## EXECUTIVE SUMMARY

The characteristics and the use of different forms of silver, including silver salts, silver oxides and silver materials appearing as silver wires, silver particles (Ag-NP) and others, were considered in view of potential human and environmental exposure and health effects. In addition, the use of nanosilver as a biocide in consumer and health-care products was reviewed considering current knowledge on bacterial susceptibility and resistance.

### Life cycle

Silver is present in different chemical oxidation states (metallic silver [$Ag^0$] or silver cations [most common $Ag^+$]) in consumer products and in the natural environment. In consumer products silver compounds appear as salts, nano sized (between 1-100 nm) and large particles. These various silver compounds have different physico-chemical properties, such as solubility and surface charge, which may all affect their fate and biological activity.

There are an increasing variety of methods for the production and application of nanosilver. Variety in manufacturing processes may also result in variation of the composition and quality of the Ag forms used in products.

Examples of consumer products that contain nanosilver include food packaging materials, food supplements (not allowed at the moment in the EU unless specifically authorized), textiles, electronics, household appliances, cosmetics, medical devices, water disinfectants, and room sprays. Currently, products that contain nanosilver are difficult to track because products are marketed under numerous brand names, and, with a few exceptions, current labelling regulations do not specifically require listing nanomaterial as a constituent.

Considering the use of various silver compounds (nano and non-nano silver compounds) as biocides in textiles, it is estimated that the global total biocidal silver usage is approximately 0.5% of the total silver use while the use of silver in textiles is estimated as 0.1% of the global total silver use of more than 150,000 tons per year. Of the amount used in textiles, Ag-NP may constitute a fraction of about 10%.

In health-care, silver compounds are used mainly to reduce the risk of infection and re-infection of wounds in antimicrobial dressings. Silver can also be found in dental materials (amalgams, cements) used in dental restoration where silver is thought to have an anti-bacterial activity and reduce caries.

Quantitative data on the life cycle of products containing nanomaterials is generally extremely scarce and currently are based mostly on comparisons with bulk products. When evaluating end-of-life phase of products containing Ag-NP it is assumed that existing waste management options (recycling, wastewater treatment, landfilling, incineration), in an analogous way to conventional products, would be used for nanomaterial products. All silver content in non-recycled waste will ultimately end up in the environment, either as solid waste in landfills; emission from wastewater treatment plants (in effluent water or in sludge); or as residual waste from incineration plants (e.g., fly ash, slag or bottom ash). No actual measurements of incineration of nanosilver-containing products exist but depending on the size of nanomaterial, models predict a release of 25 to 100% of air-borne NPs which are effectively caught by the filter systems. In Europe, the main environmental exposure route of silver compounds in textiles and cosmetics will be through wastewater treatment plants. Silver release from wastewater treatment plants to ground and surface waters is expected to be low; however, silver release at concentrations toxic to some species is possible.

### Consumer and occupational exposure

Consumer exposure depends on the formulation, use and disposal of consumer/medical products containing nanosilver materials. When users come in direct contact with products containing Ag-NP (e.g. liquids, sprays) exposure occurs immediately, while other applications may only release NPs during wear and tear. To determine the level of exposure, more information is needed on the concentrations of Ag-NP in the product, the

size and the form in which it is present (aggregates, agglomerates, coating) and the probability of the release of Ag-NP or Ag ions from the products. It should be noted that part of the human and environmental Ag exposure is due to Ag-ion release from the Ag-NPs. Measurements of nanomaterials in consumer products and their release into the environment are therefore urgently needed. The occupational exposure to Ag-NP and silver has not been studied in full detail. Limited available data suggest that systemic silver exposure in workers employed in industries producing nanosilver is not higher when compared to other occupational settings where exposure to other forms of silver occurs. However, a further detailed evaluation of the occupational exposure is needed in order to perform an occupational risk assessment.

**Toxicokinetics**

Bioavailability of silver after oral administration of Ag-NPs was shown in one rat study; it was suggested that 1-4 % of the oral dose of silver was taken up systemically. Futhermore, after inhalation exposure, silver uptake was shown by the presence of silver in various tissues after exposure. This, however, does not rule out the possibility that the silver was taken up via the gastro-intestinal tract, after been cleared from the lung.
The main target organs for Ag-NP distribution after systemic availability are the spleen, liver and kidney while there is less distribution to other organs. Also in the testes, high levels of silver were sometimes noted. Nevertheless, even with low levels of exposure, Ag distribution was observed in most major organs. Recent data indicate that some persistence of Ag may occur in the brain and testes. For distribution of silver to the brain it is not clear whether the silver is present in the brain tissue or limited to the endothelium of the brain.
There is some evidence that ionic Ag may form silver structures at the nanoscale in vivo. Presence of Ag in feces after intravenous and subcutaneous administrations indicates that biliary excretion of Ag originating from parentally administered Ag-NP.
A limitation of toxicokinetic studies on nanosilver is that most of them used inductively coupled plasma mass spectrometer (ICP-MS) or Atomic absorption spectrometry(AAS) for detection of the Ag, so it cannot be definitively concluded that Ag-NP is distributed to the organs, since all nanoparticles need to be completely dissolved to run the analytical assays. Nevertheless, more detailed studies suggest uptake in cells/organs through a combination of cellular uptake routes like ion transportation and endocytosis of particles. This would give rise to a delivery route for Ag-NP that is different from what is known for dissolved species of silver and will thereby constitute a "nano-specific" exposure.

**Toxicity**

The best-described adverse effect in humans of chronic exposure to silver is a permanent bluish-grey discoloration (argyria or argyrosis) of the skin or eyes. In several animal toxicity studies in clinical chemistry increase of various liver enzymes was observed indicating liver toxicity after a silver nanoparticle administration. However, liver toxicity could not be observed by histopathology.
In vitro studies show that cytokine production in macrophages can be induced by nanosilver, as for many other nanoparticles. In vivo studies could not clearly show whether oral exposure to silver nanoparticles results consistently in alterations of the non-specific immune responses. However, for iv exposure it was shown that the immune system is the most sensitive target for Ag-NP toxicity. Silver containing tooth restorations (also containing non-nanosilver) have been shown to cause a positive contact allergicy reaction (shown by patch testing) in a small number of people.
In vitro, several of the studies reported genotoxic effects of nanosilver. The controversial results may be explained by differences such as in Ag-NP coating, cell type used, the cellular uptake, intracellular dissolution, genotoxicity endpoint, and the way the cells were exposed. For example, pre-dispersion in a medium before cellular exposure may result in initial dissolution of the Ag-NP, so that Ag+ is present from the beginning, contributing to (geno)toxic effect, especially in short-term exposure assays (e.g. for two hours).

As the studies available on the in vivo genotoxicity of Ag-NPs are few and concern Ag-NP of variable characteristics, further studies are required to conclude whether Ag-NP could be genotoxic in vivo. The possibility of secondary genotoxic effects associated with inflammation and oxidative stress induced by silver nanoparticles has not been studied. Such studies might be justified since short-term exposure of mice and rats to several different sizes and doses of Ag-NP through inhalation and intraperitoneal injection was reported to induce oxidative stress and inflammation.
It should be stressed that, in many studies, the release of dissolved silver has been suggested to be the main cause of toxicity (in humans, in the environment and in hygienic applications), but that in spite of this observation an increasing number of studies finds that this release cannot alone account for the toxic effects observed.
Current risk assessments are mainly based on the development of argyria. In workers, the threshold limit value for metallic silver is 0.1 mg/m³ and 0.01 mg/m3 for silver salts. For the general population the World Health Organization (WHO) set a 'No Observable Adverse Effect Level'-(NOAEL) related to the sum of all exposure routes of 5 µg/kg bw (body weight)/d. Recently, for Ag-NP a NOAEL (for rats) was observed, based on a 90 day oral exposure of 30 mg/kg bw/d; this assessment was based on signs of liver toxicity.

**Environmental toxicity**

Nanosilver undergoes several transformations when it is released into the environment. After aggregation and agglomeration, the important ones are dissolution and subsequent speciation, such as formation of silver chloride and silver sulphide. Silver sulphide is particularly important because it is highly stable; sulphide is available in wastewater treatment plants and also in many freshwater bodies. The chemical species that are present determine the bioavailability and toxicity of silver in the environment. A large fraction of the silver released to freshwater bodies sorbs to suspended particulate matter and is transferred to the sediments, where it may be stored; undergo transformations, accumulation, or resuspension depending on physical, chemical, and biological conditions.
Soil conditions are complex and variable; therefore, generic predictions on the environmental fate of silver are extremely difficult to make. Bioavailability of Ag-NP in soils depends on both particle and soil properties. In general, the speciation of silver makes the mobility of ionic silver in soil very limited; however, (nano)particles may behave differently. Although, experiments of AG-NP retained in sewage sludge showed very little leaching of silver (particle and/or ions) to water.
There is much debate in the literature regarding the adverse effects caused by Ag-NP exposure on environmental species. Although it is generally accepted that dissolution of Ag-NPs does account for at least a degree of toxicity observed under Ag-NP exposure, effects cannot always be fully attributed to the measured dissolved fraction of silver. It is now becoming progressively evident that although certain Ag-NPs may show low solubility in certain media and conditions, there may be release of ions, following contact with biological receptors, which can be sustained over a long period. Two important points need to be taken into consideration. Firstly, not all conventional methods used to assess Ag-NP solubility are able to reflect Ag+ availability and, secondly, assessing the dynamic interactions between Ag-NPs and biotic receptors, including the sustained delivery of Ag+ is probably complex and not yet studied.

**Bacterial resistance**

There is evidence of an effect of Ag-NP on the composition of bacterial flora and on the bacterial adaptation associated with certain conditions and uses. Similar to ionic silver, bacterial resistance has been demonstrated for Ag-NP as well. However, evidence is often fragmentary and focused on few specific cases. There is a paucity of information on the resistance mechanisms to Ag-NP. Some of the genetic basis of bacterial resistance to ionic silver has been well documented, notably the expression of well-characterised efflux systems. Recent transcriptomic and proteomic data suggest that a decrease in oxidative

damage by regulation of anaerobic respiration is important. Exposure to ionic silver and Ag-NP produces a stress-response and affects gene expression.
More data is needed to better understand bacterial response to ionic silver and Ag-NP exposure. Regarding the hazard associated with the dissemination of a resistance mechanism following the use of Ag-NP, no documentation is available at this moment, and this in fact represents a serious gap of knowledge.

# 1. BACKGROUND

Silver (Ag) nanomaterials (nanosilver) are widely used today for their antibacterial activity. In medical care nanosilver has been used, for example, as an antibacterial agent in wound dressings (Wijnhoven et al. 2009b) such as bandages to protect patients with severe burns against infections. It has also been used in catheters to prevent the formation of infectious biofilms (Rai et al. 2009, Silver 2006). It can be expected that, with prices of medical applications of nanosilver decreasing, their use will increase. Nanosilver has also been used in consumer products such as sports textiles, other textiles, washing powder and deodorants, where nanosilver should reduce undesired odours.

Recent review papers suggest that at the current level of exposure nanosilver may not be hazardous to humans and may result in low internal exposure (Nowack et al. 2011, Ahamed et al. 2010, Johnston et al. 2010, Christensen et al. 2010). However, data is insufficient to carry out a full risk assessment (Wijnhoven et al. 2009b).

In addition, indirect adverse effects on human health may occur via an increasing resistance of micro-organisms against silver, including nanosilver and silver-based compounds. This may limit the usefulness of nanosilver in medical devices and other medical applications (Landsdown et al. 2007, McDonnell 1999, Khan et al. 2011). Furthermore, silver can be present in different forms (metallic – nanosized or not – and salts), and it is not clear how these different forms of silver influence its antimicrobial properties, a possible increase of antimicrobial resistance (AMR) and the healing process of e.g. burn wounds (Gravante et al. 2009). Recent reviews and publications proposed to use a combination of nanosilver with usual antibiotics for the treatment of specific infectious diseases caused by resistant bacteria (Abeylath et al 2008, Pissuwan et al2010, Bolla et al 2011).

## 2. TERMS OF REFERENCE

The SCENIHR is asked to assess whether the use of nanosilver, in particular in medical care and in consumer products could result in additional risks compared to more traditional uses of silver. Furthermore, the SCENIHR is asked to assess whether the use of nanosilver to control bacterial growth could result in resistance of micro-organisms.

Specifically, the SCENIHR is asked to address the following questions:

1. What may be the implications of the widespread use of nanosilver for human health and the environment? Please consider direct, as well as indirect effects occurring via the distribution into the environment (e.g. from use in appliances, discarding dental material, washing out from textiles, etc.). Does this change the existing assessments for silver in general?

2.Could the widespread use of nanosilver, in particular in medical care and in consumer products, increase the risk of selecting Ag resistant micro-organisms? Could the widespread use of nanosilver create cross-resistance in micro-organisms?

3. To what extent may the widespread use of nanosilver and the possible increase of resistant micro-organisms reduce the nanosilver's efficacy?

4. Are there any other safety, health and environmental effects of nanosilver?

In the assessment, the SCENIHR is asked to consider the following:

Please consider the entire life-cycle of products containing nanosilver (manufacture, use, waste, etc).

To this end, the SCENIHR shall document the mechanisms by which micro-organisms could develop resistance to silver, and detail the circumstances that favour resistance.

While the focus of the mandate should be on nanosilver, please consider and distinguish as appropriate between the different forms of silver (e.g. salts – metallic – ion, wires, nanosized or not, etc.) and identify the forms which exert the effects described in the questions above.

### 2.1. Methodology

This Opinion of SCENIHR is concerned with the analysis of the evidence for the potential for nanosilver to have adverse effects on human health and/or the environment. Recent scientific evidence has been reviewed to determine whether it justifies any reason for concern in regard to health risks associated with the use of nanosilver.

The SCENIHR has considered evidence derived from a wide variety of sources, including peer-reviewed scientific and medical literature and published reports of institutional, professional, governmental and non-governmental organisations. In common with the usual practice of SCENIHR Working Groups, no reliance has been made on unpublished work or publicly available opinions that are not scientifically based.

In a major review of the evidence for or against causation of disease or adverse effects, it is necessary to take into account the generally accepted criteria for causation. SCENIHR has recently published a memorandum on the weight-of-evidence approach to the evaluation of risks and hazards (SCENIHR, 2012). The criteria considered are: (i) the establishment of temporal relationship between exposure and outcome; (ii) the statistical evaluation of an effect; (iii) the evidence of a dose-response relationship; (iv) the plausibility and specificity of any association; and (v) the coherence of any putative association with existing knowledge.

In the weight of evidence approach, lines of evidence or hypothesis for causality are evaluated based on the supportive studies. When a line of evidence is consistently

supported by various studies (i.e. evidence is independently reproduced in different studies) causality is likely between the observed effect and exposure to the substance. Strength and weaknesses of the studies evaluated are considered. The weight of evidence can be categorized as follows:

***Strong overall weight of evidence***: Coherent evidence from human and one or more other lines of evidence (in particular mode/ mechanistic studies) in the absence of conflicting evidence from one of the other lines of evidence (no important data gaps).

***Moderate overall weight of evidence***: good evidence from a primary line of evidence but evidence from several other lines is missing (important data gaps).

***Weak overall weight of evidence***: weak evidence from the primary lines of evidence (severe data gaps).

***Uncertain overall weight of evidence***: due to conflicting information from different lines of evidence that cannot be explained in scientific terms.

***Weighing of evidence not possible.*** No suitable evidence available.

The evidence for the presence of a causal relationship between exposure to nanosilver and adverse effects on health or the environment is discussed in the chapters below.

# 3. SCIENTIFIC RATIONALE

## 3.1. Introduction

Recently, several reviews on human and/or environmental effects of silver nanoparticles and other silver compounds have been published ((Wijnhoven et al. 2009b, Christensen et al. 2010, Stensberg et al. 2011, Schäfer et al. 2013)
This introduction aims at highlighting the most important information to be found in these reviews. In the subsequent sections of the scientific rationale more recent insights and a more detailed overview is given on the most relevant topics mentioned.

Silver and silver products have been known for thousands of years for their prestige (jewellery & tableware) and effect in hygiene (Silver 2006, Silvestry-Rodriguez et al.2007, Wijnhoven et al. 2009b). With the expansion of the photographic industry, the use of silver increased significantly in the 20$^{th}$ century, but with the development of electronic photography the use of silver decreased considerably (Wijnhoven et al. 2009b). However, photographic use of silver still represents 8% of the worldwide silver use (Silver Institute 2012), whereas biocidal silver represents only a very small fraction of worldwise silver use (less than 1%) (Windler et al. 2013).

Nowadays silver is increasingly used in a wide range of applications. For example, in sanitation of drinking water, cooling towers, recreational waters, textiles, plastics, sunscreens and other cosmetics, food and dietary supplements, antimicrobial surfaces and medical applications (Silver 2006; Silvestry-Rodriguez 2007; Maillard and Hartemann 2012). Moreover, because of their unique properties, silver nanoparticles are also used in electronics, optics, biosensing and catalysis (Ahamed et al.2010).

Hand in hand with the growing use of consumer products containing silver, a shift in the human and environmental exposure is expected.

TOXICITY

The best-described adverse effects in humans of chronic exposure to silver are a permanent bluish-gray discoloration (argyria/argyrosis) of the skin or eyes. Besides these discolorations, exposure to soluble silver may produce damage to liver and kidney, irritation of the eyes, skin and respiratory tract. Bio-distribution studies in rats and mice demonstrated that Ag NPs administered by inhalation, ingestion or intra-peritoneal injection were subsequently detected in blood and caused toxicity in several organs including the liver and brain. Moreover, some studies indicated that Ag-NPs exerted developmental and structural malformations in model organisms (Ahamed et al. 2010, Wijnhoven et al. 2009b, Christensenet al. 2010, Stensberg et al. 2011).

*In vitro*, Ag-NPs are toxic to cells derived from skin, liver, lung, brain, vascular system and reproductive organs (somewhat in contrast to the relative low toxicity *in vivo*). Some studies have shown that Ag-NPs have the potential to affect genes associated with cell cycle progression, DNA damage and apoptosis in human cells at non-cytotoxic doses (Ahamed et al. 2010).

ENVIRONMENTAL TOXICITY

The increasing use of Ag-NPs in consumer and medical applications implies that they will find their way into the environment. The activity that makes them desirable as an antimicrobial agent could also pose a threat to the microbial communities in the environment (Sweet et al. 2011). Although the use of silver in photography was found not to affect the performance of the biological wastewater treatment plants (Pavlostathis and Maeng 1998), the use of nanosilver may raise a new concern. Nanosilver may behave differently in the environment and may have a potential for adverse effects on environmental species (Choi and Hu 2009; Stensberg et al. 2011; Sweet et al. 2011; Lowry et al. 2012).

Exposure measurements and models show that environmental concentrations for total silver in different environmental compartments are at the range of ng/L (for water) and mg/kg (for soil and sediments) (Blaser et al. 2008). These levels may cause effects in aquatic organisms, but the current understanding of the hazards of Ag-NPs in aquatic systems is still very limited and it is not possible to draw general conclusions (Fabrega et al. 2011).

BACTERICIDAL ACTIVITY AND RESISTANCE

Ionic silver has been used for centuries as an antimicrobial agent because it has a broad spectrum of activity and forms less toxic by-products compared to, for example, chlorine. In addition, synergistic effects with several other biocides have been described (Silver et al. 2006). Today, there is an increase in the application of Ag-NPs, which have been observed to have a better bactericidal activity (ng/L concentrations) than ionic silver (µg/L concentrations) (Rhim et al. 2006; Lok et val. 2006; Fernandez et al. 2010a,b; Marambio-Jones and Hoek 2010; Su et al. 2011).

The mechanism of lethality of both ionic silver and Ag-NPs involves membrane-disruption, although the exact mechanism of interaction with bacteria is still debated. Silver-ion release from Ag-NPs is considered to play an important role. In addition, the presence of Ag-NPs in bacterial membranes may lead to severe membrane disruption. Oxidative damage may also play a role in the bactericidal activity of Ag-NPs.
As with other biocides, bacterial resistance (as defined in the SCENIHR opinion on Biocides, 2009) to ionic silver and Ag-NPs has been described. A number of mechanisms have been observed: decrease in ionic silver and Ag-NPs accumulation, increased efflux, conversion of ionic silver to its metallic form, and increase in anaerobic respiration in Ag-NPs exposed bacteria. (Costerto et al. 1974; Lok et al. 2006; Gou et al., 2010; Sintubin et al., 2011; Du et al., 2012; Maillard and Hartemann 2012).

### 3.2. Forms and characteristics of silver

Silver in products (consumer, medical and/or technical products) appears in different chemical species. The product can contain (pure) metallic silver ($Ag^0$) or silver ions (most common $Ag^{1+}$, $Ag^{2+}$ or $Ag^{3+}$) in various silver compounds. Nanosilver, indicated in this opinion as Ag-NP, is defined as particulate matter, as aggregate or agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions are in the size range 1 - 100 nm (EU 2011). Those silver materials with a particle size larger than 100 nm are indicated as bulk silver.

Already before the development of modern nanotechnology, a variety of preparations containing nanosilver particles and ionic silver was available on the market as algaecides and antimicrobial treatments. Colloidal silver (a colloid consisting of silver particles suspended in liquid) products remain available in many countries as dietary supplements and homeopathic remedies. For example, the product Silver Algaedyn (registered with the US EPA in 1954) contains silver (0.8% Ag) of a particle size from 20 to 110 nm, that classifies the product as nanosilver. Other products contain micro-sized aggregates of silver nano particles.

Chemical appearance

Silver (Ag, atomic number 47, atomic weight 107.8682 u), in its metallic form, is a ductile material, whose melting point is 961.93°C, boiling point is 2212°C, specific gravity is 10.50 (20°C). The most common oxidation state of silver is $1^+$, however $2^+$ (for example in $AgF_2$), and $3^+$ (in $KAgF_4$) are found as well.

Naturally occurring silver is composed of the two stable isotopes $^{107}Ag$ (51.8% natural abundance) and $^{109}Ag$ (48.2%). In addition, twenty-eight radioisotopes have been

characterized with varying, short half-lives.

Silver and silver compounds (including salts)

Although it is usually found in ores with less rare metals, such as copper, lead, and zinc, silver was apparently discovered in nugget form - called native silver, around 4000 B.C. It is also found in ores containing arsenic, sulphur, antimony and chlorine such as argentite, horn silver, chlorargyrite and pyrargyritein (Helmenstine 2010).

Silver is found as pure metal or silver compounds. On the nanoscale, silver and silver compounds can be produced in various forms, i.e. nanoparticles, nanowires, and quantum dots. In the following paragraphs, the physical and chemical characteristics of bulk and nanosilver are concisely described.

Pure (metallic) bulk silver has a brilliant white metallic lustre and possesses the highest electrical and thermal conductivity together with the lowest contact resistance of all metals. Silver is slightly harder than gold and it is more reactive than gold and platinum. Silver is stable in pure air and water, although it tarnishes upon exposure to ozone, hydrogen sulphide, or air containing sulphur due to the formation of silver sulphide (*CRC* 2000). Silver has a high optical reflectivity when compared to other metals (Edwards & Petersen 1936).

Metallic silver has a face-centred cubic structure (fcc). The packing factor (the volume of atoms in the entire unit cell volume) is 0.74 for fcc crystals. The face-centred cubic structure has atoms located at each of the corners and the centres of all the cubic faces (the image below).

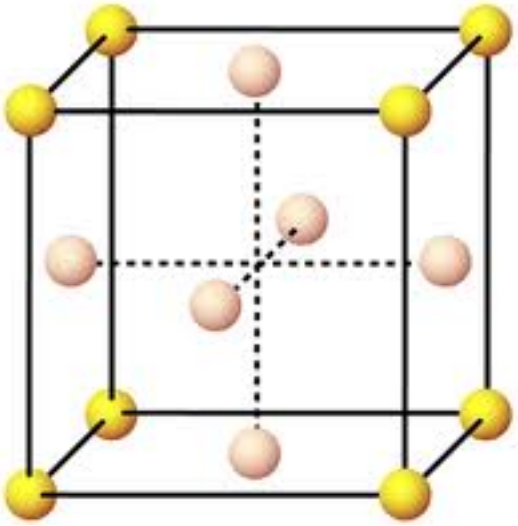

Fig. 1. Unit cell of a face-centred cubic structure (The balls indicate the positions of the silver atoms).

The surface of materials plays an important role in reactivity. Since nanomaterials contain relatively more surface atoms (actually most atoms are on the surface) the surface properties become more influential. The surface offers various adsorption sites for molecules which have different local symmetries and lead to different coordination geometries. Depending upon the site occupied, an adsorbant is likely to be bonded to one, two or four metal atoms. The bonding determines the impact of the nanomaterial on its environment

Solubility of several silver compounds in water under different temperatures at standard atmospheric pressure is given in Table 1. No values were reported for temperatures of 50 and $70^0$C.

**Table 1.** Solubility of several silver compounds in water at different temperatures*.

| Compound | Formula | $0^0$C | $10^0$C | $20^0$C | $30^0$C | $40^0$C | $60^0$C | $80^0$C | $90^0$C | $100^0$C |
|---|---|---|---|---|---|---|---|---|---|---|
| Silver acetate | $AgC_2H_3O_2$ | 0.73 | 0.89 | 1.05 | 1.23 | 1.43 | 1.93 | 2.59 | | |
| Silver Bromate | $AgBrO_3$ | | 0.11 | 0.16 | 0.23 | 0.32 | 0.57 | 0.94 | 1.33 | |
| Silver Chlorate | $AgClO_3$ | | 10.4 | 15.3 | 20.9 | 26.8 | | | | |
| Silver fluoride | AgF | 85.9 | 120 | 172 | 190 | 203 | | | | |
| Silver nitrate | $AgNO_3$ | 122 | 167 | 216 | 265 | 311 | 440 | 585 | 652 | 733 |
| Silver perchlorate | $AgClO_4$ | 455 | 484 | 525 | 594 | 635 | | | | 793 |

*The units of solubility are given in grams per 100 grams of water. Compounds that are soluble only in minute amounts are not shown in the table. [Reference IUPAC-NIST Solubility Database, Version 1.0 NIST Standard Reference Database 106]

Silver is present in different chemical species (metallic silver ($Ag^0$) or silver ions (most common $Ag^+$) in consumer products and in the natural environment. In consumer products silver compounds appear as nano-forms, larger bulk materials and/or colloidal particles. These various silver compounds have different physico-chemical properties, such as solubility and surface charge, which all may affect their fate and biological activity.

## 3.3. Life cycle stages of nanosilver

### 3.3.1. Production

There are indications that nanosilver has always existed in nature while deliberate production of nanosilver has been practised for more than a hundred years. Depending on the production methods used, various sizes and forms of nanosilver may be produced. The main challenge in production of nanosilver, and nanomaterials in general, is the control of their physical properties. One example is the melting temperature, which is size dependent and could be below 200°C for some Ag-NP, while the bulk silver melting temperature is 962°C (Qin et al. 2007).

In general, the production methods of nanoparticles have been classified as top-down and bottom-up. Physical methods such as milling or attrition, repeated quenching and photolithography are usually involved in the top-down strategies while bottom-up techniques start with a silver salt precursor that is reduced in a chemical reaction.

Nanosilver to be used in commercial products is generally produced according to the bottom-up techniques, which can tune particle size and shape as well as functionalize the nanosilver with capping agents that makes it suitable for specific applications.

Synthesis methods can also be grouped under conventional and unconventional methods. Conventional synthesis methods include the use of citrate, borohydride, two-phase (water-organic) systems, organic reducers, and inverse micelles in the synthesis process. Unconventional methods include laser ablation, radiocatalysis, vacuum evaporation of metal, and the Svedberg method of electrocondensation.

The reactants included in the wet chemistry techniques mostly contain a silver salt precursor, a reducing agent, a solvent and a capping agent. The morphology and surface

chemistry of the synthesized silver nanoparticles are governed by the chemical nature of the capping agents, the molar ratio of that agent to the silver salt, the redox potential of the reducing agent, the stirring speed and the temperature of the synthesis reaction (Gulrajani et al. 2008). Of all mentioned parameters, the concentration of the stabilizer has the highest influence. The solvent or the capping agents are occasionally used as reductants for the silver salt precursor (Tolaymat et al. 2010).

A concern with the wet chemical techniques (including colloidal production) in producing Ag-NPs is the accumulation of residual chemicals in the nanoparticles dispersion at the end of the synthesis processes. These impurities may have an impact on their technical performance (e.g. in medicine, catalysis, sensing devices) as well as on potential toxicity. The impurities usually include ionic silver since the reduction efficiency is not 100 % (El Badawy et al. 2010). The presence of impurities disables the capability of determining the actual concentration of metallic nanosilver in dispersion (Tolaymat et al. 2010). In addition, the potential presence of residues of chemical stabilizers, organic solvents, preserving agents, and chemical precursors should be taken into account.

