RISK AND HEALTH IMPACT ASSESSMENT
TRACE ELEMENTS AND NANOMATERIALS

EVALUATION OF THE TYPES, EFFICIENT USE AND HEALTH RISKS OF APPLICATION OF SILVER-BASED BIOCIDES TO PROVIDE ANTIMICROBIAL PROPERTIES TO FACE MASKS APPLIED DURING THE COVID-19 CRISIS

Intermediate report AgMask COVID-19 project 2022

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EXECUTIVE SUMMARY

Silver is authorized, under certain conditions, as biocide under the Biocidal Products Regulation (BPR, Regulation (EU) 528/2012). Consequently, some face masks, mostly reusable textile comfort face masks, are treated with silver-based biocides and are marketed on the Belgian market. In this study silver-based biocides were identified and characterized in a selection of face masks available for the general population. To evaluate whether the observed biocides can present a potential health risk, a preliminary risk analysis was performed.

Determination and characterization of silver-based biocides in face masks

In situ analysis of silver based biocides in face masks using electron microscopy imaging and EDX, combined with total silver measurement using ICP-MS demonstrated variable amounts and diverse types of silver-based biocides in the examined face masks. Four different types of silver-based biocides were demonstrated: (i) Ag^+ ions, (ii) metallic Ag^0 nanoparticles (NP) distributed in the matrix of the fibres, (iii) Ag NP and large silver particles at the surface of, or close to cotton fibres in face masks containing polycationic polymers, and, (iv) a coating consisting of metallic silver releasing Ag^+ ions, Ag^0 NP and large silver particles. Based on these data, an initial risk assessment was performed.

Assessing potential health risks

An acceptable exposure level (AEL_{mask}) of 25 µg metallic and ionic silver was determined per mask based on maximal occupational exposure levels and assuming an “intensive use” scenario (maximum use of 8 hours, replacing face mask after 4 hours)[2].

Silver was detected in 13 face masks. In 9 face masks, only Ag^+ ions were detected as silver biocide, while four face masks contain Ag^0 NP, Ag^+ ions, and/or non-nanoparticulate silver. Comparison of the measured amount of total silver in the masks with this AEL_{mask} indicated that seven out of nine face masks with a silver biocide based on Ag^+ ions only, can be considered as safe, while two face masks with a silver biocide based on Ag^+ ions exceeded the AEL_{mask} and require a more refined risk evaluation. The amount of silver in three masks that contain Ag^0 NP, Ag^+ ions, and/or non-nanoparticulate silver exceeded the acceptable exposure level. In one mask, Ag^0 NP were observed as a contamination of a nanoparticulate CuO biocide.

It has to be noted that this is a theoretical risk, and not a proven one based on real exposure data. Many uncertainties with regard to the degree of exposure remain and in our assessment we have taken a prudent (conservative) toxicological approach. However, when Ag^0 NP are present in face masks, our approach could also lead to an underestimation of the risks due to a higher toxicity of the Ag^0 NP. A calculation of the risk is not possible since the specific amount of Ag^0 NP cannot be determined when present in combination with other forms of silver. Per case an in depth risk analysis needs to be undertaken to account for the different forms of silver that are potentially released from face masks treated with the applied silver based biocides.

In one face mask, however, the amount of silver is so high that a health risk cannot be excluded, even if we consider that only a small fraction of silver biocide is released and inhaled.

Ongoing and future research

In the next phase of the project we will advance in the development of methodologies to assess the characterization and exposure of silver based biocides. Specifically, our aim is to assess the inhalation of silver based biocides from face masks by mimicking real life conditions or by developing alternative techniques to measure the release of silver biocides from face masks.

The developed methodologies and the presented results form a basis for the development of better, safer face masks that are based on the safe-by-design principle. The data will support identifying the technical specifications of face masks fit for both private and professional application. In the course of the current project, we have identified several major challenges related to the analysis, characterization and risk assessment of silver biocides in face masks, and which go beyond the scope of the ongoing research project:

- In general, scientific data on the presence and types of silver based biocides in face masks, their characteristics, the exposure and the risks for the population is limited.
- Methodologies for characterizing silver based biocides in face masks are time consuming and expensive. Consequently, within the current project only a subsample of the face masks on the market was analyzed. A systematic market study should be undertaken. Furthermore, efforts are needed to build capacity for the characterization and analysis of biocides, and particularly those based on nanoparticles in consumer goods and medical devices.
- Methods for the determination of the relative amounts of different forms of silver biocides in face masks should be developed.
- Key information about the toxicity of silver based biocides and particularly approaches for risk assessment of combinations of several types of biocides are missing. More toxicity and medical research is needed to assess the risk of vulnerable populations, especially children and persons with underlying disorders.
- Reference methods, reference materials including the appointment of a reference laboratory for the analysis and characterization of nanoparticles are needed for quality control of face masks and enforcement of legislation.
- Besides silver based biocides, many other types of biocides, such as the observed CuO NP, are being applied in face masks. It is important to detect and evaluate these biocides, and combinations thereof, in view of (nano)safety.
- The reported information urges for in depth research of the applications of silver-based biocides in face masks, and of (nano)technology applications in face masks in general. Phasing out applications that can be unsafe, product development based on the safe-by-design principle, and implementing regulatory standards, and guidelines taking in account nanosafety concerns can avoid possible future consequences caused by a poorly designed nanotechnology in consumer products, while maintaining nanotechnology’s important potential to improve (medical) products such as face masks.

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ABSTRACT

In situ analysis of silver based biocides in face masks using electron microscopy and EDX, combined with total silver measurement using ICP-MS or ICP-OES demonstrated the presence of varying amounts and different types of silver-based biocides in a selection of face masks on the Belgian market and intended to be worn by the general public. Following types of silver-based biocides were demonstrated: (i) Ag^+ ions, (ii) metallic Ag^0 NP distributed in the matrix of the fibers, (iii) Ag NP and large silver particles at the surface of, or close to cotton fibres in face masks containing polycationic polymers binding Ag^+ ions, and (iv) a coating consisting of metallic silver releasing Ag^+ ions, Ag^0 NP and large silver particles. For metallic and ionic silver, an acceptable exposure level (AEL_{mask}) of 25 µg per mask was established based on occupational exposure levels and assuming an intensive exposure scenario considering subchronic exposure of the general adult population.

Comparison of the measured amount of total silver in the masks with this AEL_{mask} indicated that seven out of nine face masks, with a silver biocide based on Ag^+ ions only, can be considered as safe. The two other face masks with a silver biocide based on Ag^+ ions require a more refined risk evaluation.

The amount of silver in the four masks that contain Ag^0 NP, Ag^+ ions, and/or non-nanoparticulate silver exceeded the AEL_{mask}. Per case an in depth risk analysis needs to be undertaken to account for the different forms of silver that are potentially released from face masks treated with the applied silver-based biocides.

ABBREVIATIONS

ACGIH	American Conference of Governmental Industrial Hygienists
AEL	Acceptable Exposure Level
AEL_{mask}	Acceptable Exposure Level per mask
Ag	Silver
Ag^0	Metallic silver
Ag^+	Silver ions
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
BMD	Benchmark dose
$BMCL_{10}$	Benchmark dose low level
COVID-19	Coronavirus disease 2019
CP	Constituent particles
E 171	Titanium dioxide as a food additive
ECHA	European chemical agency
EDX	Energy dispersive X-ray spectroscopy
EFSA	European Food Safety Authority
EM	Electron Microscopy
EU	European Union
FFP2	Free Flight Phase 2 face mask following EU standard EN 149-2001
FPS	Federal Public Service (FPS) Health, Food Chain Safety and Environment
HAADF	High angle annular dark field
HEC	Human Equivalent Concentration
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma atomic emission spectroscopy
LC_{50}	Concentration of a chemical that kills 50% of the test species
LD_{50}	Dose of a chemical that kills 50% of the test species
MAK	the German Committee for the determination of occupational exposure limits
N	Number
NIOSH	US National Institute for Occupational Safety and Health
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
NP	Nanoparticles
OSHA	Occupational Safety and Health Administration
RIVM	Dutch National Institute for Public Health and the Environment
SEM	Scanning electron microscopy
STEM	Scanning transmission electron microscopy
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
v:v	volume-to-volume ratio

INTRODUCTION

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Context

This report presents intermediate results of the research project "Evaluation of the types, efficient use and health risks of application of silver-based biocides to provide antimicrobial properties to face masks applied during the COVID-19 crisis."

This project aims to generate information essential for risk analysis, risk management, and for implementation of control measures. Its general objectives are to:

- characterize the silver-based biocides in face masks *in situ;*
- estimate the exposure by inhalation to silver ions and to silver (nano)particles;
- assess the main external factors that determine the release of silver-based biocides;
- determine the possible health risks of the application of silver-based biocides in different types of face masks available on the Belgian market.

The preliminary risk analysis in this project focuses on possible health risks associated with silver-based biocides applied to specific (types of) face masks, assuming an "intensive exposure" scenario, as advised by the Belgian government, and subchronic exposure of the general adult population. Alternative exposure scenarios are out of the scope of this study.

Types of silver based biocides applied in face masks

Treatment of textiles with silver (Ag) based biocides gives the textiles broad spectrum antibacterial and antiviral properties[1–3]. They are therefore applied to various types of medical textiles, ranging from aprons to face masks. Certain types of face masks offered on the Belgian market and online, are also treated with silver[4–11]. Examples are the Silvadur™ treated face masks, distributed by the Belgian federal government, and masks distributed by certain municipalities.

In general, silver can be present in the face masks under different forms:

- Certain biocides applied to treat textiles contain silver ions (Ag^+), in the form of silver salts. A disadvantage of such treatment with silver ions from silver salts is that they are released rapidly and washed away rapidly, limiting antimicrobial effects in time[12].
- Alternatively, textiles are treated with nanoparticles consisting of metallic silver (Ag^0)[13]. Because of the high volume-specific surface area of such nanoparticles, silver ions are released slowly from the surface of these particles and antimicrobial activity is maintained for a longer period of time.
  A disadvantage is that when silver nanoparticles are released from the textiles and taken up by living organisms, nanoparticle-specific types of toxicity, which are less known as the toxicity related to silver ions, may occur[13].
- The textile fibers can also be coated with a thin layer of metallic silver.
- Finally, the latest generation of silver-based biocides is based on polycationic polymers that bind silver anions[14]. This approach aims at a slow release of silver ions, avoiding nanoparticle specific toxicity.

When selling face masks, the application of a silver-based biocide is, because of its antimicrobial effect, proposed as a positive sales argument.
Various mechanisms of action of the antimicrobial activity of silver have been described, including interaction with bacterial cell membranes, the gradual release of silver ions that interact with proteins and inhibit essential cell functions, the interaction with DNA and the generation of reactive oxygen species.[15] Literature data suggests, however, that exposure to silver, particularly in the form of nanoparticles, can negatively impact health,[16–18], depending on the type of silver- based biocide, its stability during application and cleaning, and the type of exposure of the users.
A literature survey regarding the toxicity of silver applied as biocide is provided in annex (page 34). This literature survey details the identification, the toxicokinetics, the mode of action, the toxicity, the target organs, the occupational exposure limits and the health values of silver.
Knowing the type of silver-based biocide treatment, as well as its concentration, its occurrence as a coating or inside the fibers, and the resulting inhalation exposure is essential to evaluate the quality of the face masks and possible health risks.

Inhalation exposure due to release of silver ions and/or (nano)particles from face masks treated with silver-based biocides

In the literature, the problem of release of silver (nanoparticles) from textile is mainly viewed in the context of the development of resistance by bacteria and from an ecological point of view, for example, washing of the textile is examined as a source of silver in wastewater[12,13,19]. Literature data show that washing of fabrics treated with silver- based biocides, leads to a release of ions and/or particles from these fabrics[12,13,19,20]. For nanoparticles, this does not reduce antimicrobial effectiveness to a large extent, because excess amounts are applied at the start, while even concentrations in the order of 2 µg/g nanosilver can control bacterial growth in textiles[13].

