (measured using the comet assay) in 35 silver jewellery workers (mean age 31.7 ± 8.4 years) exposed to silver particles or silver nanoparticles and 41 non-exposed healthy subjects (mean age 29.42 ± 7.4 years). The exposed group were reported to work for at least 4 hours a day, however, no further information is given by the authors on exposure conditions. A statistically significant increase in DNA damage, measured as endogenous mononuclear leukocyte DNA strand breaks, was reported in the silver workers (mean 15.4 versus 7.48 [arbitrary units] in the control group). Workers were also significantly more likely to have an increased oxidative stress index, increased ceruloplasmin levels and decreased total thiol measurements. As an acute phase reactant, ceruloplasmin concentration increases during periods of inflammation, infection and trauma. In addition, thiols play an important role in mediating oxidative stress, and reduced levels in the workers was shown to correlate with an increased oxidative stress index. The authors conclude that exposure of workers to silver particles increases oxidative stress, leading to inflammation and decreased levels of thiols; the subsequent DNA damage results from both the direct interaction of silver and the overproduction of reactive oxygen species (ROS). The findings of this study, however, should be interpreted with care as there are a number of study limitations, particularly in terms of the small number of participants, and the lack of direct measurements or estimate of exposure for either the workers or the control population.

3.3.3.6 Carcinogenicity

No studies could be identified regarding the possible carcinogenic activity of silver or silver nanoparticles in humans. The USEPA has determined that silver is not classifiable as to human carcinogenicity.

3.4 Animal toxicity studies

3.4.1 Toxicokinetics

3.4.1.1 Absorption

Quantitative absorption data are available for silver from a limited number of oral studies using radiolabelled ^{110}Ag (as silver nitrate or silver acetate) in monkeys, dogs, rats and mice (Furchner et al., 1968). Absorption has been estimated to be around 6% in monkeys, 10% in dogs, 2% in rats and < 0.5% in mice. As for humans, absorption is also linked to transit time in these species (Furchner et al., 1968).

A study in dogs exposed by inhalation to metallic silver particles (median aerodynamic diameter of approximately 0.5 μm) estimated absorption to be 3.1% (0.8 μg) (Phalen & Morrow 1973).

Absorption of silver nitrate across intact skin has been demonstrated in guinea pigs and is similar to that of intact human skin, with approximately 1% of the applied dose being absorbed within 5 hours (Wahlberg 1965).

3.4.1.2 Distribution

It is clear that silver (largely irrespective of the route of exposure or form) can distribute widely within the body and has been shown to cross the placenta, and potentially the blood-brain barrier in experimental animals. In addition to skin, silver has been detected in liver, kidneys, brain, spleen, eyes, muscles, blood, small intestine, stomach, lungs, bladder, prostate, tongue, teeth, salivary glands, thyroid, parathyroid, heart, pancreas and duodenum (Hadrup & Lam, 2014).

Particular targets for silver deposition are the small and large intestines, stomach, liver and kidneys (Loeschner et al., 2011). Of particular note is deposition of silver within the glomerular basement membrane in kidneys, and potentially within glial cells and neurons in some brain regions including the hippocampus and pons (Hadrup & Lam, 2014). Other lines of evidence suggest that silver is deposited within the lining of the blood-brain barrier and does not cross over (Hadrup & Lam, 2014).

Ionic silver and silver nanoparticles administered through the oral route are distributed in a similar way. Van der Zande et al. (2012) compared an oral dose of 9 mg of ionic silver per kg bw per day (administered as silver nitrate) with an oral dose of 90 mg of nanoparticulate silver per kg bw per day for 28 days (the nanoparticle sizes were 15 and 20 nm). The authors reported the majority of the silver in the stomach and small and large intestines, followed by (in descending order) the liver, spleen, testes, kidneys, brain, lungs, blood, bladder and heart. In the same study, less deposition was observed following silver nanoparticle administration than following ionic silver administration. However, the differences were pronounced for all organs.

Silver nanoparticle inhalation (in various doses and exposure periods) studies in rats have shown that silver is distributed to 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 et al., 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 silver nanoparticles in natal rats reported concentrations of almost 20 µg total silver/g in the cerebellum (the only tissue investigated) following administration of 1 mg silver nanoparticles (20–30 nm) per kg bw for 21 consecutive days (Yin et al., 2013). Silver was also found to be widely distributed following intravenous administration (Lankveld et al., 2010; Dziendzikowska et al., 2012).

3.4.1.3 Metabolism

Silver deposition in cells is caused by precipitation of insoluble silver salts, such as silver chloride and silver phosphate which are transformed to soluble silver sulfide albuminates (Berry & Galle 1982). The sulfides can bind to or complex with amino or carboxyl groups in ribonucleic acid, DNA, and proteins, or can be reduced to metallic silver by ascorbic acid or catecholamines (Danscher 1981).

3.4.1.4 Excretion

Faecal and urinary levels of silver, post-exposure, were measured in two 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 silver nanoparticles compared to silver 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 et al. (2012), who reported that over 99% of the daily dose was excreted in faeces. Based on the higher faecal excretion and lower absolute levels seen in organs in animals orally exposed to ionic silver or silver nanoparticles, it would seem that silver nanoparticles are less bioavailable than ionic silver (Loeschner et al., 2011; van der Zande et al., 2012; Hadrup & Lam, 2014).

Van der Zande et al. (2012) reported a generally rapid reduction in tissue silver concentrations following 28 days of ingestion. In most tissues, silver concentrations were already significantly reduced (to below 50% of the immediate post-exposure levels) just one week following cessation of exposure and approached a return to control levels in most samples within 12 weeks. 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. Lee et al. (2013) looked at clearance of tissue-accumulated silver from rats administered 10 or 25 nm citrate-stabilized silver nanoparticles administered (by gavage) either 100 or 500 mg/kg per day for 28 days, followed by up to 4 months recovery. While the clearance half-times differed according to dose and gender; liver, spleen and kidney elimination showed similar clearance trends. Silver concentrations in the testes and brain (i.e. tissues with a biological barrier), however, did not decrease to control levels, even after a 4-month recovery period.

Following inhalation of metallic silver particles (average aerodynamic diameter of 0.5µm) in dogs, the predominant route of clearance was reported as dissolution of the silver and transport through the blood to the liver. A proportion of silver particles were also cleared by the mucociliary escalator and swallowed. Approximately 90% of the inhaled dose was excreted in the faeces within 30 days of exposure (Phalen & Morrow 1973).

No studies could be identified assessing the excretion of silver or silver nanoparticles by animals following dermal exposure. However, once absorption through the skin and distribution to bodily tissues occurs, it can be expected that elimination would be similar to that via oral or inhalation exposure, that is, primarily via the faeces, with minimal amounts excreted in the urine.

3.4.2 Acute toxicity

A number of studies have reported no adverse acute effects as a result of silver or silver nanoparticle ingestion.

Tamimi et al. (1998) investigated the acute toxicity of an anti-smoking mouthwash containing silver nitrate as the active ingredient. The oral median lethal dose (LD₅₀)⁷ in rats was found to be 280 mg of silver per kg bw and in rabbits, 800 mg of silver per kg bw.

Orally administered nanoparticulate silver was not toxic to mice or guinea pigs at acute doses of up to 5000 mg/kg bw (Maneewattanapinyo et al., 2011). The authors found no mortality or signs of toxicity throughout a 14-day post treatment 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.

Following a large single dose (2.5 g) of silver nanoparticles (13 nm) 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 silver nanoparticles.

Korani et al. (2011) conducted an acute dermal toxicity study in guinea pigs, exposed to either 1000 or 10 000 µg silver nanoparticles/mL, with observations following exposure for 14 days. A dose-dependent reduction in thickness in the epidermis and papillary layers of skin was observed.

3.4.3 Repeat dose toxicity

3.4.3.1 Systemic effects

In a drinking water study, Sprague-Dawley rats were administered silver nitrate at doses of 6, 12 and 24 mM for 60 weeks. Death occurred in 3 of 12 rats at the highest dose within 2 weeks and this group was discontinued; the group receiving 6 mM silver nitrate was also discontinued after 12 weeks, although the reasons for this are not clearly stated by the author. A decreased body weight gain was observed in the remaining group and a NOAEL of 181.2 mg/kg bw per day has been reported by the ATSDR (1990) for this study. In a further study, albino rats were administered silver (as silver nitrate) as a 0.25% solution (in drinking water; equivalent to a dose of 222.2 mg/kg bw per day) for up to 8.5 months. Deaths were recorded between 23 and 37 weeks, which the authors propose may have been related to decreased weight gain during the same period. A lowest-observed-adverse-effect level (LOAEL) of 222.2 mg/kg bw per day has been reported by the ATSDR (1990) for this study.

Patlolla et al. (2015) conducted an oral study in which rats were administered silver nanoparticles at doses between 5–100 mg/kg bw per day in deionized water for 5 days. A dose-related increase in ROS concentration was seen, with the two highest doses being statistically significant when compared with controls. Statistically significant increases in activity (at the two highest doses) were also seen for alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. Lipid hydroperoxide (a marker of cellular injury and death) in serum also increased in a dose-dependent manner when compared to the control group. Histopathological damage was seen to the liver at doses of 25 mg/kg bw per day and above.

⁷ The dose required to kill half the members of a test population after a specified test duration.

Kulthong et al. (2012) administered silver nanoparticles to rats at concentrations between 0 and 100 mg/kg bw per day for two weeks. No differences were seen in body weight or liver weight between groups and no effect was seen on plasma levels of serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase nor in hepatic cytochrome P450 enzyme activity. It should be noted, however, that while the silver nanoparticles were purchased as being < 100 nm in diameter, analysis by the authors showed an average size of 181 nm. Similarly, Kim JS 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 per day of citrate-coated silver nanoparticles (10 nm), and Van der Zande et al. (2012) reported that there was no hepatotoxicity or immunotoxicity in a 28-day feeding study in rats exposed to silver nanoparticles (< 20 nm non-coated; < 15 nm PVP-coated) at 90 mg/kg bw per day or silver nitrate at 9 mg/kg bw per day.

Conversely, Kim et al. (2008) reported significant dose-dependent changes in alkaline phosphatase and cholesterol values in male and female rats in a 28-day feeding study with silver nanoparticles administered at doses of 30, 300 or 1000 mg/kg bw per day (60 nm, suspended in carboxymethylcellulose). This led the authors to suggest that exposure to levels of silver nanoparticles greater than 300 mg/kg may result in slight liver damage. Hadrup et al. (2012a) examined the oral sub-acute toxicity of 14 nm silver nanoparticles (stabilised with PVP) and silver acetate in rats. Doses of 2.25, 4.5 or 9 mg/kg bw per day of silver nanoparticles or 9 mg/kg bw per day of silver acetate were given daily, by gavage, for 28 days. The authors found no toxicological effects following silver nanoparticle 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. The authors also conducted a metabolomics investigation of the rat urine obtained 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. Silver nanoparticle ingestion led to an increase in both metabolites, while silver acetate only increased allantoin levels. As both silver nanoparticles and silver acetate altered urine composition this suggests that female rat physiology was affected by silver ingestion (Hadrup et al., 2012b).

Jeong et al. (2010) and Shahare et al. (2013) reported adverse effects of silver nanoparticles on the intestinal mucosa following consumption. In rats administered with silver nanoparticles up to 1000 mg/kg bw per day (60 nm in carboxymethylcellulose) by oral gavage for 28 days, a dose-dependent increase in silver nanoparticles in the lamina propria (connective tissue under the epithelia) in both the small and large intestine and also in the tip of the upper villi in the ileum and protruding surface of the fold in the colon were found (Jeong et al., 2010). In addition, silver nanoparticle-treated rats showed higher numbers of goblet cells that had released their mucus granules resulting in more mucus materials in the crypt lumen and ileal lumen; they also showed an abnormal mucus composition in the intestinal goblet cells.