Finally, new physical methods (such as the spark method) allow the production of metallic nanosilver dispersions without chemical contaminants, and directly in matrices/solutions other than water. This field is led by laser ablation enables the generation of liquid-dispersed nanosilver that excels in the quality (e.g. uniformity of size, shape, chemical properties, etc.) and the quantity of the particles produced.

With this advancing variety of methods for the production of nanosilver, its applications are likewise increasing. However, this variety in production methods will also result in a variation of the quality of the Ag-NP used in products.

### 3.3.2. Uses of nanosilver

Examples of consumer products that contain nanosilver include food packaging materials, food supplements (these two applications are not allowed in the EU unless authorized)[1], textiles, electronics, household appliances, cosmetics, medical devices, water disinfectants, and room sprays. Currently, tracking products that contain nanosilver is difficult because the products are packaged under numerous brand names, and, with a few exceptions (Regulation (EC) No 1223/2009 on cosmetic products; Regulation (EU) No 1169/2011 on the provision of food information to consumers; Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food), current labelling regulations do not require that the nanomaterial be listed specifically as an ingredient.

On a quantitative basis, data on the life cycle of products containing nanomaterials is generally extremely scarce. Therefore, life cycle assessment of products with nanomaterials currently relies mostly on comparisons with bulk products and material flow modelling (Asmatulu et al. 2012; Nowack et al. 2012). Hischier and Walser (2012) reviewed the existing literature and found only 17 studies, out of which only two dealt with silver. These two studies considered the life cycle of only a few selected products and in one of them only part of the life cycle was considered. Burkhardt et al.(2011) investigated the use of various silver species as biocides in textiles, in which the main form of silver used is silver chloride. They also provide estimates of the amount of Ag-

[1] In 2008, the *EFSA Panel on Food Additives and Nutrient Sources added to Food* had stated its inability to assess the safety based on the submitted dossier. Accordingly, these products should not be on the market: Inability to assess the safety of a silver hydrosol added for nutritional purposes as a source of silver in food supplements and the bioavailability of silver from this source based on the supporting dossier, Scientific Statement of the Panel on Food Additives and Nutrient Sources added to Food (ANS), adopted on 26 November 2008, The EFSA Journal (2008) 884, 1-3.

NPs used in textiles in relationship to the use of other forms of silver, and other uses of silver in various applications (chemical industry, photography, silverware). Globally, total biocidal silver is approximately 0.5% of the total silver use and the use of silver in textiles is estimated as 0.1% of the global total silver use. Of the amount used in textiles, Ag-NP constitutes a fraction of approximately 10% (Burkhardt et al. 2011).

#### 3.3.2.1. Consumer uses

The market for consumer products containing nanomaterials is rapidly increasing worldwide. According to the EU Commission staff working paper nr 572 (2012) "Any estimates of the market size need to be taken with a certain degree of caution, although the general patterns of the estimates (i.e. order of magnitude of tonnage and market value, and relative size of market between the various materials) seem to be rather reliable. According to market data from SRI consulting, the global quantity of nanomaterials marketed annually is around 11.5 million tonnes, with a market value of roughly 20 bn. The market is dominated by two very widespread commodity materials, i.e. carbon black (9.6 million t), and synthethic amorphous silica (1.5 million t). Other nanomaterials with significant amounts on the market include aluminium oxide (200 000 t), barium titanate (15 000 t), titanium dioxide (10 000 t), cerium oxide (10 000 t), and zinc oxide (8 000 t). Carbon nanotubes and carbon nanofibres are currently marketed at annual quantities of several hundreds of tonnes (other estimates go up to a few thousands of tonnes). Nanosilver is estimated to be marketed in annual quantities of around 20 tonnes. In addition, there is a wide variety of nanomaterials which are either still at the research and development stage, or which are marketed only in small quantities, mostly for technical and biomedical applications."

According to the (US) Woodrow Wilson database , which has been updated very recently (http://www.nanotechproject.org/cpi/, accessed October 29th, 2013), the number of consumer products that are claimed to contain nanomaterials has increased from 54 products in 2005 to 1628 products in 2013. It is expected that this increase has continued until now and will further continue in the near future. There is a small set of materials explicitly referenced in nanotechnology consumer products. Silver is the most common material mentioned in the product descriptions followed by carbon, titanium (including titanium dioxide), silica, zinc (including zinc oxide), and gold.

Consumers may be exposed to nanosilver via products,i.e. in food, consumer products and medical products (http://www.nanotechproject.org/inventories/silver/; accessed September 11, 2013). Non-medical uses include home consumer products and disinfection of water and equipment, which tremendously expand the range of products containing silver and Ag-NPs. During the second half of the 20$^{th}$ century, silver was also used as a disinfectant, especially in conjunction with hydrogen peroxide. The applications of silver and Ag-NPs are listed in Table 2. The efficacy of numerous commercial products for the food industry, private swimming pools, surface and equipment disinfection were based on claims evidencing a synergistic effect. In fact, it has been demonstrated (Hartemann et al. 1995) that silver acts as a catalyst in the Fenton reaction for producing free hydroxyl radicals.

**Table 2** Examples of silver and Ag-NPs applications

| | |
|---|---|
| Healthcare | Wounds dressings, textiles, antiseptics, hospital beds and furniture |
| Home consumer products | Fabric conditioners, baby bottles, food storage containers and salad bowls, kitchen cutting boards, vacuum cleaners, disposable curtains and blinds, tableware, independent Living Aids - bathroom products, furniture (chairs), kitchen gadgets and bath accessories, dishwashers, refrigerators and washing machines, toilet tank levers, sink stoppers, toilet seats, pillows and mattresses, food storage containers, other containers, ice cube trays and other plastic kitchenware, hair and other brushes, hair straighteners, combs, rollers, shower caps.<br>Toothpaste, cosmetic deodorants, toothbrushes, tissue paper, epilators, electric shavers<br>Pet shampoos, feeders and waters, litter pans, pet bedding and shelter, paper, pens and pencils, ATM buttons, remote control, handrails (buses), computer keyboards, hand dryers, wireless voice communicators with badge and the sleeves, yoga mats, coatings for use on laptop computers, calculators, sheet protectors, name badges and holders, shop ticket holders, media storage products, laminating film, report covers and project folders, photo holders, diaries and agendas, office accessories, transparency film, collapsible coolers |
| Clothing and fabrics | Baby clothes, underwear, socks, footwear, various fabrics and vinyls, bath towels, quilts, sleeping bags, bed linens, pillows, quilts, mattress protectors and towels |
| Food | Packaging, nanobiotic poultry production |
| Construction | Powder coating (door knobs), wall paints, air conditioning, epoxy resin floor, PVC wall cladding, antimicrobial flooring, metal suspended ceiling systems, window blinds and shading systems, shelving systems, decorative wood laminates, electrical wiring accessories, notile panels (alternative to standard tiling), hygienic laminated surfaces, wallpaper, borders and murals, carpet and carpet underlay, seals (door for cooler doors and freezer cells, tank lids, mixers and (bread) kneading machines, hospital doors, vibrating screens/vibrosieves in the pharmaceutical industry |
| Disinfectants | Agricultural disinfectants, industrial disinfectants, aquaculture disinfectants, pool disinfectants |

***Food***

Examples of applications of nanosilver in the food chain can be categorised in different stages of food production. Table 3 (previously reported in Bouwmeester et al. (2007)) summarizes applications, which have been identified for the individual stages of the food production chain. The migration limit for silver from packaging products into food is 0.05 mg/kg.

**Table 3** Summary of applications of nano-silver in the food production chain

| Chain phase | Application | Nanotechnology | Function |
|---|---|---|---|
| Processing of food | Food preparation equipment | Incorporated nanosized silver particles | Anti-bacterial coating of food handling devices |
| Conservation | Refrigerators<br>Storage containers | Incorporated nanosized silver particles | Anti-bacterial coating of storage devices |
| | Food products | Nanosized silver sprays | Antibacterial action |
| | Packaging materials | Incorporation of active nanosilver particles | Oxygen scavenging, prevention of growth of bacteria |
| Food consumption | Supplements | Colloidal metal nanoparticles | Claimed to enhance desirable uptake |

For food supplements with nanosized silver, product labels contain statements about the function like "Purifying and conservation of unknown targets", "Supporting the immune system" and "Helpful against severe illness". Since these statements have not been evaluated by, for instance, the European Food Safety Authority (EFSA) and/or the European Medicines Agency (EMA), the claim of certain activities associated with these products (and thus the product itself) cannot be substantiated and may be illegal, especially for health-promoting claims.

***Consumer products***

For consumer products, previously published information of Wijnhoven et al. (2009a; b; 2010) has been used to create a list of products currently on the European market. The list of products has been updated with new products in formerly mentioned sources or new publications/websites. The most important sources for new products are listed in table 4.

**Table 4** Important inventories for consumer products containing nanomaterials

| Databases/ inventories | |
|---|---|
| Project on Emerging Nanotechnologies the Woodrow Wilson International Centre for Scholars | www.nanotechproject.org (Accessed September 11, 2013)* |
| German BUND database (Friends Of The Earth Germany) | www.bund.net (Accessed September 11, 2013) |
| ANEC - European consumer voice in standardisation - nano silver product inventory 2011/2012 | www.anec.org/(Accessed September 11, 2013) |
| Danish product website | http://nano.taenk.dk/(Accessed September 11, 2013) |

* the recent update has not been included

The applications of nano-silver in consumer products can be categorised in the following main categories and subcategories of products. The number of products per product (sub) category (based on sources mentioned above as of August 2012) is summarized in table 5.

**Table 5** Consumer product categories in which nanosilver is applied as well as the number of products in the related product category

| **Product category** | **Number of products ( % of total)** | **Subcategory** | **Number of products** |
|---|---|---|---|
| Appliances | 23 (9.5) | large appliances | 15 |
| | | laundry and clothing care | 8 |
| Home furnishing and household products | 46 (19) | packaging | 4 |
| | | cleaning products | 15 |
| | | coatings | 14 |
| | | cooking | 1 |
| | | paint | 12 |
| Personal care and cosmetics | 68 (28) | hair care | 25 |
| | | skin care | 27 |
| | | oral hygiene | 4 |
| | | baby care | 12 |
| Motor vehicles | 8 (3) | coatings/ cleaning | 8 |
| Textiles and shoes | 71 (29) | coating | 4 |
| | | clothing | 49 |
| | | other textiles | 18 |
| Filtration, purification neutralisation and sanitisation | 10 (4) | water filtration and purification | 6 |
| | | air filtration and purification | 4 |
| Health care | 11 (4,5) | wound dressings | 4 |
| | | other health care products | 7 |
| Electronics and computers | 1 (0.5) | computer hardware | 1 |
| Miscellaneous | 3 (12) | miscellaneous | 3 |
| Toys and games | 2 (0.8) | toys | 2 |

The consumer product market is changing very rapidly, especially that of cosmetic products. Therefore, it should be noted that this list of consumer products claiming to contain nanosilver gives an overview at a given moment (August 2012) and therefore may not be complete.

The overview gives a clear indication of the kind of products in which nanosilver is mostly used in the European market. The product categories "Personal care and cosmetics" as well as "Textiles and Shoes" are the main consumer product categories in this inventory.

Similar ratios of nanosilver containing products within different product categories have been found in a recent inventory of ANEC/BEUC (accessible via http://www.beuc.org/content/Default.asp?PageID=2142; accessed September 11, 2013). In the Danish inventory, the majority of registered products with nanosilver are hair care products, such as hair dryers and straighteners (irons) (38/108) and food supplements (13/108). In addition, various textile products (20/108) have been reported. These results are in line with the other inventories analysed.

### Textiles

Because of its antibacterial properties nanosilver is widely used in medical and functional textiles, such as anti-bacterial fabrics which claim to prevent infection or deodorise. (Mantovani and Zappelli 2009). Also, the use of nanosilver in more common textiles like household cleaning textiles, sportswear, gloves, socks, underwear and anti-odour clothes have been reported.

### Paints/ coatings / pigments

Various paint products with nanosilver have been reported, and Mueller and Nowack (2008) assumed that paints are amongst the most important sources for Ag nanoparticles that are released in the environment (when compared to other sources like textiles, cosmetics, cleaning agents, metal products and plastics). Kaegi et al. (2010) have provided direct evidence for the release of nanosilver in the environment, as they demonstrated leaching of Ag nanoparticles from exterior paints into the aquatic environment. About 30% of the Ag-NPs initially contained in the paint were lost within one year of exposure. However, Ag-NPs from paints are most likely not released as individual nanoparticles.

Regarding optical properties, the embedding of nano-silver and nanoparticles from other metals in transparent materials can be tuned to create optical filters that work on the basis of nanoparticle absorption. Due to metallic surface plasmons, nano-silver absorbs light at a characteristic wavelength, 435 nm purple, which leads to a yellow colour (Fang 1998). This was first applied in the colouring of glassware hundreds of years ago. Without knowing the reasons, people grinded silver and gold to the nano-scale to give church windows a permanent, non-fading yellow and red colour.

Currently conductive nano-inks are established as another application of nanosilver with high filling degrees used to print highly precise continual conductive paths on polymers.

In the EU, no nanomaterials are admitted for use in plastics that come into contact with food (European Commission 2011), unless authorized. In the US, in contrast, the use of nanosilver in food containers is possible (EPA 2010, von Goetz et al. 2013), but has to be registered.

### Biocidal products

Nanosilver is used for its biocidal properties during production to treat consumer articles before their use, which may result in residual presence of nanosilver on the surface of these products. In addition, nanosilver is sometimes intentionally included in consumer products for preventing bacterial contamination during their use. According to Hong et al. (2013), nanosilver is also considered as a potential additive to animal feed.

In Europe, the placing on the market of biocidal products is currently regulated by the Biocides Directive 98/8/EC, which is replaced by the Biocides Regulation (EC) No. 528/2012 by September 2013. Silver-containing active substances (SCAS) were identified and therefore included in the second phase of the review programme for biocidal active substances (Reg. (EC) No. 1451/2007), and are currently evaluated within the European Union for use in disinfectants and as preservatives.

Following inclusion of the SCAS in Annex I of 98/8/EC (or approval according to Biocidal Products Regulation-BPR), corresponding nanosilver biocidal products must be authorised in Member States. During evaluation, already registered products may remain on the national markets.

**Miscellaneous use**

In addition to the use of manufactured nanosilver, it should be noted that nanosilver may result from processes in which other forms of silver were used, i.e. in photography.

A typical photographic film contains tiny crystals of very slightly soluble silver halide salts such as silver bromide (AgBr) commonly referred to as "grains". The grains are suspended in a gelatine matrix and the resulting gelatine dispersion, traditionally referred to as an "emulsion," is melted and applied as a thin coating on a polymer base or, as in older applications, on a glass plate. [Mees&James, 1966.]

The free silver produced during light exposure is referred to as the "latent image". The grains containing the free silver in the form of a neutral tetramer ($Ag_4^o$) are readily reduced by chemicals referred to as "developers" forming deposit of free silver producing dark areas. All photographic emulsions contain grains of different sizes.

#### 3.3.2.2. Health-care uses

Silver has been used for centuries mainly for the disinfection of water until the 17th century when silver was described as an essential multipurpose medicinal product (Silver et al. 2006), (Edwards-Jones 2009). In the late 19th century one-percent silver nitrate solution was introduced as eye drops in newborns, and in 1901 it was used for internal antisepsis (Russell and Hugo 1994). In the 1920s the use of charged ionic silver solutions (i.e. electro-colloidals) as antibacterial agents was approved by the U.S. Food and Drug Administration (FDA). The *British National Formulary* (2012) lists the use of silver nitrate (40-95%) for external use on warts, verruca, umbilical granulomas, over-granulating tissue, cauterization and silver sulphadiazine (1%) for the 'prophylaxis and treatment of burn wounds, as an adjunct to short-term treatment of infection in leg ulcers and pressure sores, adjunct to prophylaxis of infection in skin graft donor sites and extensive abrasions' (BNF 2011).

**Current use of silver compounds**

Maillard and Hartemann (2012) recently reviewed the antimicrobial activity of silver and Ag-NPs, their toxicity and applications. Ag-NPs are mainly used in consumer and personal care products. They concluded that the wide use of silver and AgNPs (at a low concentration) in applications such as fabrics, textiles and other surfaces may appear controversial and will remain controversial as long as the benefits have not been addressed, measured and justified appropriately.

The use of silver for healthcare applications (wounds dressings, antiseptics, hospital beds and furniture) has been briefly reviewed by Edwards-Jones (2009) and its use in pharmaceutical applications is listed in Table 6.

**Table 6** List of preparations using silver according to the Martindale (2002).

| | |
|---|---|
| Silver acetate, silver borate, silver allantoinate, silver zinc allantoinate, silver carbonate, silver chloride, silver chromate, silver glycerolate, colloidal silver iodide, silver lactate, silver manganite, silver nylon polymers | Antiseptic (similar use to silver nitrate) |
| Silver nitrate (1%) | Prophylaxis of gonococcal ophthalmia neonatorum (neonatal conjunctivitis) |
| Silver protein<br>Colloidal silver | Antisepsis; eye drops and mucous membrane |
| Silver sulphadiazine (1%) | Prevention and treatment of infection in severe burns<br>Eye treatment of *Aspergillus* infections |

Nowadays, the use of ionic silver in catheters is still a matter of debate (Leoneet al.2004; Edwards-Jones 2009). Ionic silver and Ag-NPs are now used in a number of dressings (Silver et al. 2006; Toy and Macera 2011; Rigo et al. 2013). The antimicrobial activity of ionic silver, sustainability and decreased interference with dressings, decreased toxicity, and increased wound healing and fluid handling can be improved with combination with appropriate polymers (Maillard and Denyer 2006a,b). Kostenko et al. (2010) and Toy and Macera (2011) review a number of commercially available ionic silver and Ag-NPs dressings.

More recently, an international consensus on the appropriate use of silver dressing in wounds was published (International Consensus 2012) Here it was calculated that 0,0008 % of the global annual silver production is used in wound dressings; they recommend that the roles of antimicrobial dressings including silver dressings should be used to "reduce bioburden in acute or chronic wounds that are infected or are being prevented from healing by microorganisms" and as "an antimicrobial barrier for acute and/or chronic wounds at high risk for infection and re-infection." "Silver dressing should be reserved for use in wounds with or at risk of high bioburden or local infection." Examples of these types of wounds are burns, traumatic wounds and diabetic ulcers (Table 6) (Maillard and Hartemann 2012).

## Silver in dental products

Silver is used in dentistry with silver amalgams (Silver 2003) and precious alloys because of its physical properties.

Silver filling materials were historically used as a root canal obturation material (silver points); however, modern techniques and improved materials provide the clinician with much better options. Silver points have been shown to corrode spontaneously in the presence of serum and blood due to an unstable electrochemical behavior. Corrosion byproducts can also cause irreversible staining of the tooth structure and surrounding tissues (argyrosis). Silver points lack plasticity, and the consequent failure to flow and conform to the shape of the root canal system makes them less favorable as filling materials.

It has been suggested that nanosilver be incorporated as an antimicrobial in many dental materials, such as polymeric filling materials, cements, denture base materials, artificial teeth etc. One recent study on antibacterial activity found that no inhibitory activity was observed for two endodontic materials, after incorporation of nanosilver in a dental filling, whereas a third product showed enhanced inhibitory activity following the addition of Ag-NPs (Rodrigues Magalhães et al. 2012).It is not clear if the inhibitory activity is due solely to the Ag-NPs.

Silver particles were also added to experimental dental composites to reduce caries (Durner et al. 2011). The aim of the study was to show whether silver nanoparticles can lead to higher amounts of elutable substances. Compared to controls more camphorquinone (CQ), ethoxylated bisphenol-A-dimethacrylate (BisEMA) and triethylene glycol dimethacrylate (TEGDMA) were eluted when silver nanoparticles were added to the composite. Silver nanoparticles may influence the polymerization process in dental materials and lead to an increase in elutable substances.

A recent review (Peng et al. 2012) examined the therapeutic use of silver compounds in dentistry and the outcomes from clinical trials as well as mode of action and biocompatibility. *In vitro*, *in vivo* and clinical evidence has demonstrated that silver compounds are viable agents for preventing and arresting caries both in the primary and permanent dentition; however they are associated with adverse tooth discoloration and some report that pulp irritation may occur. The mode of action of silver compounds on carious tooth tissues is thought to include inhibition of the demineralization process as well as anti-bacterial activity. The authors of this review concluded that silver compounds have been shown to be an effective anti-caries agent; however, there is an incomplete understanding of how silver compounds prevent caries.

**Miscellaneous use**

On the internet, medical devices (claimed) to contain nanosilver can be found. However, no scientific papers about this kind of applications were found in the literature. Therefore, such applications are not considered for this Opinion.

Disinfection of rooms, medical equipment and ambulances by aerosolisation of hydrogen peroxide (5%) with silver-ion (dry mist) has been developed using the experience acquired with anthrax decontamination with a satisfying activity (Andersen et al. 2006; Shapey et al. 2008). However, until now nano-silver has not been used for this application and the safety of this kind of disinfection for the users is currently under review.

## Conclusions

Silver compounds have been used for centuries in health care products as an antiseptic.

Currently, the use of silver in consumer products – including nanosilver - is increasing (http://www.nanotechproject.org/cpi/Accessed October 29th, 2013). Most applications claim to have hygienic and/or disinfecting properties on the skin, hair, surfaces, equipment and water; others refer the capacity to reduce odours from clothing. It was estimated by Burkhardt et al.(2011) that globally the total biocidal silver is approximately 0.5% of the total silver use.

In textiles, the use of silver is estimated as 0.1% of the global total silver use. Of the silver used in textiles the Ag-NP constitutes only a fraction of approximately 10% (Burkhardt et al. 2011).

In health-care, the use of silver compounds is seen as an antimicrobial barrier to reduce the risk for infection and re-infection of wounds. Silver can also be found in dental materials (amalgams, cements) used in dental restorations. Here, silver is thought to reduce caries due to its anti-bacterial activity.

### 3.3.3. Waste handling of nanosilver containing products

Given that there are no provisions for the sorting of products containing nanomaterials, it is assumed that existing waste-handling infrastructures would be used for nanomaterial products in an analogous way as conventional products (Asmatulu et al. 2012). Quantitative data for the end-of-life phase of products containing nanomaterials is, however, very limited with only two studies identified to date (Burkhardt et al. 2011; Hischier and Walser 2012).

Due to the extremely low data availability, any information addressing waste handling must be considered preliminary and should be used accordingly with great caution. Despite this, thanks to extensive research and particularly modelling activity in Switzerland, the limited knowledge on nanosilver is among the best of all nanomaterials (see Burkhardt et al. 2011; Nowack et al. 2012 and references therein). These two studies provide a comprehensive review of existing knowledge on Ag-NP release from textiles and end-of-life assessment.

Based on the number of consumer products listed in the Woodrow Wilson database alone, Asmatulu et al. (2012) considered recyclability the largest potential of end-of-life fate. This is, however, not well substantiated and should rather be regarded as speculative. Nanomaterials can either be recycled by melting or by more advanced technologies for separation and purification (Asmatulu et al. 2012; Walser et al. 2012). Burkhardt et al. (2011) reviewed the available techniques that may be used to identify textiles containing silver and to recover the silver from textiles at the end of their use phase. They concluded that the reliable detection of silver in commercial textiles is technically challenging and that it is unlikely that recovery of silver from textiles will be economically feasible.

All non-recycled waste will end up in the environment. One of the major ways of disposal are landfills, which are regulated by Council Directive 99/31/EC (http://ec.europa.eu/environment/waste/landfill_index.htm, accessed February 5, 2013). This directive aims at preventing and reducing any adverse effects of landfills on the environment, with some exceptions for islands and isolated settlements. Briefly, it implies that any waste must not be deposited untreated. Treatments include separation technologies, incineration and sewage treatment plants.

Some of the waste (apart from recycled materials) is subjected to incineration. Nothing is known on actual incineration measurements of nanosilver-containing products, yet a number of extrapolations can be made from an important study on CeO2 NP in a state-of-the-art waste incineration plant (Walser et al. 2012). In the worst-case scenario they found that the final release of CeO2 NP via air was negligible, due to effective capture in the filter systems Depending on the size nanomaterial, models predict a release of 25 to 100% of air-borne NPs which are effectively caught by the filter systems (Hischier and Walser 2012). Nothing is known on actual incineration measurements of nanosilver-containing products, yet a number of extrapolations can be made from an important study on $CeO_2$ NP in a state-of-the-art waste incineration plant (Walser et al. 2012). In the worst-case scenario they found that (1) the final release of $CeO_2$ NP via air was negligible, due to effective capture in the filter systems, (2) between 53% and 81% of the NP were found in the slag. In both situations the particles were loosely bound to the surface of other burned waste but otherwise they appeared unmodified. Since both filters and slag need to be disposed of, the authors concluded that incineration plants are not an effective end-of-life treatment. Importantly, they also found that the processes in an incineration plant cannot sufficiently be predicted by a small-scale experiment. The temperature in the incinerator furnaces varies between 800-1300°C. One has to keep in mind, however, that the melting temperature of some Ag-NP is only 200°C while bulk silver melts at 962°C. It has to be noted that slag and filters will, next to landfills, also end up in construction materials.

A part of silver in consumer products, in particular cosmetics, textiles and food, will end up in wastewater treatment plants. Here, the vast majority will effectively bind to solid matter and be converted to $Ag_2S$. Yet it has to be kept in mind that Ag-NP or the resulting products such as $Ag_2S$ will undergo various transformation processes in natural environments, such as stabilisation in dispersions, formation of bound residues or release of silver ions (Nowack et al. 2012, and references therein).

Silver release from wastewater treatment plants to ground and surface waters is expected to be low; yet silver release at concentrations toxic to some species is still possible. (Nowack et al. 2012, and references therein; http://www.umsicht.uni-bremen.de/index%20engl.htm; Accessed September 11, 2013).

Following treatment in wastewater treatment plants, the resulting sludge in many European countries will end up in arable soils (Nowack et al. 2012, and references therein). Due to the above-mentioned transformation processes, a distinction of Ag-NP and other forms of silver at this stage is impossible. At current levels of use of silver in consumer products, silver concentrations in wastewater sludge are unlikely to pose any risk to soil organisms. However, it has to be kept in mind that repeated sludge applications will result in silver accumulation which may reach toxic levels. This is currently studied by the UMSICHT project (http://www.umsicht.uni-bremen.de/index%20engl.htm; accessed September 11, 2013).

### 3.3.4. Conclusions

There is a multitude of synthesis methods for nanosilver that can be grouped under conventional and unconventional methods. Conventional synthesis methods include the use of citrate, borohydride, two-phase (water-organic) systems, organic reducers, and inverse micelles in the synthesis process. Unconventional methods include laser ablation, radiocatalysis, vacuum evaporation of metal, and the Svedberg method of electrocondensation. With this advancing variety of methods for the production of nanosilver, its applications are likewise increasing. However, this variety in production methods will also result in a variation of the quality of the Ag-NP used in products.

Examples of consumer products that contain nanosilver include food packaging materials and food supplements (neither of which is allowed in the EU unless authorized), textiles, electronics, household appliances, cosmetics, medical devices, water disinfectants, and room sprays. In addition, there is the professional use of silver and nanosilver in dentistry, for wound dressings and general hygiene. The main indication for the use of (nano)silver is its antibacterial or biocidal activity, which is why silver compounds have been used for centuries in health care products as an antiseptic.

At the end of the life cycle, products become waste material. Quantitative data for the end-of-life phase of products containing Ag-NP is very limited and it needs further research. It is assumed that existing waste handling infrastructures would be used for nanomaterial products in an analogous way as conventional products.

All non-recycled waste will end up in the environment. Some of this waste is subjected to incineration. Nothing is known on actual incineration measurements of nanosilver-containing products but depending on the nanomaterial, models predict a release of 25 to 100% of air-borne NPs which are effectively caught by the filter systems.

Silver compounds in textiles and cosmetics will end up in wastewater treatment plants. Silver release from wastewater treatment plants to ground and surface waters is expected to be low, but a certain fraction of silver will pass treatment plants and reach surface water bodies. Concentrations will be low and toxic effects to aquatic organisms are unlikely, but cannot be excluded.