To our knowledge, no data are available demonstrating silver (nanoparticle) release while wearing silver containing face masks, but the absence of release, e.g. tested as a quality parameter, has not been demonstrated either. The possible release of silver in the form of ions and/or nanoparticles from face masks, that may result in exposure by inhalation, remains an important knowledge gap although it is essential information for a formal risk assessment.

METHODS

Selection of face masks

This intermediate report focuses on a selection of face masks intended to be worn by the general public. This selection contains reusable face masks distributed by the Belgian federal government and by regional governmental instances, reusable face masks from several commercial suppliers, and, for comparison, single use face masks from several commercial suppliers. The selection aims to include different types of biocides available on the market (Table 1). These same masks were also evaluated regarding the presence of titanium dioxide (TiO_2) particles[21].

ICP measurement of Ag

For total Ag analysis, the different masks or layers were homogenised by cutting them into small pieces using scissors and mixing the cuts manually. Two digestion methods were applied, depending on the material of the mask or layer. If the mask consisted of several layers with woven and non-woven textiles, the layers were digested separately. If the mask consisted only of non-woven textiles, the entire mask was homogenised.

Woven textiles (cotton, polyester or other synthetic fibres) were digested (closed microwave digestion) in a 4:1 (by volume) mixture of nitric acid and sulphuric acid at 220°C in a Mars 6 microwave (CEM, USA). This method was adopted from the application note for polyethylene terephthalate digestion[22]. The non-woven, synthetic textiles from the masks needed higher temperatures for complete digestion, and the method had to be adapted to the light fibres that were not easily wetted. Therefore, a method was developed together with the application specialists of the Belgian CEM supplier (BRS, Drogenbos). The method uses first a charring step in concentrated sulphuric acid at 260°C in iPrep vessels (CEM, USA), followed by a digestion step in concentrated nitric acid at 200°C. Both methods were in-house validated following ISO17025.

After dilution of the digests, the total Ag concentration was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) for elevated (>1 mg/kg) and by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for lower Ag concentrations. Quantification of Ag by ICP-OES was performed at wavelength 328.068 nm (Varian 720, Agilent technologies) using an external calibration. Quantification of Ag by ICP-MS (Varian 820) was performed for a m/z 107, using a matrix matched calibration. All samples were prepared and analysed in duplicate.

In situ TEM characterization

The *in situ* transmission electron microscopy (TEM) characterization method aims to detect, localize, and measure the size, morphology, agglomeration state, and elemental composition of (nano)particles in sections of the face masks.
A sample preparation methodology supporting on the methods of Gashti et al.[23], Lorenz et al.[24], Hebeish et al.[25] and Joshi et al.[26] for scanning electron microscope (SEM) and TEM analysis of textiles was set up. Semi-thin sections of individual layers of face masks were prepared by embedding them in an epoxy resin, followed by sectioning using ultramicrotomy (Figure 1).
Face masks were analysed by High Angle Annular Dark Field (HAADF) – Scanning Transmission Electron Microscopy (STEM) combined with Energy Dispersive X-ray spectroscopy (EDX) using a 200kV Talos F200S G2 TEM and Velox software (Thermo Fisher Scientific) (Figure 1).
The size of constituent particles identified to be silver by EDX was estimated by manual measurement.

The mass of Ag^0 NP at the fibre surface in AgMask-15 was determined using the method developed in the TiO_2Mask project to determine the mass of TiO_2 particles at the fibre surface[21].

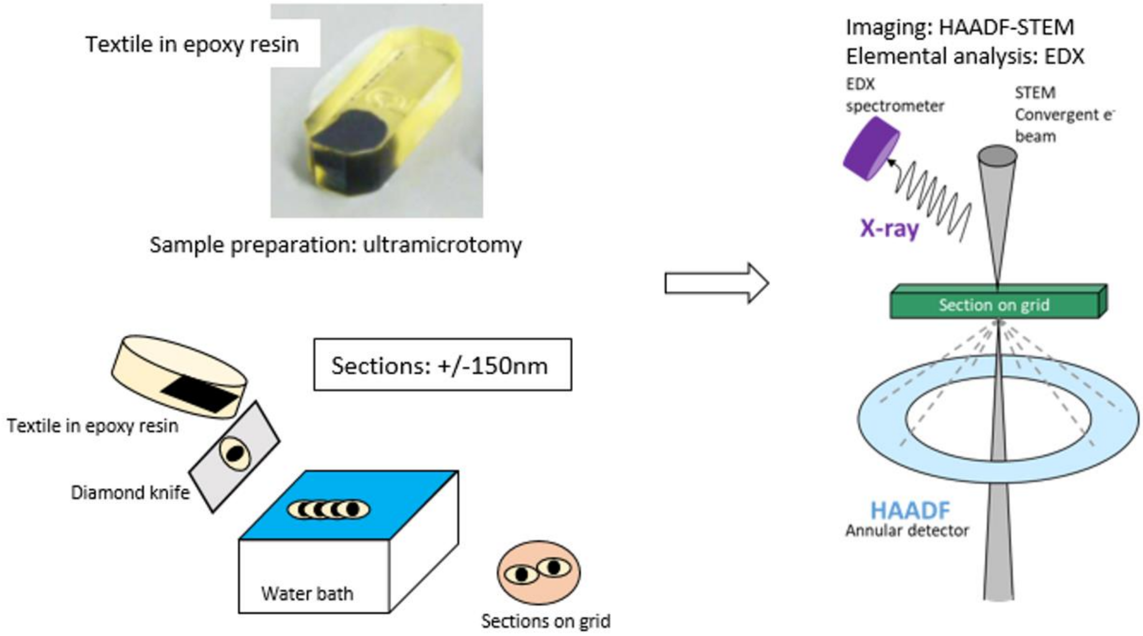

Figure 1 Illustration of the method applied for in-situ TEM characterization of face masks.

Table 1 Overview of the selected face masks

Reference	Type[a]	Type of silver-based biocidef	CE logo	Layers	Composition[c]
AgMask-01[e]	SU CM	No silver-based biocide (negative control)	N	External Central Internal	Non-woven fabric Non-woven fabric Non-woven fabric
AgMask-02	RU CM	Silver ions	N	External Central Internal	Polyester Non-woven fabric 100 % cotton
AgMask-03	RU CM	Silvadur™ antimicrobial technology: polykationic polymer releasing silver ions	N	External Central Internal	100% polyester 65% polyester and 35% cotton 65% polyester and 35% cotton
AgMask-04 [e]	SU PM	No silver-based biocide (negative control)	Y	External Central Internal	Non-woven fabric Non-woven fabric Non-woven fabric
AgMask-05 [e]	SU CM	No silver-based biocide indiated (negative control)	N	External Central Internal	Non-woven fabric Non-woven fabric Non-woven fabric
AgMask-06[d]	RU PM	2 woven layers metallized with pure silver (99.9%)	N	External Central 1 Central 2 Central 3 Internal	Polyamide, polypropylene, elastane, cotton Non-woven fabric Non-woven fabric Non-woven fabric Polyamide, polypropylene, elastane, cotton
AgMask-07[d, e]	RU PM	No silver-based biocide (negative control)	Y	External Internal	Cotton Cotton
AgMask-08	RU PM	2 woven layers metallized with pure silver (99.9%)	Y	External Central 1 Central 2 Central 3 Central 4 Internal	Polyamide, polypropylene, elastane, cotton Non-woven fabric Non-woven fabric Non-woven fabric Non-woven fabric Polyamide, polypropylene, elastane, cotton
AgMask-09	RU MM	2 woven layers metallized with pure silver (99.9%)	Y	External Central 1 Central 2 Central 3 Central 4 Internal	Polyamide, polypropylene, elastane, cotton, non-woven fabric [b]
AgMask-10 [e]	SU MM	No silver-based biocide (negative control)	Y	External Central Internal	Non-woven fabric Melt-blown fabric Non-woven fabric
AgMask-11 [e]	SU MM	No silver-based biocide (negative control)	Y	External Central Internal	66% non-woven fabric, 34% polypropylene melt-blown [b]
AgMask-12	SU PM	No silver-based biocide (negative control)	Y	External Central 1 Central 2 Internal	29.41% non-woven fabric, 14.71% melt-blown fabric, 14.71% melt-blown fabric, 26.46% hot air cotton, 14.71% non-woven fabric [b]
AgMask-13	RU CM	Nano-silver fibres releasing silver ions	N	External Central Internal	Polyamide Woven fabric Cotton
AgMask-14	RU CM	Nano-silver filter	N	External Central Internal	Cotton Cotton Cotton
AgMask-15	RU CM	AGS-20 silver-silica composite that contains nanosilver sintered onto amorphous silicon dioxide	Y	External Internal	Polyamide Polyamide
AgMask-16	RU CM	CuO impregnated (control for EDX element detection)	N	External Central 1 Central 2 Central 3 Internal	Polyester, cotton, non-woven fabric [b]
AgMask-17	RU CM	Cotton with silver nanoparticles	N	External Internal	50 % linen, 50% cotton [b]
AgMask-18	RU CM	Microfiber with silver ions	N	External Internal	Polyester, polyamide, elastane Polyester, polyamide, elastane
AgMask-20	RU CM	Nanofiber with active silver	N	External Central Internal	Non-woven fabric Nanofiber membrane Non-woven fabric
AgMask-21[d]	RU PM	Silver nanoparticles	N		crochet designer masks
AgMask-22	RU CM	Information not available	N	External Internal	Polyester Cotton
AgMask-23 [e]	RU CM	Information not available	N	External Central Internal	Polyester Non-woven fabric Cotton
AgMask-24	RU CM	Information not available	N	External Internal	Polyester, polyamide Polyester, polyamide

[a] SU CM: single-use community mask, RU CM: reusable community mask, SU PM: single-use protective mask, RU PM: reusable protective mask.RU MM: reusable medical mask, SU MN: single-use medical mask. The classification (community mask, medical mask or protective mask) is made based

on available information on the packaging or by expert opinion, but this does not mean that masks are compliant with the specific legislation. Sciensano did not evaluate the masks on their compliance.

[b] General composition. The composition of individual layers is not specified.

[c] As indicated by the producer.

[d] These, three masks were originally selected and later omitted from further analysis. AgMask-06 is a prototype mask that later was marketed in a slightly modified form as AgMask08. AgMask-07 is a cotton mask (negative control) of which only few copies were available. AgMask-21 is a crochet designer masks with a pore size that does not meet the criteria of a functional face mask.

[e] The face masks AgMask-01, AgMask-04, AgMask-05, AgMask-07, AgMask-10, AgMask-11 and AgMask-23 were selected as negative control samples. Presence of silver biocide was not indicated.

[f] Type of silver-based biocide as advertised on the packaging, the vendor's or producer's website or on the prescription.

Approach for risk assessment

CALCULATION OF ACCEPTABLE EXPOSURE LEVELS PER MASK FROM OCCUPATIONAL EXPOSURE LIMITS

Occupational exposure limits (OELs) for exposure to Ag^+ ions, as reported by NIOSH[27]:

- German MAK (Maximum Workplace Concentration) values[28] : inhalable fraction silver salts: 10 µg/m³
- ACGIH threshold limit values: metal dust and fume, inhalable fraction soluble : 10 µg/m³
- NIOSH and OSHA : identical values for soluble and insoluble silver : 10 µg/m³

Occupational exposure limits for exposure to metallic silver, as reported by NIOSH[27]:

- German MAK (Maximum Workplace Concentration) values[28]: inhalable fraction metallic silver: 100 µg/m³
- ACGIH threshold limit values (2001): metal dust and fume, inhalable, insoluble fraction: 100 µg/m³
- NIOSH and OSHA : identical values for soluble and insoluble silver: 10 µg/m³ [27]

In this study, a conservative approach is chosen and soluble and insoluble silver are not differentiated, in line with the recent OEL values of NIOSH[27]. An overall occupational exposure limit of 10 µg/m³ is applied in the calculations.
This intermediate report focuses on a selection of face masks intended to be worn by the general public. The general public population is more variable than the professionally active population. Therefore, the default assessment factor of 5 applied for the professional population in the setting of the OEL needs to be adapted to the general population for which a default assessment factor of 10 is used. Therefore an additional safety factor of 2 is introduced. This is in line with the ECHA guidance[29].