In mice administered silver nanoparticles (3–20 nm) up to 20 mg/kg bw per day by oral gavage for 21 days, a significant decrease in body weight was seen in all treatment groups compared to the control on days 14 and 21 (despite no difference in food consumption between the groups). The maximum weight loss was observed in the 10 mg/kg bw per day group. In this treatment group (results are not given for the others) there was damage to the microvilli in the small intestine. The authors suggest that silver nanoparticles interact with the protective layer of the glycocalyx and other structural elements of the microvilli of the intestinal absorptive cells causing structural changes, which results in the alteration of membrane permeability and the destruction of the microvilli. In addition, it was also suggested that the

epithelial cells of the gastrointestinal tract are destroyed leading to the observed decrease in body weight, although they do not comment as to why the effects are more pronounced in the 10 mg/kg group compared to the 20 mg/kg group (Shahare et al., 2013).

In a 28-day silver biodistribution study, rats were administered two sizes (10 and 25 nm) of citrate-stabilized silver nanoparticles by oral gavage at doses of 100 or 500 mg/kg bw per day (Section 3.4.1.4). The authors reported evidence of liver toxicity, based on an increase in cholesterol in male rats at both doses for the 10 nm particles and at the lowest dose for the 25 nm particles. In females, an increase in alkaline phosphatase and aspartate aminotransferase was reported for both doses of the 10 nm particles and at the highest dose of the 25 nm particles. One case of bile duct hyperplasia was observed among the male rats treated with the 10 nm silver nanoparticles, with a non-significant increase in inflammatory cell infiltration (Lee et al., 2013).

Sardari et al. (2012) reported adverse effects on liver, spleen and kidney in rats fed 1 and 2 mg of silver nanoparticles (70 nm) per kg bw per day by gavage for 30 days. Within the spleen, red pulp was decreased in rats treated with high doses of silver nanoparticles, while the number of lymphocytes (white pulp) was increased. The authors reported pathological changes to the kidney including necrosis of glomerular cells, bowman capsule, and proximal tubules. Inflammation of the parenchymal cells was seen in the liver and intracellular space enlargement was observed in the hepatic lobules, in addition apoptosis (programmed cell death) was reported around the central vein.

A study exposing rats to two sizes of silver nanoparticles (14 and 36 nm) in water by oral administration (ad libitum–535 µg/mL) over 55 days was conducted by Espinosa-Cristobal et al. (2013). Daily ingestion was found to average 157 mg/kg bw per day and no changes were seen in individual behaviour, body weight, sociability and food consumption between the test groups and the control. The clinical chemistry and haematology conducted only showed a significant difference (at 55 days) in blood urea nitrogen concentration for the smaller silver nanoparticles (14 nm) tested, leading the group to suggest that the smaller silver nanoparticles altered the normal glomerular filtration from the kidneys. A number of other parameters were found to be different from the control group but only after 25 days of exposure and were within normal values at the end of the study.

Ebabe Elle et al. (2013) suggested that silver nanoparticles led to liver damage by altering the regulation of lipid metabolism. Rats fed 500 mg/kg bw per day of silver nanoparticles (20 nm) by gavage for 81 days were found to have significantly elevated cholesterolemia and LDL-cholesterol and lowered triglycerides. They also found increased liver and cardiac superoxide anion production and raised liver inflammatory cytokines.

In a sub-chronic oral study in rats (Kim et al., 2010) using 30, 125 and 500 mg/kg doses of silver nanoparticles (60 nm) 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 silver nanoparticles compared to the control animals. As in the short-term study (Kim et al., 2008), 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 (enlargement).

Yun et al. (2014) administered citrate stabilized silver nanoparticles (< 20 nm) to rats by gavage daily over a 13-week period (highest dose of 1030.5 mg/kg bw per day). They reported increases in serum alkaline phosphatase and calcium as well as lymphocyte infiltration in the liver and kidney, suggesting liver and kidney toxicity.

Thakur et al. (2014) investigated the impact of 5–20 nm spherical silver nanoparticles (20 µg/kg bw per day) administered by repeated gavage (90 days) on male rats. No overt signs of toxicity recorded as deaths, changes in body weight or behavioural changes were seen.

In a 14-day feeding study, 20 nm silver nanoparticles (0.1 and 0.2 mg/kg bw per day) were administered to mice by oral gavage and the effects on erythrocytes and tissues examined (Shrivastava et al., 2014). The group found a significant decrease in body weight in both test groups compared to the control (despite the relatively low doses given) and also found a number of statistically significant differences in various blood and urinary biochemical variables indicative of oxidative stress, including elevated ROS, blood glutathione (high dose only), glutathione peroxidase, glutathione-S-transferase and urinary 8-hydroxy-2'-deoxyguanosine (a biomarker of DNA damage). The levels of ROS increased significantly in all of the tissues examined (brain, liver, kidney and spleen) at the higher concentration and in all except brain at the lower concentration. Hepatic and renal toxicity was evident from liver and kidney function tests.

In a 28-day feeding study in mice, Park et al. (2010a) found that silver nanoparticles (42 nm) at the highest dose given (1 mg/kg per day) 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.

A number of inhalation studies have been performed with silver nanoparticles (typically 12–18 nm in size) in rats and mice via whole body inhalation chambers. The degree of toxicity observed is considered to be dependent on the duration of exposure. With short-term exposure (up to 28 days) no significant adverse effects have been reported (Ji et al., 2007; Hyun et al., 2008; Sung et al., 2011; Stebounova et al., 2011). In contrast, some longer-term exposure studies (≥ 90 days) suggest a dose-dependent toxicity of silver nanoparticles to lungs and liver in rats exposed via whole body inhalation to silver nanoparticles (18–19 nm) at low (49 µg/m³), medium (133 µg/m³) or high (515 µg/m³) doses for six hours a day, five days a week for 13 weeks (Sung et al., 2008, 2009). Although a small degree of recovery of lung function following a 12-week rest period was apparent, an exposure-related lung function decrease in males (exposed to the highest silver nanoparticle dose) persisted during the recovery period (Song et al., 2013).

There are relatively few *in vivo* animal studies that assess the potential dermal toxicity of silver application (Samberg et al., 2010; Korani et al., 2011; Maneewattanapinyo et al., 2011; Kim JS et al., 2013). In the main, reported studies have evaluated the use of silver-impregnated wound dressings. Kim JS et al. (2013) conducted dermal toxicity/irritation tests using citrate-coated 10 nm silver nanoparticles. Rats were exposed for 24 hours to up to 2000 mg/kg bw per day 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 silver nanoparticles. In a skin sensitization test using 20 guinea pigs, a single animal showed some erythema, suggesting that the tested silver nanoparticles could be classified as a weak skin sensitizer. In pigs dosed topically with a solution of silver nanoparticles (0.34–34 µg/mL) for 14 consecutive days, microscopic observations showed intracellular and intercellular epidermal oedema at the lowest dose, and severe intracellular and intercellular epidermal oedema with focal dermal inflammation and the highest dose (Samberg et al., 2010). Korani et al. (2011) assessed dermal toxicity in guinea pigs exposed

to either 1000 or 10 000 µg/mL of silver nanoparticles, five times a week for 13 weeks. Toxic skin responses were noted which were dose and time-dependent.

3.4.3.2 Neurotoxicity

Evidence to show that silver crosses the blood brain barrier remains equivocal, however, even in absence of silver in the extracellular fluid of the brain, silver-induced neurotoxic effects may occur via secondary molecules that are released from the periphery (Hadrup & Lam, 2014). Silver appears to have some neurotoxic effects in rats. Rungby & Danscher (1983) reported hyperactivity in rats administered 0.01% silver nitrate in drinking water for 4 months. Hypoactivity was found to be induced in mice following withdrawal of silver nitrate in drinking water at a dose of 14 mg/kg bw per day, that had been previously administered for 125 days (Rungby & Danscher, 1984).

Hadrup et al. (2012c) assessed the effect of silver nanoparticles (14 nm stabilized with PVP) and silver acetate on the levels of brain neurotransmitters. The authors reported that silver nanoparticles (4.5 and 9 mg/kg bw per day) and silver acetate (9 mg/kg bw per day) increased the brain dopamine concentration after 28 days of oral administration. In contrast to the results seen after a 28-day exposure period, after 14 days of exposure, dopamine concentration was decreased by silver nanoparticles (at concentrations of 2.25 and 4.5 mg/kg bw per day), leading to the suggestion that there are differential effects of silver on dopamine depending on the length of exposure. In the 28-day exposure, brain noradrenaline levels were significantly increased only by silver acetate (9 mg/kg bw per day) and brain 5-hydroxytryptamine was increased only by silver nanoparticles (9 mg/kg bw per day).

Skalska et al. (2015) administered rats with silver nanoparticles (10 nm stabilized in sodium citrate, 0.2 mg/kg bw per day), silver citrate (0.2 mg/kg bw per day) or saline, over a 14-day period,. Both types of silver were found to result in ultrastructural pathological changes in the forebrain cortex and hippocampus of the treated animals, with the synaptic degeneration being greater in the hippocampus region.

No studies could be identified to address potential neurotoxic effects of silver or silver nanoparticles in animals following inhalation or dermal exposure.

3.4.3.3 Reproductive and developmental toxicity

Pregnant female rats administered 50 mg of silver chloride per animal (corresponding to 190 mg of silver per kg bw per day) orally during gestation days 1–20 showed increased post-implantation lethality. The incidence of visceral damage in the offspring was considerably higher compared to the control group and all offspring died within 24 hours of birth (Shavlovski et al., 1995).

Pregnant rats were treated with silver nanoparticles (< 10 nm suspended in carboxymethylcellulose, maximum concentration 1000 mg/kg per day) for 14 days during their pregnancy and the impact on fetal development determined on day 20 of gestation. There were no significant differences between the groups during the feeding stage. On *post mortem* examination, there were signs of oxidative stress in maternal hepatic tissues at 100 mg/kg and above (decrease in liver catalase and glutathione reductase activities), but no evidence of developmental toxicity (Yu et al., 2013). Hong et al. (2014) also failed to find any impacts of reproduction/development (mating, fertility, implantation, delivery and fetuses) in rats fed up to 250 mg/kg bw per day of citrate-stabilized silver nanoparticles (7.9 nm) for up to 52 days. They also did not show any differences in haematology, serum biochemical investigation or histopathological analysis.

In a study by Mathias et al. (2015), prepubertal male rats were fed either 15 or 30 µg/kg bw per day of silver nanoparticles for 35 days from postnatal day 23 to postnatal day 58 and then sacrificed 44 days later. While no changes were seen in growth and the animals showed no changes in sexual behaviour and serum hormone concentrations, silver nanoparticle exposure delayed the onset of puberty and reduced the acrosome and plasma integrities, reduced the mitochondrial activity and increased the abnormalities of the sperm in both treatment groups.

In male rats administered silver nanoparticles at 20 µg/kg bw per day by oral gavage for 90 days, histopathological changes in the testes were seen. These were reported as: disorganization of the normal appearance of the testis with varying degrees of atrophy in the seminiferous tubules; depletion of germ cells and germinal cell necrosis in spermatogonia; degenerative changes in form of necrosis and severe vacuolisation in sertoli cell cytoplasm; and vacuolated Leydig cells (Thakur et al., 2014). As only one dose of silver nanoparticles was utilised in this study, a LOAEL of 20 µg/kg bw per day only can be derived.

Kovvuru et al. (2015) reported that in mice, maternal ingestion of 500 mg/kg bw per day of silver nanoparticles (PVP-coated) for 5 days during the post-coitum period induced DNA deletions in developing embryos. They also reported irreversible chromosomal damage in bone marrow and double strand breaks and oxidative DNA damage in peripheral blood and/or bone marrow.

In mice administered 0.03% silver nitrate in the drinking water for 1 month (corresponding to 23 mg of silver/kg bw per day), changes in ovarian nuclear and cytoplasmic cell morphology were reported (Hadek, 1966).

No studies could be identified to address potential reproductive or developmental effects of silver or silver nanoparticles in animals following inhalation or dermal exposure.

3.4.3.4 Immunotoxicity

Reported effects of silver or silver nanoparticles on the immune system following oral administration are variable. Lymphocyte infiltration was reported in mice fed a single, very high dose (125 g/kg bw per day) of 13 nm nanoparticulate or 2–3.5 µm micro-particulate silver (Cha et al., 2008). Silver induced an autoimmune condition in the genetically susceptible H-2s mouse strain following the administration of 0.5 g of silver nitrate/L in the drinking water (which corresponded to 47 mg of silver per kg bw per day) for 10 weeks (Havarinasab et al., 2009). In a 28-day oral study, administration of nanoparticulate and ionic silver at doses up to 9 mg/kg bw per day were associated with a decreased thymus weight (Hadrup et al., 2012b). Park et al. (2010a) observed increases in plasma concentrations of interleukin 1 (high dose), interleukin 4 (high dose), interleukin 6 (middle and high dose), interleukin 10 (all doses), interleukin 12 (middle and high dose) transforming growth factor b (middle and high dose) and immunoglobulin E (high dose), as well as an increase in cell infiltration in the kidney cortex (high dose) following oral exposure of mice to 42 nm silver nanoparticles at doses of 0.25, 0.5 and 1 mg/kg bw per day.