## 3.4. Human health

### 3.4.1. Exposure

***Consumer exposure assessment***

To assess (consumer) exposure to nanomaterials in consumer products, the identification of the consumer products containing nanomaterials is only a first step. Next, the products that claim to contain nanomaterials have to be investigated further on the actual presence of nanomaterials, as was done in a former pilot study (Oomen et al. 2010, see below). Various characteristics of the nanomaterials in products have to be analyzed in detail, like chemical entity, shape of the nanomaterial and concentration. However, it is also important to know the product form and whether the particles are free or fixed, and consequently if they can be released during use. These and other exposure characteristics, previously identified by Wijnhoven et al. (2009a) and described in table 7 below, are essential for a robust exposure assessment as they determine the potential human exposure to the nanomaterial contained in the product.

**Table 7** Important characteristics for exposure assessment (adopted form Wijnhoven et al. 2009a)

| **Important characteristics for the exposure assessment** | | Comments |
|---|---|---|
| **Nanomaterial in consumer product** | Chemical entity of nanomaterial | Actual composition of material |
| | Shape of nanomaterial (in product) | Thin films and coatings<br>Composite<br>Solid particle<br>Hollow particle<br>Other particle<br>Aggregates<br>Agglomerates |
| | Product form | Spray<br>Powder<br>Liquid<br>Dispersion<br>Solid/ coating |
| | Free / fixed | Free particles<br>Fixed inside matrix |
| | Concentration | Mostly unknown (based on mass?) |
| **Application** | Direct/ indirect exposure | Direct exposure to nanomaterials in the product or indirect via release of particles out of the product |
| | Indoor/ outdoor use | Inside or outside a small space |
| | Event duration | < 5 min<br>5 min-1 hr<br>1 hr - 1 day |

| | | |
|---|---|---|
| | Frequency of events | > 1x/ day<br>1x/ day- 1x/ week<br>1x/ week - 1x/ month<br>1x/ month - 1 x /year |
| | Number of users in population | <10%<br>10-50%<br>50-90%<br>> 90% |
| **Exposure route** | | inhalation<br>dermal<br>oral<br>combination |

The characteristic of main importance is the way the nanomaterials are incorporated into the product (e.g. free nanoparticles or nanomaterials integrated into larger scale structures or fixed in a matrix) in combination with the application of the product (with either direct or indirect human exposure, via release of particles out of the product). With respect to the location of nanomaterials in the product, Hansen et al. (2007) have proposed a general framework for categories of all nanomaterials to aid hazard identification of these materials (based on the location of the nanomaterials in the system/ material). According to Hansen et al. (2007) nanomaterials can be grouped into three main categories, two of which deal with nanostructures on surfaces and in the matrix of a product, whereas the third category deals with the various uses of nanoparticles. This third category is the most relevant for consumer products.

When the combination between location and application of the product is made, products containing free nanoparticles with direct human exposure (e.g. food supplements or sunscreen products) are considered to have a high potential exposure. Conversely, products in which nanomaterials are integrated into larger scale materials with indirect human exposure (e.g. food storage bags or computers) are considered to have a low potential exposure. High potential exposure means that there may either be a high probability of exposure, or a probability of high exposure, or both. It is stressed that the qualification of 'high' and 'low' potential exposure should be interpreted in relative, but not absolute terms. Furthermore, the route of exposure is also very important for the ultimate internal exposure to nano-silver. Inhalation exposure via sprays or oral exposure of food supplements is considered to have the highest risk. In table 8 below, the potential exposure to nanosilver is indicated for various applications.

**Table 8** Ranking of the potential human exposure to nano-silver

| **Category** | **Sub category** | **Exposure route** | **Potential Exposure*** |
|---|---|---|---|
| Food and beverages | Cleaning | Inhalation/ dermal | High |
| | Cooking utensils, coatings | Dermal | Low |
| | Storage | Dermal | Low |
| | Supplements | Oral | High |
| Personal care and cosmetics | Skin care | Dermal | High |
| | Oral hygiene | Oral | High |
| | Cleaning | Dermal | High |
| | Hair care | Dermal | Low? |
| | Baby care | Dermal | High? |
| | Wound dressings | Dermal | High |
| | Over the counter products | Dermal? | High? |
| Textile and shoes | Clothing | Dermal | ? |
| | Other textiles | Dermal | ? |
| | Toys | Dermal/ Oral | ? |

| Category | Sub category | Exposure route | Potential Exposure* |
|---|---|---|---|
| Electronics | Personal care | Dermal | Low |
| | Household appliances | Dermal | Low |
| | Computer hardware | Dermal | Low |
| | Mobile devices | Dermal | Low |
| Household products/ Home improvement | Cleaning | Inhalation/ dermal | High |
| | Coating | Dermal | High ?? |
| | Furnishing | Dermal | Low |
| | Furnishing/ coating | Dermal | Low |
| Filtration, purification, neutralization, sanitization | Filtration | Inhalation | ? |
| | Cleaning | Inhalation/ dermal | High |

* "High" indicates either a high probability of exposure, or a possibility of high exposure, or both.

"Low" indicates either a low probability of exposure, or a possibility of low exposure, or both.

"?"indicates that there is no sufficient information available.

The fact that a product category is ranked with either a high or low potential exposure in above-mentioned table should not be seen as evidence for absolute high exposures or the lack thereof, but as an indication of potentially high exposures (Wijnhoven et al. 2009a; Dekkers et al. 2007). These rankings are based on the current knowledge and may need modifications when new information becomes available. Another point of interest for the future may be the cumulative exposure via various Ag-NP and Ag containing products; currently no information on this is available.

In the paper of Hansen et al. (2008), the grouping of consumer products according to location of the nanomaterial, has led to the identification of three exposure categories;

1. *Expected to cause exposure*; humans come in direct contact with these products. 'Nanoparticles in liquids' and 'airborne particles' are in this exposure category.

2. *May cause exposure*; although the nanoparticles in the products are not meant to be released, a certain amount of wear and tear must be anticipated. 'Surface bound nanoparticles' are in this category.

3. *No expected exposure to the consumer*; expected negligible exposure because the nanoparticles are encapsulated in the product. 'Nanoparticles suspended in solids' are in this category.

In conclusion, to determine the risk for exposure, more information is needed on the concentrations of nano-Ag in the product, the size and the form in which it is present (aggregates, agglomerates) and the probability of release of (nano) $Ag^+$ from the products. Measurements of nanomaterials in consumer products and possible release in the actual conditions of usage are therefore urgently needed.

*Measurements of nanosilver in consumer products*

In the past few years, more publications on the actual measurement of nanosilver in consumer products have become available. In the study of Oomen et al. (2011), 8 different consumer products were screened for the presence of nanosilver. The products were a food container, socks, indoor wall paint, a cuddly toy, a T-shirt, wound dressing, a toothbrush and a deodorant. Only in the socks and the wound dressing could the

presence of nanosilver be demonstrated. However, it was impossible to be conclusive on the absence of nanomaterials in the other products, since only a small part of the product could be analysed and, furthermore, the techniques used in this pilot study were not validated for consumer products.

On the basis of the exposure potential described above, consumer products were categorised in three different groups.

1. Spray type of products (expected to cause exposure)

Nazarenko (2011) analyzed the presence of nanosilver in several spray type products using TEM, ICP-MS, SMPS, and APS. However, no nanosilver particles were detected after spraying even though the product (liquid) itself contained the nanosilver particles. Most probably, aggregation of nanoparticles occurs in the air during or after spraying. Quadros and Marr (2011) studied three different products with silver i.e. an anti-odour spray for hunters, a surface disinfectant and a throat spray and concluded that the products emitted 0.24-56 ng of silver in aerosols per spray action. They conclude that normal use of silver-containing spray products carries the potential for inhalation of silver-containing aerosols. However, it is difficult to determine the size of the silver (nano)particles within the sprayed aerosols. It was suggested that nasal cavity or upper respiratory tract would be the target organ for the exposure via inhalation.

2. Liquid type of products (expected to cause exposure)

For this category, one publication reports the potential presence of nanosilver in consumer products as toothpaste, shampoo and detergents (Benn et al. 2010). In the products, the total amount of silver is determined by nitric acid digestion. With the use of a 100 nm filter, it was concluded that the majority of the silver released by these products in water, is released as particles, or is associated with particles, larger than 100 nm (Benn et al. 2010).

3. Solid matrix of products (nanomaterials at the surface)

*Textiles*

The Danish EPA analysed various textile products containing (nano-)silver available on the Danish market. In total, 16 products were analysed using ICP-MS and ICP-AES and 15 out of 16 products contained silver. Twelve products containing silver were analysed further for the presence of nanoparticles using scanning elctron microscopy (SEM) and energy dispersive X-ray (EDX) spectroscopy. In 4 out of 12 products, silver particles (>200 nm, 2 products) or threads (structure like particles, 2 products) were detected. In terms of EU definition of nanomaterials, the silver particles or threads detected would not be considered as nanomaterials. Migration of silver from products to artificial saliva, sweat or waste water after washing varied significantly between various fabrics (0.02~84%). They concluded that despite the potential for migration or leaching of silver out of the products, there is little risk of biological effects in the environment resulting from the use of nanosilver in textiles (Tonning et al. 2012).

Also in the studies of Benn et al. (2008, 2010), silver nanoparticles were released into water from 11 of 16 consumer products such as (sock)fabrics, toothpaste, shampoo, detergents, etc. when analysed using SEM with EDX and ICP-OES. In contrast to the silver particles released from liquid products as mentioned above, the majority of silver released from the fabrics passed through the 20-nm filter. This could be nanoparticles in addition to dissolved silver ions (Benn et al. 2010).

Finally, Lorenz et al. (2012) investigated another eight different commercially available silver-textiles during a washing and rinsing cycle. Three of the textiles contained nanosized silver (as labelled or confirmed by the manufacturer), another used a metallic silver wire and four contained silver in undeclared form. The total silver content ranged from 1.5 to 2925 mg Ag/kg. Only four of the investigated textiles leached detectable amounts of silver, of which 34-80% was in the form of particles larger than 450 nm. This is in line with the study of Geranio et al. (2009) in which a predominant fraction of

released particles > 450 nm was also found after washing of various silver nanotextiles (Geranio et al. 2009).

In addition, The Dutch Institute for Food Safety (RIKILT, Wageningen, Netherlands) published a report in 2011 (in Dutch) on washing out Ag from textiles. They reported levels of silver of 1.8–520 mg/kg in seven different textiles. The silver is present in agglomerates of primary nanoparticles of 50-300 nm. Most frequently, silver particles are agglomerates applied as coating on the textile fibres. In one product, the total surface of the textile fibres is coated with silver nanoparticles. This product contains the highest amount of silver. The four fabrics that have undergone a washing test all released silver, in the form of silver ions, silver particles and agglomerated silver particles. The amount of released silver varies between products, depending of the amount of silver present in the product and the level of silver bound to the textile fibres. Three products underwent 10 washings and released 11, 44 and 100% of the silver respectively from the fabrics. (Peters et al. 2011)

Recently, a study has been published by Von Götz et al (2013a), in which dermal exposure to (nano)Ag has been modelled based on migration data of Ag nanoparticles from textiles into artificial sweat (Von Götz et al. 2013a). The exposure assessment was based on data from a T-shirt and trousers, because no migration from dissolved and particulate silver (<450 nm) has been found from the socks investigated in this study. The conclusion from this study is that in contrast to total silver, dermal exposure to nanosilver particles, agglomerates and aggregates from functionilized textiles remains a noteworthy potential pathway when compared to other exposure routes.

*Silver in miscellaneous contact materials*

Apart from textile products, Nanosilver is found in (solid) food storage containers (currently not allowed in the EU). A recent paper by Von Götz et al. (2013b) showed that 2 out 4 food containers actually contain silver in an amount of 10-37 mg Ag/kg. Migration experiments show that one of the products has a maximal migration of 30 ng Ag/cm$^2$. This amount decreased in additional use cycles resulting in a maximum cumulative release of 34 ng Ag/cm² after 3 use cycles.
A worst-case exposure of 4.2 µg Ag can result from storage of food in a new Ag-doped box of normal size (calculated for 30 ng/cm$^2$ migration from a 140 cm$^2$ surface).This was considered by the authors to be a low amount, when compared to the general background exposure to silver (drinking water contains 0.1-9 µg Ag/L and also food contains trace amounts of Ag). The contribution of Ag-NPs in this exposure is unknown.

In another paper by Echegoyen and Nerín (2013), the results of migration studies (with different simulant solutions and times) in three commercial nanosilver plastic food containers were shown in order to gain more insight regarding the release of nanosilver into food. Migration solutions were evaluated by ICP–MS and SEM–EDX analysis and silver in dissolved form and silver as nanoparticles were analyzed. Silver migration was observed for all samples studied, with the total silver migration values ranging between 1.66 and 31.46 ng/cm2 (lower than the permissible limits). Size and morphology of the silver nanoparticles changed for the different samples (ranging between 10 and 60 nm) and migration of other nanosized materials was also confirmed.

*Products for children*

Finally, a study on the release of ionic and particulate silver from nanotechnology-based consumer products for children has been published (Quadros et al, 2013). During realistic use of a number of consumer products (plush toy, fabric products, breast milk storage bags, cleaning products, humidifiers and accessories), measurements were taken of the release of Ag into water, orange juice, milk formula, synthetic saliva, sweat, urine, into air and onto dermal wipes. The main conclusions of this study are:

- aerosol concentrations of Ag were not significantly elevated during product use
- fabrics, a plush toy, and cleaning products were most likely to release silver
- silver leached mainly via dissolution and was facilitated in media with high salt concentrations
- levels of silver to which children may potentially be exposed during the normal use of these products is predicted to be low, and bioavailable silver is expected to be in ionic rather than particulate form.

In the same report, Quadros et al. (2013) tried to put the leakage of silver into perspective. They compared the amount of silver released from products to the results of a study rats by Kim et al (2007), on the toxicity of nanosilver in rats after oral administration. They calculated a equivalent dose of 1230 mg/day for "slight" liver damage in a 10 kg child for short-term exposure. The amount of silver leached per kilogram of each product that was studied was at most 18.5 mg Ag/kg product. They concluded that therefore the quantity of silver to which children would maximally be exposed is lower than the calculated equivalent dose.
In conclusion, the reported levels of silver release from silver-containing consumer products in different studies appear to be inconsistent, which probably is due to differences in methodology used. Moreover, the absence of silver may be also related to the fact that silver was only claimed to be present, but was not actually present in the products.

***Occupational exposure***

Several occupational exposure limits and guidelines exist for silver, but the values for each depend on the form of silver as well as the individual agency making the recommendations (Drake and Hazelwood 2005). For instance, the American Conference of Governmental Industrial Hygienists (ACGIH) has established separate threshold limit values for metallic silver (0.1 mg/$m^3$) and soluble compounds of silver (0.01 mg/$m^3$). On the other hand, the permissible exposure limit (PEL) recommended by the Occupational Safety and Health Administration (OSHA) and the Mine Safety and Health Administration (MSHA) and the recommended exposure limit set by the National Institute for Occupational Safety and Health (NIOSH) is 0.01 mg/$m^3$ for all forms of silver.

With the use of nanosilver in an increasing number of applications, new or additional occupational exposure can be expected. In principle, this includes exposure towards non-nanoscaled and nano-scaled silver used and generated during production respectively, exposures to the nanoform during packaging, mixing & loading of the material during manufacture of treated articles as well as their handling and packaging. Application by, for example, spraying or roll-on application of nanosilver-containing products (e.g. paints) and secondary tasks including cleaning etc. provides another source of exposure as does manipulation and handling of treated materials, e.g. cutting and sewing nanosilver treated textiles. Finally, indirect exposures can occur for consumers from treated surfaces and articles. Consequently, dermal and inhalative exposures are expected for workers by a variety of pathways.

Lee et al. (2011) reported the results from workplace air monitoring in nanosilver production facilities. The silver metal concentrations ranged from 0.00002 to 0.00118 mg/$m^3$, depending on the type of workplace, the operation state and the manufacturing method. When silver nanoparticles were generated using induced coupled plasma, particle numbers reached 535-25,022 per $cm^3$ at the workplace outside the reactor, but the range of particle sizes was wide, presumably as the result of agglomeration or aggregation. When a wet method was used for manufacturing, particle numbers in the workplace air were significantly lower with 393 to 3526 particles per $cm^3$.

Inhalation exposures during production, post-production handling and processing in a test facility were reported by Demou et al. (2008) for microcomposite powder in which Ag-NP were sintered onto silica microparticles. Monitoring of particle numbers using SMPS in the size range from 6 to 673 nm showed an increase from a background of approx. 9,000 per $cm^3$ to a maximum of 50,000 per $cm^3$ during production and a maximum of 15,000 per $cm^3$ during processing and handling. These results are difficult

to interpret, especially as measurements were limited to particle size and numbers but did not include determination of silver content/concentration. Nevertheless, the data was interpreted in order to indicate a potential for breakaway of Ag-NP from the microcomposite (US EPA Decision Document AGS-20).

The levels and particle size distributions of dust produced in dental laboratories were measured after cutting, grinding and polishing silver-containing alloys, gypsum, porcelain and denture base materials, by means of light- and electron-microscopy as well as Coulter-counting techniques. The measurements revealed a high proportion of respirable dust (particles smaller than 5 micrometers) associated with these operations (Brune and Beltesbrekke 1980; Brune et al. 1980)

In breathing air close to the workplace, the mercury and silver contents exceeded the threshold limit values (0.01 mg/m³ TWA, time weighted average or 0.03 mg/m³ STEL short-term exposure limit according to ACGIH[2]) for short-term exposure by factors of 60 to 400 in cases when local ventilation was not in use. With efficient local exhaust systems enabling a dust reduction of about 94%, the short-term exposure limit values for mercury and silver were exceeded by factors of about 4 and 20 respectively. Mercury and silver were assayed quantitatively by means of nuclear chemical analysis. A major part of the amalgam dust consisted of respirable particles. The collected dust comprised about 80% amalgam and 20% particulate matter from grinding equipment (wheels and stones) according to scanning electron microscopy and energy-dispersive X-ray spectroscopy (EDX) measurements (Brune & Beltesbrekke, 1979). It has to be remarked that published information about occupational exposure to silver in dentistry is confounded by the concomitant exposure to mercury "as in silver amalgam". No recent information about silver exposure alone could be identified.

Information from health surveillance was published for two male individuals with a 7-year history of work in nanosilver manufacturing. In individual no. 1 an exposure of 0.35µg/m³ was estimated accompanied with a level of 0.34 µg/L in blood and 0.43 µg/L in urine. In individual no. 2 the estimated exposure was 1.35 µg/m³ accompanied with 0.30 µg/L in blood and not detectable (< 0.1 µg/L) in urine. Parameters from clinical chemistry and haematology were reported to be within the normal range (Lee et al. 2012).In another study, blood silver levels were not detectable (<0.1 µg/L) in 11 of 15 and 0.2 µg/L in the remaining 4 of 15 workers of an unexposed control group, while ranging from 0.1 to 20 µg/L in a group of 98 silver exposed workers (Armitage et al. 1996).

#### 3.4.1.1 Conclusions

The exposure to any substance including nanomaterials depends on the amount present in a product and the possibility for release or leakage out of the product. To estimate exposure levels, information is needed on the concentrations of Ag-NPs in the product, the size and the form in which it is present (aggregates, agglomerates) and the probability of release of (nano) $Ag^+$ from the product. The availability of these data is limited for the moment: reported levels of silver released from silver-containing consumer products in different studies are inconsistent, which is probably due to differences in methodology used. Measurements of nanomaterials in consumer products are therefore urgently needed.
In order to evaluate human exposure to Ag-NP, a simple categorization of exposure possibilities to Ag-NPs in products as proposed by Hansen et al. (2008) may be helpful. The categorization includes: *Expected to cause exposure*, *May cause exposure*, *and Not expected exposure to the consumer.* The type of product (spray, liquid, solid) largely

2

American Conference of Governmental Industrial Hygienists

determines the likelihood for exposure. However, reported levels of silver released from silver-containing consumer products in different studies are inconsistent, which is probably due to differences in methodology used.
Occupational exposure to silver and silver particles –mainly via airborne material – has not been studied in full detail. A further detailed description of the occupational exposure is needed in order to perform an occupational risk assessment.

### 3.4.2. Toxicokinetics of silver

General

The health risk from applications of nanosilver is obviously related to the potential exposure of individuals to the nanomaterial. If there is no absorption, the potential risks from external uses are limited to possible local effects at the site of exposure, mainly lung, GI-tract, and skin. However, data on absorption and internal systemic exposure of nanomaterials is limited and may vary depending on physicochemical properties of the individual particles and the local barriers present in different organs. For the skin, the uptake of nanomaterials is generally considered to be very low to absent. Systemic availability following oral and inhalative exposure is also generally considered to be lower for nanomaterials than for molecules. Limited absorption of nanomaterials complicates studies of organ distribution and elimination – other aspects of toxicokinetics.

Systemic (IV) administration

The blood clearance and tissue distribution of three different sizes (20 nm, 80 nm, and 110 nm) of citrate buffer-stabilized spherical nanosilver was investigated in rats by Lankveld et al. (2010). After single and repeated IV administration of 1 ml/animal (~25 µg/mL, approx. 0.1 mg/kg bw/d) for all three nanosilver sizes (measured as Ag by ICP-MS) a rapid blood clearance was observed during the first 10 minutes after administration. After a single administration, the liver and spleen were the two major organs for Ag distribution. A gradual increase was observed after the repeated (5 times) administrations, and on day 6, one day after the last administration, Ag was demonstrated in all investigated organs including lungs, liver, spleen, kidneys, heart, brain, and testes. The highest levels were observed in liver and spleen with lower levels in lung and kidneys. The 20 nm Ag-NP were mainly distributed in the liver, followed by the kidneys and spleen, whereas the larger 80 nm and 110 nm Ag-NP were mainly distributed in the spleen followed by the liver and lungs. Additionally, low levels of Ag could be detected in the blood. The 20 nm Ag-NP showed in general a lower organ distribution than the 80 and 110 nm Ag-NP, but a higher level in the kidneys. Therefore, this could imply that some excretion occurred for the 20 nm Ag-NP or significant distribution occurred in organs that were not examined in the study. From day 6 to day 17 there was a gradual decrease in organ Ag levels for all three sizes of NPs, although on day 17, Ag was still detectable at low levels in the kidneys and brain, and at considerable levels in the liver, spleen and lungs (Lankveld et al. 2010).

Park et al. (2011) evaluated the bioavailability and tissue distribution of 7.9 nm Ag-NP (citrate coated) after a single IV administration of 1 and 10 mg/kg bw in rats until 96 h after administration using ICP-MS for Ag detection. Tissue distribution was only determined in the lungs, liver and kidneys. In all three organs Ag could be detected up to 96 h after administration with the highest levels present in the liver. In the liver and lungs, a gradual decrease in Ag content was noted, whereas in the kidneys the Ag level remained similar between 24 h and 96 h (Park et al. 2011). The concentration of Ag was much higher in the feces compared to the urine, indicating predominant excretion via the bile.

A similar study using intravenous administration was performed by Xue et al. (2012) in mice with silver nanoparticles of 12-20 nm (average 15nm, coating not stated) at considerably higher doses of 7.5, 30 and 120 mg/kg bw. This study also found the

highest levels of Ag (ICP-MS measurement) in the spleen followed by the liver, lungs and kidneys, whereas distribution to other organs (brain, heart, muscle) was relatively low. A gender-related difference in biokinetics was suggested since in male mice the half life time in blood ($T_{1/2}$) was 15.6 h compared to 29.9 h in female mice. So, there appeared to be a faster blood clearance in male mice. After a single administration of Ag-NP there was a gradual decline in Ag content in various organs, although Ag was still detected on day 14 after administration with the highest Ag level in the spleen. In the spleen, there was only a limited decline in Ag content that was mainly observed in male mice. In females, for the lungs and kidneys, significant higher levels were observed on day 14 compared to male mice (Xue et al. 2012).

After a single intravenous administration of doses of 0.5 and 5 mg/kg Ag-NP (average by TEM 7.9 nm, citrate coated) in rabbits, serum kinetics and tissue distribution were followed for 28 days by Lee et al. (2012). The silver content in organs was determined by ICP-MS. A rapid decline in blood silver concentrations during the first 24 hours was followed by a slower elimination with $t_{1/2}$ of 12 and 16 days at high and low dose, respectively. Depending on the dose administered, Ag could still be detected in the blood on day 28 after injection, although the results indicated that 90% of the initial Ag-NPs in serum were eliminated. Silver accumulation was noted in all organs investigated: liver, kidneys, spleen, lung, brain, testes, and thymus. Regarding the high dose, the highest Ag level was observed in the liver followed by the spleen for all days evaluated (days 1, 7 and 28). Lower levels were observed in the kidneys/testes, lungs, thymus and brain. Regarding excretion, more silver seemed to be excreted via the feces (17 and 8 % of dose by day 7 at low and high dose, respectively) than via the urine (less than 0.05 % of dose by day 7) indicating biliary excretion of Ag and/or Ag-NP (Lee et al. 2012). The extent of excretion indicates that more than 80 % of the dose was still within the organism after one week.

Inhalation

Two repeated dose inhalation toxicity studies over 28 and 90 days respectively were published that included investigations into systemic uptake from the lung (Ji et al. 2007, Sung et al. 2009). Silver nanoparticles were generated by evaporation/condensation of Ag source material using a small ceramic heater, which produced particles in the size range of 2 – 65 nm. The exposure doses for the 28 d study were: low, (0.48 µg/$m^3$), medium (3.48 µg/$m^3$), and high (61.24 µg/$m^3$), with the silver nanoparticles having a geometric mean diameter (GMD) size of 11.9 nm, 12.4 nm, and 14.7 nm, respectively. For assessment of tissue distribution the organ silver content was determined by AAS. After 28 days of inhalation, a significant dose-dependent increase of Ag content was observed in the lung, with an indication for reduced clearance at the top dose (0.06 mg/m³). Similarly, dose dependent increases in Ag content were observed for the liver, olfactory bulb and brain. Both for male and female animals, the silver distribution was in the same order of magnitude (Ji et al. 2007). In a 90-day follow up study with 19 nm Ag-NP and higher concentrations of 0.049, 0.133 and 0.515 mg/$m^3$ Ag could be demonstrated in the liver, kidneys, olfactory bulb, brain and blood in addition to the lung, confirming uptake of Ag as either Ag-NP or Ag ions (Sung et al. 2008). The presence of Ag in the blood after inhalation might be explained by translocation via three possible routes, being ingestion and uptake in the GI-tract (after removal from the lung by the mucociliary escalator), via the drianing lymph nodes, or via translocation into the blood via alveolar epithelial cells (Oberdörster 1988). Besides uptake via the blood, presence in brain might be explained by uptake via the nasal epithelium and the olfactory nerve (Oberdörster et al. 2004). Some differences were observed between male and female rats, with female rats having a higher Ag content in the kidneys compared to the male rats (Sung et al. 2009).

Spark generated Ag-NP with a size of 4-10 nm were used in an inhalation study in rats by Takenaka et al. (2001). After 6 hours of inhalation exposure for 1 day and a concentration of 0.133 mg/$m^3$, tissue levels were followed by ICP-MS measurement of Ag up to 7 days. Directly after exposure and on day 1 after exposure, the highest Ag level

was observed in the lungs whereas in the liver, spleen, brain and heart, low concentrations of Ag were measured indicating systemic availability of the Ag. Lung burden decreased dramatically in time and on day 7 only 4% of the initial burden remained in the lung.

A 14-day inhalation study (exposure 5 days a week) was performed in mice using 10 (range 5-15 nm) nm Ag-NP, which were administered via nebulizing a Ag-NP dispersion in an inhalation chamber with an average concentration of 3.3 mg/$m^3$ (range 2.4-4.0 mg/$m^3$) (Stebounova et al. 2011). Directly after exposure Ag could only be detected in the lung tissue itself, whereas levels in the heart, liver, and brain were below detection limit. The amount in the lung was approximately 4% of the nominal Ag dose administered, which was calculated to be 803 µg/g lung. The amount of Ag measured in the lung decreased when measured at 3 weeks after exposure, at which time two of five mice had no detectable Ag in the lung.

Oral

Many studies have addressed the fate of Ag-NP after oral administration (Kim 2008; Tang 2009; Jeong 2010; Kim 2010; Par 2010; Loeschner 2011; Van der Zande 2012; Hadrup 2012; Lee et al., 2013).