Using the default short-term inhalation rate of 1.25 m³/hour for adults recommended by the Human Exposure Expert Group[4] and followed by the experts of ECHA[8] and EFSA[30], the acceptable amount of silver that can be inhaled during an exposure period of 8 hours per day, or Acceptable Exposure Level (AEL), is 50 µg.

This estimation is calculated following the equation below:

$$AEL = \left(\frac{\text{OEL}}{Additional\ Safety\ factor}\ \text{x}\ V_{air}\ \text{x}\ 8\right)$$

Where V_{air} is the volume of air inhaled by an adult in 1 hour.

Exposure scenario

The Belgian Government officially recommends to use a new mask every 8 hours[31]. When a mask is dirty or humid, it needs to be replaced. If people have to talk a lot, for example school teachers, it is recommended to use a new mask every 4 hours. Therefore we consider a realistic usage of 2 masks a day.
For an acceptable amount of Ag that can be inhaled during an exposure of 8 hours per day of 50 µg, the **acceptable amount of releasable Ag per mask, AEL_{mask}, equals** 50 µg/2 = **25 µg.**

For Ag^0 NP, OELs were reported ranging from 0.1 µg/m³ [32] to 0.9 µg/m³ [27].
Based on this, AEL_{mask} for exposure to Ag^0 NP, will be 11 and 100 times, respectively, lower than for exposure to Ag^+ ions and metallic silver. Hence, applying the scenario and calculations as described above, the AEL_{mask} for exposure to Ag^0 NP, ranges from 0.25 µg to 2.3 µg.

For the preliminary risk evaluation, the total amount of silver biocide per mask was compared to the AEL_{mask}. Considering the total amount of silver biocide per mask is a conservative approach. In reality, only the fraction of the mask in contact with the mouth, the nose and the region around it, will probably release silver that may be inhaled, not the entire mask. In addition, the calculations for the entire mask assume no interference of the different layers on silver exposure.

RESULTS AND DISCUSSION

Types and amounts of silver based biocides

ICP-OES analysis (for concentrations higher than 1 mg/kg) and ICP-MS analysis (for concentrations lower than 1 mg/kg) allowed to measure the total amount of silver, present as particles and/or as ions, in the layers of face masks (Table 2).

Textile fibres were detected and identified in the sections of the layers of all masks by HAADF-STEM analysis[21]. STEM-EDX analysis previously demonstrated the presence of titanium dioxide (TiO_2) particles in most synthetic fibres[21]. In addition, STEM-EDX analysis allowed to analyze the presence and properties of silver (nano)particles *in situ*. Using the combination of these methods, varying amounts and different types of silver-based biocides were demonstrated to be applied in the selection of face masks.

Significant amounts of silver, indicating the application of silver-based biocides, were demonstrated in 13 face masks. In nine of these face masks, silver, but no Ag^0 NP, was demonstrated, indicating that the silver biocide is largely present as Ag^+ ions. Four face masks (AgMask-03, AgMask-08, AgMask-15 and AgMask-16) were shown to contain Ag^+ ions, a Ag^0 coating and/or Ag^0 NP.

Because the amount of silver in certain of the former nine masks is relatively low (Table 2), it cannot be excluded that for some of these face masks, Ag^0 NP remain undetected because their number is below the detection limit of the applied TEM analysis method. The amount of silver in AgMask-17, advertised to contain silver nanoparticles bound to cotton, is, for example, so low (2.8 µg/mask) that this claim could not be confirmed.
The applied approach does not allow to differentiate application of Ag^+ ions from the newer generation of silver-based biocides based on (polymeric) fibres that bind Ag^+ ions. In the face masks AgMask-13, AgMask-14, AgMask-18 and AgMask-20, silver ions are claimed to be released from fibres or filters by the producer. In these masks, no silver particles were observed by EM confirming the claims that the silver biocide is largely present as Ag^+ ions.
In face mask AgMask-03 (Figure 5), the so-called Silvadur™ technology aims at a slow release of silver ions[14]. This silver-based biocide consists of polycationic polymers that bind silver anions. *In situ* EM analysis showed that at least a fraction of the total amount of silver (37 µg per mask) is present as particles observed at the surface or close to the cotton fibres (Figure 6). Near-spherical, silver nanoparticles with sizes ranging from 13 to 27 nm and larger near-spherical silver nanoparticles with sizes ranging from 55 to 115 nm (Table 2; Figure 6) were observed. In addition, larger irregularly shaped silver particles ranging from 500 nm to 1 µm were detected outside of the textile fibres, or close to the surface of polyester and cotton fibres (Figure 7). It needs to be further examined whether these were released from the fibres or can be explained as a sample preparation artefact resulting from displacement of particles by the diamond knife during ultra-thin sectioning.

In face mask AgMask-15, silver is present in both the external (83 µg) and the internal (82 µg) layers. The detected Ag-based biocide consists of metallic Ag NP distributed in the polyamide matrix of the fibers (Figure 2, Figure 3, Figure 4). The observed silver NP are near-spherical (medium spericity) and their size ranges from 11 nm to 58 nm (Table 2), which is in agreement with Egger et al. [33] and with the EPA registration data for the applied biocide HeiQ AGS-20[34]. EDX allowed to differentiate the silver nanoparticles, which are mostly isolated, from the often agglomerated TiO_2 particles, which are also present in the fibres[21] (Figure 3). It remains to be investigated further whether the silver particles are present in the fibres of the AgMask-15 face mask as the silver-silica composite that contains nanosilver sintered onto amorphous silicon dioxide[33,34], or whether they are broken off from the composite.

Assuming that all silver is present as Ag^0 NP, 0.91% (0.76 µg) and 0.54 % (0.69 µg) of the Ag^0 NP are present at the fibre surface of the external and the internal layers of AgMask-15, respectively.

In face mask AgMask-08, the total amount of silver is very high (>110 mg/layer). This is explained by the coating of the woven fibres of the external and internal layers with metallic silver (Figure 8 and Figure 9, A-D), with an approximate average thickness of 650 nm. Parts of this coating, ranging from 500 nm to 3.5 µm in size, were observed outside of the fibres, probably because they detached (Figure 9, A-H). A high amount of Ag nanoparticles was observed on the coating (just inside and outside of the fibres) (Figure 10). In addition, Ag nanoparticles were observed inside the fibres, mainly around or on the larger agglomerated TiO_2 particles (Figure 9, I-L). These silver nanoparticles have medium sphericity (near-spherical) and their size ranges from 10 to 20 nm (Table 2). It can be assumed that these metallic (Ag^0) particles are formed *de novo* by reduction of Ag^+ ions, migrating into the fibre from the external silver coating, at the (catalytic) negatively charged surface of the TiO_2 particles.

AgMask-16 contains the Argo9825 preservative and antimicrobial agent for use in the manufacture of cellulose, polymer, plastic, and textile products. According to its EPA registration file[35], this biocide is based on CuO (93.337%), and further also contains Zinc (0.313%), Silver (0.007%) and other ingredients (6.343%). This mask was originally selected as a control of the specificity of EDX element detection. STEM-EDX analysis confirmed that this mask contains many CuO NP. It also showed that the minor amount of contaminating silver (0.007%) is, at least partly, present as Ag^0 NP (Figure 12), which can be clearly distinguished from the CuO particles.

The face masks AgMask-01, AgMask-04, AgMask-05, AgMask-07, AgMask-10, AgMask-11 and AgMask-12 were selected as negative control samples. ICP-OES and TEM analyses confirmed that the masks AgMask-01, AgMask-04, AgMask-05, AgMask-07, AgMask-10 and AgMask-11 do not contain detectable amounts of silver or silver particles. AgMask-12 did however contain 7.3 µg of Ag suggesting that a silver-based biocide was applied although it was not indicated on the product information. Because no Ag^0 NP were detected using EM, it is assumed that the applied biocide is based on Ag^+ ions. In AgMask-23, no silver (biocide) was detected.

Preliminary risk evaluation

To objectify the possible risk of exposure of consumers to silver-based biocides from face masks intended to be worn by the general public, Table 2 compares the total amount of silver per face mask with the AEL_{mask} (25 µg).

Seven of nine face masks (AgMask-02, AgMask-12, AgMask-13, AgMask-17, AgMask-20, AgMask-22, and AgMask-24) where no Ag particles were observed by EM and where the silver biocide, quantified by ICP-OES, was hence assumed to be (largely) present in its ionic form, contain not enough silver to exceed AEL_{mask}. Considering (i) that a conservative approach is applied based on the occupational exposure limit of 10 µg/m^3 for soluble (ionic) and insoluble (metallic) silver, as determined by NIOSH[27], (ii) that an additional safety factor of 2 is introduced, taking into account the higher variability of the general public than the professionally active population, and (iii) that an intensive use exposure scenario (8 hours per day, 2 masks) is evaluated, the applied amount of Ag^+ ions can considered to be safe for these masks.

The amount of Ag^+ ions in the face masks AgMask-14 and AgMask-18 exceeds the AEL_{mask}, by 3.5 and 7 times, respectively, indicating that a health risk cannot be excluded and that a more refined risk evaluation is required.

In AgMask-16, the CuO based, EPA-registered Argo9825 preservative and antimicrobial agent is applied. This masks was shown to contain a small number of Ag^0 NP, although the mass fraction of silver in the Argo9825 biocide is minute (0.007%). The amount of Ag^0 NP is so much lower than the amount of observed CuO NP, that possible health risks of CuO are more relevant than those of Ag. The characterisation and risk analysis of the CuO particles is out of the scope of this research project.

Comparison of the total amount of silver with AEL_{mask} indicates that also for the face masks AgMask-03, AgMask-08 and AgMask-15, health risks cannot be excluded. These masks contain a combination of Ag^+ ions, Ag^0 NP, and for AgMask-03 and AgMask08, non-nanoparticulate silver. Technically, it is not possible to measure the relative amounts of these different types of silver-based biocides when they simultaneously occur in a face mask. Consequently, it is difficult to accurately analyze the risk when ionic silver is present in combination with Ag^0 NP and non-nanoparticulate silver. In a conservative approach, assuming the worst-case scenario that all silver biocide is present in its nanoparticulate form, the values in Table 2 indicating the number of times that the amount of silver exceeds AEL_{mask} need to be multiplied with a factor 100, which overestimates possible risks.

The amount of silver in AgMask-03 marginally (factor 1.5) exceeds the AEL_{mask} estimated for inhalation exposure to ionic and metallic silver (25 µg). In this mask, a fraction of the amount of Ag is, however, present as Ag NP that are not bound to a polymer matrix, and hence are susceptible to release. The OEL for silver nanoparticles, depending on the source, is 11 to 100 times lower than for ionic and metallic silver (see "Literature survey regarding the toxicity of silver": "Occupational exposure limits", page 37). Hence, the current risk assessment might underestimate possible health risks.

The amount of silver in AgMask-15 exceeds the AEL_{mask} estimated for inhalation exposure to ionic and metallic silver (25 µg) 6.6 times. Silver is present as Ag^0 NP that are mixed with the fibre matrix (Figure 3), as the observed TiO_2 particles. In this case, migration of the silver particles, with a size in the order of 32 nm and completely incorporated in the fibre polymers of the face mask, can be excluded by theoretical considerations: only particles smaller than 5 nm can migrate in the polymers constituting the face masks[36]. Particles at the fibre surface might, however, be released when they are subjected to abrasion or to aerodynamic forces. Direct measurement of released particles is problematic because, to our knowledge, no standardized methods are available to determine whether particles are released from face masks during normal use, and to determine which amount of silver is released.
Therefore, an indirect approach to compare the mass of particles at the fibre surface with AEL_{mask}, which was first developed in the TiO_2Mask project[21], was applied to compare the mass of Ag^0 NP at the surface of the textile fibres of AgMask-15 with AEL_{mask}. Assuming that all silver is present as Ag^0 NP (0.91% (0.76 µg) and 0.54 % (0.69 µg) of the Ag^0 NP are present at the fibre surface of the external and the internal layers of AgMask-15, respectively), the total amount of Ag^0 NP at the fibre surface is estimated to be 1.45 µg. Safe levels expressed as AEL_{mask} for Ag^0 NP range between 0.25 µg and 2.3 µg (See "Calculation of acceptable exposure levels per mask from occupational exposure limits" on page 19). Therefore, a health risk related to the presence of Ag^0 NP cannot be excluded and further refinement on the release of the Ag^0 NP is necessary.