Conversely, Van der Zande et al. (2012) observed no immunotoxicity following the oral administration of silver nanoparticles (15 and 20 nm) at 90 mg/kg bw per day or ionic silver at 9 mg/kg bw per day for 28 days.

No studies could be identified to address potential immunotoxic effects of silver or silver nanoparticles in animals following inhalation or dermal exposure.

3.4.3.5 Genotoxicity (*in vivo*)

A number of *in vivo* genotoxicity studies have been carried out with ionic silver and silver nanoparticles in rats and mice. Those using the comet assay, micronucleus assay and chromosome aberration test are summarized in Table 7 below. With regards to genotoxicity testing, the Organisation for Economic Co-operation and Development has produced a series of test guidelines for genetic toxicology which includes the following battery of tests:

- bacterial reverse mutation test (e.g. the Ames test) TG 471;
- *in vivo* mammalian alkaline comet assay (single cell gel electrophoresis assay) TG 489;
- mammalian micronucleus assay (*in vivo* TG 474; *in vitro* TG 487);
- mammalian chromosome aberration test (*in vitro* TG 473, *in vivo* TG 475, TG 483); and
- mammalian gene mutation assay (*in vitro* TG 476, *in vivo* TG 488).

Many of the test guidelines have recently been updated and a new genetic toxicology guidance document is in draft form (OECD, 2015). While it is acknowledged that some substances, including nanomaterials, may require special modifications to the test guidelines, no guidance is provided on this within the test guidelines. It is becoming increasingly clear, for example, that the Ames test is not effective at assessing the genotoxic potential of nanoparticles as the results following challenge with a variety of nanoparticles have predominantly been negative, although nanoparticles have been shown to produce positive genotoxic responses from *in vitro* mammalian cell test systems (Landsiedel et al., 2009; Doak et al., 2012). In addition, the comet assay is an “indicator” test which detects primary DNA damage, but not the consequences of the damage. The DNA measured in the comet assay may, for example, lead to cell death or it may result in DNA repair (which can result in return the DNA to its original state or may result in a mutation). The micronucleus assay and chromosome aberration test both test for chromosomal aberrations resulting from exposure to the test chemical.

Of the 11 studies outlined in Table 7, five used rats and six used mice and, most studies analysed bone marrow samples. Doses of silver nanoparticles ranged from 0.01 mg/kg bw per day (Taveres et al., 2012) to 1000 mg/kg bw per day (Kim et al., 2008; Kim JS et al., 2011). The comet assay was used in eight of the studies. In rats, Patolla et al. (2015) found a dose-dependent increase in DNA damage in the comet assay (significant at 50 mg/kg bw per day and above) in a 5-day oral feeding experiment, while Dobrzynska et al. (2014) saw no significant effect on bone marrow leukocytes after a single intravenous injection (maximum concentration 10 mg/kg bw). In mice, the comet assay gave more consistent results with five out of the six studies showing an increase in DNA damage following exposure to silver nanoparticles using a variety of routes of administration. Asare et al. (2015) found no effect in mouse liver, lung or testes following a single intravenous dose (5 mg/kg) of 20 nm silver nanoparticles. The micronucleus assay gave mixed results, where two of the five tests were positive. Both of the positive tests were in bone marrow tissue from rats; one using a 5-day oral administration and one using a single intravenous injection. There is some suggestion that different cells within the same tissue (Dobrzyńska et al., 2014) and different tissues (Li Y et al., 2013) may display different susceptibility to genotoxic effects. Dobrzyńska et al. (2014), for example, found a statistically significant increased frequency of micronuclei in erythrocytes from bone marrow, following exposure to silver nanoparticles, but not reticulocytes; although other authors have reported negative results in rat bone marrow erythrocytes (e.g. Kim JS et al., 2011). Chromosome aberrations were seen in each of the three studies that employed this test; two in rats (Patolla et al., 2015; El Mahdy et al., 2014) and one in mice (Ghosh et al., 2012). The most frequently noted aberrations varied by study. One study (Li Y et al., 2013) used the Pig-a assay (a relatively new *in vivo* gene mutation test – not shown in Table 7)

in mice exposed to 5 nm PVP silver nanoparticles by intravenous injection and reported no effect over the control.

There was no evidence of genetic toxicity in male or female rats based on an analysis of micronucleus induction from bone marrow, following inhalation exposure of rats to silver nanoparticles at levels up to 515 µg/m³ over a 90-day period (Kim JS et al., 2011). Dong et al. (2013) exposed rats to silver nanoparticles at an inhalation level of 381 µg/m³ 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.

No studies could be identified to assess genotoxic effects of silver or silver nanoparticles following dermal exposure in animals.

3.4.3.6 Carcinogenicity

Silver is not classified as a human carcinogen. Fibrosarcomas have been induced in rats following subcutaneous imbedding of silver foil; imbedded silver metal foil appeared to produce fibrosarcomas with a latent period of 275 days in 32% of implantation sites (Oppenheimer et al., 1956). However, the relevance of this to humans is uncertain and may reflect solid-state carcinogenesis in which even insoluble solids such as plastic have been shown to result in local fibrosarcomas (Coffin & Palekar, 1985). Both positive (Schmahl & Steinhoff, 1960) and negative (Furst & Schlauder, 1977) results for tumorigenesis have been reported following subcutaneous and intramuscular injection, respectively, of colloidal silver in rats. However, the relevance of these routes of exposure to humans is, again, unclear (ATSDR, 1990). No studies on carcinogenicity from silver nanoparticles were identified.

Table 7: Genotoxicity testing of silver nanoparticles

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	Micronucleus assay	Chromosome aberration test
Kim et al., 2008	Rat	Bone marrow	CMC 60 nm, NP	28 days, oral (unstated admin.)	30, 100, 1000 mg/kg bw/day	-	No significant effect. A small, dose-related, increase in polychromatic erythrocytes was seen in male rats. A small increase was also seen in polychromatic erythrocytes in female rats for 2 of the tested doses. None of the increases, however, were statistically significant when compared to the control.	-
Patolla et al., 2015	Rat	Bone marrow	10 nm, naked NP	5 days, oral (using feeding needles)	5, 25, 50, 100 mg/kg bw/day	All doses caused a dose-dependent increase in DNA damage, the 50 and 100 mg/kg doses produced statistically significant increases.	A dose-dependent increase in micronucleus frequencies was seen. The 50 and 100 mg/kg doses gave statistically significant increases.	A dose-dependent increase in chromosome aberrations was observed. Chromatid gaps and breaks were the most frequently noted aberrations.
Kim JS et al., 2011	Rat	Bone marrow	18 nm, naked NP	90 days inhalation	30, 300, 1000 mg/kg bw/day	-	No statistically significant differences were seen in the erythrocytes.	
El Mahdy et al., 2014	Rat	Bone marrow	9 nm, PVP NP	28 days ip injection	1, 2, 4 mg/kg bw/day	-	-	Statistically significant chromosome aberrations were seen at all tested concentrations. Centromeric attenuations were the most frequent structural aberration observed.

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	Micronucleus assay	Chromosome aberration test
Dobrzynska et al., 2014	Rat	Bone marrow	20 nm, naked NP	Single iv injection, animals killed at 24 h, 1 and 4 weeks	5, 10 mg/kg bw	No significant effect was seen on bone marrow leukocytes.	A significantly increased frequency of erythrocyte micronuclei was seen 24 hours after exposure to both 5 and 10 mg AgNP/kg bw. The enhanced frequency was also seen at 1 and 4 weeks post-exposure. No impact was seen on reticulocytes.	-
Awasthi et al., 2015	Mouse	Liver	5 nm, NP	Single oral dose (by oral intubation) (animals killed at 3 and 24 h) Weekly (5 weeks) oral dose (by oral intubation)	50, 100 mg/kg bw 10, 20 mg/kg bw/day	A significant difference in all comet assay parameters at both 3 and 24 h for the single 100 mg/kg dose. For the multiple exposure mice, significant damage was seen for both 10 and 20 mg/kg doses.	- -	- -
Ghosh et al., 2012	Mouse	Bone marrow	120 nm (ave), NP	Single ip injection	10, 20, 40, 80 mg/kg bw	An increase in DNA damage (over the control) was seen. There was no clear dose-response relationship.	-	A significant increase (cf. control) in the frequency of aberrant cells and number of breaks per cell was seen.
Tavares et al., 2012	Mouse	Blood	19 nm (ave), citrate NP	Single ip injection, blood taken at 1, 6, 12 and 24 h	10, 25, 50 µg/kg bw	Limited effects, with only the lowest dose (10 µg/kg) producing a significant increase in DNA damage cf. the control.	-	Aberrations were mainly chromatid breaks. No clear dose-response relationship.

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	Micronucleus assay	Chromosome aberration test
Al Gurabi et al., 2015	Mouse	Liver	44 nm (ave), NP	Single ip dose (animals killed at 24 or 72 h)	26, 52, 78 mg/kg bw	Significant damage seen at all doses. At the lowest dose (26 mg/kg) damage was only significant after 72 h.	-	-
Li Y et al., 2013	Mouse	Bone marrow	5 nm, PVP NP	Single iv injection	0.5, 1, 2.5, 10, 20 mg/kg bw	-	No effect seen on reticulocytes.	-
Li Y et al., 2013	Mouse	Bone marrow, liver	15-100 nm, PVP NP, 10-80 nm, silicon NP	Single or 3 day iv injection	25 mg/kg bw	Following a 3-day exposure, no increase in liver DNA damage was seen in the standard comet assay. A significant increase in DNA damage, however, was seen in the enzyme modified assay for both PVP and silicon AgNP, suggesting that AgNP can cause oxidative DNA damage.	No effect seen on reticulocytes.	-
Asare et al., 2015	Mouse	Liver, lung, testes	20 nm, NP	Single iv dose (animals killed at 1 and 7 days)	5 mg/kg bw	No significant effect in any tissue.	-	-

ave–average; bw–body weight; ip–intraperitoneal; iv–intravenous; NP–nanoparticles

3.4.4 *In vitro* toxicity

There has been a marked increase in the number of *in vitro* studies investigating the potential toxicity of ionic silver and silver nanoparticles in recent years. A wide range of cell types from human, rat, mouse, hamster and porcine origin have been investigated, including cells derived from: blood (e.g. Zhang et al., 2013); brain (e.g. Haase et al., 2012); 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). It is possible that secondary cells (i.e. cancer-derived or immortalized cell lines) may not provide useful information in terms of silver or silver nanoparticle toxicity on normal undifferentiated cells, which are most relevant to human exposure scenarios, as these may have multiple molecular pathways that are deregulated (Franchi et al., 2015). Thus, the emphasis in this document is data derived from toxicological studies that have utilized primary cells.

The interpretation of *in vitro* data in terms of its relevance to human exposure scenarios is unknown and so, for clarity, a summary of findings is provided below, with full details provided in Appendix B.