In a 28-day oral repeated dose toxicity study Kim et al. (2008) investigated Ag-NP (60 nm) in rats. Ag content in organs was determined by AAS. A dose dependent accumulation of Ag content was observed in all tissues examined (blood, brain, kidney, liver, lung, stomach and testes). The highest levels were observed in the stomach followed by the liver, kidneys and lungs. The spleen was not investigated. In the kidneys a gender related accumulation was noted with a twofold higher Ag content in female rats when compared to male rats, while in other organs the Ag content was similar. In an additional study it was demonstrated that the higher Ag content in the female kidneys was present in all regions of the kidney including cortex, outer medulla and inner medulla. Most of the Ag appeared to be located in the basement membranes (Kim et al. 2009). Histology after a 28-day oral repeated dosing revealed the presence of luminal and surface particles, and silver nanoparticles in the tissue of the gastro-intestinal tract (Jeong et al. 2010).

In a 90-day oral repeated dose toxicity study with 56 nm Ag-NP of the same manufacturer, similar results were obtained as in the 28-day oral repeated dose toxicity study with the detection of Ag in all organs investigated and a gender difference in kidney accumulation of the Ag (Kim et al. 2010).

Loeschner et al. (2011) evaluated the tissue distribution of Ag by ICP-MS in a 28-day oral repeated dose study in rats. The organ distribution pattern for Ag-NP (size 14 nm for about 90% of the particles, and 50 nm for about 10% of the particles) and silver acetate (AgAc) was similar, although the absolute silver content after Ag-NP was lower. The Ag-NPs were stabilized in a polyvinylpyrrolidone (PVP) aqueous dispersion. Approximately 11% of the silver was reported to be present in a "non-nanoparticulate" form, possibly as silver ions. In addition to the GI-tract the highest silver levels were noted in kidneys followed by the liver, and low levels in the lungs and brain were observed for both Ag-NP and AgAC. A fraction (< 0.1 % of dose) of the daily dose was excreted via the urine within 24 h and the majority, respectively 63% and 49% via the feces (Loeschner et al. 2011).

In addition to the IV administration (see above) Park et al. (2011) also investigated the fate of orally administered Ag-NP (7.9 nm) in rats. Similar to the IV administered Ag-NP Ag was observed in the liver, lungs and kidneys but at much lower levels. The majority of the Ag remained present in the feces. A comparison of the AUC (area under the curve, blood conc.) of 5792 and 27570 vs. 70 and 1166 min*µg/ml for IV vs. oral administration at low and high dose respectively, would suggest an oral bioavailability of Ag from the tested Ag-NP in the range of 1-4 %.

Van De Zande et al.(2012) also compared Ag-NP with an ionic Ag (silver nitrate, in a 28 day oral repeated dose toxicity study in rats. Ag-NP of <15nm (core size) coated with PVP (PVP-Ag-NP) administered at a dose of 90 mg/kg bw, were compared to non-coated Ag-NP size <20 nm (90 mg/kg bw) and ionic silver ($AgNO_3$, 9 mg/kg bw). Both silver particle preparations contained approximately7 % of the silver in its ionic form but PVP-Ag-NP degraded further (up to 45% within 7 days). Ag detection was performed with single particle ICP-MS (SP-ICP-MS). The highest silver blood values were obtained using $AgNO_3$ with 7-10 fold lower relative levels after Ag-NP or PVP-Ag-NP administration when normalized to dose. At one week after the last treatment, Ag content in the blood was reduced to a non-detectable level. Similar results were obtained for the feces. When results were normalised against the daily silver dose administered, the highest levels in organs were observed one day (day 29) after the $AgNO_3$ treatment, with much lower levels after Ag-NP and PVP coated Ag-NP treatment.

This difference practically disappeared for most organs except the spleen and testis when organ content was normalized against the content of ionic silver in the Ag-NP preparations. There was no difference between the levels and organ distribution for the PVP coated and noncoated Ag-NP. The highest organ levels were noted in the GI-tract and much lower levels in the internal organs. For the organs, similar levels were observed for the liver, spleen and testes, while lower levels were present in the kidneys, brain and lungs. In wash out groups, the reduction for Ag content was followed up for 84 days after the start of the experiment. For all three types of Ag ($AgNO_3$, PVP Ag-NP, and noncoated Ag-NP), a decrease in silver content was demonstrated by AAS in most organs examined on day 36, while complete clearance was approached on day 84. However, in some organs, Ag could still be detected on day 84 (brain, testes, kidneys, and spleen). Remarkably, using SP-ICP-MS silver nanoparticles could be observed in various organs (liver, spleen); Ag-NP were not only detected in Ag-NP exposed rats, but also in $AgNO_3$ exposed rats, demonstrating the formation of nanoparticles from $Ag^+$*in vivo* that are probably composed of silver salts (Van De Zande et al. 2012).

Lee et al. (2013) investigated potential persistence of Ag in organs after a 28-day repeated oral exposure of Sprague-Dawley rats to 10 nm and 25 nm Ag-NP. A low (100 mg/kg) and high (500 mg/kg) dose was investigated. Ag distribution was determined directly after the oral administrations on day 28 and after a recovery period of 1, 2 and 4 months. On day 28 Ag could be detected in the blood and all organs investigated (liver, kidneys, spleen, testes, and brain). In the recovery period of 1, 2 and 4 months after the Ag administrations, no Ag was detected in the blood. Expressed as ng/g tissue, highest Ag levels were present in the spleen and ovaries followed by the kidneys and liver. Lowest levels were noted for the brain and testes. For the liver, spleen and kidneys in female rats, higher levels were present compared to male rats. In most tissues evaluated the Ag content gradually decreased with time. However, for the brain and testes the Ag clearance was less when compared with the other organs. In all organs at month 4 Ag could be detected, especially in the high dose groups. The size of the Ag-NPs did not affect the clearance from the various organs. Blood biochemistry revealed increases in liver enzymes indicating some liver toxicity, but most of the hepatotoxicity markers returned to normal after 2 months recovery.

When mice were treated for 14 days by oral administration of Ag-NP (22 nm, 42 nm, and 71 nm) the Ag-NP were distributed to organs including the brain, lungs, liver, kidneys and testes, while large Ag-particles (323 nm) were not detected in those tissues (Park et al. 2010). The highest level was noted for the 22 nm Ag-NP in the kidneys followed by the testes and liver, while Ag levels in the lungs and brain were in the same order of magnitude as the liver. Regarding the 42 nm, Ag-NP highest levels were observed in the testes and lungs followed by the brain, whereas for the 71 Ag-NP, highest levels were observed in the liver while much lower levels were observed in the kidney and brain. In the non-treated control group and the animals treated with Ag-NP of 323 nm, no Ag could be detected in the various organs. Ag was determined by ICP-MS (Park et al. 2010).

Other exposure routes

Tang et al. (2009) evaluated the tissue distribution of Ag-NP (50-100 nm) and silver microparticles (2-20 µm) after subcutaneous (SC) injection in rats. Ag content was determined by ICP-MS. Silver was demonstrated in the blood, brain, liver, spleen, lungs, and kidneys up to 24 weeks after SC injection. Translocation was higher for the Ag-NP compared to the silver microparticles; 0.15% versus 0.02% respectively of the injected dose. The highest distribution was observed in the liver followed by the kidneys, spleen and brain/lungs. The relative high presence of Ag in the feces compared to the urine indicated more biliary excretion than urinary excretion. No significant Ag levels were observed in the heart, uterus, ovaries adrenal gland and femur. TEM identified particles in the lumen of blood vessels and various tissues (kidney, liver, spleen, brain, lung) suggesting the diffusion of Ag-NPs. However, in addition to the particles, the presence of Ag itself was not further identified (i.e by energy dispersive X-ray (EDX) spectroscopy) in the observed structures. The presence of these structures in the brain neurons does warrant further research in this area.

In view of its application as an antimicrobial agent in wound dressings, the possibility for skin penetration of silver during the use of silver containing wound dressings was investigated (Trop et al. 2006, Vlachou et al. 2007, Larese et al. 2009). Vlachou et al. (2007) evaluated the use of Acticoat™ wound dressings containing silver-ion releasing coatings on patients with relatively small burns using ICP-MS for Ag detection. Serum levels of Ag were observed ranging from 0 to 53.1 µg/L with a mean of 10.9 µg/L. indicating some uptake via the wounds. In all patients investigated on day 3 and day 6 of the treatment, some Ag was detected in the blood serum with a range of 1.1 - 226.0 µg/L. In a case report, using another type of coating, Trop et al. (2006) described one patient treated for burn wounds with elevated Ag content in blood and urine after the use of a silver-containing wound dressing, suggesting systemic availability and urinary excretion of the Ag originating from the wound dressing.

Experimental data on intact and damaged skin *in vitro* using the Franz diffusion method showed that silver nanoparticle absorption was very low but detectable (Larese et al. 2009). The experiment was performed with full thickness human skin obtained as surgical waste using electro-thermal AAS for Ag determination. Silver nanoparticles were observed by TEM in the stratum corneum of the skin.

Conclusions on toxicokinetics

Bioavailability of silver after oral administration of Ag-NPs in rats was shown and in one study it was calculated to be in the range of 1-4 % of the oral dose. Additionally, after inhalation exposure, silver uptake was demonstrated in view of the presence of silver in the lung and in various tissues as well. However after inhalation, depending on the particle dimensions, uptake via the GI- tract cannot be excluded. Uptake of silver from wound dressing over burned skin results in significant serum levels, while uptake over intact skin could not be quantified.

The main target organs for Ag-NP distribution after systemic availability are the spleen, liver and kidneys, whereas there is less distribution to other organs. Furthermore, high levels of silver were sometimes noted in the testes. Nonetheless, albeit with low levels, Ag distribution was demonstrated in most major organs including the brain. For distribution of silver to the brain it is not clear whether the silver is present in the brain tissue or limited to the endothelium of the brain. Recent data indicates that some persistence of Ag may occur in the brain and testes, although its significance for toxicity is not known. There is some evidence that ionic Ag may form Ag particles at the nanoscale *in vivo*. Sex differences (females having higher Ag levels) were observed in several papers investigating different routes of administration i.e inhalation and oral administration. In view of the presence of Ag in feces after IV and SC administrations, there are indications of biliary excretion of Ag originating from administered Ag-NP.

A limitation of toxicokinetic studies of nanosilver is that most studies used ICP-MS or AAS for detection of the Ag, so it cannot be definitively concluded that Ag-NP are distributed to the organs.

### 3.4.3. Hazard

General toxicity

A critical review of studies examining exposure to the various forms of silver was conducted to determine if some silver species are more toxic than others (Drake and Hazelwood 2005). The adverse effects of chronic human exposure to silver are a permanent bluish-gray discoloration (argyria or argyrosis) of the skin or eyes. Most studies discuss cases of argyria and argyrosis that have resulted primarily from exposure to the soluble forms of silver. Besides argyria, exposure to soluble silver compounds may produce other toxic effects, including liver and kidney damage, irritation of the eyes, skin, respiratory, and intestinal tract, and changes in blood cells.

Silver may present a toxic hazard to exposed workers. To define the potential risks of long-term exposure to silver a cross-sectional investigation was conducted of 27 Caucasian males occupationally exposed to primarily insoluble silver compounds and 27 controls (Pifer et al. 1989). Physical examination and electron microscopy of skin biopsies revealed no cases of generalized argyria. Measurements of facial discoloration, judged from colour photographs by panels of laymen and physicians, showed no significant difference between the two groups. Although 29% of the silver workers and none of the controls exhibited ocular silver deposition, optometric and contrast sensitivity tests revealed no significant deficits in visual performance. The kidney and respiratory findings were normal (confounded by smoking and alcohol consumption) in both populations. Despite the increased presence of silver in the blood, feces, and hair of the recovery workers versus the controls, there was no evidence that chronic silver exposure adversely affected the health of these employees.

Ionic silver (silver sulphadiazine) has a long history as an antimicrobial agent used in human health care. Walker and Parsons (2012) reviewed the published literature on how ionic silver may enter the body from exposure to silver-containing wound care products and its eventual metabolic fates in an assessment of the safety during normal use of these products in wound care. Following the application to breached skin there appears to be little evidence of localised or systemic toxicity.

Intraorally, exposure to silver-containing particles generated by polishing dental amalgam fillings may give rise to "amalgam tattoos" of the oral mucosa (ICD-9 Code Diagnosis 729.6 Amalgam tattoo).

Amalgam tattoo is an iatrogenic lesion caused by traumatic implantation of dental amalgam into soft tissue and is the most common localized pigmented lesion in the mouth. In a study of a mass screening oral examination in the United States, it was found in about 0.4-0.9% of the adult population and in Sweden in about 8%. Clinically, amalgam tattoo presents as a dark grey or blue, flat macule located adjacent to a restored tooth. Most are located on the gingiva and alveolar mucosa followed by the buccal mucosa and the floor of the mouth. Microscopic examination reveals that amalgam is present in the tissues in two forms: as irregular dark, solid fragments of metal or as numerous, discrete fine, brown or black granules dispersed along collagen bundles and around small blood vessels and nerves. In most lesions, it is present in both forms. The biologic response to the amalgam is related to particle size, quantity and elemental composition of the amalgam. Large fragments often become surrounded by dense fibrous connective tissue. Smaller particles are associated with mild to moderate chronic inflammatory response with individual macrophages engulfing small amalgam particles. Occasionally, the reaction takes the form of a foreign body granuloma in which macrophages and multinucleated giant cells are present. Some of the multinucleated

giant cells also contain amalgam particles. Diagnosis of amalgam tattoo is usually obvious from the location and clinical appearance (Buchner 2004).

Pigmented areas of the oral mucosa can also be associated with silver-containing corrosion products of dental alloys used for prosthetic restorations (Vencklikova et al. 2011). Silver-containing electron-dense particles (Ag-EDPs) are frequently found in pigmented areas. Eight patients with diagnosed signs of localized argyria were investigated in this study. Biopsies from distinctly pigmented gingival areas were subjected to histological examination, electron microscopy and x-ray microanalysis. Elemental composition of Ag-EDPs determined by x-ray microanalysis showed mainly silver in combination with sulphur or selenium or a combination of both chalcogens.

In vitro toxicity / cytotoxicity

The cytotoxicity of Ag-NPs varies between studies. Both high cytotoxicity (2-5 µg/mL; Hussain et al. 2005; Soto et al. 2007, Park et al. 2011a) as well as almost no cytotoxicity (up to 100 µg/mL) have been reported (Samberg et al. 2012). The apparent conflicting results on the cytotoxicity of Ag-NPs may partly reflect differences among the studies in the type of Ag-NPs tested (primary particle size, coating, surface charge, solubility), cell type (uptake, sensitivity), and the technique used for exposing the cells such as dispersion method (dispersing agent, sonication, agglomeration, etc.) and exposure duration, resulting in variable exposure to Ag ions and/or Ag-NPs as well as their characteristics that may be important for toxicity (Ellegaard-Jensen et al. 2012; Nymark et al. 2013).

Park et al.(2011a) showed a dose-dependent inhibition of the development of contracting cardiomyocytes for three different sizes of nanosilver (20 nm, 80 nm, and 113 nm diameter) in an embryonic stem cell assay. The 20 nm silver nanoparticles were the most potent in this assay, but they were not as potent as ionic silver. The effects on stem cell differentiation were observed at doses lower than those associated with cytotoxicity as determined by a decrease in metabolic activity.

Immune responses and allergies

*In vitro* nanosilver induced the generation of various inflammatory markers in the macrophage cell line RAW 264.7 (Park et al. 2011).Low increases compared to control cells were observed for interleukin (IL)-1α, IL-1β, IL-10 (fold increase 2x-6x), medium increases for IL-6, macrophage inhibitory protein (MIP)-1α, MIP-1B (fold increae 16x-27x), while high increases were observed for tumor necrosis factor (TNF)α (191x), MIP1 (202x) and granulocyte-colony stimulating factor (G-CSF) (2625x), the 20 nm particles being most potent compared to the 80 nm and 113 nm silver nanoparticles. In addition, other *in vitro* studies showed that cytokine production in macrophages can be induced by nanosilver (Martinez-Gutierrez et al. 2012, Park et al. 2011)(Also, human peripheral blood mononuclear cells produced IL-6 and IL8 after exposure to Ag-NP, whereas TNFα production was not affected (Greulich et al. 2011)

In contrast, in an intravenous 28-day repeated dose toxicity study in rats with 20 nm and 100 nm nanosilver, no cytokine level changes were found – although ex *vivo* cytokine induction and production by isolated spleen cells were affected (De Jong et al. 2013). In this study, Wistar rats were treated with a maximum dose of 6 mg/kg body weight, which was well tolerated by the animals. However, both for 20 nm and 100 nm Ag-NP growth retardation was observed during the treatment. A severe increase in liver and spleen size and weight was noted in line with earlier publications (Lankveld et al. 2010; Tang et al. 2009; Van Der Zande et al. 2012; Lee et al. 2012; Xue et al. 2012). The spleen proliferation was due to an increased cell number. Both T and B cell populations showed an increase in absolute cell number, whereas the relative cell numbers remained constant. During the histopathological evaluation, brown and black pigments indicating Ag-NP accumulation were noted in the spleen, liver, and lymph nodes. Ag-NP was also

detected incidentally in other organs. Clinical chemistry indicated liver damage (increased alkaline phosphatase, alanine transaminase, and aspartate transaminase) that could not be confirmed by histopathology. Hematology showed a decrease in several red blood cell parameters. General toxic effects included a decrease in body weight and an increase in liver and spleen weight, the organs in which Ag is mostly distributed.

The most striking toxic effect was the almost complete suppression of the natural killer (NK) cell activity in the spleen at high doses. Other immune parameters affected were: decreased interferon and interleukin (IL)-10 production by concanavalin-A stimulated spleen cells, increased IL-1b and decreased IL-6, IL-10 and TNF-a production by lipopolysaccharide stimulated spleen cells, increase in serum IgM and IgE, and increase in blood neutrophilic granulocytes. For the increase in spleen weight, a critical effect dose (5% change compared to controls) of 0.37mg/kg body weight (b.w.) could be established. The lowest critical effect dose (CED) for a 5% change compared to control animals was observed for thymus weight (CED05 0.01 mg/kg b.w.) and for functional immune parameters, i.e. decrease in NK cell activity (CED05 0.06 mg/kg b.w.) and LPS stimulation of spleen cells (CED05 0.04 mg/kg b.w.). The most sensitive parameters for an adverse effect were a decrease in thymus weight and effects on functional immune parameters like suppression of NK cell activity and effects on spleen lymphocyte stimulation. These results show that after intravenous administration for nanosilver, the most sensitive parameters for potential adverse responses were effects on the immune system.

In addition, contact allergy to silver can occur. Laine et al. (1997) patch-tested 118 patients with oral lichenoid lesions (OLL) topographically related to dental fillings to reveal contact allergy to restorative materials. Eighty patients (67.8%) displayed positive patch-test reactions to metals of dental filling materials: 76 reactions were found to various mercury compounds, 4 to sodium aurothiosulphate, 3 to stannic chloride and 2 to silver nitrate. The positive patch-test reactions appeared more commonly in patients with restricted contact lesions (85.1%, type-1 lesions) compared to patients with lesions exceeding to the adjacent areas (38.6%, type-2 lesions).

Several immune parameters were determined in a 28-day repeated dose oral toxicity study of nanosilver (Van Der Zande et al. 2012). Blood IgM and IgG levels, and proliferation activity, cytokine production, and natural killer cell activity of isolated spleen cells were not affected, although silver was demonstrated to be present in the spleen. Therefore, it was concluded that the oral exposure to silver nanoparticles did not result in alterations of the non-specific immune responses *in vivo*. Notably, the results of *in vivo* cytokine production is in contrast to several *in vitro* studies showing that cytokine production in macrophages *in vitro* assays can be induced by nanosilver as indicated above.

Genotoxicity

A number of studies on the genotoxicity of Ag-NPs were identified. Issues mentioned above for equivocal results on cytotoxicity are probably also relevant for genotoxicity tests of Ag-NPs. The studies may not all be directly comparable, as Ag-NPs of variable characteristics have been tested in different studies. The differences particularly concern particle size and coating. It was pointed out by Park et al. (2010) that "most data on the toxicity of Ag-NPs have been generated using nanoparticles modified with detergents to prevent agglomeration, which may alter their toxicities". Detergents or coating material may not always have been identified in the studies. As explained in greater detail below, the words "coating not indicated" are used when the paper did not comment on the presence or absence of coating. Sometimes the chemicals associated with Ag-NPs may themselves be genotoxic - DNA damage in spleen cells and sperm abnormalities observed in BALB/c mice after intraperitoneal injection of Ag-NPs (primary particle size 9±6 nm, 9.1-10.7 µg Ag/kg b.wt) were reported to be due to anionic surfactant (not

identified) used to stabilize the particles (Ordzhonikidze et al. 2009). Some Ag-NPs have been reported to contain toxic contaminants that can be removed by washing (Samberg et al. 2010).

*In vitro genotoxicity studies*

Ag-NPs (average particle size 10 nm; coating not indicated; tested up to 500 µg/plate) were not mutagenic in a bacterial reverse mutation test with histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium* or tryptophan-requiring strain (WP2uvrA) of *Escherichia coli* in the presence and absence of a metabolic activation system (Kim et al. 2012, 2013). No induction of mutations was either seen in *Salmonella* strains TA98, TA100, TA102, TA1535 or TA1537 when 5nm Ag-NPs (coating not indicated) was tested up to a toxic dose (76.8 µg/plate) (Li et al. 2012). In general, bacterial mutagenity tests are not considered relevant for testing nanoparticles, due to limited uptake.

*In vitro* studies with various forms of Ag-NPs in human and mammalian cells have consistently shown induction of DNA damage at low, non-cytotoxic doses. Three studies on Ag-NPs in human bronchial epithelial BEAS 2B cells showed an induction of DNA damage, as assessed by the comet assay after exposure for 2 h (Ag-NPs <100 nm, no coating indicated; 190-762 µg/mL without S9 mix (Supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 g for 20 minutes in a suitable medium; this fraction contains cytosol and microsomes - Duffus et al 2007), 293-1172 µg/mL with S9 mix) (Kim et al. 2010), 24 h (Ag-NPs 43-260 nm, coating not indicated; 0.01-10 µg/mL) (Kim et al. 2011a), and 4 h and 24 h (Ag-NPs 42.5±14.5 nm; coated with 15 wt% PVP; 16-48 µg/cm$^2$ i.e., 61-182 µg/mL) (Nymark et al. 2013). In the last study (Nymark et al. 2013), DNA damage increased in a dose-dependent manner from 8 up to 24 µg/cm$^2$ (20.4-91.2 µg/mL) but was not further increased at higher doses; as the PVP-coated Ag-NPs showed very low solubility to the medium used, the DNA-damaging effect was probably not due to $Ag^+$ ions in the medium but associated with cellular uptake of Ag-NPs.

A dose-dependent increase in bulky DNA adducts was described in human lung adeno-carcinoma A549 cells following a 24-h exposure to 0.5-15 µg/mL PVP-coated (0.2 % PVP) Ag-NPs (30-50 nm primary particles, 149±34 nm in cell medium) for 24 h (Foldbjerg et al. 2011). The formation of bulky adducts was inhibited by a pre-treatment with the antioxidant N-acetyl-*L*-cysteine. Ag-NPs induced higher increases in reactive oxygen species (ROS) than $AgNO^3$, suggesting that the Ag-NP-induced ROS were not solely due to $Ag^+$ release to the medium.

Starch-coated Ag-NPs (6-20 nm) induced DNA damage, as measured by the comet assay (treatment time not given), and micronuclei, as assessed by the cytokinesis block assay (100 and 200 µg/mL; 70 h), in normal human lung IMR-90 fibroblasts and human glioblastoma U251 cells in a dose-dependent manner (AshaRani et al. 2009). In the fibroblasts, a significant increase in DNA damage was observed at 25 µg/mLand higher doses, with no further elevation after 100 µg/mL. In the glioblastoma cells, DNA damage increased steadily (first significant effect at 50 µg/mL). DNA damage repair was supposed to be the reason for the $G_2$/M cell cycle arrest observed. The Ag-NPs also caused damage to mitochondria and increased the production of reactive oxygen species (ROS) in a dose-dependent manner. Transmission electron microscopic (TEM) analysis indicated Ag-NPs in mitochondria and the nucleus. Suggested mechanisms for the genotoxic effects were direct interaction of Ag-NPs with DNA or disruption of the mitochondrial respiratory chain by Ag-NPs leading to ROS production and interruption of ATP synthesis.

In human mesenchymal stem cells (hMSCs), Ag-NPs (43-260-nm; coating not indicated) induced a dose-dependent (0.01-10 µg/mL, 24 h) formation of micronuclei (MN) in BEAS 2B cells both in the presence and absence of cytochalasin B (Kim et al. 2011a). A somewhat higher effect of Ag-NPs (10 µg/mL) was seen without cytochalasin B (4.6-fold increase of MN compared tocontrols) than with it (2.8-fold increase of MN compared to

controls). Cytochalasin B, (applied in the *in vitro* micronucleus assay to prevent cytokinesis and, thereby, identify, as binucleate cells, cells that have divided after the treatment) was added simultaneously with Ag-NPs, which may have reduced particle uptake, as cytochalasin B inhibits the polymerisation of actin important for particle uptake processes. The Ag-NPs aggregates were wrapped with an endocytic vesicle within the cytoplasm and nucleus of BEAS-2B cells. Induction of DNA damage and micronuclei by Ag-NPs was reduced by scavengers, especially superoxide dismutase but also mannitol, sodium selenite, and catalase. When a modified comet assay, using a post-treatment with FpG (formamidopyrimidine DNA glycosylase) or ENDOIII (endonuclease III) to transform oxidative DNA adducts to strand breaks, was applied, a higher level of DNA-damage was observed in cells treated with Ag-NPs (10 µg/mL tested) compared with similar treatments without the endonucleases. FpG-treated cells showed greater oxidative DNA damage than ENDO III-treated cells. The results suggested that ROS generated by Ag-NPs increased oxidative DNA damage in both purines and pyrimidines, with a more pronounced effect on purines. Furthermore, in the oxidative stress assay, Ag-NPs significantly increased ROS. These results suggested that Ag-NPs have genotoxic effects in BEAS 2B cells and that oxidative stress stimulated by Ag-NPs may be an important factor in their genotoxic effects. However, treatment of BEAS 2B cells with the PVP-coated Ag-NPs did not increase structural chromosomal aberrations (tested up to 182 µg/mL in 24-h treatment and 20.4 µg/mL in 48h treatment) or micronuclei (tested up to 182 µg/mL, 48-h treatment) where cytochalasin B was added at 6 h after starting the Ag-NP treatment (Nymark et al. 2013); Ag-NPs inhibited mitoses at doses higher than 20.4 µg/mL and reduced the cytokinesis block cell proliferation index in a dose-dependent manner.

A 24-h treatment with Ag-NPs (approximate size 58.9 nm, no coating indicated) induced a dose-dependent increase in DNA damage (comet assay; 0.01-10 µg/mL) and micronuclei (0.1-10 µg/mL; with and without S9 mix) in Chinese hamster ovary CHO-K1 cells (Kim et al. 2013); again, a higher micronucleus induction was seen without Cyt-B than with Cyt-B. Ag-NPs (average size 10 nm, no coating indicated) did not increase the frequency of CHO-K1 cells with structural chromosomal aberrations, polyploidy or endore duplication after a continuous 24-h treatment (0.488-1.953 µg/mL) or a 6-h pulse treatment with (7.813-31.25 µg/mL) or without (0.977-3.906 µg/mL) a metabolic activation system (S9 mix) (Kim et al. 2012).

In mouse lymphoma L5178Y $tk^{+/-}$ cells, Ag-NPs (<100 nm, no coating indicated) induced DNA damage (comet assay) without S9 mix (449-1797 µg/mL) and with S9 mix (942-3770 µg/mL) but not mutations (tested up to 2500 µg/mL for 3 h) (Kim et al. 2010).

Leukocytes in human peripheral blood treated with synthesized uncoated crystalline Ag-NPs (40-60 nm) showed an increase in DNA damage in the comet assay after 5min and 3h treatments (Flower et al. 2012).

In Ntera2 human testicular embryonic NT2 cells, Ag-NPs (nominal size 20 nm, coating not indicated) and especially sub-microsized Ag particles (nominal size 200 nm, coating not indicated) induced DNA damage (tested doses 12.5-100 µg/mL) as measured by the comet assay, especially without using FpG (Asare et al. 2012); no induction of DNA damage was seen in primary testicular cells from wild-type or 8-oxoguanine DNA glycosylase knock-out ($mOgg1^{-/-}$) mice after treatment with these particles (tested up to 100 µg/mL).