The amount of silver in AgMask-08 exceeds the AEL_{mask} largely, by a factor of 9402. This mask contains a coating of non-nanoparticulate metallic silver at the particle surface, and Ag^0 NP at the surface of the coating and of the agglomerated TiO_2 particles in the fiber matrix. Assuming that the latter Ag^0 NP are formed *de novo* by reduction of Ag^+ ions, migrating into the fibre from the external silver coating, at the (catalytic) negatively charged surface of the TiO_2 particles implies that the concentrations of Ag^+ ions are relatively high. Probably, the additional risk from the presence of these Ag^0 NP is low because these Ag^0 NP in the fibres are mostly surrounded by a polymer matrix. They are not likely to be released[36], as opposed to the Ag^0 NP that were shown detaching from the Ag^0 coating of the fibres (Figure 10, Figure 11). It remains to be examined whether the combination of Ag^+ ions with TiO_2 particles, with known catalytic properties, could result in *de novo* formation of Ag^0 NP in other masks, where the concentrations and the (reaction) environment are different. Although further research is necessary, this observation illustrates, however, that the combination of silver ions and TiO_2 must be critically examined to avoid unintended formation of Ag^0 NP.

AgMask-08 contains four identical central layers similar to those of the control masks such as AgMask-01. These layers are expected not to contain silver particles. The relatively high amount of silver detected in these central layers (208 µg) is assumed to originate from contamination from the surrounding layers with very high silver content. STEM-EDX analyses detected only two particles in the four inner layers of AgMask-08.
Representative SEM images of AgMask-08 (Figure 11), combined with EDX, show that the fibers of the external layers of this mask are nearly completely coated with a silver coating. This coating was damaged or incomplete in many areas: Figure 11A and Figure 11B show that the Ag^0 coating is released from the fiber surface at the pressure points of the woven fabric. Where the coating is detached, many Ag^0 NP are observed. Their size ranges from approximately 16 nm, the minimal size that readily can be detected with this SEM configuration, to 100 nm. The coating appears to be made up of Ag^0 NP: zooming in on the coating revealed that the coating consists of numerous finely packed or compressed nanoparticles (Figure 11D). If the nanoparticulate composition of the coating is taken in account, a 11 to 100-fold lower AEL_{mask} should be applied, such that the amount of silver in AgMask-08 exceeds the AEL_{mask} largely, by a factor between 103422 and 940200. This, and the observation that Ag NP are released, implies that a health risk of face masks of this type is highly probable and requires action.

The intermediate results of this project support the conclusions of Blevens et al.[37] that although many face masks have substantiated claims to contain silver with antiviral and antimicrobial properties, “these certifications or patents are not enough to determine credibility, and stricter regulations by federal agencies on product testing for manufacturers that make claims are necessary to ensure the efficacy of the product advertised, as well as a cloth face mask inhalation standard.”
These results further support the opinion of ANSES stating that “it is impossible to reach a single conclusion that can be generalized to all silver nanoparticles with regard to their identification, the evaluation of their dangerousness, their antibacterial activity and the possible phenomena of bacterioresistance, whatever the planned or existing applications”[8].
In general, these results support ANSES’s recommendations[8] to limit the use of silver nanoparticles (production, processing, use) to applications whose usefulness has been clearly demonstrated and for which the balance of benefits for human health in relation to the risks for the environment is positive.

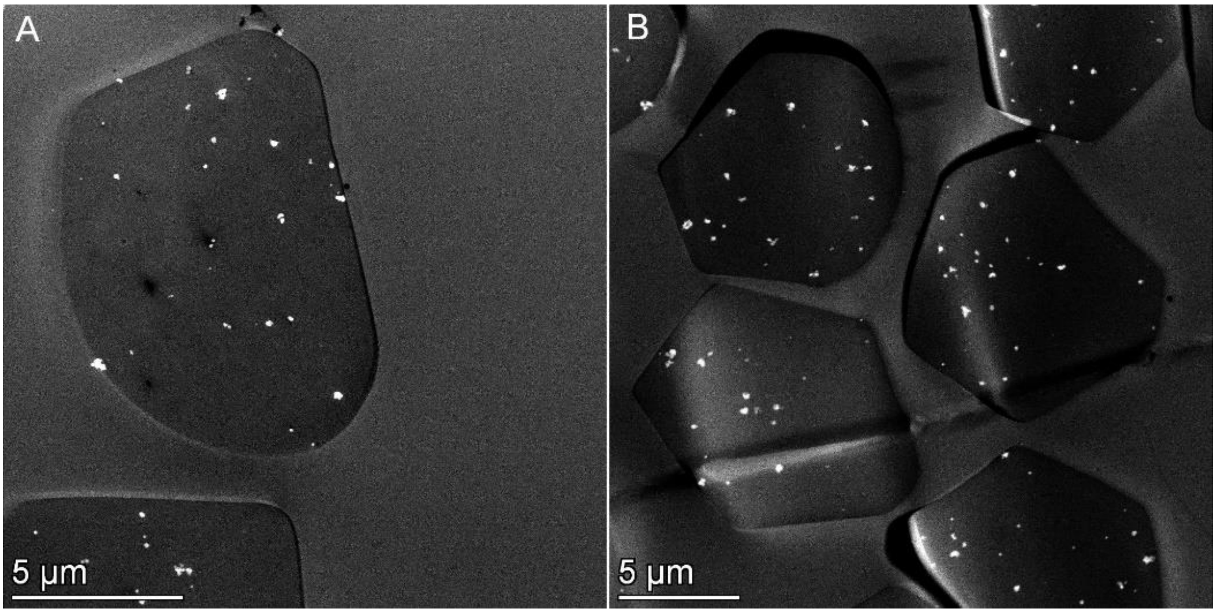

Figure 2 Representative STEM images showing cross sections of fibres observed in the face mask AgMask-15, with (A) the external layer, and (B) the internal layer.

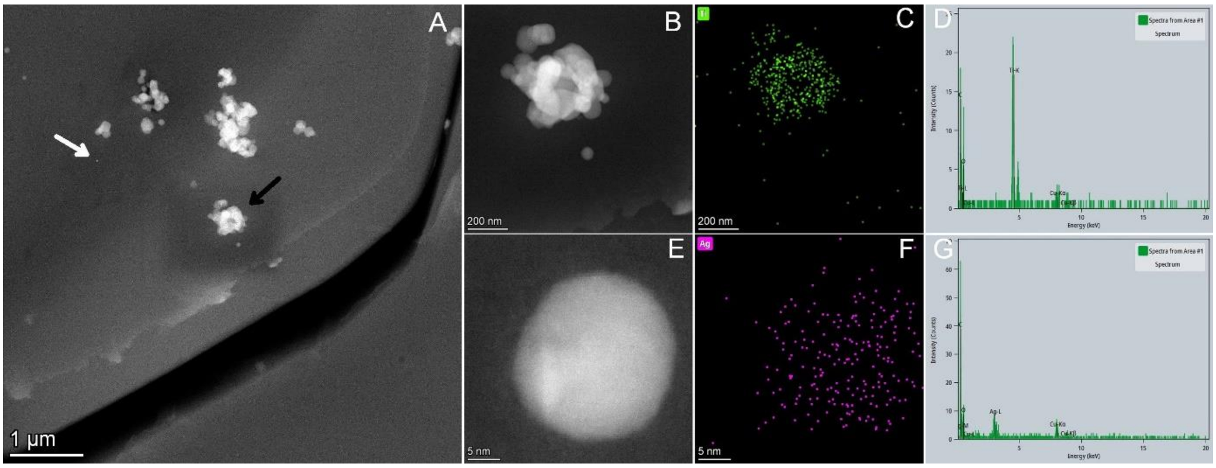

Figure 3 Representative STEM-EDX analysis of particles in a section of a polyamide fibre in AgMask-15, with (A) HAADF-STEM image showing silver (white arrow) and titanium dioxide(black arrow) particles, (B-G) STEM-EDX analysis of the particles indicated in A with (B, E) higher magnification STEM images of the particles, (C, F) the corresponding spectral images of Ti and Ag obtained by EDX and (D, H) the EDX spectra.

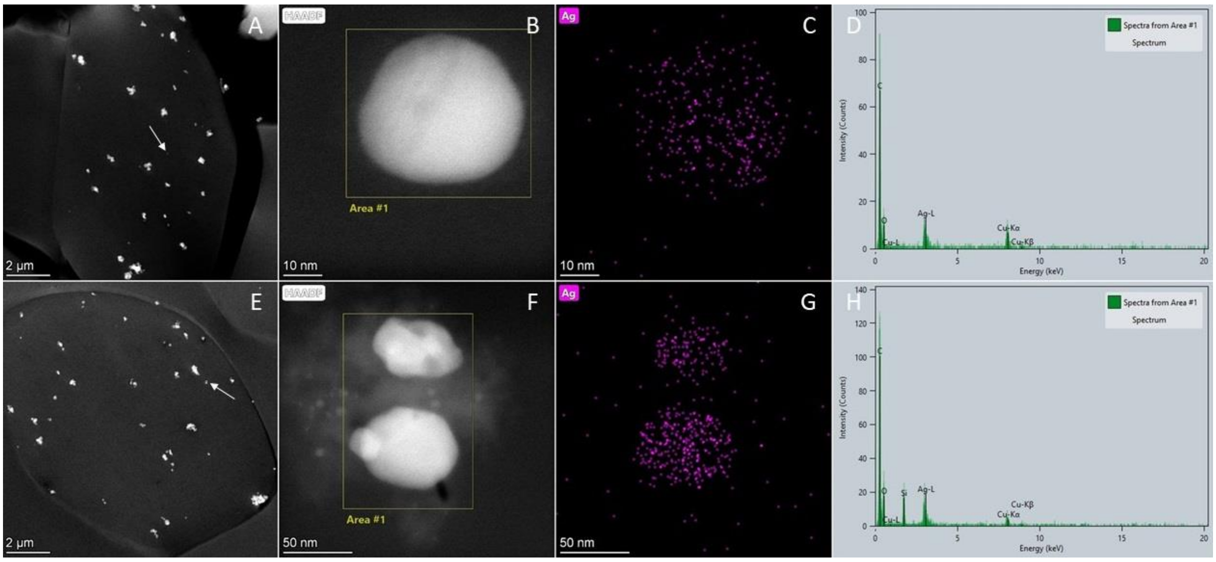

Figure 4 Representative STEM-EDX analysis of particles in a polyamide fibre in AgMask-15, with (A, E) HAADF-STEM images of a section of polyamide fibres showing particles, (B-D, F-H) STEM-EDX analysis of the particles indicated in A and E (white arrow) with (B, F) higher magnification STEM images of the particles, (C, G) the corresponding spectral images of Ag obtained by EDX and (D, H) the EDX spectra of the area indicated by the yellow box in B and F.