- Ionic silver and silver nanoparticles cause toxic effects in a wide variety of cell types from a range of organs and tissues. The degree of toxicity varies according to the form of silver (ionic silver or silver nanoparticles), the silver nanoparticle size and coating and the sensitivity of individual cell types.
- In the liver, although toxic effects are reported, primary cells seem to be much more resilient to the effects of silver than secondary cell lines.
- Silver is cytotoxic to lung macrophages and fibroblasts and also brain cells, including astrocytes and neurons. Based on work using cells derived from the rat adrenal medulla it has been suggested that both ionic silver and silver nanoparticles are developmental neurotoxicants. The majority of studies identified relating to the gut are based on secondary cancer-derived cell lines and have suggested that silver is cytotoxic to colon epithelial cells (Caco-2, HT29 and SW480), although there is a suggestion that uptake of silver by intestinal cells (HT29) is less than other cells and that the production of mucin may be protective. Normal colon mucosal epithelial cells (NCM460) seem to be less susceptible to the impact of silver nanoparticles than the secondary cell lines.
- Baby hamster kidney cells and human embryo kidney cells (HEK293) are sensitive to silver nanoparticles, with DNA damage seen in HEK293 cells detected following exposure to 1 µg silver nanoparticles per mL.
- In the blood, silver nanoparticles can cause platelet aggregation *in vitro* (human platelets) and *in vivo* (rats following intravenous or intratracheal administration) and has been shown to result in rupture of human red blood cells. Silver nanoparticles have also been shown to be cytotoxic to human blood mononuclear cells at concentrations as low as 1 µg/mL.
- Silver shows cytotoxicity in macrophages and keratinocytes and also affects zinc and selenium metabolism in keratinocytes and skin fibroblasts.
- Potential reproductive effects of silver have been shown, *in vitro* with a decrease in oocyte maturation and inhibition of the proliferation of spermatogonial stem cells.
- The comet assay and micronucleus test, in particular, indicate that silver nanoparticles (and ionic silver) may be genotoxic, with results dependent upon both the size and type of silver nanoparticles and also the sensitivity of the cell type although, as noted earlier, there is no regulatory approved comet assay for *in vitro* use and damage can be reversible.

- Generally, exposure of cells to silver (ionic silver and silver nanoparticles) results in oxidative stress; in susceptible cells this can result in a range of toxic effects including apoptosis, changes in gene expression and DNA damage. Different cell sensitivity is likely to be due to a range of factors and includes protection produced by mucin (e.g. HT29 cells) and intracellular antioxidant levels.

3.4.5 *In vitro* to *in vivo* extrapolations

Monteiro-Riviere et al. (2013) looked at the impact that pre-incubation of silver nanoparticles with a number of different proteins (albumin, IgG and transferrin – to form protein-complexed nanoparticles) had on the uptake of silver nanoparticles by human epidermal keratinocytes. Silver nanoparticle association with serum proteins significantly modulated silver uptake compared to native silver nanoparticle uptake. Shannahan et al. (2015) also examined how the formation of a protein corona as a result of exposure to a biological environment can impact on the uptake and also toxicity of silver nanoparticles; this group used citrate-coated silver nanoparticles (20 nm) and two rat cell types. The silver nanoparticles were incubated with human serum albumin, bovine serum albumin, high-density lipoprotein or water (control) to form a protein corona. Silver nanoparticles readily associated with human serum albumin, bovine serum albumin and high-density lipoprotein and, in each case, there was an increase in the hydrodynamic size of the silver nanoparticles. The addition of the protein corona decreased cellular uptake of silver nanoparticles and, at higher concentrations (25 and 50 µg/mL), reduced cytotoxicity.

3.5 Vulnerable populations

No information on the possible impact of silver or silver nanoparticles on vulnerable populations was identified.

3.6 Summary of the safety and toxicity of silver

The findings from identified *in vivo* studies relating specifically to the oral route of exposure are summarized below.

- Metallic silver is inert, and absorption is determined by ionization (under oxidizing conditions) to release the biologically active ionic silver, which is absorbed into the systemic circulation. Absorption rates of 18% have been reported for orally administered silver in humans, and between 0.4 and 18% in other mammals. Ionic silver binds strongly to metallothionein, albumins, and macroglobulins and is distributed to all tissues in the descending order of: stomach and small and large intestines, liver, spleen, testes, kidneys, brain, lungs, blood, bladder and heart. Silver deposition can occur through precipitation of insoluble silver salts, which are transformed to soluble silver sulfide albuminates. Excretion occurs via the bile and urine.
- There are a limited number of toxicity studies in humans relating to the toxicity of ionic silver or silver nanoparticles following exposure by any route. Of those available, no substantial toxicity has been reported.
- There are a limited number of experimental oral toxicity studies for silver. In the rat, silver (as silver nitrate) has moderate acute toxicity and is slightly toxic following acute exposure in the rabbit. Silver nanoparticles are considered to have slight to no acute toxicity via the oral route.
- There are a limited number of animal studies relating to the toxicity of silver following repeated exposures. A NOAEL of 181.2 mg/kg bw per day has been determined for silver based on reduced body weight gain in Sprague-Dawley rats.

- There are a large number of animal studies in rats (principally), mice and guinea pigs, relating to the toxicity of silver nanoparticles following repeated oral exposure. These have shown evidence of a dose dependent toxicity of silver nanoparticles related to a number of endpoints, namely: death, decreases in body weight, hypoactivity, altered neurotransmitter levels, altered liver enzymes, altered blood values, enlarged hearts, immunological effects, increased sperm abnormalities; delayed onset of puberty. Histopathological changes to liver, kidney, spleen, intestine, epidermis and brain tissue have also been reported.
- Of the studies identified, the most sensitive toxicological endpoint relates to histopathological changes in the testes in male rats. A LOAEL of 20 µg/kg bw per day has been derived for this endpoint.
- No *in vivo* genotoxicity studies carried out with silver in humans or animals could be identified. Although *in vitro* findings suggest that ionic silver may be genotoxic, the relevance of these findings to humans is unknown.
- Genotoxicity studies have been carried out *in vivo* with silver nanoparticles in the rat and in mice, at repeated oral doses between 5 and 1000 mg/kg bw per day for up to 35 days. The findings suggest that silver nanoparticles administered by the oral route may induce DNA damage at the chromosomal level, becoming clastogenic at higher levels. However, there is no indication of direct mutagenicity.
 - In the rat and mouse, a dose-dependent increase in DNA damage was seen using the comet assay, which was significant at 50 and 10 mg/kg bw per day and above in rats and mice respectively.
 - A dose-dependent increase in micronucleus formation has been reported in rats, which reached significance at a dose of silver nanoparticles of 50 mg/kg bw per day and above.
 - A dose-dependent increase in chromosome aberrations has also been observed in rats administered silver nanoparticles at doses between 5 and 100 mg/kg bw per day. Chromatid gaps and breaks were the most frequently noted aberrations.
 - The mode of action for silver nanoparticle toxicity is at present undefined, however it is closely related to its transformation in biological and environmental media:
 - nanosilver particles can interact with membrane proteins and activate signalling pathways, leading to inhibition of cell proliferation;
 - nanosilver particles can enter the cell through diffusion or endocytosis to cause mitochondrial dysfunction, generation of ROS, leading to damage to proteins and nucleic acids inside the cell, and finally inhibition of cell proliferation; and
 - both the ionic and nano-form of silver can interact with sulfur containing macromolecules such as proteins.

4. Environmental considerations

Environmental considerations are largely beyond the scope of this report; however, it has been noted that release of silver and silver nanoparticles (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 silver salts and silver nanoparticles to selected environmentally relevant test organisms as well as target organisms. Table 8 shows the median L(E)C₅₀ or MIC data for silver nanoparticles and silver salts.

Table 8: Median L(E)C₅₀⁸ for all organisms except bacteria and median MIC for bacteria for silver nanoparticle and silver salts (adapted from Bondarenko et al., 2013)

Group of organisms	Median L(E)C ₅₀ /Minimum inhibitory concentration			
	AgNP (mg/L)	Number of data	Ag salts (mg/L)	Number of data
<i>Target</i>				
Algae	0.36	17	0.0076	10
Bacteria	7.10	46	3.3	27
Protozoa	38	7	1.5	3
<i>Non-target</i>				
Crustaceans	0.01	17	0.00085	8
Fish	1.36	17	0.058	4
Nematodes	3.34	21	4.8	4
Mammalian cells <i>in vitro</i>	11.3	25	2	18

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

⁸ LC₅₀ – median lethal concentration: the concentration required to kill half the members of a test population after a specified test duration.

EC₅₀ – half maximal effective concentration: the effective concentration of a chemical that causes half of the maximum response in a test population after a specified test duration.

5. Discussion

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

5.1 Efficacy

In many of the studies reported in Section 2, it is often difficult to determine the efficacy of the silver component (especially in the studies outlined in Sections 2.2 and 2.3) 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).

5.1.1 Copper/silver ionization in hospital water systems

Copper/silver ionization is often used for microbial control (especially against *Legionella* spp.) particularly in hospital hot water distribution systems. In well-run systems (where ion concentrations are monitored and kept at recommended levels) most studies have shown that *Legionella* spp. are reduced to low levels and that implementation of the ionization system markedly reduces the number of cases of nosocomial Legionnaires' disease.

5.1.2 Ionic silver in drinking water

Studies on the bacterial inactivation resulting from ionic silver added to water have shown that LRVs vary widely (generally between 3 and 7), with some bacteria being more sensitive (i.e. more easily killed or inactivated) than others. Generally, long contact times are required to reduce bacterial concentrations. In addition, the majority of studies spiked water samples with laboratory grown bacteria. Studies using silver on harvested rainwater, for example, typically showed poorer LRVs (0.4–2.9). The use of laboratory grown bacteria, (which tend to be “less virulent and hearty than wild microbial consortiums”– Madrigan et al., 2000) may, thus, overstate the effectiveness of treatment.

Two non-bacterial studies were identified. De Gusseme et al. (2010) showed a 3 log₁₀ reduction in bacteriophage after 2 hours of exposure to a high silver concentration (5 mg/L). Abebe et al. (2015) found that silver nitrate-treated *C. parvum* oocysts were significantly less infective in mice than untreated oocysts.

The role of water chemistry is also likely to be important in determining the efficacy of silver as a disinfectant in real world conditions. Silver forms numerous salts with low water solubility and silver ions are easily sequestered by anions commonly found in natural waters including chloride, bromide, carbonate and phosphate. Even at low concentrations which do not induce silver precipitation, chloride and phosphate have been shown to hinder the bioavailability and mitigate the antibacterial activity of silver ions (Xiu et al., 2011).

5.1.3 Silver nanoparticle applications

The exploratory drinking-water applications (principally employing filtration) identified used a wide range of media/matrices (e.g polyurethane foam, paper and polystyrene beads). The majority of studies tested efficacy against bacteria (typically *E. coli*), with values up to 7 log₁₀ reduction being reported (range 1.6–7.6). De Gusseme et al. (2010) showed that biogenic silver nanoparticles produced a 4 log₁₀ reduction in bacteriophage after three hours and murine norovirus after 30 minutes; this was in stark contrast to chemically-produced silver nanoparticles, which showed no inactivation. Abebe et al. (2015)

found that silver nanoparticle-treated *Cryptosporidium* oocysts showed some reduction in mouse infectivity compared to non-treated oocysts.

5.1.4 Silver-coated ceramic filter applications

A number of studies have considered the impact of silver coating on ceramic filters. In terms of the efficacy of the silver within silver-coated ceramic filters, it seems likely that the type of silver employed (silver nitrate or silver nanoparticles), how it is applied (painted on, dipped or fired in) and in what concentration the silver is applied affects both the bacterial removal and the effluent silver concentration. Overall, however, many of the studies which compared silver-coated and non-coated filters have not shown convincing benefits of silver nitrate or silver nanoparticle application, as shown in Table 9.

Table 9: Comparison of bacterial removal efficiencies of silver-coated and non-coated ceramic filters

Silver type	Bacterial removal cf. non-coated filter	Reference
AgNP	No significant difference	van Halem et al., 2007
Unspecified	Significant improvement only after filtration of 5000+ litres	Wubbels et al., 2008
AgNP	Variability of results for both coated and non-coated filters was too great to reach a conclusion	Bielefeldt et al., 2009
AgNO ₃	Overall, silver-coated filters outperformed non-coated filters, however, individual results were very variable	Bloem et al., 2009
AgNO ₃	No significant difference	Brown & Sobsey 2010
AgNP	Slight improvement over non-coated filters, although only likely to be statistically significant at 17% sawdust content	Kallman et al., 2011
AgNP	No significant difference	Zhang & Oyanedel-Craver, 2013
AgNP/AgNO ₃	AgNP was found to be more effective than AgNO ₃ , although source of clay was also an important factor	Rayner et al., 2013

While some studies (e.g. Bloem et al., 2009) have shown that silver application can improve microbial removal (e.g. by between 1.8 and 3.3 log₁₀ reduction in *E. coli*) compared to non-treated ceramic filters, silver-coated ceramic filters (even from the same manufacturer) seem to show great variability in bacterial removal. A number of studies have shown that water chemistry may greatly affect the longevity of the treatment effect (Bielefeldt et al., 2013; Mittelman et al., 2015). Of the silver-coated ceramic filter studies which considered virus reduction, viruses were poorly removed and did not meet the 3 log₁₀ reduction required for 2-star performance classification (WHO, 2016).