In human mesenchymal stem cells (hMSCs), Ag-NPs (mean diameter 46 nm for single particles, 404 nm for agglomerates; coating not indicated) induced DNA damage (comet assay) after 1-h, 3-h and 24-h treatment in dose-dependent (0.1-10 µg/mL) and time-dependent manner (Hackenberg et al. 2011). The same doses of Ag-NPs also induced chromosomal aberrations in these cells after a one-hour treatment. Mostly chromatid-type aberrations were formed.

In human hepatoma HepG2 cells, Ag-NPs (7-10 nm, stabilized with polyethylenimine) accelerated cell proliferation and induced genes associated with cell cycle progression at

doses below 0.5 µg/mL, while cytotoxic effects were seen at 2-3 µg/mL and a clear increase in micronuclei at 1 µg/mL (only dose tested), Ag-NPs showing a much stronger effect than $Ag_2CO_3$ (Kawata et al. 2009). As cysteine (an effective $Ag^+$ ligand) abolished the micronucleus induction only partially, the authors concluded that both $Ag^+$ ions and NPs contributed to the genotoxic effect observed.

Flow cytometric evaluation of micronuclei in human lymphoblastoid TK6 cells after exposure to Ag-NP showed a dose-dependent induction of micronuclei by a 28-h treatment with 5nm Ag-NPs (coating not indicated), with a significant effect at 25 and 30 µg/mL, doses that showed the highest toxicity allowed for the assay (Li et al. 2012).

Ag-NPs (20 nm, uncoated, 0.1-3 µg/mL) did not induce mutations in mouse embryonic MEF-Lacz fibroblasts. Larger uncoated Ag-NPs (80 nm and 113 nm) produced a slight increase in mutation frequency which was not, however, statistically significant and occurred at toxic doses (1-50 µg/mL) (Park et al. 2011a).

Induction of micronuclei was also observed in human breast carcinoma HeLa cells after a 24-h treatment with 20-60 mg/mL of Ag-NP hydrogel (containing 2000 ppm uncoated Ag-NPs of 3-30 nm, sterile water, glycerine, carbomer, and triethanol amine; resulting in Ag NP doses of 40-120 µg/mL), followed by a cell wash and a 18-h incubation with Cyt-B (Xu et al. 2012). Comparison was made to cultures treated with 60 mg/mL of the hydrogel component alone. DNA microarray studies indicated that thousands of genes were up- or down-regulated after a 48-h exposure to Ag-NP hydrogel, and GO pathway analysis suggested that 14 theoretical activating signalling pathways were associated to up-regulated genes and three signal pathways to down-regulated genes. The authors suggested that the balance between anti-ROS response and DNA damage, chromosome instability, and mitosis inhibition might play important roles in silver-NP induced toxicity and that inflammatory factors were likely to be involved in the toxic effects of Ag NP hydrogel complex-induced toxic effects via the JAK-STAT signal transduction pathway and immune response pathway.

Various mechanisms may be responsible for the *in vitro* genotoxicity of Ag-NPs, such as leaching of ionic $Ag^+$ from the particles in the culture medium, induction of reactive oxygen species (ROS) due to particle penetration through the cell membrane, and positive surface charge of Ag-NPs allowing interaction with negatively charged DNA (Johnston et al. 2010; El Badawy et al. 2011; Kim et al. 2011a; Nymark et al. 2013). As the solubility of Ag particles is expected to depend on the available surface area, the size and coating of the particles is expected to affect the efficiency of ion release and the overall toxicity of the particles (Ahamed et al. 2008, 2010; Johnston et al. 2010; Kawata et al. 2009). The release of ions from the NPs could be more efficiently performed inside the cells than in the exposure medium, either due to the coating being degraded by the cell (Schrand et al. 2008), or due to the Ag-NPs being ionized in the cells by a Trojan-horse type mechanism (Park et al. 2010; Nymark et al. 2013).

*In vivo genotoxicity studies*

In the *Drosophila melanogaster* wing spot test (wing somatic mutation and recombination test), Ag-NPs (average diameter by transmission electron microscopy 38.67 nm, hydrodynamic diameter in suspension 45.39 nm; coating not indicated), supplied to third instar larvae at a concentration of 0.1–10 mM induced a small but statistically significant increase in the frequency of total spots, while silver nitrate was negative. Ag-NPs appeared to function mainly by inducing somatic recombination (Demir et al. 2012).

A clear dose-dependent increase in DNA damage, studied by the comet assay, was observed in blood leukocytes of Wistar rats after intravenous administration of bare Ag-NPs (size range 13±1 – 35±1 nm by TEM, 13±2 – 40±2 nm by XRD; 4, 10, 20 and 40 mg/kg b.wt; for 32 days at 5 days' interval) (Tiwari et al. 2011) Comet tail length in the 40, 20, 10, 4 mg/kg groups was, respectively, 5.0, 4.7, 2.6, and 1.4 times higher than in

controls, indicating a significant increase in DNA damage at all doses of Ag-NPs. ROS in blood serum was elevated in the high-dose group.

Negative results were obtained in Sprague-Dawley rats in the bone marrow erythrocyte micronucleus test after a 28-day oral exposure to Ag-NPs (average particle size 60 nm, coating not indicated) at daily doses of 30, 300 and 1000 mg/kg b.wt and sampling 24 h after the last administration(Kim et al. 2008) and after a 90-day inhalation exposure to Ag-NPs (18 nm, coating not indicated; 0.7, 1.4 and 2.9 x $10^6$ particles/$cm^3$,6 h/day) (Kim et al. 2011b). In the oral study (Kim et al. 2008), Ag was observed to accumulate in several tissues (including the blood) in a dose-dependent manner, and a slight dose-related increase of micronucleated polychromatic erythrocytes was present in male rats, although the increase was not statistically significant.

Conclusions on genotoxicity studies

*In vitro*, the genotoxic effect of nanosilver was demonstrated in several studies. However, the genotoxic effects of Ag-NPs seen *in vitro* may also be a consequence of effects seen only *in vitro* (Walmsley 2005) and probably depend on Ag-NP coating and cell type (Park et al. 2009; Nymark et al. 2013). Also, the way the cell exposure is performed probably affects the results. Pre-dispersion in medium before exposure may result in initial dissolution of the Ag-NPs, so that more soluble $Ag^+$ is present from the beginning, especially in short-term exposure assays (e.g. two hours). Table 9 summarizes the *in vitro* genotoxicity studies performed with Ag-NPs in mammalian cells.

As the studies available on the *in vivo* genotoxicity of Ag-NPs are few and concern Ag-NPs of variable characteristics, further studies are required to conclude whether Ag-NPs could be genotoxic *in vivo*, preferably after representative route of exposure. Such studies should also address site-of-contact mutagenicity

**Table 9**Genotoxicity studies performed with Ag-NPs in cultured mammalian cells *in vitro*.

| Material | Particle size | Cell system | Treatment time | Genotoxicity endpoint, assay | Result | Lowest positive dose or highest negative dose (µg/ml) | Reference |
|---|---|---|---|---|---|---|---|
| Polyvinyl pyrrolidone coated (PVP, 0.2 %) Ag-NPs | 30-50 nm primary particles, 149±34 nm in cell medium | Human lung adenocarcinoma A549 cells | 24 h | Bulky DNA adducts | + | 0.5-15 | Foldbjerg et al. 2011 |
| Starch-coated Ag-NPs | 6-20 nm | Normal human lung IMR-90 fibroblasts | ND | DNA damage, comet assay | + | 25 | AshaRani et al. 2009 |
| Starch-coated Ag-NPs | 6-20 nm | Normal human lung IMR-90 fibroblasts | 70 h | Micronuclei, cytokinesis block assay | + | 100 | AshaRani et al.2009 |
| Starch-coated Ag-NPs | 6-20 nm | Human glioblas-toma U251 cells | ND | DNA damage, comet assay | + | 50 | AshaRani et al. 2009 |
| Starch-coated Ag-NPs | 6-20 nm | Human glioblas-toma U251 cells | 70 h | Micronuclei, cytokinesis block assay | + | 100 | AshaRani et al. 2009 |
| Ag-NPs (no coating indicated) | <100 nm | Human bronchial epithelial BEAS 2B cells | 2 h | DNA damage, comet assay | + | 190 (-S9 mix)<br>293 (+S9 mix) | Kim et al. 2010 |
| Ag-NPs (no coating indicated) | 43-260 nm | Human bronchial epithelial BEAS 2B cells | 24 h | DNA damage, comet assay | + | 0.01 | Kim et al. 2011a |
| Polyvinyl pyrrolidone coated (15 wt%) Ag-NPs | 42.5±14.5 nm | Human bronchial epithelial BEAS 2B cells | 4 h<br>24 h | DNA damage, comet assay | + | 61 | Nymark et al. 2013 |
| Polyvinyl pyrrolidone coated (15 wt%) Ag-NPs | 42.5±14.5 nm | Human bronchial epithelial BEAS 2B cells | 24 h<br>48 h | Micronuclei, cytokinesis block assay | - | 182 | Nymark et al. 2013 |

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|---|---|---|---|---|---|---|---|
| Polyvinyl pyrrolidone coated (15 wt%) Ag-NPs | 42.5±14.5 nm | Human bronchial epithelial BEAS 2B cells | 24 h<br>48 h | Chromosomal aberrations | - | 182 (24 h)<br>20.4 (48 h) | Nymark et al. 2013 |
| Ag-NPs (no coating indicated) | 43-260-nm | Human bronchial epithelial BEAS 2B cells | 24 h | Micronuclei, with cytichalasin B (cytokinesis block assay) and without it | + | 0.01 (both assays) | Kim et al. 2011a |
| Ag-NPs (no coating indicated) | 43-260-nm | Human bronchial epithelial BEAS 2B cells | 24 h | Oxidative DNA damage, comet assay using FPG and ENDO III endonucleases | + | 10 (only dose tested) | Kim et al. 2011a |
| Ag-NPs (no coating indicated) | Approximately 58.9 nm | Chinese hamster ovary CHO-K1 cells | 24 h | DNA damage, comet assay | + | 0.01 (±S9 mix) | Kim et al. 2013 |
| Ag-NPs (no coating indicated) | Approximately 58.9 nm | Chinese hamster ovary CHO-K1 cells | 24 h | Micronuclei, cytokinesis block assay and without cytochalasin B | + | 0.1 (±S9 mix) | Kim et al. 2013 |
| Ag-NPs (no coating indicated) | Average 10 nm | Chinese hamster ovary CHO-K1 cells | 24 h<br>6 h (pulse) | Chromosomal aberrations, polyploidy, endoreduplication | - | 1.953 (24 h)<br>31.25 (6 h, +S9 mix)<br>3.906 (6 h, -S9 mix) | Kim et al. 2012 |
| Ag-NPs (no coating indicated) | <100 nm | Mouse lymphoma L5178Y $tk^{+/-}$ cells | ? | DNA damage, comet assay | + | 449 (-S9 mix)<br>942 (+S9 mix) | Kim et al. 2010 |
| Ag-NPs (no coating indicated) | <100 nm | Mouse lymphoma L5178Y $tk^{+/-}$ cells | 3 h | Mutations, mouse lymphoma assay | - | 2500 | Kim et al. 2010 |
| Uncoated crystalline Ag-NPs | 40-60 nm | Human peripheral blood leukocytes | 5 min<br>3 h | DNA damage, comet assay | + | ? | Flower et al. 2012 |
| Ag-NPs (no coating indicated) induced DNA damage | 20 nm (nominal) | Ntera2 human testicular embryonic NT2 cells | | DNA damage, comet assay especially without using FpG (formamido-pyrimidine-DNA glycosylase) | + | 12.5 | Asare et al. 2012 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Sub-microsized Ag particles (no coating indicated) | 200 nm (nominal) | Ntera2 human testicular embryonic NT2 cells | ? | DNA damage, comet assay especially without using FpG (formamido-pyrimidine-DNA glycosylase) | + | 12.5 | Asare et al. 2012 |
| Ag-NPs (no coating indicated) induced DNA damage | 20 nm (nominal) | Primary testicular cells from wild-type or 8-oxoguanine DNA glycosylase knock-out ($mOgg1^{-/-}$) mice | ? | DNA damage, comet assay | - | 100 | Asare et al. 2012 |
| Sub-microsized Ag particles (no coating indicated) | 200 nm (nominal) | Primary testicular cells from wild-type or 8-oxoguanine DNA glycosylase knock-out ($mOgg1^{-/-}$) mice | ? | DNA damage, comet assay | - | 100 | Asare et al. 2012 |
| Ag-NPs (no coating indicated) | 46 nm (mean of single particles) 404 nm (mean of agglomerates) | Human mesenchymal stem cells (hMSCs) | 1 h<br>3 h<br>24-h | DNA damage, comet assay | + | 0.1 | Hackenberg et al. 2011 |
| Ag-NPs (no coating indicated) | 46 nm (mean of single particles) 404 nm (mean of agglomerates) | Human mesen-chymal stem cells (hMSCs) | 1 h | Chromosomal aberrations | + | 0.1 | Hackenberg et al. 2011 |
| Ag-NPs (COATING?) | SIZE? | Human hepatoma HepG2 cells | ? | Micronuclei, cytokinesis block assay | | >0.5 | Kawata et al. 2009 |
| $Ag_2CO_3$ | NA | Human hepatoma HepG2 cells | ? | Micronuclei, cytokinesis block assay | | ? | Kawata et al. 2009 |
| Ag-NPs (no coating indicated) | 5 nm | Human lymphoblastoid TK6 cells | 28 h | Flow cytometric micronucleus test | + | 25 | Li et al. 2012 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ag-NPs (uncoated) | 20 nm | Mouse embryonic MEF-Lacz fibroblasts | ? | Gene mutations | - | 3 | Park et al. 2011a |
| Ag-NPs (uncoated) | 80 nm | Mouse embryonic MEF-Lacz fibroblasts | ? | Gene mutations | - | 50 | Park et al. 2011a |
| Ag-NPs (uncoated) | 113 nm | Mouse embryonic MEF-Lacz fibroblasts | ? | Gene mutations | - | 50 | Park et al. 2011a |
| Ag-NP hydrogel (2000 ppm uncoated Ag-NP, sterile water, glycerine, carbomer, and triethanol amine) | 3-30 nm (Ag-NP) | human breast carcinoma HeLa cells | 24 (+ 18 h after wash) | Micronuclei, cytokinesis block assay | + | 40 (20 mg/ml of hydrogel; comparison with hydrogel without Ag-NPs) | Xu et al. 2012). |

Reproductive and Developmental Toxicity

Available information on toxicity of nanosilver and silver to mammalian reproduction and development is limited. Full guideline studies in rats, mice or rabbits specifically addressing reproductive toxicity of nanosilver could not be identified.

*In vitro Reproductive and Developmental Toxicity*

*In vitro* studies using the embryonic stem cell test (EST) showed that Ag-NPs (20 nm, 80nm, and 110 nm, uncoated) as well as $AgNO_3$ were able to inhibit the development of contracting cardiomyocytes (Park et al. 2011a). Inhibitory activity was already observed in doses as low as 10 µg/mL. The Ag ions were slightly more toxic than the Ag-NPs, whereas the larger Ag-NPs (80 nm and 110 nm) were less toxic.

Several studies identified reproductive effects of nanosilver in ecotoxicity studies (Lu et al. 2013). Effects on embryo development and reproduction were reported in a wide array of species including zebrafish (*Danio rerio*), Japanese medeka (*Oryzias latipes*), *Caenorhabditis elegans* and Fathead minnow (*Pimephalespromelas*) as summarised by Ahamed et al. (2010). Increased incidences of abnormalities and malformations in embryos, hatching delay and increased mortality as well as reproductive failure were described. Additionally, in Drosophila an effect on reproduction success was observed (Philbrook et al. 2011).

In cultures of C18–4 mouse spermatogonia-like cells, reduced cell viability was observed with concentrations from 10 µg/mL of 15 nm Ag-NP and increased apoptosis was reported from 5 µg/mL (Braydich-Stolle et al. 2005). These observations were confirmed by Asare et al. 2012, who reported cytotoxicity of 20 and 200 nm sized Ag-NP in murine primary testicular cells from the lowest tested concentration of 10 µg/mL. These results indicate that testicular cells are sensitive for the toxic effects of Ag-NP, although it is questionable whether this also indicates reproductive toxicity.

*In vivo Reproductive and Developmental Toxicity*

Very recently, the results of a combined repeated-dose toxicity study with the Reproduction / Developmental Toxicity Screening Toxicity Test according to OECD TG 422 for Ag-NP became available (Hong et al. 2013). Citrate capped Ag-NP (8 nm) were applied by gavage at doses of 62.5, 125 and 250 mg/kg bw/d to males 14 days prior to mating and 28 days during and after mating, and to females 14 days prior to mating, during mating and gestation, and during 4 days of lacatation. This resulted in an overall duration of exposure of 42 days for males and up to 52 days for females. No treatment-related maternal, developmental or reproductive toxicity was reported. Silver levels in liver were more than 5-fold lower than expected from data reported by Kim et al. (2008) and more similar to those reported by Lee et al. (2013a) at the dose of 100 mg/kg bw/d. Therefore, dosing may not have been high enough to reliably detect potential reprotoxic properties. OECD test guideline 422 recommends three dose levels with the highest dose inducing toxic effects or a limit dose test with 1000 mg/kg bw/d.

Reproductive and developmental effects of single oral applications of Ag-NP (20nm) on gestational day (GD) 9 were evaluated in mice at doses of 10, 100 and 1000 mg/kg bw (Philbrook et al. 2011). There were no obvious signs of maternal toxicity, including behavioural changes or weight loss throughout the 10 days post-exposure. Litter size, maternal weight gain from GD 9 to GD 19 and mean fetal weights and lengths also did not differ from the control group. However, there was a significant increase in the number of non-viable fetuses from 3.3 % in the controls to 9.6 / 5.5 / 6.1 % at 10 / 100 / 1000 mg/kg b.w. Ag NPs. However, the effect was statistically significant only for the 10 mg/kg b.w. dose group ($p<0.05$) and a clear dose-response was lacking. The authors speculated that this may have been due to extensive particle aggregation and reduced

bioavailability at the higher doses. A major limitation of this study is the single dosing at GD 9.

In relation to the effect of silver on reproduction, the timing of the exposure may be of importance as demonstrated by the results obtained with silver chloride (Shavlovski et al. 1995). Pre- and postnatal death was reported to increase in rats following application of 250 mg/kg bw silver chloride with the diet from GD 1 to GD 20 (Shavlovski et al. 1995). Interestingly, rats treated from GD 7 to GD 15 only showed normal pregnancy. Post-implantation loss was reported as 9.6 % in controls, 5 % in animals treated from GD 7-15 and 36 % in animals treated from GD 1-20. All 33 newborn animals from another group of 5 animals treated throughout pregnancy died post-partum (control 5.9 %). In addition, an increased incidence of abnormal foetuses was reported for the group treated with silver chloride throughout gestation with 34.7% of fetuses with cryptorchism (control 1.3 %, GD7-15: 0 %) and 30.6 % hydronephrosis (control 5.3 %, GD7-15: 3.9 %). Complementing mechanistic investigations suggested replacement of copper from ceruloplasmin, reduced copper concentrations in the placenta and fetuses, and reduced SOD activity in embryonal tissues as the cause of the effect.

Following repeated oral exposure of Fisher 344 rats over 90 days to 500 mg/kg bw/d nanosilver (NAMATECH) suspended in 0.5 % methylcellulose, the weight of ovaries remained unaffected (Kim et al. 2010). Notably, a slight, statistically significant ($p<0.05$) increase in the weight of left testes of 0.51±0.01 g vs. 0.47±0.02 g in control was observed (right testes: 0.49±0.02 vs. 0.47±0.02 g), but histopathology of the organ was not performed according to the report. Silver levels in testes were in the same order of magnitude as those in the livers of treated animals (Kim et al. 2010: 0 / 6.6 / 12 / 24 µg/g testes wet weight at 0 / 30 / 125 / 500 mg/kg bw/d), but the analytic method could not distinguish between silver species (Ag-NP and $Ag^+$). Oral 28-day studies in rats also suggest that silver reaches the testes (Kim et al. 2008; Lee et al. 2013b). In the study of Kim et al. (2008), the following silver levels were reported in testes vs. liver: 0.1 vs. 0.02 µg/g in controls, 1.2 vs. 0.5 µg/g at 30 mg/kg bw/d, 3.6 vs. 8.7 µg/g at 300 mg/kg bw/d and 7.4 vs. 71 µg/g at 1000 mg/kg bw/d. Unfortunately, no histopathological analysis was performed in this study. In the study of Lee et al. (2013b), testicular levels of Ag determined following 28-day oral exposure of rats towards 100 and 500 mg/kg bw/d of 10 and 25 nm sized Ag-NP as well as after 1, 2, and 4 month of follow up. Tissue silver levels were around 0.4 ppm in the lower dose and around 0.8 ppm in the higher dose with little or no detectable decrease during the 4-month post-exposure observation period. No alterations in the testes tissue were found at histopathology. Interestingly, however, the overall NOAEL in this study was 500 mg/kg bw/d, while a LOAEL of 125 mg/kg bw/d was established in previous studies based on liver toxicity (Kim et al. 2008 and Kim et al. 2010) and tissue silver concentrations (liver and testes) were lower, suggesting that there may be differences in potency or silver release between the Ag-NP batches tested (Lee et al. 2013b).

In contrast to the studies described above, no such silver accumulation was observed in testes in a 5-day study using iv injection of different Ag-NPs by Lankveld et al (2010) (NanoComposix, mean size 20 / 80 / 110 nm). A gradual decrease in testicular Ag levels was observed after the five-day repeated administrations and low levels of Ag were still detectable in the testes 12 days after the last IV administration at day 17. Recent evidence from a 28-day oral study in rats with post-exposure follow-up over 1 and 8 weeks suggests that clearance of silver from testis is very slow, regardless of whether silver or nanosilver was applied (Van der Zande et al. 2012). Also for mice, Park et al. (2010) reported similar or higher organ levels of silver in testes compared to liver following repeated oral exposure to 1 mg/kg bw/d Ag-NPs with a mean size of 22 and 42 nm, while no silver was detectable in testes for 71 and 323 nm sized particles.

In a subchronic inhalation study in rats with concentrations of 0.06 mg/m$^3$ *in situ* generated Ag-NPs (~12 nm), ovaries, testes and epididymis were examined histologically, but no lesions were observed (Ji et al. 2007). Unfortunately, silver levels in

these organs were not reported. As the NOAELs of 100 µg/m$^3$ and 117 µg/m$^3$(based on liver and lung toxicity) in subchronic inhalation toxicity studies are significantly higher (Sung et al. 2009; Song et al. 2013), an absence of effects in the male reproductive organs at 60 µg/m$^3$in the study by Ji et al. (2007) would, however, not be sufficient to exclude those as possible targets of silver toxicity.

#### 3.4.3.1 Conclusions on hazards

Silver and nanosilver are clearly shown to have toxic potential although toxicity in general in humans seems to be low. In in-vitro studies, Ag-NPs are cytotoxic and have genotoxic DNA damaging capacity, and show developmental toxicity. In-vivo genotoxicity and developmental toxicity studies could not confirm or negate the effects of Ag-NP. Although Ag uptake and possible persistence in the testes was observed, histopathology did not reveal specific testicular toxicity. Liver toxicity is indicated by the effect of Ag-NPs on various liver enzymes. *In vivo*, effects on the immune system were observed both regarding allergy to Ag itself, but also in repeated dose toxicity studies regarding effects on cytokine production and on non-specific immune responses like natural killer cell activity. These immune effects warrant further studies exploring the functionality of the immune system after exposure to Ag-NPs.

## 3.5. Environmental health

### 3.5.1. Introduction

Nanosilver is released from a variety of sources in a variety of different forms. Silver released from applications in contact with water (textiles, pipes, personal care products etc.) reaches the sewer system. Forms of silver identified by Lorenz et al. (2012) for releases from textiles, for example, include Ti/Si-AgCl nanocomposites, AgCl nanoparticles, large AgCl particles, nanosilver sulphide and metallic nano-Ag with AgCl being the most frequently observed chemical form. If the sewer system is connected to a wastewater treatment plant (WWTP), approximately 95% of the silver entering the WWTP sorbs to sewage sludge and may then be transferred to fields with the sludge. The fraction passing the WWTP enters the surface water. Accordingly, silver levels need to be predicted for soil treated with sewage sludge and for freshwater receiving WWTP effluents. These levels then need to be compared to threshold concentrations for adverse effects in various species such as bacteria, earthworms, algae, daphnids, and fish. It is important that threshold concentrations for adverse effects are determined for all environmentally relevant forms of silver, not only for ionic silver and metallic silver nanoparticles.

### 3.5.2. Exposure

Silver concentrations have been measured in water bodies and sediments in several parts of the world, mainly in Europe and North America. This includes regions with background levels or moderate contamination as well as sites with exceedingly high silver contamination from, e.g., mining activities. Typical levels in freshwater moderately affected by human activity are around 1–10 ng/L (Wen et al. 1997; 2002; Tappin et al. 2010; Lanceleur et al. 2011). Waters affected by untreated wastewaters, photographical industry or mining show higher concentrations that are in the range of 100 ng/L to 1 □g/L (Wen et al. 2002; Lanceleur et al. 2011).

Silver concentrations in river sediments are on the order of 1 mg/kg for industrialized regions (Gobeil 1999; Lanceleur et al. 2011) and may reach up to 10 or 100 mg/kg at highly contaminated sites (Dissanayake et al. 1983; Lanceleur et al. 2011). The geogenic background level is around 0.07 mg/kg (Pais and Benton Jones 1997).

#### 3.5.2.1. Fate and behaviour of silver in freshwater

In surface waters, silver is present as the monovalent ion and as sulfide, bicarbonate or sulfate salts or adsorbed onto organic or inorganic material (Purcell and Peters 1998). Furthermore, silver will form complexes with chloride (Fortin and Campbell 2000) or even more stable complexes with thiosulfate (Hiriart-Baer et al. 2006). Aquatic silver toxicity has been found to depend on the free $Ag^+$ ion concentration as well as other dissolved chemical species of silver. For silver salts as well as for metallic silver ($Ag^0$), solubility and the composition of the water are of utmost importance for determining the detrimental effects of silver (Ratte 1999). Since different species of silver will have different bioavailability to biological organisms, an evaluation of silver speciation is of outmost importance for the assessment of toxicity.

In the ENRHES review, Stone et al. (2010) reviewed all available literature on nanosilver up until December 2009. It was concluded that the environmental fate and behavior of nanosilver was relatively unknown. Since then, a couple of studies have focused on the transformation of Ag-NPs as reviewed by Levard et al. (2012). Engineered Ag-NPs will most often have an inner core of metallic silver ($Ag^0$) with usually an organic coating. These coatings will stabilize Ag-NPs against aggregation and keep the particles suspended (Levard et al. 2012). Uncoated Ag-NPs will be electrostatically stable because of the presence of negative charges on their surface due to hydroxide, oxide or sulfide groups (Levard et al. 2012) and the addition of dissolved stabilizers such as TWEEN-20. However, Gao et al.(2009) demonstrated that the dispersion of Ag-NPs is greatly influenced by the water chemistry: in natural waters[3], Ag-NPs aggregation is influenced by pH, dissolved organic carbon (DOC), suspended particulate matter (SPM) and natural organic matter (NOM) and electrolyte concentrations. Li et al. (2010) have shown that Ag-NPs aggregate in the presence of electrolytes such as NaCl, $NaNO_3$ and $CaCl_2$.

Under aerobic conditions metallic sliver can be oxidized through silver/hydrogen peroxide intermediates leading to $Ag_2O$ and subsequent dissolution in water to $Ag^+$ (Levard et al. 2012; Liu and Hurt 2010). The influence of the chemical composition of the water on particles sizes, dissolution and stability of nanosilver dispersions is however difficult to predict. A number of models have been developed to describe this dissolution process, but currently no model takes in account all parameters believed to play a role in the dissolution process, e.g. particle size, state of aggregation, particle coating, water chemistry and influence of natural organic matter. Especially the latter may play a major role in reducing the dissolution of Ag-NPs in water due to a rapid formation of an NOM coating of the silver particles (e.g. Gao et al. 2009).

Most of the presently available models agree that the dissolution rate increases with decreasing particle diameter, and evidence is building up that Ag-NP dissolution is more controlled by the initial Ag-NPs size than by the aggregation (Kent & Vikesland 2012). Since dissolution kinetics may be size-related (nonlinearily), dynamic testing and modeling of metal uptake and toxicity may be the way forward to take into account the non-equilibrium behavior of Ag-NPs dissolution during toxicity testing (Veltman et al. 2010).