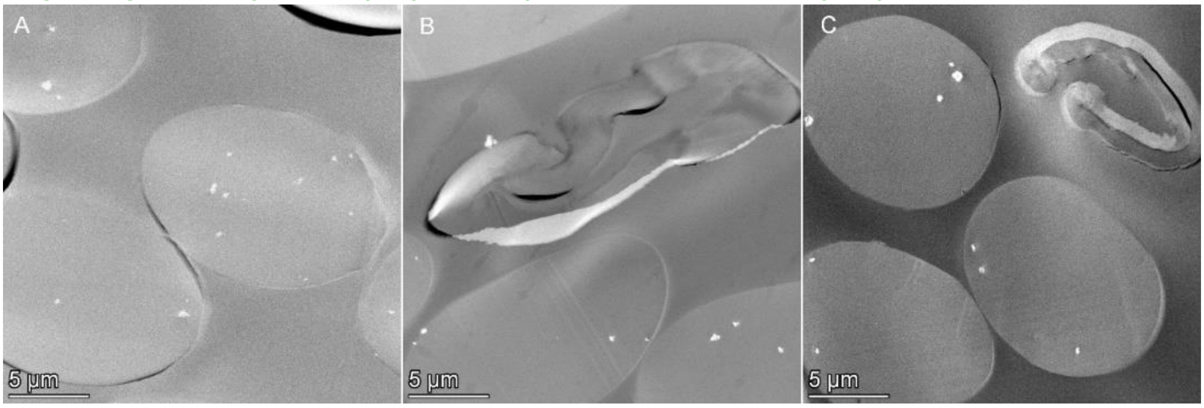

Figure 5 Representative STEM images showing cross sections of fibres observed in the face masks, with (A) the external layer (polyester), (B) the middle layer (polyester and cotton) and (C) the internal layer (polyester and cotton) of AgMask-03. Polyester fibres contain TiO_2 particles[21].

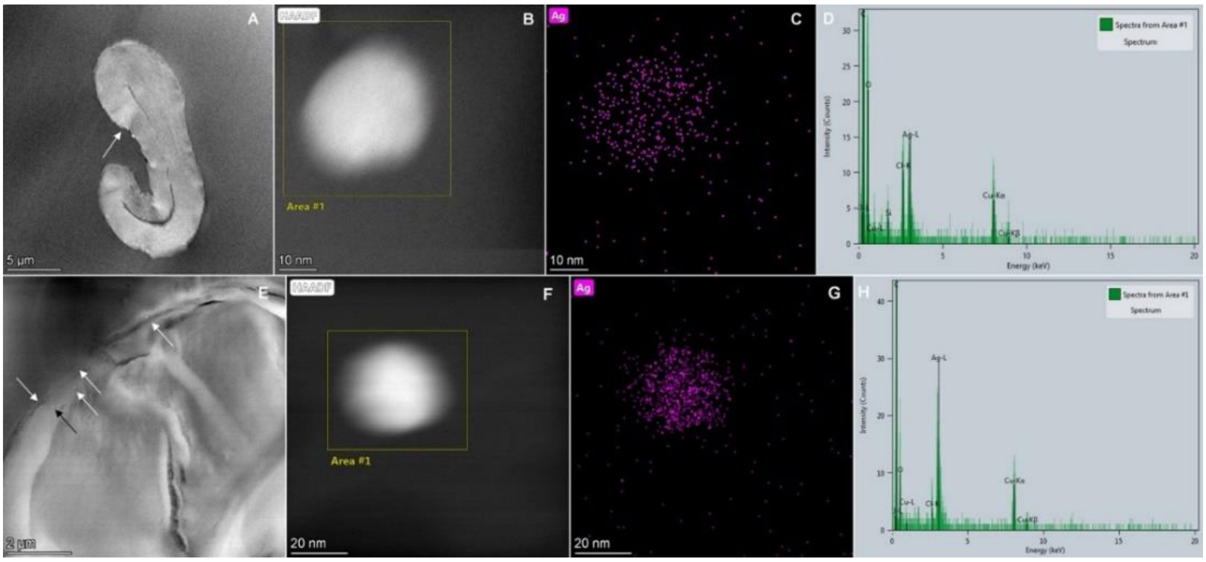

Figure 6 Representative STEM-EDX analysis of nanoparticles at the surface of cotton fibres in AgMask-03, with (A, E) HAADF-STEM images of a section of cotton fibres showing particles, (B-D, F-H) STEM-EDX analysis of the particles indicated in A (white arrow) and E (black arrow) with (B, F) higher magnification STEM images of the particles, (C, G) the corresponding spectral images of Ag obtained by EDX and (D, H) the EDX spectra of the area indicated by the yellow boxes in B and F.

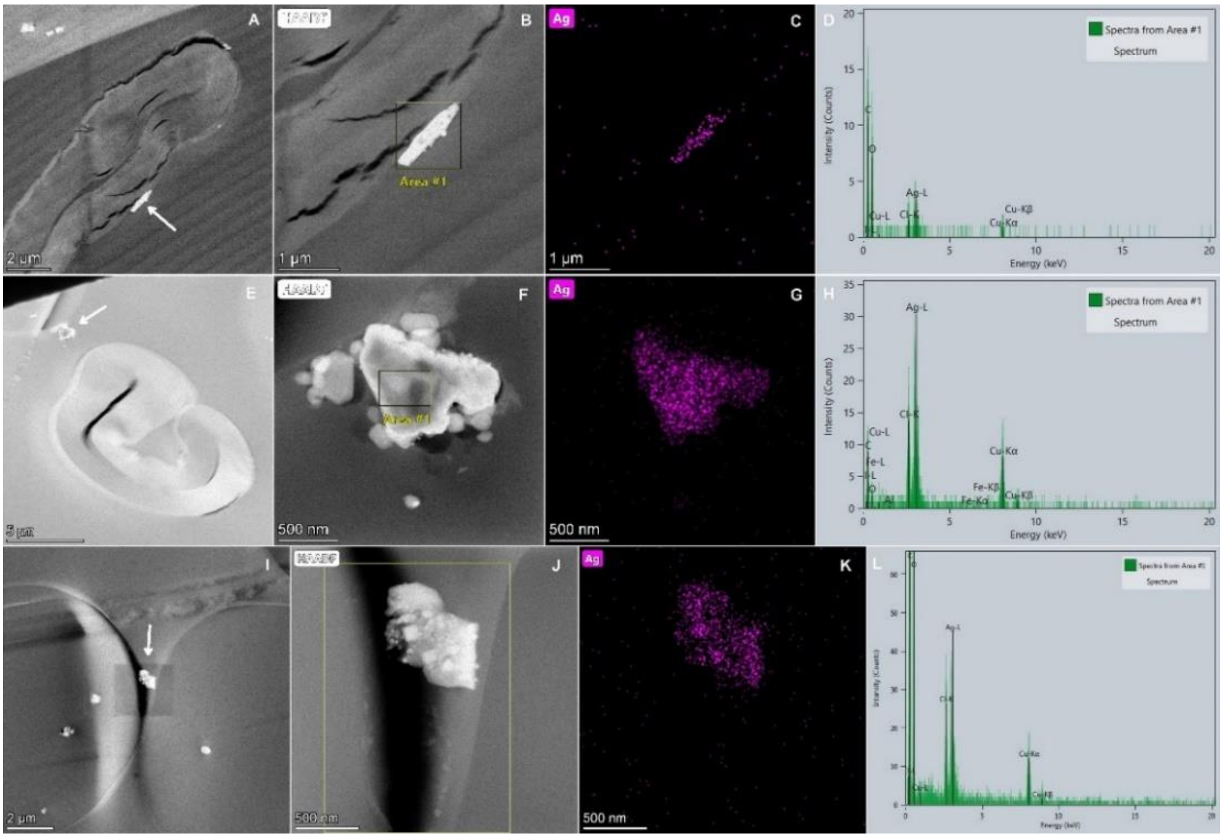

Figure 7 Representative STEM-EDX analysis of particles at the surface of a cotton fibre (A-D) and polyester fibres (E-L) in AgMask-03, with (A, E, I) HAADF-STEM image of a section of cotton and polyester fibres with particles at the surface, (B-D, F-H, J-L) STEM-EDX analysis of the particles indicated in A, E and I (white arrow) with (B, F, J) higher magnification STEM image of the particles, (C, G, K) the corresponding spectral image of Ag obtained by EDX and (D, H, L) the EDX spectrum of the area indicated by the yellow box in B, F and J.

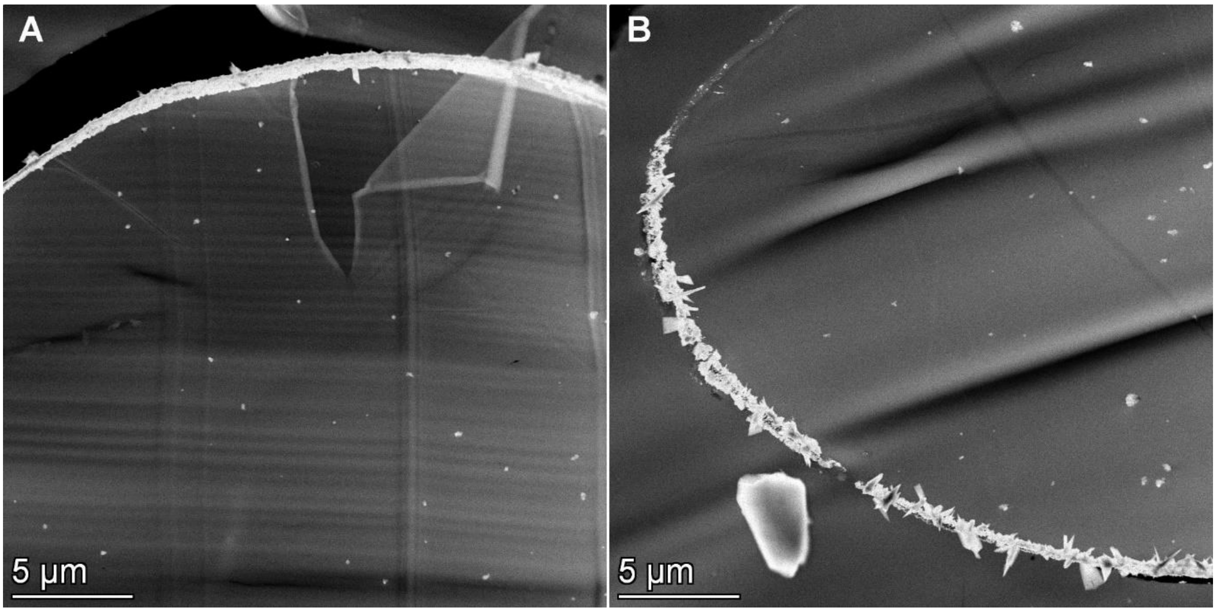

Figure 8 Representative STEM images showing cross sections of fibres observed in the face masks, with (A) the external layer, and (B) the internal layer of AgMask-08