5.1.5 General points for potential drinking-water applications

Although an initial glance at the results suggests that silver may reduce microbial contamination in water (in some circumstances), there are a number of limiting factors that need to be considered, including:

- There is an emphasis on bacterial testing (this has been highlighted above).
- Few studies have tried to assess the silver applications in field conditions, using both turbid and non-turbid water and realistic contact times (and this may account for the diversity of reported results and the higher reported LRVs seen in the laboratory compared to field studies). Of the two reported silver-containing water treatment products tested against the WHO Scheme, one using colloidal silver and one using silver-treated ceramic filters, neither met requirements for effective household water treatment performance (i.e. providing comprehensive protection).
- There is a lack of consideration that silver may be acting as a bacteriostat and the impacts of silver leaching on the mid- to long-term performance of the product. Few studies have looked at regrowth (i.e. the possibility that silver is acting as a bacteriostatic rather than bactericidal agent in the low concentrations required for drinking-water applications – i.e. $\leq 100 \mu\text{g/L}$), or the presence of silver in stored filtered water and the mode of action of silver at low concentrations is unclear.
- There is no clear accounting for the presence of potentially toxic contaminants in applications using silver nanoparticles (which may, at least in some instances, be the cause of microbial inactivation). Silver nanoparticles can be synthesized in a variety of ways, some of which use toxic reagents. It is often not clear from the studies on silver nanoparticle applications whether adequate steps were taken to remove these contaminants before efficacy testing.

5.2 Toxicity

It is clear that 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. Animal *in vivo* study results suggest a range of toxic effects including decreases in body weight, histopathological changes to a number of organs and tissues, alterations to serum enzymes and neurotransmitter levels, increased sperm abnormalities, delayed onset of puberty and indications of genotoxicity. *In vivo* and *in vitro* studies have, however, produced an array of often conflicting information, which means that drawing clear conclusions about silver toxicity is difficult, although there are a number of reasons for the conflicting results as illustrated in the following sections.

5.2.1 Silver nanoparticles

There are numerous different methodologies for the synthesis of silver nanoparticles; they can be produced in a wide range of sizes and shapes and stabilized with a variety of capping agents, and these factors alone make generalizations difficult.

5.2.1.1 Synthesis and capping

Chernousova & Epple (2013) have noted that the reproducible laboratory synthesis of silver nanoparticles 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. In addition, a number

of studies have shown that the choice of capping or stabilizing agent can change the toxicity of silver nanoparticles.

5.2.1.2 Size

There are a number of techniques for determining silver nanoparticle size; those most commonly used are transmission electron microscopy and dynamic light scattering. Transmission electron microscopy 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 silver nanoparticle dispersion prior to drying). Dynamic light scattering 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 silver nanoparticles also depends on the medium in which they are suspended, with Bouwmeester et al. (2011), for example, finding larger hydrodynamic sizes for silver nanoparticles when they were suspended in cell culture medium, compared to water. In the review sections and below, usually only the primary size of the silver nanoparticles has been reported to avoid over complicating the text.

Some studies have suggested that smaller silver nanoparticles are more toxic to mammalian cells than larger nanoparticles and microparticles. Carlson et al. (2008), for example, found that 15 nm carbon-coated silver nanoparticles caused more toxicity than 50 nm carbon-coated silver nanoparticles in rat alveolar macrophages and Li et al. (2012) reported similar results for PVP-coated silver nanoparticles (25, 35, 45, 60 and 70 nm) in human lung fibroblasts. Liu et al. (2010) found that small PVP-coated silver nanoparticles (5 nm) were more toxic to four different cell lines than both ionic silver (silver nitrate) and larger particles, as shown in Table 10.

Table 10: Half maximal effective concentration for cell mortality in four different cell lines (Liu et al. 2010)

Cell line	Half maximal effective concentration (EC ₅₀)			
	AgNO ₃ (µg/mL)	AgNP-5 nm (µg/mL)	AgNP-20 nm (µg/mL)	AgNP-50 nm (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

Adapted with permission from Liu W et al., Impact of silver nanoparticles on human cells: effect of particle size, Nanotoxicology. Copyright 2010 Taylor and Francis.

Dasgupta et al. (2015) reported that 60 nm silver nanoparticles were more toxic to both A549 (lung carcinoma cells) and HCT116 (colon carcinoma cells) than 85 nm silver nanoparticles, manufactured using the thermal co-reduction. However, this does not seem to be universally the case as Powers et al. (2011), for example, found that larger PVP-coated silver nanoparticles (50 nm) had greater effects on DNA synthesis and caused a higher degree of oxidative stress in PC12 cells than the smaller PVP-coated particle (10 nm). Park et al. (2010b) reported greater cytotoxicity of 70 nm silver nanoparticles 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 ionic silver concentration, but reduced silver nanoparticle concentration (Pratsinis et al., 2013). In a review of toxicity data on

mammalian cell lines, Bondarenko et al. (2013) found that when plotting $L(E)C_{50}$ data for PVP-coated silver nanoparticles (to avoid coated versus non-coated toxicity issues) against the primary size of the silver nanoparticles, no correlation was seen ($R^2=0.1$); plotting the data from Liu et al. (2010) resulted in a correlation of $R^2=0.4$, while plotting data from just one study on A549 cells (Liu et al., 2010) revealed a correlation of $R^2=0.81$. This demonstrates how difficult it is to make generalizations about the toxicity of silver nanoparticles to mammalian cells.

5.2.1.3 Experimental quality

There are numerous pitfalls that await the unwary silver nanoparticle researcher. These include lack of characterization of the silver nanoparticles, gradual release of silver ions from the dissolved silver nanoparticles 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 silver nanoparticles, and interference of silver nanoparticles with the toxicity tests.

In order to improve comparability between studies it is important that the silver nanoparticles used are adequately characterized. It has been suggested that complete characterization of silver nanoparticles 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 silver nanoparticle characterization is increasingly being reported, and it is clear that where commercial silver nanoparticles are utilized, 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 silver nanoparticle and previously stored silver nanoparticles on human mesenchymal cells. The aged silver nanoparticles were found to be considerably more toxic than those that were freshly prepared, with the silver nanoparticles that had been prepared 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 silver nanoparticles. The difference in toxicity was attributed to differing amount of released silver 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 stabilize silver nanoparticles 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 microbiological contamination), they found that both the nanoparticles and their solvents contained variable levels of endotoxin (to which many immune cells are especially sensitive). As noted above, some of these aspects may also impact on disinfection efficacy studies.

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 (4–15 nm) 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).

5.2.2 *In vivo* toxicity

The studies outlined in Section 3.4, largely focus on the effects of silver nanoparticles (with some comparisons with silver salts). A number of dose-dependent animal toxicity findings have been reported including death, weight loss, hypoactivity, altered neurotransmitter levels, altered liver

enzymes, altered blood values, enlarged hearts and immunological effects (Hadrup & Lam, 2014). While many studies show no negative impacts, toxicological effects (histopathological changes in the testes) following chronic oral administration in rats were seen at a silver nanoparticle concentration of 20 µg/kg bw per day.

There has been a recent increase in interest in the possible genotoxic effects of silver. Although a number of studies have been reported only three of the *in vivo* studies looked at oral exposure. Two of these found possible genotoxic effects (Awasthi et al., 2015; Patolla et al., 2015) of which one reported a dose-dependent increase in chromosome aberrations at 5 mg/kg bw (the lowest dose examined) and above. A human study of silver jewellery workers (Aktepe et al., 2015) found evidence of possible DNA damage (based on the comet assay), but gave no information about working conditions or exposure to silver and other possible contaminants.

5.2.3 *In vitro* toxicity

Primary cells are more representative of tissue. They can be expected to reproduce the normal response of normal individuals (Oostingh et al., 2011) and therefore 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 from different donors) are required for each assay, making standardization 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 silver nanoparticles than primary cells and this has led to the exploration of silver nanoparticles 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 silver nanoparticles; however, the majority of publications do show reduced cell viability and increased ROS generation following silver nanoparticle 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 silver nanoparticles, 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 silver nanoparticles may act on different cellular targets and may differentially affect specific intracellular pathways depending on the cell types used. Chernousova & 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. Conclusions

It is difficult to draw any strong conclusions about the efficacy of silver (ionic silver and silver nanoparticles) in drinking-water treatment because of the wide range of approaches used in the various studies reviewed. The studies have used different types of silver (silver salts versus silver nanoparticles; capped silver nanoparticles versus bare silver nanoparticles; differently sized silver nanoparticles; silver nanoparticles created using different synthesis methods), different methodologies, different cells and microorganisms, different concentrations of test organisms and exposure for different time periods.

In drinking-water treatment applications, silver (ionic silver, experimental silver nanoparticle applications and silver-coated ceramic filters [ionic silver and silver nanoparticles]) has generally only shown to be effective against bacteria (i.e. 1.6 to 7.6 \log_{10} reductions), most notably *E. coli*, with relatively long contact times. Based on the current available evidence, which is particularly limited for viruses and protozoa, silver does not appear to meet the WHO minimum performance recommendations for POU treatment products, which require effectiveness for two of the three pathogen classes. This is, partly, because of the paucity of data documenting performance efficacy against these classes of microbes in water. In the one study on protozoan parasite reduction by silver, there was only limited effectiveness on *Cryptosporidium* infectivity and a \log_{10} reduction was not documented. For silver ions and nanoparticles, only one study on bacteriophage reduction in water has been reported, with effective \log_{10} reduction (i.e. 3–4 \log_{10} reductions) by ions and “biogenic” silver (zerovalent silver nanoparticles on a bacterial carrier matrix) but not by chemically-produced nanoparticles. It should also be noted that two silver containing products have failed the WHO evaluation scheme for household water treatment products, one a colloidal silver added to water and the other a silver-treated ceramic filter. Furthermore, it should be noted that relatively long contact times were required for effectiveness, which would reflect conditions where water would need to be stored.

Silver in combination with copper (copper/silver ionization) has been used to successfully suppress the growth of *Legionella* bacteria in plumbing (principally hospital) systems.

The body of evidence on safety seems to suggest that silver (in ionic form or as silver nanoparticles) 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. Most of the evidence on the toxicity of silver comes from *in vitro* studies. However, there is accumulating evidence from mammalian *in vivo* data, especially with silver nanoparticles, that suggest that exposure to silver may result in toxic effects in exposed subjects, given sufficient dosage and lengths of exposure. In particular, available data indicate that silver nanoparticles have potential to damage DNA, although the potential for genotoxicity or DNA damage with silver nanoparticles requires further investigation as to its significance for humans.

In summary, the current evidence is sufficient to indicate that:

- Silver has not demonstrated significant capability to be considered a candidate for primary disinfection of drinking water.
 - There are insufficient data to document that it acts against a broad spectrum of pathogenic organisms. Performance efficacy has been adequately documented only for some bacteria and not for viruses and protozoan parasites. The impact of water chemistry is often neglected in efficacy studies, and further, long contact times are generally required.
- Silver/copper continuous ionization systems can be effective supplemental disinfectants to control *Legionella* regrowth and reduce legionellosis risks in hospital hot water plumbing

systems at concentrations well below current recommended drinking-water concentrations. The hot water system is not considered to be drinking water, so human exposure from consumption is minimal. This approach is favoured by the long contact times that are achieved in those systems. Proper operation and maintenance and periodic monitoring is required to assure continued performance.

- In some studies, at least, silver may be toxic to mammalian cells *in vitro*, and there is an indication that some toxic effects can also be seen from *in vivo* animal studies.

It should also be noted that in its current applications in POU household water treatment devices, as a supplement or amendment to microporous filters, it is difficult to determine if silver is acting as a bacteriostat or bacteriocide.