In the presence of reduced sulphur, silver has a very strong tendency to form silver sulfide; silver sulphide is thermodynamically extremely stable. Even under oxic conditions, freshwater may contain metal sulfide clusters that make sulfide available for

[3]Bodies of water present in nature (lakes, seas, rivers, etc.)

reaction with silver (Rozan et al. 2000). The sulphidation process will both alter the surface charge and dissolution rate of Ag-NPs (Levard et al. 2012), and also affect their reactivity, mobility and toxicity (Bianchini et al. 2002; Levard et al. 2012; Reinsch et al. 2012). Silver sulfide and organic silver-sulfide compounds are very stable and represent an important type of environmentally relevant silver species. A recent study by Lowry et al. (2012) demonstrated that 18 months after addition on Ag-NPs to freshwater mesocosms the speciation of Ag in sediments was indeed dominated by $Ag_2S$ and Ag complexed with reduced S in organic matter (Ag-sulfhydryl). However, Lowry et al. (2012) also found that some of the added Ag added to the mesocosms was taken up by plants, fish and insects showing that Ag originating from the NPs was bioavailable, probably because of incomplete or slow sulphidation.

In freshwater bodies, silver in both metallic and sulfidic form will sorb to suspended particulate matter (SPM) in the water column. Because the SPM settles to the sediment and silver sorbed to SPM, silver is transferred to the sediment. The higher the SPM concentration, the greater the sedimentation mass flow of silver is. An environmental fate modeling study on silver in the Rhine River that used realistic silver emissions as input found silver concentrations in the water column in the range of 20 ng/L to 150 ng/L and concentrations in the sediment around 4 mg/kg (Blaser et al. 2008); furthermore, Blaser et al. (2008) found that approximately 95% of the silver that is released to the river is present in the sediment. The results reported by Blaser et al. (2008) are based on silver emissions from a wide range of silver uses; biocidal applications of silver account for approximately 10% of these emissions and nanosilver, in turn, represents approximately 30% of all biocidal silver applications (Burkhardt et al. 2011; Windler et al. 2013).

#### 3.5.2.2. Fate and behaviour in soils

Basically, silver in soils behaves as in aquatic systems, i.e. particle aggregation, agglomeration, transport, speciation and bioavailability of silver and other metal (oxide) nanoparticles are strongly affected by particle size, coating, pH, ion concentration and exposure duration (Darlington et al. 2009; Coutris et al. 2012). Coutris et al. (2012) compared silver nitrate, uncoated and citrate-coated Ag-NP in two different soils and concluded "that some Ag-NPs can act as a continuous source of bioaccessible Ag, while $AgNO_3$ is rapidly immobilized in soil". In a study with PVP-coated Ag-NPs in 16 soils, dissolution could only be detected in six of them, probably due to their higher organic matter content (Cornelis et al. 2011).

When compared to aquatic systems, Ag bioavailability in soils is affected by a number of additional or soil-specific factors. In the following, key points of a recent review (Tourinho et al. 2012) are summarized and supplemented.

- Depending on texture, soils provide a large surface area. The solid components (minerals, organic compounds) are typically negatively charged so that any dissolved cations will be more or less readily adsorbed (e.g. by clays) or complexed (mostly chelation by organic acids). However, also negatively charged Ag-NP can sorb to the edges of Fe- and Al-based clays (Cornelis et al. 2012).

- Accordingly, NP transport in soils is favoured by a lipophilic or negative surface charge of the particles and (mostly) hindered by a positive charge. Thio et al. (2011) demonstrated that citrate- and polyvinylpyrrolidone (PVP)-coated Ag-NP were highly mobile on silica surfaces over a wide range of pH and ionic strength conditions. Mobility was further enhanced in the presence of natural organic matter in the solution.

- NOM has often been found to stabilize NP dispersion (e.g Fabrega et al. 2009, Akaighe et al. 2011), yet it can also hamper NP mobility (Coutris et al. 2012) and contribute to agglomeration in positively charged soil surfaces (e.g. clay's crystal edges). At low pH or dry conditions, organic acids from NOM may be predominantly

hydrophobic, with according affinity for lipophilic surfaces of many NP coatings, e.g. polyvinylpyrrolidone (PVP). Such organic coatings can be used as substrates by microorganisms, i.e. the stability of NP with such coatings is limited. Organic acids from NOM have even been shown to form Ag-NPs via reduction of $Ag^+$ at environmentally relevant conditions (Akaighe et al. 2011).

- In aerated soils, the soil solution is a thin film between solid particles and soil air from which diffusion occurs. Thus, above the groundwater table the conditions in the solution are usually highly oxic. In highly organic soils with sufficient nutrient availability this oxygen concentration will be readily consumed by decomposer activity. Additionally, $CO_2$ formed during decomposition reaches concentrations several orders of magnitude above those in the atmosphere, resulting in high concentrations of dissolved $HCO_3^-$ and thus much lower pH values than in surface waters.
- Soils are extremely heterogeneous, and distribution of solvents is often restricted due to isolation of the particles by soil air. Even on a very small scale (within single soil aggregates), both oxic and anoxic conditions can occur.
- Soil chemistry, sorption and complexation are not static but highly dynamic due to temporal variation in climate and biological activity. In temperate climates, soils below freezing depth are metabolically active year-round, although activity peaks at moderately warm and sufficiently humid conditions. This means that, depending on the respective conditions, nanoparticles are likely to be (dis-)aggregated, (re)dissolved, precipitated and even formed anew (Akaighe et al. 2011).
- Changes in water availability due to weather and plant uptake result in highly variable ion concentrations. Plant root exudates and respired $CO_2$ lower the soil pH, which affects also ion sorption. Nutrient uptake by plants and microorganisms and ion excretion by animals and microorganisms affects the reaction equilibria in the soil solution. So do a number of root exudates which form complexes, such as citrate.
- The permanently ongoing decomposition of organic matter in soils accordingly alters the chemistry of the soil solution.

In summary, soil conditions are so complex and variable that predictions on the environmental fate of whatever chemical are generally extremely difficult, if not an impossible task. Relevant biological test systems are more important and endpoints that integrate these uneven conditions such as community metabolic activity or survival and reproduction of animals that move through and feed on soil. In general, silver ions are not very mobile; however, particles may behave differently.

### 3.5.3. Effects of Ag-NP on aquatic systems

For silver salts, concentrations in the range of 1.2 - 4.9 µg/L (expressed as free $Ag^+$) have been found to be lethal for representative species of aquatic plants, invertebrates and fish (Eisler 1996).

Ag-NP toxicity to aquatic organisms has recently been reviewed by Stone et al. (2010), Fabrega et al. (2011) and Lapresta-Fernández et al. (2012). In the following section, the focus will be directed towards the so-called base set organisms (i.e., fish, daphnia and algae) used in environmental risk assessment of chemicals. Furthermore, the toxicity towards microorganism will be dicussed due to the potential effects of silver nanoparticles to waste water treatment plant organisms.

Published work reporting the effects of Ag-NP in freshwater sediments is limited. As observed in other environments, results so far indicate that observed effects very much depend on surface coatings, even though when assessing bioaccumulation that may not

always be the case (Coleman et al. 2013). Overall, results indicate that the toxicity of Ag-NPs to sediment organisms tend to be lower (but not necessarily insignificant) than what has been observed in water column-dwelling species.

Work by Lowry et al. (2012) reported on the fate and behaviour of poly(vinylpyrrolidone)-coated Ag-NPs in freshwater mesocosms simulating an emergent wetland environment with the Ag-NPs applied either via the water column or into the sediment. After 18 months it was observed that 70 wt % of the added Ag was found within the sediment, and most fraction was found in the compartment in which they were applied. Most silver was transformed via sulphidation (as described above) although transfer into rooted plant biomass (≈3 wt % for the sediment dosed mesocosm) was recorded. Additionally, body burdens of 0.5−3.3 $\mu$g Ag/g wet weight were found in mosquito fish and chironomids. Lowry et al. (2012) concluded that Ag from the NPs was still available even after sulphidation and even when total water column Ag concentrations were low (<0.002 mg/L).
Studies comparing the effects of two sizes (6 and 20 nm) of PVP-coated Ag-NPs, 6 nm gum arabic coated Ag-NP and $AgNO_3$ (at equivalent concentrations) on eleven species of common wetland plants exposed via soil and in aqueous environment, concluded that the soil exposures led to lower effects of Ag-NP. Effects tended to be higher on exposures to GA-Ag-NPs although it depended on taxa (Yin et al. 2012).

In summary, studies conducted to date recognise that although exposure in sediments may be different from what occurs in the water column due to higher availability of organic matter and other potential ligands for silver, it is clear that uptake still occurs and hazard is observed.

In marine systems, Ag-NP are unlikely to cause severe damage: The high salinity generally fosters NP aggregation, and in the presence of sulphide and chloride silver ions will form sparingly soluble precipitates. However, soluble $[AgCl]_{aq}$ complexes are also formed which can be highly bioavailable (Reinfelder & Chang 1999).

#### 3.5.3.1. Effects of Ag-NP on fish

A few studies have focused on the in vivo effects of Ag-NPs in fish and these show that Ag-NP are toxic to fish at different stages of development (Griffitt et al. 2009; Bilberg et al. 2012; Yeo and Kang 2008).

The studies by Griffitt et al. (2008) found LC50, 48h values of around 7 mg/L for juvenile and adult zebrafish, whereas Bilberg et al. (2010) reported LC50, 48h-values as low as 84 µg/L in a static renewal of Ag-NP. While Griffitt et al. (2008) used Ag-NP with a metaloxide coating, Bilberg et al. (2010) used PVP-coated particles. This may explain the large difference in EC50 values in these two studies, since Griffitt et al. (2008) report a 0.07% dissolution of Ag after 48 h and Bilberg et al. (2012) found that about 40% of the added Ag was present as silver ions.

In tests with zebrafish embryos, Yeo and Kang (2008) reported decreased hatching rates, weak heart beats, edema and abnormal notochords after exposure to 10-20 nm Ag-NPs for 48 in concentrations of 10-20 µg/L. The study by Asharani et al. (2008) used starch/BSA coated Ag-NPs (5-20 nm in nomial sizes) in concentration of 5-100 mg/L and zebrafish embryos were exposed for 72 h. Sub-lethal effects were found in the lowest concentration (5 mg/L) and lethal concentrations in the range of 25-50 mg/L. Phenotypic changes were observed in concentrations above 50 mg/L when the fish were exposed to Ag-NPs, but not for Ag+ (added as $AgNO_3$). However, although the concentrations of Ag-NPs and $AgNO_3$ are not comparable (Ag-NPs concentration was 2000 times higher than $AgNO_3$ concentration) the nanoparticle effect claimed in the paper remains to be proven.

In Japanese medaka (Japanese rice fish, Oryzias latipes), Ag-NPs (average size 49.6 nm by TEM; coating not indicated) induced p53, glutathione S-transferase (GST) and

cytochrome P450 1A (CYP1A) genes at the mRNA level, which was interpreted to suggest DNA damage, and oxidative and carcinogenic stress. Silver nitrate induced genes involved in inflammation, metallic detoxification responses, and overall stress response (Chae et al. 2009). The findings indirectly suggest oxidative stress and DNA damage, although they do not prove that DNA was actually damaged.

#### 3.5.3.2. Effects of Ag-NP on daphnia

Only a few studies have been carried out with pelagic invertebrates (Fabrega et al. 2011; Stone et al. 2010). In the study by Griffitt et al. (2008) the toxicity of oxide-coated Ag-NP was studied in 48 hour static tests with adult Daphnia pulex and Ceriodaphnia dubia neonates. The average particle diameter in dispersion was measured to be 44.5 nm and LC50, 48h-values for Ag-NP reported to be 40 µg/L for D. pulex adults and 67 µg/L for *C. dubia* neonates. After the 48 hours of incubation it was found that less than 0.07% by mass of the original dose of Ag-NP was found in the dissolved form.

For citrate coated Ag-NPs the studies by Asghari et al.(2012), Allen et al. (2010), and Kennedy et al.(2010) reported EC50,48h and LC50,48h values from 1.1-11 µg/L. For PVP-coated Ag-NPs Zhao & Wang (2012) found LC50,48h values in the same range (2.0 µg/L), but other authors found LC50,48h values up to 97 µg/L (Kennedy et al., 2010). In the review by Fabrega et al. (2011) it is noted that toxicity testing with invertebrates shows that nanoparticle capping, ionic strength and the presence of organic matter in the test media are important parameters for Ag-NPs toxicity.

Bianchini et al. (2002) investigated the effect of sulphide on the toxicity of silver in Daphnia magna. Sulphide was found to reduce the toxicity of ionic silver; when the silver concentration exceeded the sulphide concentration, the acute toxicity of the free silver ion was reduced according to the amount of sulphide present.

#### 3.5.3.3. Effects of Ag-NP on algae

The few studies that have been conducted to assess the effects of Ag-NP on algal growth and photosynthesis generally find EC50-values below 1 mg/L. In a growth inhibition test with freshwater green algae *P. subcapitata* Griffitt et al. (2008) found an EC50, 96h of 190 µg/L for metaloxide coated Ag-NP. Using the same algal species McLaughlin and Bonzongo (2012) found that water chemistry and especially presence of organic matter play a major role in the toxicity of Ag-NP. They suspended Ag-NPs powder in pure water and found sizes of 145 ± 2.9 nm and a zeta-potential of −36.8 ± 0.56 mV, In different water types, sizes of Ag-NPs and zeta-potentials of dispersions increased, with moderately hard water as the extreme with sizes of 395 ± 50.9 in very unstable dispersions with zeta potentials of −4.38 ± 0.47. Correspondingly, McLaughlin and Bonzongo (2012) found EC50 (96h) ranged from 4.6 µg/L in algal culture medium to 192 µg/L in a wetland water sample. In a study of polyvinylpyrrolidone-coated Ag-NPs, Kennedy et al. (2010) stress the importance of determining the dissolved fraction of silver and reports EC50 values around 20 µg/L for two different sizes of Ag-NPs. These studies all find EC50-values well below 1 mg/L, demonstrating that based on tests with *P. subcapitata* (one of the algal species recommended in the ISO standard and OECD guideline tests for algal growth inhibition), Ag-NPs should be classified as very toxic towards algae.

This level of toxicity is confirmed by the study of Navarro et al. (2008a) who, for carbonate-coated Ag-NP, found EC50-values ranging from 355 µg/L after 1 hour and decreasing to 92 µg/L after 3-5 hours in tests with the green algae Chlamydomonas reinhardtii. Expressed in terms of free $Ag^+$, EC50 (1h) was 3.6 ± 0.5 µg/L and EC50 (5h)0.9 ± 0.08 µg/L. Particle sizes were 25 ±13 nm, and a ζ-potential of -36.6 ±3.2 mV was measured. In a set of parallel experiments, cysteine was added to Ag-NP test dispersions. With 5 and 10 µM of this ligand added, EC50, 1h-values were found to have

increased to 6.1 µg/L and 6.6 µg/L respectively. Navarro et al. (2008) concluded that the measured $Ag^+$ in Ag-NPs dispersions could not alone explain the toxicity observed, and that it therefore is likely that nanoparticles acted as a source for delivering the toxic $Ag^+$ ion formed when algae interact with the Ag-NP in the dispersion.

#### 3.5.3.4. Effects of Ag-NPs on the microbial community

Very few studies have investigated the effects of Ag-NPs on bacterial community composition. However, a Canadian team (Das et al. 2012a,b) has recently reported the loss of bacterial phenotype diversity during the exposure of natural bacterioplankton to Ag-NPs: Ag-NPs affect bacterioplankton community compositions, not just bacterioplankton metabolism and production. Consequently, emergence of rare bacteria phylotypes under high Ag-NPs exposure suggests that some bacteria could adapt to the conditions induced by Ag-NPs (Das et al. 2012b). Significant changes in abundance, function, and community composition of microorganisms following a single-dose Ag-NP treatment were also observed in a long-term terrestrial mesocosm field experiment (Colman 2013).

This effect on the bacterial community diversity has also been observed by Doiron et al. (2012), who reported on changes in marine bacterial richness in marine microcosms exposed to polymer-coated Ag-NPs and ionic silver. Other studies observed no (Bradford, 2009) or reduced effects (Colman 2012) in laboratory Ag-NP exposure experiments on stream water and sediment samples. However, such observations may be explainable by factors reducing silver toxicity in specific environments such as complexation, precipitation, aggregation, and low redox potential.

A similar effect on soil microbial community has also been reported (Gryndler et al. 2012; Kumar et al. 2012). In addition, the study of the effluent from a nanosilver producing washing machine indicated that the release exhibited negative effects on a natural bacterial community as its abundance was clearly reduced when exposed to the nanowash water (Farkas et al. 2011). Similar observations have been reported with metal oxide nanoparticles, CuO and $Fe_3O_4$, that also affected the soil bacterial community composition (Ben-Moshe et al. 2013).

### 3.5.4. Terrestrial systems - soils

A certain fraction of silver released from nanosilver applications will end up in soils, especially by sewage sludge applications. Organisms will then be exposed either by the water route (plant roots, microorganisms and microfauna living in and near the soil solution), via nutrition (microorganisms breaking down organic matter and animals feeding on them) or by body surface contact (Navarro et al. 2008). The latter is particularly relevant since the particles will mostly be sorbed at the soil matrix and most soil organisms (e.g. earthworms or nematodes) have a highly permeable skin, which is often also used for respiration (e.g. plant roots, nematodes, Collembola).

PLANTS

Citrate-coated Ag-NP adversely affected seedling growth of *Phaseolus radiatus* and *Sorghum bicolor* grown on agar at 5 mg/L and higher, with concentration-dependent bioaccumulation. However, growth reduction in soil was much less pronounced, significant effects of NP occuring only in *S. bicolor*. In tests with *Lolium multiflorum* seedlings on filter paper, gummi arabicum-coated Ag-NP and $AgNO_3$ inhibited growth at 40 mg/L, yet morphological damage occurred only for Ag-NP (Yin et al. 2011). Addition of cysteine reduced the toxicity of $AgNO_3$ but not of Ag-NP, which was explained by Ag-

NP dissolution inside the plant cells. El-Temsah and Joner (2010) regarded germination of ryegrass, barley and flax to be less sensitive to FeNP and Ag-NP than shoot growth. Tests in solutions showed particle size and plant-specific differences, with ryegrass being most sensitive (inhibitory effects on shoot length at 10 mg Ag/L). Unfortunately, particle size classes in this study were not properly characterized.

## SOIL ANIMALS AND MICROORGANISMS

Most of the few published studies on Ag-NP toxicity on terrestrial animals considered earthworms and nematodes (Tourinho et al. 2012; nematodes see under microfauna). 1000 mg Ag/kg soil affected survival of *Eisenia fetida* only when applied as $AgNO_3$, yet both salt and Ag-NP resulted in 100% reproductive failure (Heckmann et al. 2011). Schlich et al. (2012) studied the OECD standard particles NM-300K (uncoated, 15 nm, with each 4% of stabilising agents Tween-20 and polyoxyethylene glycerol trioleate). With these, the $EC_{50}$ for reproduction of *E. fetida* in RefeSol 01A ranged between 74 and 146 mg Ag / kg dry soil (compared to 42 and 47 mg/kg for $AgNO_3$). The authors also found decreasing bioaccumulation factors in earthworm tissue with increasing Ag concentrations irrespective of the Ag compound, yet silver contents of worms exposed to Ag-NPs were almost twice as high as in salt-exposed worms.

Soil type strongly affects Ag bioaccumulation and reproductive toxicity to *E. fetida* (Shoults-Wilson et al. 2011b). In this and another study (Shoults-Wilson et al. 2011a), $AgNO_3$ was eight times more toxic than PVP-Ag-NPs, and no effect of particle size or coating (PVP vs. oleic acid) was found. A most interesting study reported that *E. fetida* avoids soils contaminated with $AgNO_3$ or Ag-NP at environmentally relevant concentrations (around 10 mg/kg), which could not be explained by Ag dissolution or changes in the microbial community structure (Shoults-Wilson et al. 2011c). Gene expression patterns revealed that both PVP-Ag-NPs and $AgNO_3$ induced oxidative stress in *E. fetida*. Apparently most oxidation occurred after or during particle uptake (Tsyusko et al. 2012).

Soil microorganisms are highly sensitive to Ag-NPs, the effect increasing with decreasing particle size (e.g. Panacek et al. 2006, Radniecki et al. 2011). The nitrifier *Nitrosomonas europaea* was extremely sensitive to $AgNO_3$ and two size classes of Ag-NP, which decreased ammonia oxidation and destabilized the outer membrane (Radniecki et al. 2011). Toxicity to *N. europaea* was mainly related to colloidal stability and dissolution of Ag-NP, with PVP-coating rendering them less toxic than citrate or gum Arabic coating (Arnaout and Gunsch 2012). In a four-month incubation experiment of soil with Ag-NP (from a commercially available spray), soil microbial biomass was clearly reduced at a concentration as low as 3.2 µg Ag per g dry soil (Hänsch and Emmerling 2010). Bactericidal effects cannot solely be explained by $Ag^+$ dissolution and may be mitigated in the presence of organic compounds (Fabrega et al. 2009). Throbäck et al. (2007) observed that Ag+ reduced denitrification from soil microcosms together with an alteration of the microbial population. Murata et al. (2005) described the effect of silver concentration on the growth of soil microbial communities.

Unicellular eukaryotes and other microfauna appear to be much less sensitive to Ag-NP than bacteria, and more sensitive to ionic than to nanoparticular silver. The lethal concentration of Ag-NP against the protist *Paramecium caudatum* (25 mg/L) was 60 times higher than the corresponding concentration of ionic silver (Kvitec et al. 2009). This value is more than four orders of magnitude higher than bactericidal concentrations of the same particles (Panacek et al. 2006). Survival and reproduction of the nematode *Caenorhabditis elegans* were reduced at similar concentrations of citrate-coated Ag-NP (10 to 100 mg/L; Kim et al. 2012). The authors observed epidemic endema and burst but no particle intake. However, Meyer et al. (2010) observed growth inhibitions at

slightly lower concentrations of citrate- or PVP-coated Ag-NP, particle uptake and even transgenerational transfer. Part of the toxicity could be explained by ionic silver.

**CONCLUSION/SUMMARY OF AQUATIC ECOTOXICITY OF SILVER NANOPARTICLES**

Nanosilver undergoes several transformations when it is released into the environment. The important ones are dissolution and subsequent speciation, such as formation of silver chloride and sulphide. Silver sulphide is particularly important because it is highly stable and sulphide is available in wastewater treatment plants and also in many freshwater bodies. The chemical species that are actually present determine the bioavailability and toxicity of silver in the environment. A large fraction of the silver released to freshwater bodies sorbs to suspended particulate matter and is transferred to the sediments, where it may form reservoirs.

From the present literature, it is becoming evident that the coating of Ag-NPs plays a role in the toxicity expressed by the particles. The most commonly studied silver nanoparticles are stabilized with citrate or PVP. The presently published data on ecotoxicity of silver nanoparticles with citrate or PVP coatings ranks these (independent of size) as "very toxic to aquatic organisms" with EC50 or LC50 for fish, daphnia and algae below 1 mg/L.

As anticipated from the speciation of silver in water, dissolved silver (as ionic or complexed silver in the oxidation state +1) has in most cases higher toxicity than silver nanoparticles (in oxidation state 0). Based on the finding that Ag-NPs were less toxic than $AgNO_3$, Griffitt et al. (2008) suggest that regulations based on soluble metals may be adequate to protect aquatic life. In relation to this statement, it should be stressed that though release of dissolved silver in many studies has been found to be the main cause of toxicity, as described by Misra et al (2012), this release cannot alone account for the toxic effects observed. The assessment of effects of dissolved silver versus Ag-NPs is complicated by the fact that it is experimentally difficult to determine the factors controlling dissolution. In the literature there seems to be sufficient evidence to claim that size of Ag-NPs is inversely related to solubility. However, since sizes of Ag-NPs in dispersions are often controlled by surface modifications and dissolution is also dependent on media constituents, it is not straightforward to predict solubility of Ag-NPs in ecotoxicity tests. Hence even if the assessment of effects of Ag-NPs is done based on the dissolved concentration, this needs, at present, to be done on a case-by-case basis.

### 3.5.5. Bioaccumulation

Relatively few published studies have assessed the effects of Ag-NPs in environmental systems. Studies often report that bioacummulation was studied but in reality protocols followed do not allow for equilibrium to be reached and thus it is debatable if bioacummulation was in effect assessed (as per OECD bioaccumulation protocol approaches). Nevertheless, a few studies have attempted to assess uptake, translocation and depuration of Ag-NPs.

Studies by Garcia-Alonso et al. (2011) on the effects of citrate-capped 30-nm Ag-NPs on the marine invertebrate *Nereis diversicolor*, revealed that particles could be detected in the gut and lumen of the organism using TEM and x-ray diffraction epithelium exposed to nano-citrate-Ag-NP–spiked sediment (250 ng/g) for 10 d after a 3-d depuration period. Particle translocation across the intestinal wall was not documented in the present study. (Garcia-Alonso et al. 2011).

Another study by McTeer et al. (2013) assessed the bioavailability, toxicity, and transfer of Ag-NPs and $AgNO_3$ in two food chain organisms: the alga *Chlamydomonas reinhardtii* and *Daphnia magna*. The effects of phosphate, a potential $Ag^+$-binding ligand and a key

compound of phytoplankton productivity were evaluated. Ag-NP and $AgNO_3$ were found to accumulate at similar concentrations into microalgae during high phosphate treatment, but $AgNO_3$ accumulation was found to be higher in the low phosphate treatment. After feeding on Ag-exposed algae to either $AgNO_3$ or Ag-NP, there were no differences in the accumulation of silver by *D. magna* (McTeere et al. 2013).

The work by Coleman et al. (2013) described above indicated that Ag-NP coating and size had minimal impact on bioaccumulation potential as determined by BAF values. The authors indicated that their work suggests that Ag-NPs in sediment systems may result in bioaccumulation that is similar to micron-sized Ag. In contrast, in water-only exposures, the smaller Ag particles were bioaccumulated by *Lumbriculus variegatus* more readily than the larger Ag particles, although both particles were detected in tissues at 48 h with Field Flow Fractionation FFF-ICP-MS and SP-ICP-MS. Coleman et al. (2013) indicate the importance of incorporating particle sizing techniques in bioaccumulation assays in order to explore how NPs interact with biological systems (Coleman et al. 2013).

Another study (Yin et al., 2011) assessed the uptake and toxicity of Ag-NPs of different sizes to the common grass, *Lolium multiflorum* (seeds exposed via soaked filter papers). Results were compared with observed effects of $AgNO_3$. Results showed root and shoot Ag content increased with increasing Ag-NP exposures. Ag-NPs inhibited seedling growth, resulted in development failure and general malformations. In contrast, seedlings exposed to identical concentrations of $AgNO_3$ or supernatants of ultracentrifuged Ag-NP solutions showed no such abnormalities. Ag-NP toxicity was influenced by total NP surface area with smaller Ag-NPs (6 nm), and more strongly affected growth than did similar concentrations of larger (25 nm) NPs for a given mass. Cysteine was found to mitigate the effects of $AgNO_3$ but did not reduce the toxicity of Ag-NP treatments. The authors propose that observed effects on exposure to Ag-NPs can be directly attributed either to the nanoparticles or to the ability of Ag-NPs to deliver dissolved Ag to organisms (Yin et al. 2011).

Croteau et al. (2011) compared silver bioavailability and toxicity in the freshwater gastropod *Lymnaea stagnalis* after exposure to ionic silver and to Ag-NPs capped with citrate or with humic acid, and they found that silver form, exposure route, and capping agent influenced bioaccumulation dynamics. Snails accumulated Ag from all forms after either aqueous or dietary exposure. For both exposure routes, uptake rates were faster for $Ag^+$ than for Ag-NPs. In the diet, Ag-NPs affected digestion, which resulted in impaired growth. Depuration was higher for waterborne exposure of Ag-NPs when compared to dissolved $Ag^+$. Interestingly, depuration was slow after dietary exposures regardless of silver form, therefore leading to the view that diet may be an important exposure route in the long term (Croteau et al. 2011).

In summary, although it is still early days given the lack of relevant data, and also given current discussions at OECD regarding the appropriateness of current OECD protocols for the assessment of bioacummulation of nanoparticles (given that they have been devised for chemical substances that can be maintained in the steady state), it is clear that Ag-NPs are taken up by organisms, translocated from the gut, in certain conditions, and also depurated, to a point. There are also indications that in certain conditions body burdens are larger following exposures to Ag-NPs, compared to the equivalent levels of dissolved silver. It must be stressed, however, that much work is still required in this area of research.