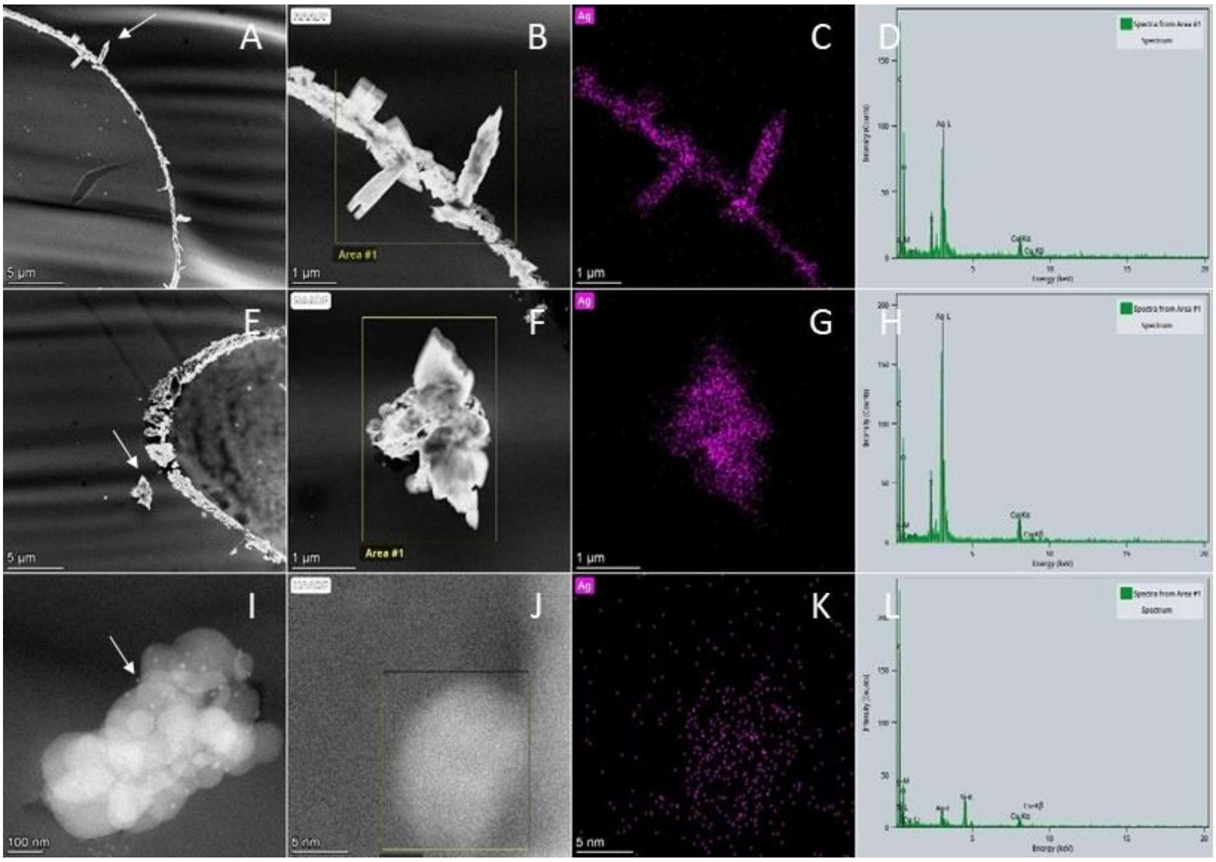

Figure 9 Representative STEM-EDX analysis of particles in a woven fabric in AgMask-08, with (A, E, I) HAADF-STEM images of a section of woven fibres showing particles, (B-D, F-H, J-L) STEM-EDX analysis of the particles indicated in A, E and I (white arrow) with (B, F, J) higher magnification STEM images of the particles, (C, G, K) the corresponding spectral images of Ag obtained by EDX and (D, H, L) the EDX spectra of the area indicated by the yellow box in B, F and J.

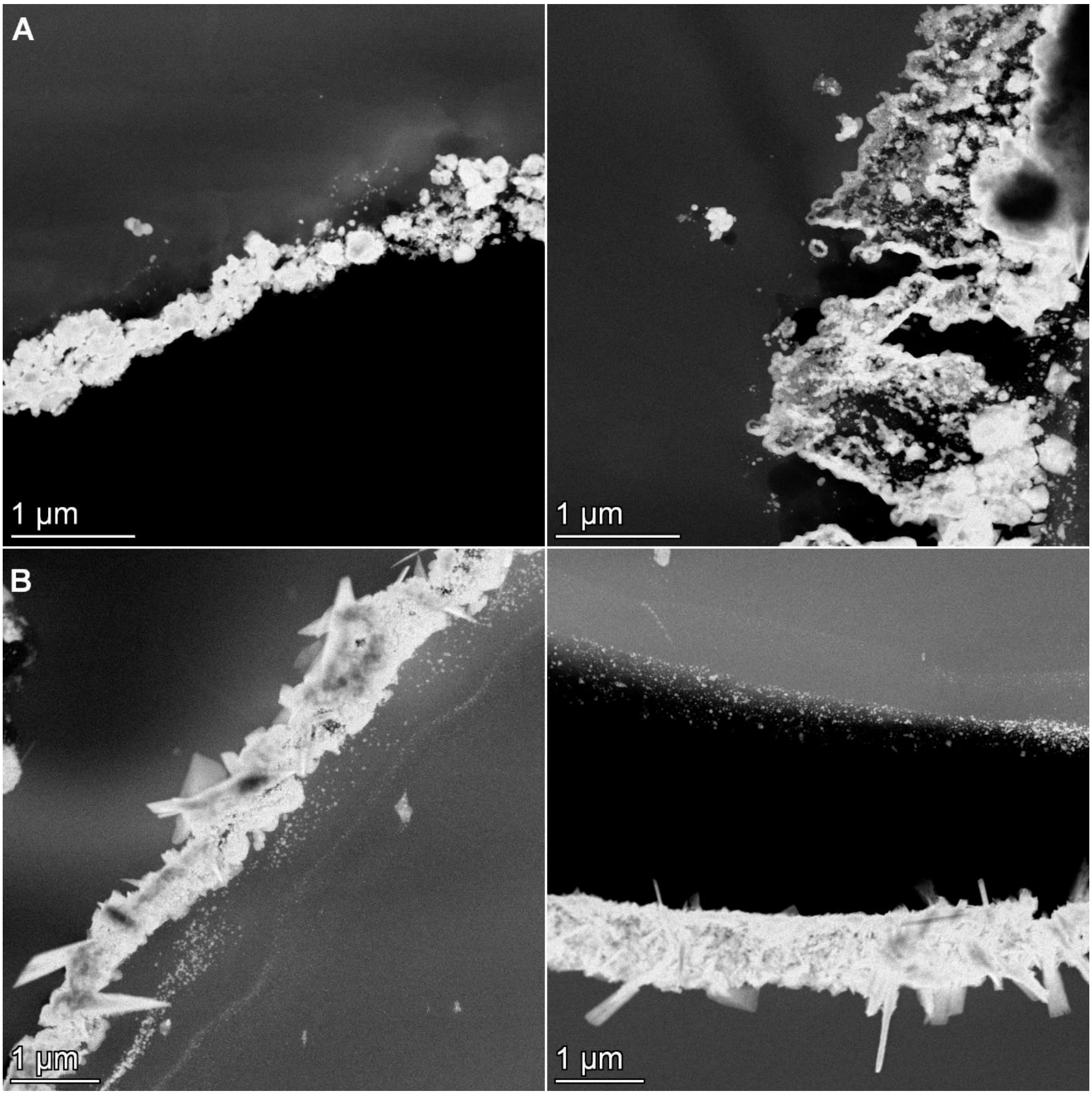

Figure 10 Representative STEM images showing the silver coating of woven fibres observed in a face mask, with (A) the external layer, and (B) the internal layer of AgMask-08.

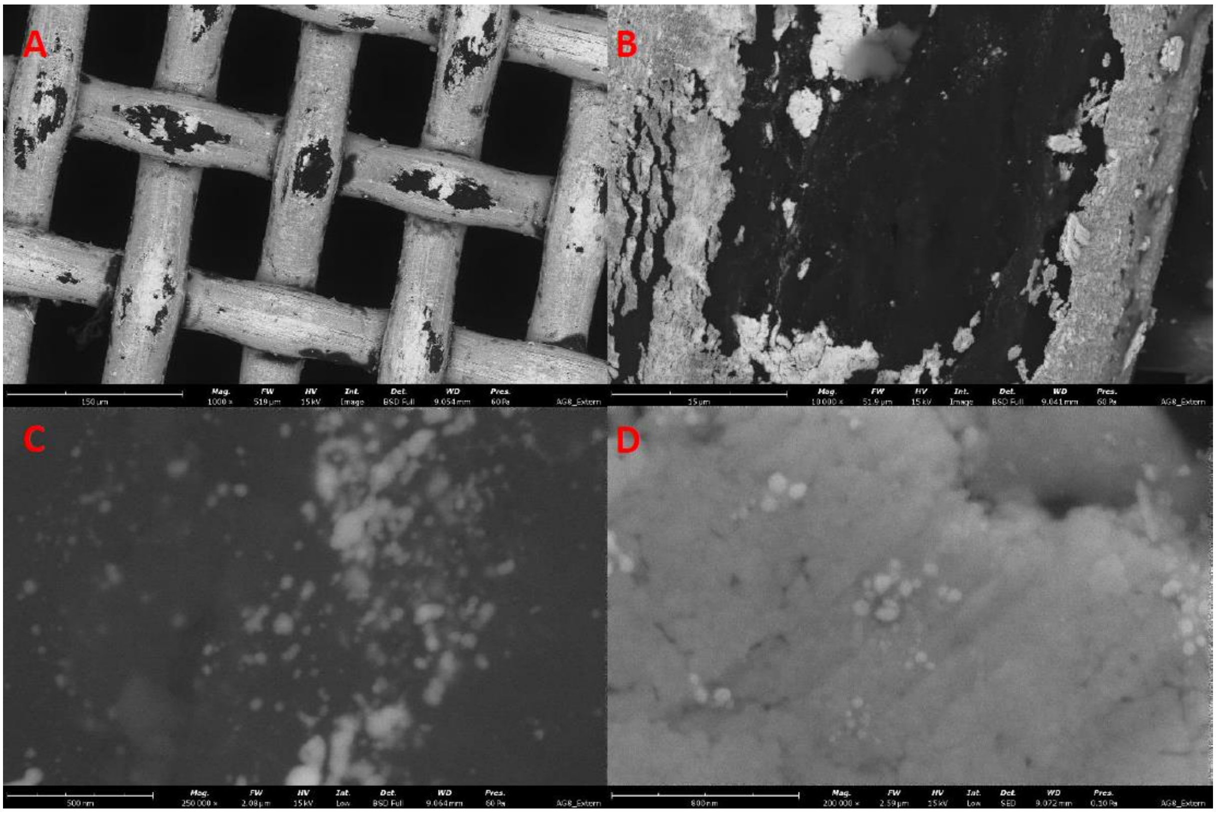

Figure 11 Representative SEM images of AgMask-08 at magnifications of 1000x (A), 10000x (B), 250000x (C) and 200000x (D). Courtesy of Thermo Fisher Scientific.