On the basis of the significant data and performance gaps in disinfection efficacy as a primary disinfectant of water, the limited data on the range of microorganisms against which it is effective and under what conditions, and the availability of widely used, well-characterized disinfectants, silver is not recommended for use as a primary disinfectant in drinking-water supplies at this time. There are also uncertainties around the toxicology, particularly with regard to human health end points. While there is no evidence that the use of silver in household water filters has either caused adverse health effects or leached excessive levels of silver into filtered water, the overall evidence base does not indicate that such supplemental use of silver in water filters improves the microbiological quality and safety of the filtered water.

7. References

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Appendix A: Disinfectant mode of action

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

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 ROS (Park et al., 2009); and
- interaction with DNA (Thurman & Gerba, 1989).

Feng et al. (2000) conducted a mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus 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 & 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 ionic silver, 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_{10} reduction, caused by the silver ion disinfection in the bacteria they studied (*E. coli* and *S. 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 silver nanoparticles 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 ionic silver from the silver nanoparticles (Li et al., 2008). A number of authors have shown that silver nanoparticles 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 silver nanoparticles on *E. coli* using both scanning electron microscopy and transmission electron microscopy. The bacteria were cultured in a liquid medium supplemented with silver nanoparticles ($50 \mu\text{g}/\text{cm}^3$) for 4 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 silver nanoparticles 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 silver nanoparticles may cause free-radical generation, leading to subsequent cell damage. Kim et al. (2007) looked at the free-radical generation effect of silver nanoparticles on microbial growth inhibition using electron spin spectroscopy. The group showed that free-radicals were generated by the silver nanoparticles and that addition of an antioxidant reduced the bactericidal efficacy of the silver nanoparticles. They suggested that the free-radicals may be derived from the surface of the silver nanoparticles.

Shrivastava et al. (2007) studied the impact of silver nanoparticles on *E. coli*, *S. aureus* and *Salmonella typhus*. The silver nanoparticles were found to be more effective against the Gram-negative bacteria. The group found that the principal antimicrobial mechanisms were silver nanoparticles 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 silver nanoparticles 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 silver nanoparticles caused toxicity via protein/membrane and oxidative damage. In their study, the silver nanoparticles released silver ions and subsequently superoxide radicals.

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Appendix B: *In vitro* toxicity of silver and silver nanoparticles

There has been a marked increase in the number of studies looking at the *in vitro* toxic effects of silver (principally silver nanoparticles) 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).

These cells came from a variety of different sources including human, rat, mouse, hamster and porcine cells. These are described in detail below and the genotoxicity studies are summarised in Table B1.

In vitro studies covering exposure to cells derived from many of the target organs identified from *in vivo* studies are outlined below. It is likely that secondary cells (i.e. cancer-derived or immortalized cell lines) may not provide useful information in terms of silver nanoparticle toxicity on normal undifferentiated cells, which are most relevant to human exposure scenarios. For example, molecular pathways in cancer-derived cells are potentially deregulated (Franchi et al., 2015). Thus, in the 2015 literature update, the emphasis is on toxicological studies using primary cells.

B1. Liver

In the studies outlined below, researchers tested different silver nanoparticles against a variety of liver cell types although, generally, these were secondary cells (i.e. cancer-derived or immortalized cell lines). Different tests were used to assess toxicity but, usually, at least one test of cytotoxicity was included. Results were expressed in a variety of ways and include measures of the half maximal inhibitory concentration (IC₅₀) and LC₅₀.

Cha et al. (2008) exposed Huh-7 (hepatoma) cells to silver nanoparticles (13 nm) 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 et al. (2009) compared the cytotoxicity of silver nanoparticles (5–10 nm) and silver nitrate to human hepatoma (HepG2) cells using three different measures of cell viability. The MTT⁹ (a tetrazolium dye) and Almar Blue tests assess cell metabolic activity (through mitochondrial function), while the lactate dehydrogenase (LDH) tests assesses membrane integrity. The IC₅₀ values for the LDH tests in both silver nanoparticles and silver nitrate were markedly lower than the other tests, suggesting

⁹ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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).

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 (tests based on the tetrazolium dyes MTT and XTT¹⁰) for HepG2 cells exposed to silver nanoparticles (5–10 nm) of between 2.75 to 3 mg/L, very similar to that reported by Kim 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 (NO) concentration; increases in lipid peroxidation and cytochrome c content; decrease in glutathione (GSH) content and a decrease in superoxide dismutase [SOD] activity). Kawata et al. (2009) investigated the effects of silver nanoparticles (7–10 nm and stabilized with polyethylenimine) and silver carbonate on HepG2 cells at concentrations below those resulting in cytotoxicity. As silver nanoparticles were found to result in significant toxicity above 1 mg/mL (although silver carbonate still appeared to be non-cytotoxic at that dose), a concentration of 1 mg/mL was used in further experiments. At that concentration, silver nanoparticles were found to significantly increase the frequency of micronucleus formation, indicating DNA damage and chromosome aberrations (silver carbonate did not increase levels above those seen in the control). In addition, exposure to silver nanoparticles also altered gene expression, including the up-regulation of stress-related genes. Sahu et al. (2015) evaluated gene expression profiles in HepG2 cells exposed to 2.5 mg/L of 20 or 50 nm silver nanoparticles for 4 and 24 hours, and found that exposure to 20 nm silver nanoparticles resulted in a transient upregulating of stress response genes (such as metallothioneins and heat shock proteins). A number of cellular pathways, including the p53 signalling pathway and NRF2-mediated oxidative stress response pathway, were also impacted by silver nanoparticle exposure.

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

In 2005, Hussain et al. showed that silver nanoparticles (15 nm and 100 nm) were toxic to immortalized rat liver (BRL 3A) cells. Silver nanoparticles resulted in a concentration-dependent increase in LDH leakage and showed significant cytotoxicity at 10–50 µg/mL. The MTT assay also showed that silver nanoparticles caused significant cytotoxicity above 5 µg/mL. In addition, the level of ROS was found to increase in a concentration-dependent manner and a significant depletion of GSH was observed relative to control cells.

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

¹⁰ (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide).

as compared to unexposed cells suggesting that antioxidant defence mechanisms were triggered by silver nanoparticles exposure.

Kulthong et al. (2012) looked at the impact of silver nanoparticles on rat liver microsomes and specifically any changes in activity in hepatic cytochrome P450 (CYP) enzyme activity. The silver nanoparticles strongly inhibited CYP2C and CYP2D activities, but had no, or less, effect on other CYP activities. The impact in microsomes was in contrast to the *in vivo* study where no toxic effects were seen and where no change in CYP activity was observed.

The potential differences between primary and secondary cells is illustrated by Faedmaleki et al. (2014), who compared the impact of silver nanoparticles on mice primary liver cells in comparison to human HepG2 cells. Cell viability was measured using MTT and HepG2 were found to be significantly more sensitive to silver nanoparticles than the primary liver cells, with an IC_{50} value of 2.7 $\mu\text{g/mL}$ (HepG2) compared to 121.7 $\mu\text{g/mL}$.

B2. Lung

A number of studies have been conducted on the toxicity of various types of silver nanoparticles (different sizes and coatings) to lung cells *in vitro*. Typically, either A549 cells (a lung carcinoma alveolar epithelial cell) or, less frequently, other cells 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 silver nanoparticles impacts on toxicity with smaller silver nanoparticles typically being more toxic than larger particles (Carlson et al., 2008; Li et al., 2012; Gliga et al., 2014).

Foldbjerg et al. (2011) compared the toxic effects of silver nanoparticles (PVP-coated, 69 nm, up to 20 $\mu\text{g/mL}$) and silver nitrate (up to 10 $\mu\text{g/mL}$) on A549 cells. Both silver nanoparticles and silver nitrate were cytotoxic (as determined by impact on mitochondrial activity), although the cytotoxic impacts of silver nitrate (EC_{50} – 6 $\mu\text{g/mL}$) were seen at lower doses than those following silver nanoparticle exposure (EC_{50} – 12.5 $\mu\text{g/mL}$). The measured toxicity of both types of silver 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 silver nanoparticles were found to induce a greater increase in ROS than the silver nitrate. In comparison to the control, ROS levels were increased almost 16-fold at 10 μg silver nanoparticles/mL, but only approximately 8-fold by the same concentration of silver from silver nitrate. This group (Foldbjerg et al., 2012) also looked at the effects of silver nanoparticles (16 nm) and silver nitrate at low (non-cytotoxic) doses on gene expression in A549 cells. Exposure to silver nanoparticles 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 silver nanoparticles (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 μg silver nanoparticles/mL for 48 hours. The silver nanoparticles caused cytotoxicity, as measured by mitochondrial function (MTT assay) and membrane permeability (LDH assay). Silver nanoparticles 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

silver nanoparticles 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 silver nanoparticles modulate the cell cycle in A549 cells. Lee et al. (2011) showed that silver nanoparticles (hydrodynamic diameter 480 nm) 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 silver nanoparticles 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 silver nanoparticle-mediated impact on S phase arrest or down-regulation of proliferating cell nuclear antigen protein, 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. AshaRani et al. (2009a) looked at the anti-proliferative activity of silver nanoparticles (6–20 nm, starch coated) in normal human lung fibroblasts (IMR-90). Electron micrographs showed that silver nanoparticles were taken up by the cells and showed a uniform distribution both in cytoplasm and nucleus. Although the silver nanoparticle-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 silver nanoparticles with glucose, lactose, oligonucleotides and combinations of these ligands in comparison with bare silver nanoparticles on A549 cells. While the modification seemed to increase the uptake of the silver nanoparticles into the cells it also acted to decrease the toxicity, with the bare silver nanoparticles 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 silver nanoparticles (25, 35, 45, 60 and 70 nm) 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 silver nanoparticle size. Gliga et al. (2014) also examined the size-dependent cytotoxicity of silver nanoparticles, using 10, 40 and 75 nm citrate-coated, 10 nm PVP-coated and 50 nm uncoated silver nanoparticles. Using BEAS-2B cells (immortalized bronchial epithelial cells), they found that only the 10 nm silver nanoparticles were cytotoxic irrespective of coating; the reason for the greater toxicity of the smaller particles was believed to be due to the release of significantly more ionic silver compared with the other silver nanoparticles.

Carlson et al. (2008) explored the possible toxicity of inhaled silver nanoparticles using rat alveolar macrophages. The toxicity of three silver nanoparticles (coated in hydrocarbon) of different sizes (15 nm, 30 nm, 55 nm) was assessed at various doses. In general, the 15 nm silver nanoparticles showed the greatest toxicity and the 55 nm silver nanoparticles showed the least toxicity (e.g. the EC_{50} for increased LDH leakage was 27 $\mu\text{g/mL}$ for the 15 nm silver nanoparticles and > 75 $\mu\text{g/mL}$ for the larger particles). The authors also found a significant increase in ROS and a correlated decrease in levels of GSH following exposure to silver nanoparticles (15 nm) and increased secretion of inflammatory cytokines/chemokines.

B3. 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 have been identified in the literature.

B3.1 Astrocytes

The ability of astrocytes to withstand silver seems to depend upon the form of the silver and the silver nanoparticle coating. Luther et al. (2011), for example, exposed primary cultures of rat astrocytes to PVP-coated silver nanoparticles (70 nm) for up to 24 hours (approximately 10 µg silver/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 silver nitrate, was found to severely compromise cell viability. This group found that the silver nanoparticles taken up by the astrocytes remained sequestered in the cells following 7 days of incubation in silver nanoparticle-free medium (Luther et al., 2012). The same robustness to silver nanoparticle toxicity was not seen when rat astrocytes were exposed to smaller, peptide coated silver nanoparticles (20 and 40 nm), where the silver nanoparticles 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, silver nanoparticles (starch coated, 6–20 nm) were found to result in cytotoxicity and genotoxicity in U251 cells (AshaRani et al., 2009a, b) and silver chloride was found to cause oxidative stress in A172 cells (Simmons et al., 2011).