## 3.6. Hazard related to bacterial resistance

### 3.6.1. Introduction

Silver salts and Ag-NPs are used in many applications for infection control, including dressings and compresses, silver and Ag-NPs containing "antimicrobial" surfaces (see section 3.2). The potential repeated exposures of bacteria to a low concentration of silver and Ag-NPs might be counterproductive; questions have been raised regarding the possibility of selecting less susceptible bacteria, and increased tolerance to silver has been documented (Maillard and Hartemann 2012). Based on a previous opinion on biocide effect on antibiotic resistance, the distribution and long-term exposure of Ag-NPs is likely to impinge on microbial adaptative response, and such a risk remains to be evaluated.

With the development of nanotechnologies and nanoparticles, novel applications based on the combination of Ag-NPs with antibiotics is being explored notably against multiple drug resistant (MDR) bacteria (Allahverdiyev et al. 2011; Huh et al. 2011; Rai et al. 2012). Limited results from clinical applications and combined anti-bacterial activity of nanomaterial-based or assisted antibiotics (nanoantibiotics) have been discussed (Huh et al. 2011). Some adverse effects have been observed with such combination of Ag-NPs/antibiotics (Bowler et al. 2012).

In summary, ionic silver has been used for a century mainly for disinfection. Ionic silver is still used in a number of specific medical applications. In addition, the number of applications containing Ag-NPs has dramatically increased over the last 10 years. The concentration used and possible long-term microbial exposure to these products are concerns in view of the possible development of bacterial resistance.

### 3.6.2. Bacterial Susceptibility to Silver and Ag-NP

Bacterial susceptibility and bioavailability

Ionic silver has a broad spectrum of antimicrobial activity against planktonic and sessile bacteria (Edward-Jones 2009; Percival et al. 2011). The activity of silver resides in ionic silver at a concentration of $10^{-9}$ to $10^{-6}$ mol/L, while $Ag^0$ is inactive.

There are an increasing number of reports on the bactericidal activity of Ag-NPs. In the literature it has been suggested that Ag-NPs have a higher bactericidal potency than ionic silver *per se* (Rhim et al. 2006; Lok et val. 2006; Fernandez et al. 2010a,b; Marambio-Jones and Hoek 2010; Su et al. 2011). Ag-NPs have also been reported to have activity against bacterial biofilms (Kostenko et al. 2010; Huang et al. 2011). The bactericidal activity of Ag-NPs can be improved with combination with polymers such as chitosan and cationic polysaccharide (Banerjee et al. 2010).

The anti-microbial activity of ionic silver and Ag-NPs depends upon its bioavailability and the type of target microorganisms. The limitations of ionic silver used for the treatment of burn wounds are the lack of penetration and neutralisation with organic matter; both these effects have been well reported. It has been estimated that the maximum concentration of available ionic sliver attainable in wounds is 1 µg/mL (Maillard and Denyer 2006b). As a result ionic silver is unlikely to eliminate bacteria already colonizing the wound.

Bioavailability can be dramatically altered by complexation, sorption and precipitation, e.g. in the presence of chloride, sulphide and phosphate, and organic matter (Xiu et al. 2012). In contrast, its activity is not severely affected by dilution (the concentration exponent of silver nitrate is 1).

Other factors affecting biocidal activity during usage

Ionic silver activity is modestly affected by temperature or a change in pH. Increase in temperature increases activity. Activity of ionic silver increases at alkaline pH, but it should be noted that some combinations such as silver sulfadiazine is unstable at alkaline pH (Maillard and Denyer 2006b). Duration of exposure is also an important parameter to consider for long-term usage devices. The use of silver in endotracheal tube was shown to prevent biofilm formation for a few days (Berra et al. 2008; Roes et al. 2008) but not in longer use (Olson et al. 2002).

Water hardness and notably the presence of divalent cations affect the bactericidal efficacy of Ag-NPs against Gram-positive bacteria in a liquid environment, possibly through an increase size of nanoparticle aggregates and perhaps a reduced binding to the bacterial surface (Jin et al. 2010). However, the presence of divalent cations seem to increase the efficacy of Ag-NPs against Gram-negative bacteria, possibly by increasing the interaction and 'local concentration' of the negatively charged silver-nanoparticle and the negatively charge lipopolysaccharide layer (Jin et al. 2010).

Biofilms of *E. coli* were found to be four times more resistant to nanosilver inhibition than planktonic cells. Nanoparticle aggregation and retarded biosorption were suggested to be responsible for some of the increase in resistance (Choi 2010). Park et al. found that Ag-NPs were taken up to a lesser extent than silver ions by *Pseudomonas aeruginosa* biofilms resulting in a reduced biofilm-inactivating efficiency of Ag-NPs compared to silver ions (Park 2013). The efficiency of Ag-NPs was significantly enhanced by stirring, which caused an increase in Ag-NP biosorption by biofilms.

The size and morphology of nanoparticles affect biocidal efficacy (Lok et al. 2007; Pal et al. 2007; Samberg et al. 2010). The nanosize scale of Ag-NPs affects the surface area of the particle that is in contact with the target bacteria. Truncated triangular nanoplates, with a high number of {111} facets were shown to be highly reactive (Pal et al. 2007). It has recently been suggested that Ag-NPs properties such as size, shape, surface coating and surface charge will affect the rate, location and/or timing of ionic silver release (Xiu et al. 2012).

In silver-impregnated dressing, the release of ionic silver is linked to the level of hydration (Lansdow et al. 2005). The release of ionic silver also depends on the polymer matrix used (Monteiro et al. 2009).

### 3.6.3. Mechanistic considerations

Ionic silver is generally considered to interact with multiple microbial target sites (Russell and Hugo 1994). Its antimicrobial activity results from its combination with, and alteration of, microbial proteins, with eventually structural and metabolic disruption (Maillard and Denyer 2006a; Silver et al. 2006). One of the major target sites for ionic silver is at the bacterial cell membrane level, where it can inhibit the proton motive force and the respiratory electron transport chain, and affect membrane permeability resulting in bacterial cell death (Percival et al. 2005; Silver et al. 2006; Edwards Jones 2009; Randall et al. 2013). It has been suggested that once the ionic silver has penetrated within the bacteria, recovery is improbable. The presence of moisture is required for the penetration of ionic silver within the bacteria, which highlights some potential issue with 'dried' silver containing 'antimicrobial' surfaces.

The microbicidal mechanism of action of Ag-NPs has been generally less studied compared to ionic silver. Different mechanisms have been described, some linked to direct membrane interaction, other with the generation of ionic silver (McQuillan et al. 2011). The mechanism of action of Ag-NPs has been linked to their ability to generate more ionic silver and to increase the production of reactive oxygen species (Xu et al.

2012), notably when combined to halides. Ag-NPs may also deliver more efficiently ionic silver to small surfaces (Wijnhoven et al. 2009b). Su et al. (2009) demonstrated that bactericidal effect of Ag-NPs immobilized on a surface was caused by the loss of membrane integrity due to reactive oxygen species, while the energy-dependent metabolism was inhibited. Similar findings were observed by Miyoshi et al. (2010) who studied Ag-NPs prepared on montmorillonite clay. In these cases, where Ag-NPs are immobilized on surfaces, the release of ionic silver from Ag-NPs does not appear to be linked with the bactericidal effect of Ag-NPs (Su et al. 2009; Miyoshi et al. 2010).

Ag-NPs (10 nm) were found to destabilise the bacterial outer membrane and release intracellular potassium, although the precise mechanisms remain unknown (Lok et al. 2006). Furthermore, Ag-NPs was shown to elicit a rapid collapse of the proton motive force and decrease cellular ATP level, presumably following the collapse of membrane potential (Lok et al. 2006). Both ionic silver and Ag-NPs seem to share a similar membrane-targeting mechanism of action, although Ag-NPs are more efficient with effective concentration in the nanogram range in comparison to the microgram range for ionic silver (Lok et al. 2006). Sondi et al. (2004) observed the accumulation of Ag-NPs in *E. coli* membrane with subsequent damage to the bacterial membrane. Xu et al. (2004) reported that Ag-NPs with size ranging up to 80 nm can accumulate within the bacteria (*Ps. aeruginosa*) and such accumulation might be related to membrane permeability. It was also found to depend on the activity of efflux pump MexAB-OprM. It should be noted that in this study, picogram concentration of Ag-NPs were used.

There might also be some important differences in interactions of Ag-NPs between Gram-negative and Gram-positive bacteria; glutathione-coated Ag-NPs were shown to penetrate the bacterial cytoplasm of *E. coli* resulting in an increased antimicrobial effect, while in *S. aureus*, the lack of penetration means an interaction limited to the bacterial surface (Taglietti et al. 2012).

Ag-NPs microbicidal mechanisms of action seems to depend on size, although conflicting data have been reported on what size range is responsible for conferring a bactericidal effect (Xu et al. 2004; Morones et al. 2005). Ag-NPs up to 80 nm can penetrate the inner and outer bacterial membrane (Xu et al. 2004), while Ag-NPs of less than 10 nm diameter cause cytoplasmic leakage by forming pores on the bacterial cell wall, but do not affect extracellular proteins or the bacterial nucleic acid (Gogoi et al. 2006).

Ag-NPs have been shown to affect bacterial motility and chemotaxis in *Pseudomonas putida* (Jimenez-Sanchez et al. 2012) and in *Bacillus subtilis* (Babu et al. 2011).

The production of free radicals in combination with $H_2O_2$ is also another way to enhance bactericidal activity for disinfecting inanimate surfaces or water (Hartemann et al. 1995; Xu et al. 2012). Likewise, a combination of nanosilver and iodine has been shown to damage bacterial cell wall and produce reactive oxygen species causing oxidation damage in the bacterial cell cytoplasm leading to bactericidal effect (Banerjee et al. 2010).

In conclusion, there is a limited number of papers describing the mechanisms of biocidal action of Ag-NPs and ionic silver. Some information from the literature is also conflicting. There is some evidence that ionic silver reacts with key proteins leading to structural and metabolic disruptions. A direct effect on the bacterial membrane permeability/stability has been proposed as an Ag-NPs-specific mechanism (Sintubin et al. 2011; Du et al. 2012). Although Ag-NPs particle properties may account for some bacterial toxicity effect, it has been suggested that the main factor conferring a bactericidal activity of Ag-NPs resides with the release of silver ions (Xiu et al. 2012). Currently, there is no consensus on the molecular mechanisms of interaction of Ag-NPs and ionic silver with bacteria.

### 3.6.4. Bacterial Resistance to Silver and Ag-NP

*In situ* evidence of bacterial resistance to ionic silver

Silver resistance in *Pseudomonas aeruginosa* associated with burn wounds was first reported by Cason et al. (1966). The development of bacterial resistance to silver nitrate (Cason et al. 1966; Cason and Lowburry 1968) prompted a change in formulation and the use of silver sulphadiazine - a combination of silver and sulphonamide (Fox 1968; Modax and Fox 1974; Modak et al. 1988). In the 1970s, outbreaks of burn wound infection or colonization by Gram-negative isolates resistant to ionic silver and silver sulphadiazine were reported for a number of bacteria; in *Enterobacter cloacae* (Gayle et al. 1978), *Providencia stuartii* (Wenzel et al. 1976), *Pseudomonas aeruginosa* (Bridges et al. 1979) and *Salmonella typhimurium*(Mchugh et al. 1975). Bacterial resistance to silver sulphadiazine developed rapidly mainly because of the antibiotic component (Klasen 2000).

A study involving 30 patients with chronic leg ulcers showed that all infections other than a single strain of Enterobacter cloacae were sensitive to 1mM of silver nitrate. They suggested that prolonged exposure to silver dressings does not lead to silver-resistant bacteria (Lansdown and Williams 2007).

Recently microbial flora from chronic leg ulcers were cultured following 3 weeks treatment with silver-based dressings, and genotypic and phenotypic analysis of 56 isolated were investigated further. The silver-based dressings were shown to have little activity against primary wound pathogens and one silver-resistant *E. cloacae* (MIC > 512 mg/L) harboured the following silver resistance determinants: *silE*, *silS* and *silP* (Sutterlin et al. 2012). Additonal *in vitro* investigations highlighted that silver-resistance was more frequent in cephalosporin-resistant isolates (Sutterlin et al. 2012).

*In vitro* evidence of bacterial resistance to ionic silver

Exposure to silver may contribute to the selection of bacteria that are intrinsically resistant to silver (Wenzel et al. 1976; Bridges and Lowburry 1977; Haefeli et al. 1984; Silver 2003; Davis et al. 2005). Emerging silver-resistance from environmental bacterial isolates has been documented in Enterobacteriaceae (Hendry et al. 1979; Kaur and Vadehra 1986; Starodub and Trevors 1989, 1990) and in *Acinetobacter baumanii* (Deshpande and Chopade 1994) under experimental conditions.

In the laboratory, high-resistance to silver (>1024 ppm) in *Escherichia coli* has been produced following repeated exposure to increasing concentration (i.e. step-wise training (Li et al. 1997).

Mechanisms of bacterial resistance to ionic silver

Although there are many standard efficacy tests to determine the efficacy of different forms of silver against bacteria, these tests rely on the use of large bacterial population (millions of bacteria). There are no standardised tests to investigate specific interactions of ionic silver and Ag-NPs or other biocides with a single bacterium.

The main mechanisms conferring silver resistance involve (i) reducing ionic silver penetration via a non-specific transporter, such as the ATPase CopB (Nies 1999); (ii) reducing accumulation (i.e. increasing in silver efflux)(Silver 2003); and (iii) reducing its concentration by increased neutralization and reduction of ionic silver to the inactive metallic form (Nies 1999).

For example, in a silver-resistant *Escherichia coli* produced by stepwise training, active efflux and outer membrane protein changes accounted for the high resistance of the

strain to ionic silver (Li et al. 1997). Kaur and Vadehra (1986) observed a similar ionic silver uptake between a *Klebsiella* strain resistant to silver (70 µg/mL) compared to an ionic silver-sensitive parent strain (10 µg/mL). Since the ionic silver uptake of spheroplasts of both strains was also similar, the difference in susceptibility was attributed to a change in bacterial cell membrane composition. The activity of succinate dehydrogenase was also reduced in the silver-resistant strain (Kaur and Vadehra 1986). Starodub and Trevors (1989, 1990) observed differences in ionic silver binding to and accumulation in *Escherichia coli*, between a silver-resistant isolates (1 mM silver nitrate) and a silver-sensitive construct derived from the isolate cured of its plasmid conferring silver resistance. The formation of an inactive, insoluble silver sulphide following the chelation of silver by the sulfhydryl groups of metal-binding proteins has also been described (Liau et al. 1997), while exopolysaccharide could be involved in reducing the concentration of ionic silver (Miao et al. 2009).

Bacterial adaptation to Ag-NPs

The nanoparticles associated with the bacterial cell wall, appearing to interact with the outer and inner membranes, and then dissolve to release $Ag^+$ into the bacteria and affect a transcriptional response enhancing the silver ion stress response in *E. coli* (McQuillan et al., 2011). When naphthalene-degrading *Pseudomonas putida* cells were exposed to Ag-NPs they exhibited tortuous movement (motility) and a repellent response (Jimenez-Sanchez et al., 2012). Proteomic analysis showed induced expression of a number of envelope proteins (OmpA, OmpC, OmpF, OppA and MetQ) in *E. coli* as a result of Ag-NPs (10 nm) exposure (Lok et al. 2006).

Silver nitrate treatment of *Bacillus cereus* induced a reduction in their motility and chemotactic activity compared to the control (unexposed) bacteria. In addition, global transcriptomic analysis reported that about 10% of the genes (included in the microarray) representing chaperones, transporters, membrane proteins, replication, etc, were differently regulated following exposure (Babu et al. 2011).

A recent study tried to determine the effect of colloidal silver present in orange juice dispersion against bacteria using a simulated digestion protocol (a model using enzymes, buffers and conditions to mimic digestion). When planktonic bacterial cultures and biofilms (obtained with a prototypical *E. coli* strain that is present in the GI tract) were exposed to this material, differences in antibacterial action were observed between liquid cultures versus biofilms, digested versus undigested silver, and differences between digested silver nitrate versus silver colloids (Sooresh et al. 2012).

In conclusion, these data clearly demonstrated the effect of Ag-NPs on the distribution of bacterial flora and on the bacterial adaptation in certain conditions and uses; however, these data are fragmentary and focused on few specific cases.

Ag-NPs might be effective against ionic silver–resistant bacteria although such activity against silver resistant bacteria needs to be better explained. Furthermore, bacterial resistance to Ag-NPs has been observed as well (Lok et al. 2007; Samberg et al. 2010; Hsu et al. 2010). Overexpression of detoxification enzymes and membrane repair related proteins have also been suggested as mechanisms involved in Ag-NPs resistance (Gou et al., 2010). A reduction of Ag-NPs penetration or accumulation might be explained partly by the interaction of Ag-NPs with the outer membrane of Gram-negative bacteria: the presence of lipopolysaccharide might induce electrostatic repulsion with negatively charged silver nanoparticles (Costerto et al. 1974) while phosphomonoester function group and carboxyl groups could complex with ionic silver (Guine et al. 2006).

Recently, Xiu et al. (2012) observed that a low concentration of various Ag-NPs increased the viability of resting *E. coli*, and suggested that these observations resulted from bacterial exposure to released ionic silver concentrations (ranging from 3-7.9 µg/L) which might have enhanced bacterial fitness (Ag-NPs studied: PEG-Ag-NPs-3nm, 2.2 mg/L;

PEG-Ag-NPs-5 nm, 1.8 mg/L; PEG-Ag-NPs-11 nm, 2 mg/L; PVP-Ag-NPs-20 nm, 16.4 mg/L; PVP-Ag-NPs-40 nm, 5.7 mg/L; PVP-Ag-NPs-80 nm, 6.7 mg/L). Schacht et al. (2013) reported that Ag-Nps (≥20-80 µg/mL) extended the lag phase of *Cupriavidus necator*, but bacteria treated with Ag-NPs 20-60 µg/mL resulted in higher maximum growth rates despite initial repression of growth.

Transcriptome analysis of *E. coli* exposed to Ag-NPs revealed that copper/silver resistance genes including *cusBCF*, *copA* and *cueO* were unregulated, together with genes involved in anaerobic respiration, decreasing oxidative damage (Du et al., 2012). In addition, it was found that the deletion of *fnr*, *fdnH* and *narH*, genes involved in intracellular oxygen availability and redox and anaerobic respiration related enzymes increased resistance against Ag-NPs (up to 500 µg/mL), while deletion of *acrA* and *glpB* increased bacterial susceptibility to Ag-NPs (Du et al. 2012).

Genetic basis of ionic silver resistance in bacteria

Ionic-silver resistance in bacteria has been often found to be encoded on plasmid (McHugh et al. 1975; Gupta et al. 2001; Davis et al. 2005; Merlino and Kennedy 2010) and has been described in a number of Gram-negative bacteria such as *P. aeruginosa*, *Pseudomonas stutzeri*, *Citrobacter* spp., *Serratia marcescens* and *Salmonella enterica* serovar Typhimurium (Silver et al. 2006). It has also been described on chromosome (Silver and Phung 1996, 2005; Gupta et al. 2001).

In ionic silver-resistant *S. enterica Typhimurium* isolated from a burns unit, ionic-silver resistance was encoded on the plasmid pMG101, also conferring multi-drug resistance (Silver 2003). The plasmid contained *silCBA* that encodes a resistance nodulation division (RND) efflux pump with homologues to that of AcrB in *E. coli* (Silver 2003), *silE* encoding for periplasmic silver-binding protein, SilE which binds ionic silver (Silver et al. 2006). SilA is an inner membrane cation pump protein while SilC an outer membrane protein (Silver et al. 2006). The silver resistance determinant has been described as unique among resistance systems since it encodes for two energetically distinct efflux pumps (Silver et al. 2006). The *sil* genes have been found to occur only on IncH incompatibility group plasmids (Silver et al. 2006). The occurrence of silver encoded plasmid in enteric bacterial isolates from a hospital was found to exceed 10% (Silver 2003). In *A. baumanii* silver-resistance encoded on a 54kb plasmid was transferred successfully to *E. coli* by conjugation. The transformed *E. coli* cells were shown to be more efficient than efflux accumulated silver ions (Deshpande et al. 1994).

In conclusion, the genetic basis of the resistance against ionic silver in bacteria has been well documented, notably the gene expression of well-characterised efflux systems. There is however a paucity of information on the resistance mechanisms to Ag-NPs. Exposure to ionic silver and Ag-NPs induces a stress-response and affects gene expression. More data is needed to better understand bacterial responses to ionic silver and Ag-NPs exposure.

Dissemination of resistance factors against silver

Plasmid-mediated metallic salt resistance is associated with co-resistance to chemotherapeutic antibiotics (McHugh et al. 1975; Gupta et al. 1999b) and as a result, silver resistance might persist in the clinical setting (Gupta et al. 2001; Johnson et al. 2006; Sutterlin et al. 2011; Stokes and Gillings 2011; Sandegren et al. 2012; Kremer and Hoffmann 2012)

Loh et al. (2009) reported that the presence of silver-resistance genes in methicillin-resistant *Staphylococcus aureus* (MRSA; 33 isolates) and methicillin-resistant coagulase-negative S *aureus* (MR-CNS; 8 isolates) isolated from wounds and nasal cavities in human and animals was low (2/33 MRSA and 1/8 MR-CNS) and restricted to a single gene (*silE*). In addition isolates with the *silE* genes remain susceptible to a silver-

containing hydrofiber wound dressing (Loh et al. 2009). Another study investigating the presence of silver-resistance genes in 172 isolates from human (112) and equine chronic wounds (60) reported that only 6 isolates, all *Enterobacter cloacae* (2 from human and 4 from horses), possessed the resistant *sil* gene cassette (Woods et al. 2009). All the silver-resistant genes were present extrachromosomally. The *sil* gene cassette in these isolates conferred a resistance of > 5 mg/L MIC, compared to a 1.25 mg/L in the *sil*-negative strains. It was further reported that a silver-containing dressing kills the *sil*-positive and -negative strains within 30 min, although the *sil*-positive strains were overall more resilient to the silver dressing (Woods et al. 2009)

Expression and over-expression of multidrug resistance efflux pumps and changes in bacteria outer membrane composition are linked to cross-resistance to unrelated antimicrobials (Maillard and Denyer 2009). This is a common mechanism for developing resistance.

The antibacterial activity of ionic silver and nanosilver, associated with the transfer of resistance mechanisms reported for silver alone suggests that genetic mobile elements (GME) represent a possible dissemination way for nanosilver resistance. One example is the description of an MDR strain, *Salmonella Typhimurium* exhibiting original antibiotic and metal ion insusceptibility pattern: resistant to silver nitrate, mercuric chloride, ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamides (McHugh, et al., 1975). The plasmid that contributes to this large MDR phenotype has been recently used for screening the antibacterial activity of silver complexes. Wright et al., (2012) reported that this GME is able to confer a reduced susceptibility against various silver carbapen complexes derivatives to a susceptible strain. This suggests that dissemination of SCC resistance could be favoured by the associated resistance gene targeting other antibacterial agents. The silver resistance mechanisms present in this plasmid comprise two efflux pump systems and silver binding proteins (Gupta et al. 1999b). At this moment, no specific studies have been carried out regarding the effect of membrane structural changes (LPS or OMP alterations) on Ag-NPs activities and the relationships between dissemination of plasmids involved in these alterations or strains exhibiting these modifications and the Ag-NPs resistance.

Co-/cross-selection of resistance:

Mühling et al. (2009) evaluated the potential link between environmental pollution by Ag-NPs and antibiotic resistance in a long-term microcosm exposure experiment in a coastal marine environment and observed no co-selection of antibiotic resistance (Mühling et al. 2009). However, this result could be due to factors reducing silver bioavailability in specific environments such as salinity and organic matter content leading to aggregation and complexation. A recent review by Reidy et al. (2013) and Mijnendonckx et al. (2013) raise concerns that the unrestricted use of nanosilver will drive the further generation and spread of antibiotic resistance in human pathogens and call for risk—benefit analysis for all Ag-NP applications.

### 3.6.5. Conclusions

Some of the genetic basis of bacterial resistance to ionic silver has been well documented, notably the expression of well-characterised efflux systems. There is a paucity of information on the resistance mechanisms to Ag-NPs, although recent transcriptomic and proteomic data suggest that a decrease in oxidative damage by regulation of anaerobic respiration is important. Exposure to ionic silver and Ag-NPs produces a stress-response and affects gene expression. More data is needed to better understand the bacterial response to ionic silver and Ag-NPs exposure. Regarding the hazard associated with the dissemination of the resistance mechanism following the use of Ag-NPs, no documentation is available at this moment and this represents a serious gap in knowledge.

These data clearly demonstrated the effect of Ag-NPs on the distribution of bacterial flora and on the bacterial adaptation in certain conditions and uses; however, these data are fragmentary and focused on few specific cases. Silver and nanosilver can contribute to the dissemination of resistance genes, and there is a growing body of evidence of such dissemination and the presence of silver-resistance genes in the environment."

### 3.7. General Conclusions

Silver compounds have been used for centuries in health care products as an antiseptic.

There is a multitude of synthesis methods for nanosilver that can be grouped under conventional and unconventional methods. Conventional synthesis methods include the use of citrate, borohydride, two-phase (water-organic) systems, organic reducers, and inverse micelles in the synthesis process. Unconventional methods include laser ablation, radiocatalysis, vacuum evaporation of metal, and the Svedberg method of electrocondensation. With this advancing variety of methods for the production of nanosilver, its applications are likewise increasing. However, this variety in production methods will also result in a variation of the quality of the Ag-NP used in products.

Currently, the use of silver in consumer products – including nanosilver - is increasing. Most applications claim to have hygienic and/or disinfecting properties on the skin, hair, surfaces, equipment and water; others refer the capacity to reduce odours from clothing. It was estimated that globally the total biocidal silver is approximately 0.5% of the total silver use. In textiles the use of silver is estimated as 0.1% of the global total silver use. Of the silver used in textiles, the Ag-NP constitutes only a fraction of approximately 10%.

In healthcare the use of silver compounds is seen as an antimicrobial barrier to reduce the risk for infection and re-infection of wounds. Silver can also be found in dental materials (amalgams, cements) used in dental restorations. Here silver is thought to have an anti-bacterial activity, reducing caries.

At the end of the life cycle, products become waste material. Quantitative data for the end-of-life phase of products containing Ag-NP is very limited and it needs further research. It is assumed that existing waste handling infrastructures would be used for nanomaterial products in an analogous way as conventional products.

All non-recycled waste will end up in the environment. Some of this waste is subjected to incineration. Nothing is known on actual incineration measurements of nanosilver-containing products but depending on the size of nanomaterial, models predict a release of 25 to 100% of air-borne NPs which are effectively caught by the filter systems.

Silver compounds in textiles and cosmetics will end up in wastewater treatment plants. Silver release from wastewater treatment plants to ground and surface waters is expected to be low, but a certain fraction of silver will pass treatment plants and reach surface water bodies. Concentrations will be low, toxic effects to aquatic organisms are unlikely, but cannot be excluded.

The exposure to any substance including nanomaterials is dependent on the amount present in a product and the possibility for release or leakage out of the product. To estimate exposure levels, information is needed on the concentrations of Ag-NP in the product, the size and the form in which it is present (aggregates, agglomerates) and the probability of release of (nano) $Ag^+$ from the product. Measurements of nanomaterials in consumer products are therefore urgently needed. In order to evaluate the potential risk the simple categorization of exposure possibilities to Ag-NPs in products as proposed by Hansen et al., (2008) may be helpful. The categorization includes: *Expected to cause exposure, May cause exposure, and No expected exposure to the consumer.* The type of product (spray, liquid, solid) largely determines the risk for exposure. However, reported

levels of silver release from silver-containing consumer products in different studies are inconsistent, which is probably due to differences in the methodology used.
Occupational exposure to silver and silver particles – mainly via airborne material – has not been studied in full detail. A further detailed description of the occupational exposure is needed in order to perform an occupational risk assessment.

Bioavailability of silver after oral administration of Ag-NPs in rats was shown and in one study it was suggested to be in the range of 1-4 % of the oral dose. Also after inhalation exposure, silver uptake was demonstrated in view of the presence of silver in various tissues, but after inhalation uptake via the gastrointestinal tract cannot be excluded.