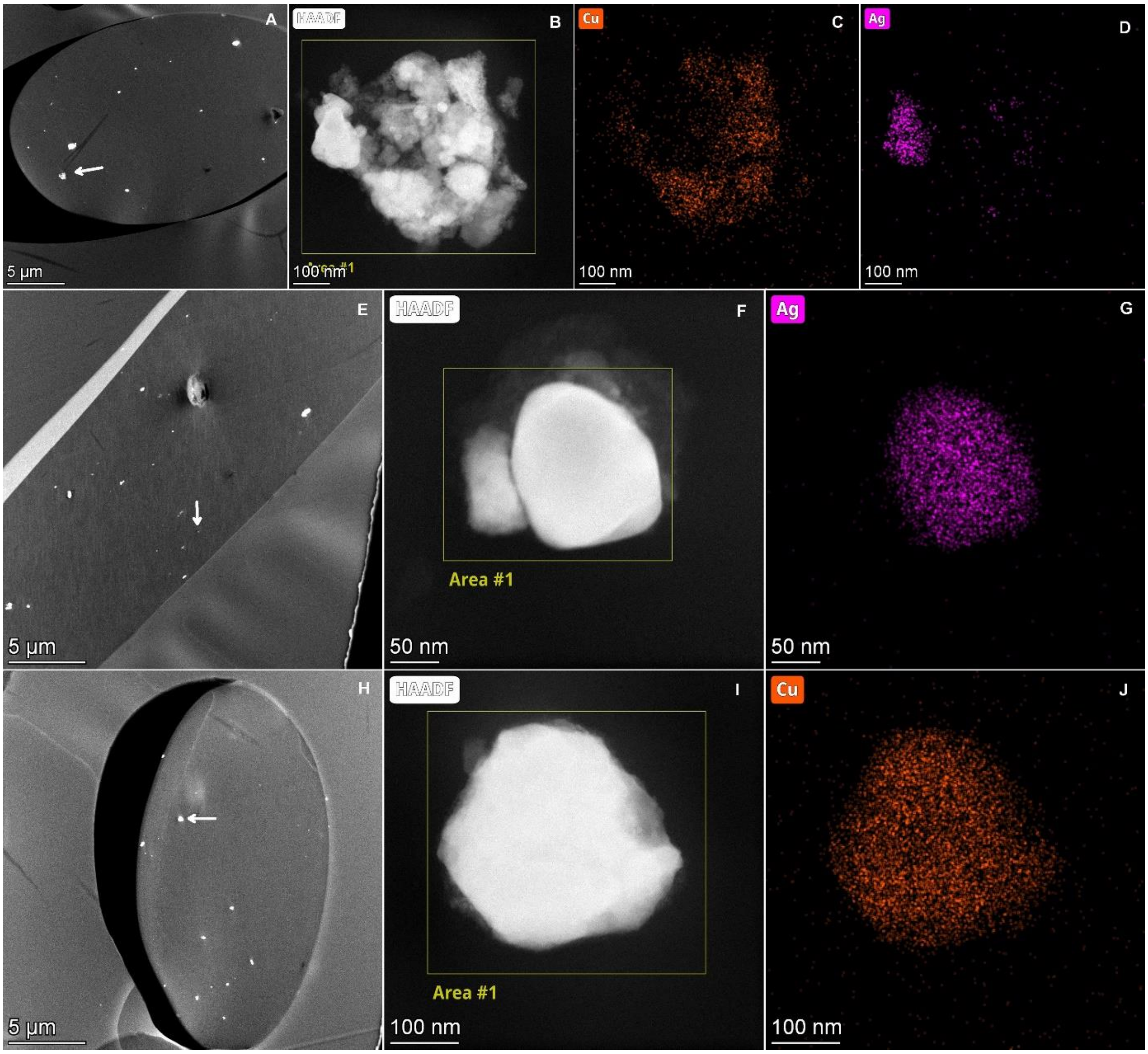

Figure 12 Representative STEM-EDX analysis of particles in non-woven fibres in AgMask-16, with (A, E, H) HAADF-STEM images of section of non-woven fibres showing particles, (B-D, F-G, I-J) STEM-EDX analysis of particles indicated in A, E, and H (white arrow) with (B, F, I) high magnification STEM images of the particles and (C-D, G, J) the corresponding spectral images obtained by EDX.

Table 2 Overview of the total amount of silver per layer and per face mask, presence of Ag particles per layer, types of the silver biocide , and comparison of the total silver content per mask with AEL_{mask} for the different types of face masks

Reference	Layers	Total Ag (µg/layer)[a]	Total Ag/mask (µg)	Ag particles[b]	Type of silver biocide	Times the amount of silver exceeds AEL_{mask}[e]
AgMask-01	External	ND	ND	N		
	Central	ND		N		
	Internal	ND		N		
AgMask-02	External	11.5	20	N	Ag^{+} ions	0.8
	Central	8.6		N		
	Internal	ND		N		
AgMask-03	External	37	37	Y	Ag^{+} ions, NP and large Ag^{0} particles	1.5
	Central	ND		Y		
	Internal	ND		Y		
AgMask-04	External	ND	ND	N		
	Central	ND		N		
	Internal	ND		N		
AgMask-05	External	ND	ND	N		
	Central	ND		N		
	Internal	ND		N		
AgMask-07	External	ND	ND	N		
	Internal	ND		N		
AgMask-08	External	112484	235044	Y	Ag^{0} coating, Ag^{0} NP, Ag^{+} ions	9402
	Central[c]	208		N		
	Internal	122352		Y		
AgMask-10	External	ND	ND	N		
	Central	ND		N		
	Internal	ND		N		
AgMask-11	External	ND	ND	N		
	Central	ND		N		
	Internal	ND		N		
AgMask-12	External	7.3	7.3	N	Ag^{+} ions	0.3
	Central 1			N		
	Central 2			N		
	Internal			N		
AgMask-13	External	3.0	13	N	Ag^{+} ions	0.5
	Central	ND		N		
	Internal	3.0		N		
AgMask-14	External	ND	87	N	Ag^{+} ions	3.5
	Central	1.3		N		
	Internal	85		N		
AgMask-15	External	83	165	Y	Ag^{0} NP	6.6
	Internal	82		Y		
AgMask-16	External	3.8	9.1	N	CuO particles NP Ag^{0} particles	0.4
	Central 1	3.3		Y		
	Central 2			N		
	Central 3			Y		
	Internal	2		N		
AgMask-17	External	1.2	2.8	N	No Ag^{0} NP observed, presumably only Ag^{+} ions	0.1
	Internal	1.6		N		
AgMask-18	External[d]	66	176	N[d]	Ag^{+} ions	7
	Internal	110				
AgMask-20	External	0.5	6.5	N	Ag^{+} ions	0.3
	Central	5.4		N		
	Internal	1.2		N		
AgMask-22	External	ND	14	N	Ag^{+} ions	0.6
	Internal	13		N		
AgMask-23	External	ND	ND	N		
	Central	ND		N		
	Internal	ND		N		
AgMask-24	External	2.3	3.2	N	Ag^{+} ions	0.1
	Internal	0.8		N		

[a] ND= not detected

[b] Ag particles are present in fibres (Y) or not present (N) as assessed by STEM-EDX analysis.

[c] AgMask-08 contains 4 identical central layers similar to those of AgMask-01.

[d] The external and internal layers of AgMask-18 are identical. So far, only the external layer was examined by STEM-EDX.

[e] Based on the AEL_{mask} calculated from the OEL for silver, as described on page 19

CONCLUSION

In situ analysis of silver-based particles in face masks using electron microscopy and EDX, combined with total silver measurement using ICP-MS or ICP-OES, demonstrated varying amounts and different types of silver-based biocides in a selection of face masks offered on the Belgian market and intended to be worn by the general public. Following types of silver-based biocides were demonstrated: (i) Ag^+ ions, (ii) metallic Ag^0 NP distributed in the matrix of the fibers, (iii) Ag NP and large silver particles at the surface of, or close to cotton fibres in face masks containing polycationic polymers binding Ag^+ ions (Silvadur™ technology), (iv) as a coating consisting of metallic silver releasing Ag^+ ions, Ag^0 NP and large silver particles.

The possible health risk of inhalation exposure of silver depends on the type and the amounts of the silver-based biocide. At this moment, no standardized methods are available to determine the amounts of the different types of silver biocides that are released from face masks during normal use. Hence, inhalation exposure cannot be measured directly, and the possible associated health risk related to application of face masks cannot be assessed directly.
Therefore, an AEL_{mask} of 25 µg was determined based on occupational exposure levels for metallic and ionic silver. Hereto, an intensive use scenario considering subchronic exposure of the general adult population was assumed. To objectify the possible risk of exposure of consumers to silver biocides from face masks intended to be worn by the general public, the AEL_{mask} was compared to the measured amount of total silver in the masks.
This comparison indicated that seven out of nine face masks with a silver biocide based on Ag^+ ions only can be considered safe. Two face masks with a silver biocide based on Ag^+ ions exceed the AEL_{mask} and a more refined risk evaluation is to be considered.
Four face masks contain Ag^0 NP in combination with other forms of the silver biocide (Ag^+ ions, and/or non-nanoparticulate silver). The amount of silver in these masks exceeded the AEL_{mask} of 25 µg for ionic and metallic silver. Per case an in-depth risk evaluation needs to be undertaken to account for the different forms of silver that are potentially released from face masks treated with the applied silver-based biocides. In one face mask, a combination of CuO and Ag^0 NP was observed. Further research is needed to conclude on the safeyy of this face mask.
The identification of both CuO NP and contaminating Ag^0 NP in an examined face mask illustrates the importance of detecting and evaluating combinations of different biocides to assure nanosafety.

The importance of wearing face masks against COVID-19 is unquestionable. Even so, the reported information urges for in depth research of the applications of silver-based biocides in face masks, and of (nano)technology applications in face masks in general. Phasing out applications that can be unsafe, product development based on the safe-by-design principle, and implementing regulatory standards, and guidelines taking in account nanosafety concerns can avoid possible future consequences caused by a poorly designed nanotechnology in consumer products, while maintaining nanotechnology's important potential to improve (medical) products such as face masks.

ANNEX

Literature survey regarding the toxicity of silver

IDENTIFICATION

Silver is identified by the CAS No: 7440-22-4 and the EC No 231-131-3. It is applied for its antimicrobial activities in water disinfectants, biocides, medical products, cosmetics, toothpastes. It is used as well in the food industry, as the food additive E 174 (food colour) and in food packaging materials[38,40,41].

TOXIKOKINETICS

Literature data suggest that AgNPs and silver ions eluted from AgNPs can be absorbed via the inhalation, oral, parenteral, or dermal routes in humans and experimental animals. Differences in rate and degree of absorption varied according to the route of exposure, particle size, degree of aggregation, dissolution rate, and/or nature of any surface coating or binding material, properties which may also be important in terms of toxicity. Biodistribution studies after AgNP exposure have shown that silver is distributed in several organs and tissues including the lung, the liver, the kidney, the spleen, the intestine, the testes, the brain and the blood after inhalation or ingestion. After inhalation and depending on the particle dimensions, uptake via the gastro-intestinal tract, through the mucociliary clearance mechanism followed by swallowing to the gut, cannot be excluded.

There is a possibility of bioaccumulation in certain target organs (e.g. brain and testes). The passage of silver (ions or nanoparticles) through blood-brain[38,42], placenta[43,44] or blood-testicular barriers[44,45] has been shown. In a subchronic (12-week) inhalation study in male and female rats dose-dependent silver concentrations were measured in lung tissue: "*The silver concentrations following 12 weeks of exposure gradually cleared from the lung tissue during a 12-week post-exposure (recovery) period, but the lung silver concentration remained statistically significantly elevated relative to controls at the highest exposure concentration of 381 µg/m^3. The lung clearance half-times (T1/2) ranged from 28.5 to 112.9 days across dose groups and sexes*"[46].

MODE OF ACTION

Induction of reactive oxygen species, interaction with proteins, interruption of intracellular O_2 reduction may be responsible of the different toxic effects observed (cytotoxicity, DNA damage, inflammation, apoptosis, necrosis, lipid peroxidation, inhibition of mitochondrial activity, …)[47,27].

"Although the biological interactions with inhaled AgNPs are not fully understood, the possible mechanisms include dissolution and release of soluble silver species, and particle transformation through sulfidation (binding with sulfur) or opsonization (binding with protein), which can stabilize the silver" [48,40,27].

GENOTOXICITY

Ag NPs produce genotoxic effects in both *in vitro* and *in vivo* assays[40]. However, a larger proportion of positive results was obtained in the *in vitro* tests. The *in vivo* oral genotoxicity studies performed do not allow a definitive assessment of the possible genotoxic hazard associated with oral exposure to Ag NPs.

Positive results have been reported *in vitro* in gene mutations tests in mouse lymphoma L5178Y assays, in comet assays (DNA breaks) and in micronucleus tests in various mammalian cell lines. Histone H2AX phosphorylation and upregulation of DNA damage response and repair proteins have also been reported. [40]

In vivo chromosomal aberrations were reported in rat bone marrow after oral, intraperitoneal or intravenous exposure to Ag NP. Both positive and negative results were shown in *in vivo* micronucleus tests in mice and rats (blood, bone marrow or liver) after oral or intravenous exposure. A majority of negative *in vivo* Comet assays (in liver, lung, testis, blood or bone marrow) were reported in mice or rats exposed orally or intravenously. The enzyme-modified versions (Fpg, Endo-III or OGG-1), which detect mainly oxidized bases, produced more positive results. Considering all of the *in vivo* results, there is no clear evidence that the genotoxic effect of Ag NPs is influenced by NPs size or coating.