B3.2 Neurons

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

B3.3 Neurodevelopment and neurogenesis

The possible impacts of silver 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. They are used as a model for neuronal differentiation. Powers et al. (2010, 2011) have looked at the impact of silver nitrate and silver nanoparticles on these cells. A one-hour exposure of undifferentiated PC12 cells to 10µM ionic silver was found to inhibit DNA synthesis and protein synthesis. Longer exposure resulted in oxidative stress and loss of viability. Ionic silver directly inhibited mitotic activity. The same concentration of ionic silver 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 has also looked at the effects of silver nanoparticles (citrate- and PVP-coated) in PC12 cells. In undifferentiated cells, citrate-coated silver nanoparticles (10 nm) 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 silver nanoparticles caused oxidative stress and impaired differentiation into the acetylcholine phenotype. In undifferentiated cells, PVP-silver nanoparticles (10 nm and 50 nm) reduced DNA synthesis; the 50nm particle size had a greater effect. All three silver nanoparticles significantly suppressed the acetylcholine phenotype, but the small PVP-silver nanoparticles enhanced differentiation into the dopamine phenotype (Powers et al., 2011). The authors suggest that their results point to the likelihood that silver and silver nanoparticles are developmental neurotoxicants.

Cooper & Spitzer (2015) used rat neuroblastoma cells (B35) and cultured adult neural stem cells from the subventricular zone from Sprague-Dawley rats to assess the sublethal effects of silver nanoparticles (1 µg/mL) on neural function. Silver nanoparticle exposure in differentiating NSC induced the formation of f-actin inclusions (indicating a disruption of actin function). The silver nanoparticle exposure in B35 cells resulted in a decrease in neurite extension and branching, thus interfering with cytoskeleton-mediated processes that are vital to neurogenesis (which is thought to play a key role in cognitive functions such as learning and memory).

B3.4 Brain endothelial cells

Two recent studies have examined the impact of silver nanoparticles 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 cellular accumulation, changes in pro-inflammatory mediators and changes in morphology and permeability following exposure to PVP-coated silver nanoparticles (25, 40 and 80 nm in size). Silver nanoparticles were found to accumulate in the cells in a size-dependent manner (with less accumulation seen for the 80 nm silver nanoparticles). The cellular association of silver nanoparticles 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 silver nanoparticles (10, 50 and 100 nm) 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 silver nanoparticles 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 silver nitrate, suggested that the ionic form was less toxic to the endothelial cells than silver nanoparticles.

B4. Gut

A number of studies have looked at the impact of silver nanoparticles 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 silver nanoparticles, rather than cell culture medium, to try and more closely simulate *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 silver nanoparticles (nominal sizes 20, 34, 61 and 113 nm) and silver ions (from silver nitrate). Concentrations of silver nanoparticles of up to 50 µg/mL (irrespective of size) reduced metabolic activity in the Caco-2 cells by less than 20%, while a concentration of 5 µg/mL silver nitrate resulted in a 70% reduction in metabolic activity. Translocation of silver derived from either silver nanoparticle suspensions or silver nitrate 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 60 nm silver nanoparticles (citrate) and silver ions (silver nitrate). 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 silver nanoparticles, in the presence of proteins, were found to survive gastric digestion and reach the intestine where they were present in large clusters and co-localized with chlorine. The chlorine was thought to be involved in connecting separate silver nanoparticles inside clusters with “chlorine inter-particle bridges”. Following intestinal digestion, the silver nanoparticles 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, sulfur and chlorine (20–30 nm in size). The authors suggest that ingestion of silver nanoparticles and silver ions results in intestinal exposure to nanoparticles, albeit with different chemical compositions. Böhmert et al. (2014) conducted some similar work, subjecting silver nanoparticles to simulated digestion (both Böhmert et al., (2014) and Walczak et al., (2013) based their digestion model on the method described by Versantvoort et al., 2005) but then examined their toxicity to Caco-2 cells. Cells were exposed to primary and digested particles as well as a digestion fluids mixture without silver nanoparticles to act as a control. It was found that silver nanoparticles seemed to overcome gastrointestinal juices in their particulate form, without forming large quantities of aggregates, and there seemed to be only a slight reduction in their cytotoxic potential following digestion. This work has been extended by also including the main food components (i.e. carbohydrates, proteins and fatty acids) in the *in vitro* digestion process to further simulate realistic conditions (Lichtenstein et al., 2015). The uptake and cytotoxicity of digested and undigested polyacrylic acid-coated silver nanoparticles were investigated in Caco-2 cells. Silver nanoparticles digested with simulated food had a similar cellular uptake to undigested ones. However, silver nanoparticles digested in the absence of food simulants had a considerably lower cellular uptake, leading the authors to suggest that without the use of food components during *in vitro* digestion, uptake may be under estimated. Hsin et al. (2008) looked at the impact of two different commercially available preparations of silver nanoparticles (1 and 100 nm) on human colon cells (HCT116). One preparation (Ching-Tai) was found to result in significant decreases in cell viability after 24 hours at 50 µg/mL, while the other (Sun-Lan) 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 silver nanoparticles.

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

The impact of silver nanoparticles (18 nm) on gene expression in HT29 cells (and human kidney cells – see below) was explored by Gopinath et al. (2010). A concentration of 11 µg/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 silver nanoparticles 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 20 nm and 200 nm silver nanoparticles 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 silver nanoparticles, 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 nanoparticle uptake. The cellular uptake of silver nanoparticles 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 & Schwab (2013) looked at the cytotoxic effects of uncoated silver nanoparticles (20–30 nm) on Caco-2 and SW480 intestinal cells. The silver nanoparticles were 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 silver nanoparticles 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 silver nanoparticles in cell culture media may be due to the stabilizing effect of foetal bovine serum in the cell culture medium.

Giovanni et al. (2015) looked at a wide range of silver nanoparticles concentrations and their impact on selected human cell models representative of tissues in oral and gastrointestinal systems (TR146 – buccal epithelial cells and NCN460 – colon mucosal epithelial cells). After 24 hours incubation, very little cytotoxicity was seen in either cell type at silver nanoparticle levels of 100 µg/mL.

B5. 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).

Hudecová et al. (2012) exposed human embryo kidney cells (HEK293) to 20 nm silver nanoparticles. Although there was clear agglomeration of the particles, the silver nanoparticles 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 µg/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 µg/mL, although DNA damage could be detected even after exposure to 1 µg/mL silver nanoparticles. Singh and Ramarao (2012) found that renal epithelial cells (A498) were sensitive to 44 nm silver nanoparticles, with a significant reduction in viability (MTT and Coomassie Blue assay) at 1 µg/mL. This group looked at five different cell lines; 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 silver nanoparticles (< 20 nm, capped with polyoxylaurat Tween) were one of the more toxic nanomaterials examined, with an LC₅₀ of between 4.5–10 µg/cm² (depending on the cell culture medium used). Silver nanoparticle 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 silver chloride caused an increase in the oxidative stress response in four out of five cell lines examined, including kidney cells – HEK293T.

Gopinath et al. (2010) used baby hamster kidney cells (BHK21) to investigate the impact of 18 nm silver nanoparticles on primary cells. Cells exposed to 11 µg/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 silver nanoparticles induced the p53-mediated apoptotic pathway.

B6. 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 silver nanoparticles (69 nm) and silver ions (from silver nitrate) on the human monocytic leukaemia cell line (THP-1). Cells were exposed for up to 24 hours: it was found that both silver nanoparticles and ionic silver 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 silver nanoparticles (EC_{50} of 0.62 $\mu\text{g/mL}$ ionic silver compared to 2.44 μg silver nanoparticles/mL). Haase et al. (2012b) also looked at the toxicity of silver nanoparticles on THP-1 cells. They used two peptide coated silver nanoparticles (20 nm and 40 nm) and found that while both silver nanoparticles were toxic to the monocytes, the 20 nm silver nanoparticles were more toxic. The toxic effect was found to increase with time, thus, the IC_{50} for 20 nm silver nanoparticles at 24 hours was 110 $\mu\text{g/mL}$, compared to 18 $\mu\text{g/mL}$ at 48 hours.

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

- control–5.4% aggregation;
- 100 μg silver nanoparticles/mL–28% aggregation; and
- 250 μg silver nanoparticles/mL–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 silver nanoparticles (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 silver nanoparticles 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 particles). Both silver nanoparticle preparations (citrate stabilized and bare particles) were significantly more haemolytic than the micron sized particles (of equivalent mass concentration). The haemolysis was related to the release of silver ions (with the silver nanoparticles releasing considerably more than the micron preparations).

Silver nitrate at various concentrations (up to 33 μM) 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 silver nitrate penetrated the blood cells and resulted in oxidation of the reduced glutathione or the formation of a silver-glutathione complex.

Barkhordari et al. (2014) explored the impact of naked silver nanoparticles (1–1500 $\mu\text{g/mL}$) on human blood mononuclear cells. MTT assays were conducted after 6 or 24 hours incubation. The percentage cell death was higher after 24 hours than 6 hours, and all concentrations of silver nanoparticles resulted in significantly more cell death than in the control cells. The greatest impact seemed to be at 500 $\mu\text{g/mL}$,

although the authors do not report whether the differences between the cells incubated with 500 µg/mL silver nanoparticles and the higher concentrations are statistically significant.

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

B7. 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 silver nanoparticles has also been investigated using an *in vitro* system.

B7.1 Skin penetration

Larese et al. (2009) looked at the penetration of silver nanoparticles through human skin using an *in vitro* test system that utilised abdominal full thickness skin obtained as surgical waste. Skin was essentially bathed in silver nanoparticles (25 nm 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, silver nanoparticle absorption through intact skin was seen. As might be expected, penetration through damaged skin was five times greater than that through intact skin. Silver nanoparticles could be seen (using transmission electron microscopy) in the stratum corneum and upper layers of the epidermis.

B7.2 Skin cells

Arora et al. (2008) used secondary human skin epithelial cells (A431) to study cellular responses induced by spherical silver nanoparticles (7–20 nm). As the IC₅₀ (XTT assay) was 11.6 µg/mL, cells were subsequently exposed to a dose roughly half of that value. At 6.25 µg/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 silver nanoparticles (10 nm). In addition to inducing high quantities of ROS, silver nanoparticles caused a disruption in the epidermal growth factor signalling response.

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

Samberg et al. (2010) looked at the cytotoxicity of bare silver nanoparticles (20, 50 and 80 nm) and carbon-coated silver nanoparticles (50 and 80 nm) to primary neonatal human epidermal keratinocytes. If silver nanoparticles 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 silver

nanoparticles or washed silver nanoparticles did not cause a decrease in cell viability, suggesting that the toxicity seen in the unwashed silver nanoparticles is a result of residual contamination from the silver nanoparticle synthesis (in this case formaldehyde). Although washed silver nanoparticles 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 silver nanoparticles (25–50 nm) caused a concentration- and time-dependent decrease in cell viability (based on mitochondrial function) in HaCaT cells at concentrations of 11 µg/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 silver nanoparticles had been washed out of the system.

Comparative silver nanoparticle (~ 65 nm) 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 assay) was the most sensitive test (HaCaT LD₅₀ at 24 hours of 51.8 mg/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 after 72 hours for HaCaT of 30.4 mg/L compared to HeLa of 0.04 mg/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; this could be an important factor in the different sensitivity to silver nanoparticles.

Srivastava et al. (2012) investigated the impact of silver nanoparticles (size unstated) and silver ions (silver sulfate) on selenium metabolism in keratinocytes (HaCaT). They found that, while there was no clear cytotoxic effect of silver nanoparticle (up to 10 µM) or silver sulfate (up to 1000 nM) exposure on the keratinocytes, silver nanoparticles and ionic silver 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.

B8. Macrophages

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

Shavandi et al. (2011) looked at the cytotoxicity of silver nanoparticles (18–34 nm) 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 NO production were seen at 0.4 ppm silver nanoparticles. Park et al. (2010) also used murine peritoneal macrophages (RAW 264.7) to examine the impact of silver nanoparticles. Silver nanoparticles 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 silver nanoparticles 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. (2010) reported that silver nanoparticles were ingested by phagocytosis, but that they were not 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 silver nanoparticles with a similar size (< 10 nm) and shape (spherical), but different coatings and surface charge were tested against two cell lines: mouse macrophage (RAW 264.7) and mouse lung epithelial cells. The same pattern of toxicity was seen in both cell lines with, essentially, the silver nanoparticles with the greater positive surface charge being more toxic. The macrophage cells were more sensitive to the silver nanoparticles than the lung epithelial cells (Suresh et al., 2012). Singh and Ramarao (2012) also found that RAW 264.7 macrophages were highly sensitive to silver nanoparticle (44 nm) toxicity, with a significant reduction in cell viability (MTT assay) seen after 72 hours exposure to 3 µg/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 µg/mL).