The main target organs for Ag-NP distribution after systemic availability are spleen, liver and kidney, whereas there is less distribution to other organs. Also in the testes sometimes high levels of silver were noted. Nonetheless, although with low levels, Ag distribution was demonstrated to most major organs including the brain. For distribution of silver to the brain it is not clear whether the silver is present in the brain tissue or limited to the endothelium of the brain. Recent data indicate that some persistence of Ag may occur in brain and testes although its significance for toxicity is not known. There is some evidence that ionic Ag may form Ag particles at the nanoscale *in vivo*. Sex differences (females having higher Ag levels) were observed in several papers investigating different routes of administration i.e inhalation and oral administration. In view of the presence of Ag in feces after IV and SC administrations, there are indications for biliary excretion of Ag originating from administered Ag-NP.

A limitation of toxicokinetics studies of nanosilver is that most studies used ICP-MS or AAS for detection of the Ag, so it cannot be definitively concluded that Ag-NP are distributed to the organs.

In laboratory studies, the genotoxic effect of nanosilver was demonstrated. However, the genotoxic effects of Ag-NPs seen *in vitro* may also be a consequence of effects seen only *in vitro* and probably depend on Ag-NP coating and cell type. Also the way the cell exposure is performed probably affects the results. Pre-dispersion in medium before exposure may result in initial dissolution of the Ag-NPs, so that more soluble $Ag^+$ is present from the beginning, especially in short-term exposure assays (e.g. two hours). Genotoxicity studies in vivo are few and concern Ag-NPs of variable characteristics. The in-vivo genotoxic studies could not confirm or negate the genotoxicity of Ag-NP. Further studies are required to conclude whether Ag-NPs could be genotoxic *in vivo*.

Silver and nanosilver are clearly shown to have toxic potential although toxicity in general in humans seems to be low. In *in-vitro* studies Ag-NPs are cytotoxic and have genotoxic DNA-damaging capacity, and show developmental toxicity. Although Ag uptake and possible persistence in the testes was observed, histopathology did not reveal specific testicular toxicity. Liver toxicity is indicated by the effect of Ag-NPs on various liver enzymes. *In-vivo*, effects on the immune system were observed both regarding allergy to Ag itself, but also in repeated dose toxicity studies regarding effects on cytokine production and on non-specific immune responses like natural killer cell activity. These immune effects warrant further studies to the functionality of the immune system after exposure to Ag-NPs.

Nanosilver undergoes several transformations when it is released to the environment. The important ones are dissolution and subsequent speciation, e.g. formation of silver chloride and sulphide. Silver sulphide is particularly important because it is highly stable and sulphide is available in wastewater treatment plants and also in many freshwater bodies. The chemical species that are actually present determine the bioavailability and toxicity of silver in the environment. A large fraction of the silver released to freshwater bodies sorbs to suspended particulate matter and is transferred to the sediments, where it may form reservoirs.

From the present literature, it is becoming evident that the coating of Ag-NPs plays a role in the toxicity expressed by the particles. The most commonly studied silver nanoparticles are stabilized with citrate or PVP. The presently published data on ecotoxicity of silver nanoparticles with citrate or PVP coatings ranks these (independent of size) as "very toxic to aquatic organisms" with EC50 or LC50 for fish, daphnia and algae below 1 mg/L.

As anticipated from the speciation of silver in water, dissolved silver (as ionic or complexed silver in the oxidation state +1) has in most cases higher toxicity than silver nanoparticles (in oxidation state 0). Based on the finding that Ag-NPs were less toxic than $AgNO_3$, it appears that regulations based on soluble metals may be adequate to protect aquatic life. In relation to this statement, it should be stressed that though release of dissolved silver in many studies has been found to be the main cause of toxicity, an increasing number of studies find that this release cannot alone account for the toxic effects observed. The assessment of effects of dissolved silver versus Ag-NPs is complicated by the fact that it is experimentally difficult to determine the factors controlling dissolution. In the literature there seems to be sufficient evidence to claim that size of Ag-NPs is inversely related to solubility. However, since sizes of Ag-NPs in dispersions are often controlled by surface modifications and dissolution is also dependent on media constituents, it is not straightforward to predict solubility of Ag-NPs in ecotoxicity tests. Hence even if the assessment of effects of Ag-NPs is done based on the dissolved concentration, this needs, at present, to be done on a case-by-case basis. There is much debate in the literature regarding the adverse effects caused by Ag-NP exposure. Although it is generally accepted that dissolution of Ag-NPs does account for at least a degree of toxicity observed under Ag-NP exposure, effects cannot always be fully apportioned to the measured dissolved fraction of silver. It is now becoming progressively evident that although certain Ag-NPs may show low solubility in certain media and conditions, following contact with biological receptors there may be release of ions, which may be sustained over a long period. Two important points need to be taken into consideration. First, not all conventional methods used to assess Ag-NP solubility are able to reflect $Ag^+$ availability and, second, assessing the dynamic interactions between Ag-NPs and biotic receptors, including the sustained delivery of $Ag^+$ is very complex.

There are a limited number of papers describing on the mechanisms of biocidal action of Ag-NPs and ionic silver. Some information from the literature is also conflicting. There is some evidence that ionic silver reacts with key proteins leading to structural and metabolic disruptions. A direct effect on the bacterial membrane permeability/stability has been proposed as an Ag-NPs-specific mechanism. Although Ag-NPs particle properties may account for some bacterial toxicity effect, it has been suggested that the main factor conferring a bactericidal activity of Ag-NPs resides with the release of silver ions. Currently, there is no consensus on the molecular mechanisms of interaction of Ag-NPs and ionic silver with bacteria.

Some of the genetic basis of bacterial resistance to ionic silver has been well documented, notably the expression of well-characterised efflux systems. There is a paucity of information on the resistance mechanisms to Ag-NPs, although recent transcriptomic and proteomic data suggest that a decrease in oxidative damage by regulation of anaerobic respiration is important. Exposure to ionic silver and Ag-NPs produces a stress-response and affect gene expression. More data is needed to better understand bacterial response to ionic silver and Ag-NPs exposure. Regarding the hazard associated with the dissemination of resistance mechanism following the use of Ag-NPs, there is growing evidence that this may be the case although more data are needed to ascertain how widespread and common this dissemination is.

Limited data also demonstrates the effect of Ag-NPs on the distribution of bacterial flora and on the bacterial adaptation in certain conditions.

## 3.8. Risk Assessment

### 3.8.1. Existing evaluations

A No Observable Adverse Effect Level (NOAEL) for humans of 0.39 mg / person/ day, (corresponding to 6.5 µg/kg bw/day, considering an adult of 60kg bw) is considered by the World Health Organization (WHO 2004). Based on these values, WHO concluded that silver levels of up to 0.1 mg/L can be tolerated in drinking water when silver compounds are used to maintain the microbiological quality of the water (WHO 2004, Guidelines for Drinking Water-Quality, Third Edition). Similarly, EFSA recommends not exceeding the group specific migration limits of 0.05 mg/L and 0.05 mg/kg in water and food, resp., based on the same information (EFSA 2004, 2005, 2006. Opinion on a 4th, 7th, 12th list of substances for food contact materials).

In the US, EPA recommends a standard of 0.1 mg/L (100 ppb) in drinking water, approximately resulting in a total absorbed dose of half the NOAEL after 70 years (Silvestry-Rodriguez et al, 2007).

For chronic oral exposure to silver, the US EPA has derived a Reference Dose (RfD) of 5 µg/kg bw/d. This value was based on the LOAEL of 1 g (total dose) observed for argyria in humans following therapeutic iv application and factors for oral absorption (4 %), body weight (70kg), lifelong exposure (70 years / 25550 days) and LOAEL-to-NOAEL extrapolation (3) (US EPA Integrated Risk Information System, Substance No. 0099, Reference Dose for Chronic Oral Exposure (RfD): Silver, 10-01-2008).

For occupational inhalation exposure situations, MAK (*Maximum Workplace Concentration)* values of 0.1 mg/m$^3$ and 0.01 mg/m$^3$that exist for metallic silver and silver salts respectively were set in Germany and Switzerland to protect against argyria (DFG/Deutsche Forschungsgemeinschaft: MAK- und BAT-Werte-Liste 2006; Suva/Schweizerische Unfallversicherungsanstalt: Grenzwerte am Arbeitsplatz 2007). These values are identical with Threshold Limit Values (TLVs) proposed by ACGIH (ACGIH 1986/Ex. 1-3), while NIOSH and OSHA derived identical values of 0.01 mg/m$^3$ for silver dust and soluble silver.

Under the U.S. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the U.S. EPA has recently evaluated an explicitly nanosilver pesticide for use as textile preservative (EPA-HQ-OPP-2009-1012-0064). As a number of data gaps were identified, authorisation of the substance was granted conditionally with the applicant having to implement a 4-year testing programme. Tier 1 of testing included, amongst other studies, subchronic oral and inhalation toxicity testing as well as a screening assay for reproductive / developmental toxicity with the specific material. To complete a provisional risk assessment, US EPA used reference values obtained in studies with other nanosilver materials: an inhalation NOAEC of 133 µg/m$^3$ based on a 90-day study in rats (Sung et al. 2009), an oral NOAEL of 0.5 mg/kg bw/d based on a 28-day study in mice (Park et al. 2010), and route-to-route extrapolation from the oral to the dermal route. An additional uncertainty factor of 10 for quality of database was included, resulting in a reference margin-of-exposure (MoE) of 1000 and 3000 for incidental and chronic exposures.

Finally, various silver-containing active substances (SCAS) are currently assessed according to the European Biocides Directive (BPD, 98/8/EC) with Sweden as Rapporteur Member State (RMS). Originally, a joint dossier was received from the European Silver Task Force (ESTF) including eight different SCAS as notified in the biocides review programme. During evaluation, the identity of these substances was redefined as elemental silver, silver nitrate, silver borosilicate glass, reaction mass of titanium dioxide and silver chloride, silver phosphate glass, silver sodium hydrogen zirconium phosphate, silver copper zeolite, silver zinc zeolite, and reaction mass of silicon dioxide and silver. Of these, at least the identities elemental silver and reaction mass of silicon dioxide and

silver contain nanoforms of the element. A draft assessment has been made available for the identity silver zinc zeolite, disclosing a read-across concept between all SCAS that uses substance-specific data as much as possible, but closes data gaps using adjusted effect levels for the silver ion released from all SCAS. Adjustment was based on comparative release data and estimated silver ion equivalents. In the draft silver zinc zeolite dossier, an ADI of 0.3 µg/kg bw/d has been derived for silver ion equivalents based on the NOAEL of 0.03 mg/kg bw/d extrapolated from a 2-year study in rats (covering biochemical evidence for liver toxicity, organ pigmentation and endometrial polyps). A value of 10 µg/kg bw was proposed as acute reference dose based on a NOAEL of 1 mg/kg bw/d silver ion equivalents extrapolated from a 90-day study in dogs.

The current occupational exposure limits do not reflect the apparent difference in toxicities between soluble and metallic silver; thus, many researchers have recommended that separate permissible exposure limits (PELs) be established (Drake and Hazelwood 2005).

In a recent review by Hadrup and Lam (2014), a Tolerable Daily Intake (TDI) value of 2.5 µg/kg bw/day for oral exposure to colloidal Ag-NPs based on significant increase of TGF-â in serum of mice exposed during 14 days to 22 nm silver particles (Park et al 2010); the derived NOAEL of 0.25 mg/kg was divided by a safety factor of 100 to calculate the TDI.
This TDI is almost 10-fold higher than the ADI calculated in the SCAS dossier, probably due to differences in solubilisation of metallic silver versus other forms of silver particles.

**Table 10** Exposure limits set by different organisations and reported NOAELs from animal and/or epidemiological studies.

| Organisation | Advice / limit set | Based on effect | Reference |
|---|---|---|---|
| WHO | 10 g intake lifetime, 0.1 mg/L(100 ppb) drinking water<br>Lifetime exposure (70 y or 25550 days):<br>- NOAEL equals 0.39 mg / person/ day or (bw = 70 kg) 0.005 mg/kg bw/d. | Argyria | WHO/SDE/WSH/03.04/1, Silver in drinking water |
| U.S. EPA | RfD 5 µg/kg bw/d<br>Based on LOAEL of 1 g total dose (4% absorption) | Argyria | US EPA Integrated Risk Information System, Substance No. 0099, Reference Dose for Chronic Oral Exposure (RfD): Silver, 10-01-2008 |
| EFSA | 0.05 mg/L and 0.05 mg/kg in water and food, resp | Argyria | EFSA 2004, 2005, 2006. Opinion on a 4th, 7th, 12th list of substances for food contact materials. |
| ACGIH | TLV:<br>- 0.1 mg/$m^3$metalic silver<br>- 0.01 mg/$m^3$ silver salts | Argyria | ACGIH 1986/Ex. 1-3 |
| DFG<br>(Deutsche | MAK (Maximale Arbeitsplatz-Konzentration )<br>BAT (Biologischer Arbeitsstoff Toleranz Wert) | Argyria | Deutsche Forschungsgemeinschaft: |

| | | | |
|---|---|---|---|
| Forschungsgemeinschaft) | - 0.1 mg/$m^3$ Metallic silver<br>- 0.01 mg/$m^3$ silver salts | | MAK- und BAT-Werte-Liste 2006; Suva/Schweizerische Unfallversicherungsanstalt: Grenzwerte am Arbeitsplatz 2007 |
| NIOSH & OSHA | 0.01mg/$m^3$ for silver dust and soluble silver | Argyria | |
| FIFRA<br>(Federal Insecticide, Fungicide & Rodenticide Act) US<br>US EPA. | Inhalation: NOAEC of 133 µg/$m^3$<br>Oral NOAEL: 0.5 mg/kg bw/ | Inhalation: Sung et al. 2009 – 90 day study rats<br>Oral: Park et al. 2010 – 28 day study | "nano"silver pesticide for use as textile preservative (EPA-HQ-OPP-2009-1012-0064) |
| EU - | In an attempt to set a standard for different silver containing forms the release of silver ion was taken as golden standard:<br>An ADI of 0.3 µg/kg bw/d for silver ion;<br>Acute reference dose 10 µg/kg bw | based on NOAEL of 1 mg/kg bw/d silver ions. | European Biocides Directive (BPD, 98/8/EC) |
| | | | |
| Experimental studies | | | |
| Oral route<br>56 nm Ag-NP<br>13 weeks (90 days)<br>F344 rats | NOAEL (no observable adverse effect level) of 30 mg/kg<br>LOAEL (lowest observable adverse effect level)<br>of 125 mg/kg | Liver toxicity | Kim et al. Particle and Fibre Toxicology 2010, 7:20 |
| Inhalation, whole body exposure<br>18–19 nm Ag-NP<br>6 h/d, 5 d/w, for 13 W<br>Sprague-Dawley rats | NOAEL (no observable adverse effect level) of 100 µg/$m^3$ | Liver & Lung toxicity | Hussain et al., Tox. Sciences 108(2), 452–461 (2009)<br>doi:10.1093/toxsci/kfn246 |
| Oral exp., ICR mice, 28 days, 22 – 42 – 71 nm | NOAEL 0.25 mg/kg ofbw/day | Liver & Lung toxicity | Park et al Environ Tox Pharm 2010<br>doi:10.1016/j.etap.2010.05.004 |

CONCLUSIONS

The safety limits set for silver, mainly for use in an occupational setting, were calculated from data of epidemiologic studies showing argyria as the most relevant and sensitive human health effect. Silver ions are considered potentially more hazardous and therefore a 10-fold difference is often observed in limits for silver metal compared to silver salts. The EU considers an ADI of 0.3µg/kg bw/d for silver ion. No specific exposure limits have been calculated for nanosilver.

### 3.9. Recommendations

In general, more information is required on the possible contribution of Ag-NP to environmental and human toxicity, and to the occurrence and mechanism of antimicrobial resistance. Current evidence from the peer-reviewed literature raises some concern on possible health effect of continuous exposure to Ag-NP. Such concerns question the increased usage of products containing Ag-NP, in particular usage in consumer product that is not linked to justified and tangible benefits. When Ag-NP are used in consumer products, care should be taken that consumer / hygienic products release sufficient silver to be functional / effective.

Currently, a specific human risk assessment for Ag-NP is not feasible as information on possible long term effects are lacking. Therefore, more studies on health effects after long term exposure are needed. Also more exposure data is necessary, as is data on all products containing Ag-NPs as data on exposure levels during use of Ag-NP containing products.

Considering the biocidal action of Ag-NP and bacterial resistance to Ag-Np several mechanisms have been described, although more evidence is required to ascertain their significance. While the release of silver ions may account for most bacterial toxicity, the molecular mechanisms of interactions of Ag-NP with bacteria is not well understood. Although bacterial resistance to ionic silver has been well documented, notably efflux systems, other resistance mechanisms as well as the possible horizontal gene transfer between bacteria – as shown for other NP – need to be investigated in more depth.

## 4. OPINION

*1. What may be the implications of the widespread use of nanosilver for human health and the environment? Please consider direct as well as indirect effects occurring via the distribution into the environment (e.g. from use in appliances, discarding dental material, washing out from textiles, etc.). Does this change the existing assessments for silver in general?*

A number of consumer and medical care products contain silver, most often for hygienic purposes. Silver in these products is used in many formulations such as nanosilver, bulk metallic silver, silver salts and silver ion exchangers. It is important to note that nanosilver represents probably less than 50% of the silver used as a biocide in consumer products. The total amount of silver released from consumer products is generally rather low. However, products that contain nanosilver are difficult to track because they are marketed under numerous brand names, and with a few exceptions, current labelling regulations do not specifically require listing of nanomaterials.

The best described, but in general considered to be relatively harmless, adverse effects in humans of chronic exposure to silver are a permanent bluish-grey discoloration (argyria or argyrosis) of the skin or eyes probably the result of the precipitation of Ag-formations at the nanoscale. Exposure leading to this condition is in general a few orders of magnitude larger compared to more recent safety studies *in vivo*. *In vivo* and *in vitro* studies have now indicated that nanosilver exposure leads possibly to genotoxicity, changes in activity of the immune system and an accumulation of silver in spleen, liver and testes. More details on the toxicity of silver (regrettably, the different forms can often not be separated) can be found in the conclusions, the executive summary and in the scientific background of this opinion.

The environmental compartments that receive silver from anthropogenic sources are mainly freshwater bodies, freshwater sediments, and soils. This environmental exposure to silver originates from many uses of silver, including a wide range of technical applications in industry, medical applications, water disinfection and more recent uses of silver in consumer products. As in the case of human exposure, nanosilver is only a small fraction of the total amount of silver entering the environment. However, it has been observed that Ag-NPs act as a source of bioavailable silver, and it cannot be excluded that Ag-NPs represent a new source of environmental exposure to silver that delivers silver to organisms in a way that is not effective for other forms of silver.

Human and environmental effects of silver are mainly linked to silver ions. In the environment, silver is effectively detoxified when reduced sulfur is present, because silver sulfide, which is formed by silver in contact with reduced sulfur, is very stable and does not release ionic silver. Large fractions of the silver reaching the environment will precipitate as silver sulfide. Unfortunately, there is limited toxicity data for silver sulphide.

In conclusion, the widespread (and increasing) use of silver containing products implicates that both consumers and the environment are exposed to new sources of silver. Human exposure is direct (food, hand-to-mouth contact, skin) and may be lifelong, while in the environment Ag-NPs may be a particularly effective delivery system for silver to organisms in soil, water and sediment and may act as sources of ionic silver over extended periods of time. Therefore, additional effects caused by widespread and long term use of Ag-NPs cannot be ruled out.

*2. Could the widespread use of nanosilver, in particular in medical care and in consumer products, increase the risk of selecting Ag resistant micro-organisms? Could the widespread use of nanosilver create cross-resistance in micro-organisms?*

There is a paucity of information on the bacterial resistance mechanisms to Ag-NP.

- Some of the genetic basis of bacterial resistance to ionic silver has been well documented, notably the expression of well-characterised efflux systems.
- Recent transcriptomic and proteomic data suggest that a decrease in oxidative damage by regulation of anaerobic respiration is important. Moreover, combined exposure to ionic silver and Ag-NP seems to produce a stress-response affecting gene expression.

More data are needed to understand better bacterial response to ionic silver and Ag-NP exposure. Regarding the hazard associated with the dissemination of the resistance mechanism following the use of Ag-NP, no documentation is available at this moment, representing a serious gap of knowledge.

Besides the development of specific resistance mechanisms to silver, studies have shown an effect of Ag-NP on the distribution of bacterial flora and on the bacterial adaptation in certain conditions and uses. However, evidence is fragmentary and only focused on a few specific cases.

Since other NP have been shown to substantially increase the horizontal gene transfer between bacteria – which is extremely relevant for developing resistance – the potential of Ag-NP to induce similar effects should be given particular attention.

*3. To what extent may the widespread use of nanosilver and the possible increase of resistant micro-organisms reduce the nanosilver's efficacy?*

As mentioned above, the paucity of information on the resistance mechanisms to Ag-NP does not allow for the formulation of a definite answer. While some of the genetic basis of bacterial resistance to ionic silver has been well documented - the expression of well-characterised efflux systems - others are still not well explored, although recent data suggest that a decrease in oxidative damage by regulation of anaerobic respiration is important. Exposure to ionic silver and Ag-NP produces a stress-response and affects gene expression. More data is needed to better understand bacterial response to ionic silver and Ag-NP exposure.

Since the mechanisms resulting in Ag-Nps resistance are not well understood, it is not possible at this time to estimate whether or not resistance will increase and spread in view of a more widespread use of nanosilver in products.

*4. Are there any other safety, health and environmental effects of nanosilver?*

The increasing use of nanosilver in several, significantly different consumer product will most probably result in lifelong human exposure via various routes (skin, textiles, oral, food preservation). Although no clear adverse health effects have been associated with silver exposure in general, no information can be found on long-term (low) exposure to silver or silver containing products in the general population.

Since the sensitivity of different bacteria to silver is significantly different, it is unclear how populations of bacteria, such as the human bacterial flora, will change in the presence of low, but significant, amounts of silver and how resistance to silver (as for other bacterial products) can become established in bacterial populations.

## 5. CONSIDERATION OF THE RESPONSES RECEIVED DURING THE CONSULTATION PROCESS

A public consultation on this opinion was opened on the website of the non-food scientific committees from 13 December 2013 to 02 February 2014. Information about the public consultation was broadly communicated to national authorities, international organisations and other stakeholders.

Twenty-eight organisations and four individuals participated in the public consultation providing input to the four main scientific questions (in total 60 contributions were received). Out of the 28 organisations participating in the consultation, there were fourteen NGOs, six public authorities and five business companies.

Each submission was carefully considered by the Working Group and the scientific opinion has been revised to take account of relevant comments. The literature has been accordingly updated with relevant publications. The scientific rationale and the opinion section were clarified and strengthened.

The text of the comments received and the response provided by the SCENIHR is available here
http://ec.europa.eu/health/scientific_committees/consultations/public_consultations/scenihr_consultation_17_en.htm

## 6. MINORITY OPINION

None

## 7. LIST OF ABBREVIATIONS

| | |
|---|---|
| AAS | Atomic Absorption Spectrometry |
| ACGIH | American Conference of Governmental Industrial Hygienists |
| Ag | Silver |
| AgAc | Silver acetate |
| AgBr | Silver bromide |
| $AgF_2$ | Silver (II) Fluoride |
| AMR | Antimicrobial resistance |
| ANEC | European Association for the co-ordination of consumer |
| APS | Aerodynamic Particle Sizer |
| ATM | Automatic Teller Machine |
| BPR | Biocidal Products Regulation |
| CED | Critical effect dose |
| CQ | Camphorquinone |
| DLS | Dynamic Light Scattering |
| DOC | Dissolved organic carbon |
| ECDC | European Centre for Disease prevention and Control |
| ECHA | European Chemicals Agency |
| EFSA | European Food Safety Authority |
| EMA | European Medicines Agency |
| ENDOIII | endonuclease III |
| EST | embryonic stem cell test |
| EU | European Union |
| fcc | face-centred cubic structure |
| FFF-ICP-MS | Field Flow Fractionation Inductively Coupled Plasma Mass Spectrometry |
| FpG | formamido-pyrimidine-DNA glycosylase |
| G-CSF | Granulocyte-colony stimulating factor |
| GI | Gastro intestinal |
| GME | Genetic mobile elements |
| GST | Glutathione S-transferase |
| hMSCs | In human mesenchymal stem cells |
| ICP-AES | Inductively coupled plasma atomic emission spectroscopy |
| ICP-MS | inductively coupled plasma mass spectrometer |
| ICP-OES | inductively coupled plasma optical emission spectrometry – which is a synonym for ICP-AES |
| IL | Interleukin |

| | |
|---|---|
| IUPAC | International Union of Pure and Applied Chemistry |
| iv | Intravenous |
| $KAgF_4$ | Potassium Silver (III) Fluoride |
| LCA | Life-cycle assessment |
| LPS | Lipopolysaccharide or endotoxin |
| MDR | Multiple drug resistant |
| MIP | Macrophage inhibitory protein |
| MSHA | Mine Safety and Health Administration |
| NIOSH | National Institute for Occupational Safety and Health |
| NIST | National Institute of Standards and Technology |
| NK | Natural killer |
| NOAEL | No Observable Adverse Effect Level |
| NOM | Natural organic matter |
| NPs | Nanoparticles |
| OECD | Organisation for Economic Co-operation and Development |
| OLL | oral lichenoid lesions |
| OSHA | Occupational Safety and Health Administration |
| PEL | permissible exposure limit |
| PVC | Polyvinyl chloride |
| PVP | polyvinylpyrrolidone |
| SPM | Suspended particulate matter |
| SC | Sub cutaneous |
| SCAS | Silver-containing active substances |
| SCCS | Scientific Committee on Consumer Safety |
| SCENIHR | Scientific Committee on Emerging and Newly Identified Health Risks |
| SCHER | Scientific Committee on Health and Environmental Risks |
| SEM-EDX | Scanning Ellectron Microscopy & X-ray Spectroscopy |
| SMPS | Scanning Mobility Particle Sizer |
| SP-ICP-MS | Single particle inductively coupled plasma mass spectrometer |
| SPM | Suspended particulate matter |
| TDI | Tolerable Daily Intake |
| TEGDMA | triethylene glycol dimethacrylate |
| TEM | Transmission electron microscopy |
| TNF | Tumor necrosis factor |
| US-EPA | United States Environmental Protection Agency |
| WHO | World Health Organization |

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## 9. GLOSSARY

| Term | Explanation |
|---|---|
| Ag-NP | Ag-NP is defined as single particle, aggregate or agglomerate having one or more external dimensions in the size range 1 - 100 nm. Silver materials with a particle size larger than 100 nm are indicated as **bulk** silver |
| Bulk silver | Silver materials with a particle size larger than 100 nm in all external dimensions |
| Capping agents | a strongly absorbed monolayer of usually organic molecules used to aid stabiliztion of nanoparticles |
| Dissolved nanosilver | AG-NP solutions – note the differences in solubilization of metallic silver versus other chemical compounds containing silver atoms or ions; the silver concentration is usually measured by ICP-MS |
| DNA | Deoxyribonucleic acidis a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms |
| DLS | Dynamic Light Scattering is a method used for measuring the particle size distribution |
| EDPs | Electron-dense particles are found in pigmented areas. Elemental composition of Ag-EDPs determined by x-ray microanalysis showed mainly silver in combination with sulphur or selenium or a combination of both chalcogens. |
| FCC | Face-Centred Cubic – a lattice structure defined in solid states of materials; bulk metallic silver has FCC structure |
| Forms of silver (chemistry) | Silver can be present in different forms such as pure metallic silver,silver salts and silver oxides |
| Forms of silver (geometry) | Bulk and nanosilver, which could be produced as nanoparticles, nanowires, and quantum dots |
| Freundlich adsorption isotherm | Freundlich adsorption isotherm is a curve relating the concentration of a solute on the surface of an adsorbent to the concentration of the solute in the liquid with which it is in contact |
| GST | glutathione S-transferase previously known asligandins, comprise a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyze the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification |
| Mesocosm | an experimental tool that brings a small part of the natural environment under controlled conditions |
| Nanoparticles | See nanodefinition published by SCENIHR (Opinion on The scientific aspects of the existing and proposed Definitions relating to products of nanoscience and |

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|---|---|
| | Nanotechnologies, 2008) |
| Nanosilver | Silver material with at least one size in the range between 1 and 100 nm. Examples are: thin films (one dimension smaller than 100 nm), fibres/wires (two dimensions smaller than 100 nm), particles (three dimensions smaller than 100 nm) |
| Toxicokinetic | The absorption, distribution, metabolism and excretion of a chemical |