Ag NP-induced oxidative stress and the resulting high level of reactive oxygen species are potential reasons for the observed DNA perturbations causing DNA breaks and oxidative adducts.
Interpretation of *in vitro* findings in comparison with those from *in vivo* studies is challenging. Study protocols and controls are highly variable across studies. Additionally, genotoxicity studies of transformed or cancer-derived cell lines may have limited relevance for predicting effects *in vivo* in animals and humans. The doses in *in vitro* studies can be much higher than those *in vivo*, and thus the *in vitro* results may not be predictive of the outcomes at much lower doses *in vivo*. Statistically significant increases in bulky DNA adducts were reported at doses from 2.5 µg Ag NP/ml. [27]
In vitro studies have shown that the predominant topic areas for cellular and sub-cellular changes induced by Ag NPs are (1) development of oxidative stress and induction of apoptosis and (2) DNA damage/genotoxicity.
Ag NPs with the smallest diameter were more effective than larger nanoparticles in mediating physiologic and toxicological changes (enhanced generation of reactive oxygen species, significant depletion of cellular reduced glutathione levels, lowered mitochondrial function)[27,38,39].

TOXICITY: TARGET ORGANS

Humans
There is a lack of long-term toxicity data, consumer exposure data, and human health effect data on Ag NPs. Only few studies have been published on worker exposures to silver nanomaterials. No adverse health effects were reported and nanomaterial exposures were not characterized in detail[27,39].

Animals: inhalation exposure
Acute or sub-chronic toxicity studies in rats and mice have identified few clinical or behavioral effects regardless the route of exposure (oral, inhalation, dermal). Particles smaller than 0.1 µm have been shown to penetrate deeply into the alveolar region, mainly by diffusion. The LC_{50} (concentration of a chemical that kills 50% of the test species) of silver nanoparticles is ≥ 3.1×10^6 particles/m^3 (750 µg/m^3)[49].
Following acute and subacute inhalation, lung inflammation ranged from nonexistent to minimal/moderate, with varying degrees of resolution over time[50–52]. Lung function deficits, where present, were transient[53]. Goblet cell hypertrophy and hyperplasia were noted in one study[54].

The main identified target organs of Ag NPs are the lung, the liver and the kidneys.

In repeated inhalation toxicity studies, Sprague Dawley rats were exposed to 0, 49, 133 or 515 µg/m^3 Ag NP (diameters ranging from 2 to 65 nm (median of 16 nm)) for 6h/day, 5 days/week for 13 weeks in a whole body inhalation chamber[55,56] or exposed to 0, 49, 117 or 381 µg/m^3 for 12 weeks followed by 12 weeks recovery[46]. These studies demonstrated the uptake of silver ions or nanoparticles in the blood and subsequent distribution to all major organs and tissues: lungs, liver, kidneys, blood, brain, spleen, eyes, testes, and ovaries[46,55,56]. Perturbation of lung function and induction of inflammatory responses were shown[44, 546,55,56]. In addition, histopathologic changes were observed in the kidney and in the liver, in which bile duct hyperplasia and necrosis were identified[56]. The LOAEL for lung function deficits in female rats was 49 µg/m^3 whereas in male rats the NOAEL was 133 µg/m^3 (Sung et al. 2008)[53]. In contrast in the study of Song et al. (2013)[44], the NOAEL for lung function deficits was 49 µg/m^3 in male rats and 381 µg/m^3 in female rats. The NOAEL for bile duct hyperplasia was 133 µg/m^3 (Sung et al. 2009)[54].

In a 13-week inhalation study[55,56], lung function deficits (decreased tidal volume, minute volume, and peak inspiration flow), inflammation responses, and alveolar accumulation of macrophages were reported in the rats[55]. In a recovery study[46], it was shown that 12 weeks after cessation of exposure, the alveolar inflammation had resolved in the female rats and had resolved in all but one of the male rats at the highest dose. Pulmonary fibrosis was not observed. Lung inflammation was an early-stage effect of minimal severity.

The $BMCL_{10}$ estimated for pulmonary inflammation (see NIOSH, 2021) was 62.8 µg/m^3, based on the pooled male and female rat data from both subchronic inhalation studies (Sung et al. 2009; Song et al. 2013). The $BMCL_{10}$ estimated for liver bile duct hyperplasia (see NIOSH 2021) were 50.5 and 92.5 µg/m^3 in female and male rats, respectively (Sung et al. 2009).

Toxicity of Ag NPs has been demonstrated *in vitro* for several different types of alveolar cells (e.g. epithelial cells and macrophages). The rat pulmonary inflammation response to Ag NPs following subchronic inhalation is an early-stage effect of minimal severity. Persistent inflammation in animals or humans can result in disease. Adverse lung and liver effects associated with exposure to Ag NPs in these rat studies were considered to be relevant to humans.

The release of ionic silver has been found to be the main cause of toxicity. Nevertheless an increasing number of studies found that this release cannot alone account for the toxic effects observed.

<u>Animals: oral exposure</u>

The oral LD_{50} of citrate capped Ag NP in rats is >2000 mg/kg bw [57].

Mice were exposed by gavage to 0.25, 0.5 or 1 mg Ag NP/kg bw per day for 28 days[58]. Liver toxicity and kidney inflammation were observed at 1 mg/kg bw per day. The NOAEL was 0.5 mg/kg bw per day.

Sprague Dawley rats were exposed by gavage to 0, 30, 300 and 1000 mg Ag NP/kg bw per day for 28 days. Significant dose-dependent changes were found in the alkaline phosphatase and cholesterol values in either the male or female rats, indicating that exposure to ≥ 300 mg of Ag NP may result in slight liver damage. The NOAEL was 30 mg/kg bw per day[59]. F344 rats were exposed by gavage to 0, 30, 125 and 500 mg Ag NP/kg bw per day for 90 days. Significant dose-dependent changes were found in alkaline phosphatase and cholesterol. A higher incidence of bile-duct hyperplasia with or without necrosis, fibrosis and/or pigmentation was observed. There was a gender-related difference in the accumulation of silver in the kidneys (2-fold increase in female kidneys compared to males). The NOAEL was 30 mg/kg bw per day [60].

REPRODUCTIVE TOXICITY

It is difficult to assess the reproductive (fertility) and developmental potential of Ag NPs due to the limitations of available data.

Oral exposure during gestation (GD6-19) of pregnant female rats to 0, 100, 300 and 1000 mg Ag NP/kg bw per day caused oxidative stress in maternal liver at ≥100 mg/kg bw per day, but did not cause developmental toxicity at doses up to 1000 mg/kg bw per day[61].

Exposure to Ag NPs resulted in testicular/sperm toxicity in males and ovarian and embryonic toxicity in females. It was reported that maternal injection of Ag NPs delayed physical development and impaired cognitive behavior in offspring. Accumulation of Ag was noted in the testes after administration of Ag NPs[55].

It was recently shown that inhaled silver nanoparticles accumulated in placental and fetal tissues in mice and were associated with an adverse pregnancy outcome[43]. An increased number of resorbed fetuses was observed in female mice exposed to 18- to 20-nm Ag NP by nose-only inhalation at 640 µg/m^3, for 4 h/day during the first 15 days of gestation. Estrogen plasma levels were reduced in the mothers, and inflammatory mediators were elevated in the lungs and placenta[43].

A Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test was conducted in rats[62]. Citrate capped silver nanoparticles were orally administered to groups of Sprague-Dawley rats at 0, 62.5, 125 and 250 mg/kg bw per day for 42 days (males: 14 days before mating, 14 days during the mating, and 14 days of post-mating; females: 14 days before mating, during the mating and gestation, and 4 days of lactation). No adverse effects have been reported. The NOAEL of cAg NPs is considered to be > 250 mg/kg bw per day for general toxicity in parent animals and in F1 pups.

Oral exposure of pregnant rats to 0, 1, 3 or 5 µg/kg bw per day during pregnancy resulted in a delay in vaginal opening and testes descent in the offspring[63].

Adult male rats were exposed to Ag NP by gavage to 0, 5.36 or 13.4 mg/kg bw per day (twice weekly) for 6 months. There was a significant decrease in testosterone level and a significant increase in the luteinizing hormone level. There were also dose-related significant decreases in superoxide dismutase activity and increases in malondialdehyde levels in the testis of the treated animals, indicating the induction of oxidative stress by Ag NPs. Sperm viability was decreased at the two doses and vacuolations were observed in Sertoli cells with disturbance in the arrangement[64] [27,38,57].

CARCINOGENICITY

There is no carcinogenicity study available[38,39].

NEUROTOXICITY

There are few neurotoxicity data available on rodents. Some motor and cognitive (memory) effects have been reported, but they need to be confirmed. Considering the potential passage of Ag NPs through blood-brain barriers and their bioaccumulation, toxic effects at the level of the central nervous system can't be excluded.

Lee et al.[60] described that in a 14-day inhalation exposure of mice to Ag NP, alterations in brain gene expression occurred. Ag NP exposure modulated the expression of several genes associated with motor neuron disorders, neurodegenerative disease and immune cell function[38,39].

IMMUNOTOXICITY

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[38,39,41,57].

OCCUPATIONAL EXPOSURE LIMITS

<u>OEL for silver nanoparticles</u>

1) An occupational exposure limit (OEL) of <u>**0.19 µg/m³**</u> for Ag NPs has recently been proposed based on the silver tissue dose and liver bile duct hyperplasia response in female rats observed in a subchronic rat inhalation toxicity study[56] and by taking the human equivalent concentration (HEC) with kinetics into consideration[18].

2) *Christensen et al.* [32]*derived OELs of* <u>***0.1–0.67 µg/m³***</u> *as an 8-hr TWA for Ag NPs, based on the rat subchronic inhalation data reported in Sung et al.* [55,56,65].

3) *The PBPK model of Bachler et al.*[66] *was also used to estimate the 45-year working lifetime Ag NP exposure concentrations that would result in tissue doses equivalent to those associated with the NOAEL or benchmark dose estimates for pulmonary inflammation or liver bile duct hyperplasia in the rat subchronic inhalation studies*[46,56]*. The estimated HECs to the rat NOAELs for lung and liver effects were 0.19 to 3.8 µg/m³ for total silver, and 6.2 to 195 µg/m³ for soluble/active tissue doses, depending on particle size (15-nm- or 100-nm-diameter Ag NPs)"* [27].

   "The lowest HEC estimate of 23 µg/m³ was used as the point of departure (PoD) to derive a recommended exposure limit (REL) for silver nanomaterials. A total uncertainty factor of 25 was applied to the PoD estimate (i.e. 23 µg/m³ / 25 = 0.9 µg/m³).

   Thus, the NIOSH REL for silver nanomaterials (<100 nm primary particle size) is <u>***0.9 µg/m³***</u> *as an airborne inhalable 8-hour time-weighted average (TWA) concentration"*[65].

4) **NIOSH** : Recommended exposure limit : 0.9 µg/m³ [27,65]

OEL for total silver (As reported by NIOSH[27])

1) MAK (Maximum Workplace Concentration) values [28]: inhalable fraction : silver salts: 10 µg/m^3, silver: 100 µg/m^3
2) ACGIH Threshold Limit Values (2001): Metal dust and fume, inhalable fraction: soluble: 10 µg/m3, insoluble: 100 µg/m^3
3) NIOSH and OSHA: identical values for soluble and insoluble silver : 10 µg/m^3 [27]

HEALTH VALUES

EPA

In a provisional risk assessment, US EPA used reference values obtained in studies with other nanosilver materials: an inhalation No Observed Adverse Effect Concentration (NOAEC) of 133 $\mu g/m^3$ based on a 90-day study in rats[56], an oral NOAEL of 0.5 mg/kg bw per day based on a 28-day study in mice[58], 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 of 1000 and 3000 for incidental and chronic exposures.

EFSA

In 2016, EFSA concluded that the available information was insufficient to assess the safety of silver (and Ag NPs) as food additive (E174).[38] Currently, no acceptable daily intake is established for E174 nor for the nanoparticle fraction present in E174.

ANSES

"In 2015, ANSES recommends limiting the use of silver nanoparticles (production, processing, use) to applications whose usefulness has been clearly demonstrated and for which the balance of benefits for human health in relation to the risks for the environment is positive. ANSES stated that it is impossible to reach a single conclusion that can be generalized to all silver nanoparticles with regard to their identification, the evaluation of their dangerousness, their antibacterial activity and the possible phenomena of bacterioresistance, whatever the planned or existing applications." [8]

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