Pratsinis et al. (2013) synthesized uncoated silver nanoparticles (6 to 20 nm) supported on inert nanostructured silica and looked at the impact of silver ion release on the viability of murine macrophages (RAW 264.7). Small silver nanoparticles (< 10 nm) released or leached larger fractions of their mass as ionic silver upon dispersion in water and this strongly influenced the cytotoxicity.

B9. Reproductive system

Tiedemann et al. (2014) assessed the impact of silver (silver nanoparticles and silver nitrate) on porcine gametes. The 11 nm bovine serum albumin coated silver nanoparticles and silver nitrate led to a significant decrease in oocyte maturation or complete arrest of maturation respectively. The silver was found to accumulate mainly in the cumulus cell layer surrounding the oocyte. None of the sperm vitality parameters assessed (motility, membrane integrity and morphology) were significantly affected by silver.

Zhang et al. (2015) exposed male mouse somatic Leydig (TM3) and Sertoli (TM4) cells to two different sizes of silver nanoparticles (10 nm and 20 nm) and examined effects on cell viability, metabolic activity, oxidative stress and apoptosis. TM3 and TM4 cells which had been exposed to silver nanoparticles for 24 hours were then used as feeder cells for spermatogonial stem cells and the impact on gene expression examined. The silver nanoparticles inhibited the viability and proliferation of both TM3 and TM4 cells by damaging cell membranes and inducing the generation of ROS. The 10 nm silver nanoparticles were found to be more cytotoxic than the 20 nm silver nanoparticles. Silver nanoparticle exposure was found to significantly down-regulate the expression of genes related to testosterone synthesis (TM3) and tight junctions (TM4). In addition, exposure of the TM3 and TM4 cells to silver nanoparticles inhibited the proliferation and self-renewal of spermatogonial stem cells.

B 10. Genotoxicity

A total of 19 *in vitro* studies are outlined in Table B1, of these the majority (14) used human cells including stem cells, bronchial epithelial cells, lymphocytes, fibroblasts and keratinocytes. A range of different silver nanoparticles were examined including naked particles and also those stabilized with citrate, bovine serum albumin, PVP, polyethylenimine, polyoxylaurat tween and polyetherimide; 2 studies (Jiang et al., 2013 and Milić et al., 2015) also looked at silver nitrate in parallel to silver nanoparticles.

The comet assay was the most frequently employed test (14 of 19 studies) and, in each case, suggested that silver nanoparticles can cause DNA damage. The lowest concentration at which DNA damage was seen was 0.01 µg/mL following exposure of human bronchial epithelial cells to 59 nm naked silver nanoparticles (Kim HR et al., 2011) and exposure of hamster ovary cells to 40–59 nm naked silver

nanoparticles (Kim HR et al., 2013). As with the *in vivo* results, there is a suggestion that sensitivity to the genotoxic effects of silver nanoparticles is cell specific (e.g. Tomankova et al., 2015). Castiglioni et al. (2014), however, found that the DNA damage in human microvascular endothelial cells exposed to 35 nm naked silver nanoparticles was reversible (when silver nanoparticles were removed from the culture medium) suggesting, in this case, that no permanent modifications occurred. In line with other (non-genotoxicity) studies, cells seem to be more sensitive to smaller silver nanoparticles (e.g. Avalos et al., 2015).

The micronucleus assay was used in 7 of the studies and was found to result in increased micronuclei in six cases. Nymark et al. (2013) reported no increase in micronucleus formation after exposure of human bronchial epithelial cells to 42 nm PVP silver nanoparticles (although positive results were reported for the comet assay). Vecchio et al. (2014) found that silver nanoparticle-induced genotoxicity, measured using the micronucleus assay, was dependent on lymphocyte sub-type and was particularly pronounced in CD2⁺ and CD4⁺ cells.

Only three studies used the chromosome aberration test. Chromosome damage was reported in a single study, where chromatid deletions and exchanges were significantly elevated following exposure of mesenchymal stem cells to 46 nm bovine serum albumin silver nanoparticles (Hackenberg et al., 2011).

One study used a gene mutation assay. Huk et al. (2015) looked at silver nanoparticles, with different surface coatings, using the HPRT gene mutation test in hamster lung fibroblast cells (V79-4). All the tested silver nanoparticles induced HPRT gene mutation, but it was shown that the stabilizing agent could play a significant role in the degree of reported toxicity, with sodium citrate and Tween being found to be mutagenic in their own right.

Table B1: *In vitro* genotoxicity studies

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	MN assay	Chromosome aberration test
Hackenberg et al., 2011	Human	Mesenchymal stem cells	46 nm, bovine serum albumin NP	1, 3, 24 h	0.01–10 µg/mL	A statistically significant dose-dependent increase in DNA damage at ≥ 0.1 µg/mL after 1 h exposure.	-	A significant increase in chromosome damage (mainly chromatid deletions and exchanges) seen at ≥ 0.1 µg/mL.
Kim HR et al., 2011	Human	Bronchial epithelial cells (BEAS-2B)	59 nm, naked NP	24 h	0.01–10 µg/mL	A dose-dependent increase in DNA damage was seen (≥ 0.01 µg/mL).	A dose-dependent increase in micronuclei was observed at ≥ 0.01 µg/mL.	-
Nymark et al., 2013	Human	Bronchial epithelial cells (BEAS-2B)	42.5 nm, PVP NP	4, 24, 48 h	2.5–240 µg/mL	A significant increase in DNA damage was seen at concentrations of ≥ 60.8 µg/mL.	No increase in micronucleus formation was seen even after 48 h exposure.	No increase in chromosome aberrations was observed.
Flower et al., 2012	Human	Peripheral blood cells	40–60 nm, naked NP	5 min, 3 h	50, 100 µg/mL	A significant increase in DNA damage was seen (≥ 50 µg/mL), with effects apparent after 5 min (leading to the suggestion that the damage is caused by the generation of free radicals).	-	-

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	MN assay	Chromosome aberration test
Tavares et al., 2012	Human	Leucocytes	19 nm, (ave) citrate NP	1, 6, 12, 24 h	10, 25, 50 µg/mL	DNA damage varied according to dose and length of exposure. The greatest level of DNA damage was seen at ≤ 6 h. No significant differences were seen at 24 h, suggesting DNA repair.	-	-
Ghosh et al., 2012	Human	Lymphocytes	120 nm (ave), NP	3 h	25, 50, 100, 150, 200 µg/mL	DNA breakage was seen at the lowest concentration administered (25 µg/mL), there was no clear dose-response relationship (with significant responses seen at 25, 50 and 200 µg/mL).	-	-
Vecchio et al., 2014	Human	Lymphocytes	10 and 70 nm, citrate and PVP NP	24, 48, 72 h	0.1, 10, 50 µg/mL	-	Unsorted lymphocytes showed an increase in micronucleus frequency when exposed to 10 µg/mL of the 10 nm citrate, 10 nm PVP and 70 nm citrate AgNP. Different lymphocyte sub-types showed different AgNP sensitivity.	-

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	MN assay	Chromosome aberration test
Ivask et al., 2015	Human	Lymphocytes	18 nm, citrate NP; 28 nm, branched polyethylenimine NP	24 h	0.1–25 µg/mL	-	In primary lymphocytes a significant increase in micronucleus formation was seen following exposure to 12.5 µg/mL citrate NP and 0.8 µg/mL polyethylenimine NP. Both cell lines examined (B- lymphocytes – WIL2-NS & T-lymphocytes – JURKAT) were more susceptible to AgNP, with increased micronucleus formation seen at 3.1 µg/mL citrate NP and 0.4 µg/mL polyethylenimine NP.	-
Kermanizadeh et al., 2013	Human	Renal proximal tubule HK-2	8–47 nm, polyoxylaurat Tween NP	4 h	1.25, 2.5, 5 µg/cm ³	DNA damage was observed (in both the standard and FPG modified comet assay) for all doses assessed.	-	-
Castiglioni et al., 2014	Human	Microvascular endothelial	35 nm, naked NP	24 h	0.05–25 µg/mL	A dose-dependent increase in DNA damage was seen. The damage was reversible on removal of AgNP from culture medium, suggesting that no permanent modifications occur.	-	-

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	MN assay	Chromosome aberration test
Franchi et al., 2015	Human	Fibroblasts	50–82 nm, PVP NP	24 h	0.01, 0.1, 1, 10 µg/mL	A significant increase in DNA damage was seen at the highest concentration tested (10 µg/mL), which was well below the IC ₅₀ value of 42.5 µg/mL.	-	-
Avalos et al., 2015	Human	Fibroblasts (dermal & pulmonary)	4.7 nm, polyetherimide NP; 4.7 nm, PVP NP; 42 nm, naked NP	24 h	0.1–1.6 µg/mL (coated NP), 0.1–6.7 nm (naked NP)	A significant and dose-dependent increase in DNA damage was seen in both fibroblast types to both sizes of AgNP. The fibroblasts were more sensitive to the smaller, coated, AgNP (increased damage at 0.1 µg/mL) compared to the 42 nm naked NP (increased damage at 0.5 µg/mL).	-	-
Tomankova et al., 2015	Human Mouse	Fibroblasts (BJ), Keratinocytes (SVK14) Fibroblasts (NIH3T3)	106 nm, naked NP; 48 nm, naked NP	6 h	1.3–2.3 mg/L (based on individual IC ₅₀ value for each AgNP and cell type)	Both types of AgNP used caused DNA damage in the human fibroblasts and keratinocytes. The mouse cells were less sensitive and there was no significant difference seen from the control.	-	-

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	MN assay	Chromosome aberration test
Szmyd et al., 2013	Human	Keratinocytes	15 nm, PVP NP	24, 48 h	12.5, 25 $\mu\text{g}/\text{mL}$	A significant increase in DNA damage seen following exposure to 25 $\mu\text{g}/\text{mL}$ AgNP. Damage levels were significantly greater after 48 h exposure.	-	-
Jiang et al., 2013	Hamster	Ovary cells (CHO-K1)	15 nm, bovine serum albumin NP; AgNO ₃	24 h	1, 5, 10 $\mu\text{g}/\text{mL}$	-	Concentrations of ≥ 5 $\mu\text{g}/\text{mL}$ significantly increased micronucleus formation above the control. 10 $\mu\text{g}/\text{mL}$ Ag ⁺ resulted in a significantly greater increase (~5-fold cf. control) in micronucleus formation than AgNP (~3 fold cf. control).	-
Kim JS et al., 2013	Hamster	Ovary cells (CHO-K1)	Citrate NP	6, 24 h	0.48–31.25 $\mu\text{g}/\text{mL}$	-	-	No effect seen.
Kim HR et al., 2013	Hamster	Ovary cells (CHO-K1)	40–59 nm naked NP	24 h	0.01, 0.1, 1, 10 $\mu\text{g}/\text{mL}$	Dose-dependent increase in DNA damage seen at ≥ 0.01 $\mu\text{g}/\text{mL}$. 10 $\mu\text{g}/\text{mL}$ AgNP caused an approximately 450% increase in DNA breakage compared to the control.	A significant increase in micronucleus formation was seen at concentrations of ≥ 0.1 $\mu\text{g}/\text{mL}$. Greater micronucleus formation was seen in tests conducted without the presence of cytochalsin B and/or S9.	-

Reference	Animal	Tissue/cell type	Silver type	Exposure duration	Dose	Comet assay	MN assay	Chromosome aberration test
Li X et al., 2013	Hamster	Embryo cells	Naked NP	24 h	10, 20, 40 µg/mL	-	An increase in micronucleus frequency was seen following exposure to ≥ 10 µg/mL AgNP. The lower concentrations increased micronucleus formation in a dose-dependent manner. The increase was not found to be dose-dependent for the highest concentration examined and it is speculated that this might have been due to cell toxicity.	-
Milić et al., 2015	Pig	Porcine kidney (PK15)	61 nm, citrate NP; AgNO ₃	24, 48 h	1–100 mg/L AgNP 1 mg/L Ag ⁺	DNA damage was seen at 1 mg/L Ag ⁺ and at ≥ 10 mg/L AgNP (after 24 h) and ≥ 5 mg/L (after 48 h).	-	-

ave–average; bw–body weight; cf–compared with; FPG–formamidopyrimidine-DNA glycosylase; ip–intraperitoneal; iv–intravenous; NP–nanoparticles

B 11. References

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