Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Silver

EC Number: 231-131-3
CAS Number: 7440-22-4

CLH-O-0000007152-82-01/F

Adopted

2 June 2022

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CLH-O-0000007152-82-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **Silver**

EC Number: **231-131-3**

CAS Number: **7440-22-4**

The proposal was submitted by **Sweden** and received by RAC on **14 September 2020.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Sweden has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **19 October 2020**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **18 December 2020**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Brendan Murray**

Co-Rapporteur, appointed by RAC: **Irina Karadjova**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **2 June 2022** by **a simple majority of all members present and having the right to vote**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	Silver massive: [particle diameter ≥ 1 mm]	231-131-3	7440-22-4	Muta. 2 Repr. 1B Skin sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H341 H360FD H317 H400 H410	GHS08 GHS07 GHS09 Dgr	H341 H360FD H317 H410		 M = 10 M = 10	
RAC opinion	TBD	Silver massive: [particle diameter ≥ 1 mm]	231-131-3	7440-22-4	Repr. 2 STOT RE 2	H361f H373 (nervous system)	GHS08 Wng	H361f H373 (nervous system)			
Resulting entry in Annex VI if agreed by COM	TBD	Silver massive: [particle diameter ≥ 1 mm]	231-131-3	7440-22-4	Repr. 2 STOT RE 2	H361f H373 (nervous system)	GHS08 Wng	H361f H373 (nervous system)			

Note - The DS originally proposed that massive and powder silver should have the same classification and therefore one entry in Annex VI to CLP (i.e., all silver > 100 nm), based on the hazards of the powder. However, RAC has concluded that massive silver warrants independent assessment for aquatic hazards and 'No Classification' is concluded for aquatic hazards of massive silver. Consequently, separate classification tables are presented for massive, powder, and nano silver.

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	Silver powder: [particle diameter > 100 nm < 1 mm]	231-131-3	7440-22-4	Muta. 2 Repr. 1B Skin sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H341 H360FD H317 H400 H410	GHS08 GHS07 GHS09 Dgr	H341 H360FD H317 H410		 M = 10 M = 10	
RAC opinion	TBD	Silver powder: [particle diameter > 100 nm < 1 mm]	231-131-3	7440-22-4	Repr. 2 STOT RE 2 Aquatic Acute 1 Aquatic Chronic 1	H361f H373 (nervous system) H400 H410	GHS08 GHS09 Wng	H361f H373 (nervous system) H410		 M = 10 M = 10	
Resulting entry in Annex VI if agreed by COM	TBD	Silver powder: [particle diameter > 100 nm < 1 mm]	231-131-3	7440-22-4	Repr. 2 STOT RE 2 Aquatic Acute 1 Aquatic Chronic 1	H361f H373 (nervous system) H400 H410	GHS08 GHS09 Wng	H361f H373 (nervous system) H410		 M = 10 M = 10	

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	Silver nano: [particle diameter > 1 nm ≤ 100 nm]	231-131-3	7440-22-4	Muta. 2 Repr. 1B Skin sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H341 H360FD H317 H400 H410	GHS08 GHS07 GHS09 Dgr	H341 H360FD H317 H410		M = 1000 M = 100	
RAC opinion	TBD	Silver nano: [particle diameter > 1 nm ≤ 100 nm]	231-131-3	7440-22-4	Repr. 2 STOT RE 2 Aquatic Acute 1 Aquatic Chronic 1	H361f H373 (nervous system) H400 H410	GHS08 GHS09 Wng	H361f H373 (nervous system) H410		M = 1000 M = 1000	
Resulting entry in Annex VI if agreed by COM	TBD	Silver nano: [particle diameter > 1 nm ≤ 100 nm]	231-131-3	7440-22-4	Repr. 2 STOT RE 2 Aquatic Acute 1 Aquatic Chronic 1	H361f H373 (nervous system) H400 H410	GHS08 GHS09 Wng	H361f H373 (nervous system) H410		M = 1000 M = 1000	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Introduction

Silver (EC 231-131-3, CAS 7440-22-4) is a soft, malleable transition metal with the highest known electrical and thermal conductivities. The CLH proposal covers all forms of elemental or metallic silver (See 1.3 below). Silver is not listed in Annex VI of CLP and has not been considered for harmonised classification and labelling previously. All hazard endpoints were open for consideration.

The classifications proposed by the Dossier Submitter (DS) cover:

- Skin Sens. 1 H317,
- Muta. 2 H341,
- Repr. 1B H360FD,
- Aquatic Acute 1 (M=10, silver),
- Aquatic Acute 1 (M=1000, nanosilver),
- Aquatic Chronic 1 (M=10, silver),
- Aquatic Chronic 1 (M=100, nanosilver).

The CLH report is based mainly on data derived from silver nanoparticles and an extensive read-across from other silver compounds such as silver salts and silver containing active substances (SCAS), supplied from various sources including the review programme for biocides under Regulation (EU) No 528/2012 (Biocides Products Regulation), the registration dossier under Regulation (EC) No 1907/2006 (REACH) and numerous published studies identified in the scientific peer-reviewed literature. There were few studies available in which the toxicity of silver in bulk form (i.e. on a scale greater than the nano) was investigated.

Scope of the Silver CLH report

The classification proposed by the DS solely covers silver with EC number 231-131-3 and CAS number 7440-22-4 (this includes silver massive-, powder- and nano-forms).

Silver and silver compounds - substance naming standardization and substances used in human health endpoint investigations:

The test substances considered relevant for the human health hazard assessment of silver by the DS, may be broadly categorised into two groups: silver metal and silver compounds, with the former subdivided into silver in bulk form and silver in nanoparticle form (see below).

- Silver (EC number 231-131-3 and CAS number 7440-22-4):
    i. Nanoforms: Elemental silver (Ag^0) nanoparticles (single grouping), covering a variety of silver sizes ≤ 100nm in the largest dimension.
    ii. Elemental silver (Ag^0) in bulk form > 100nm in any one or more dimensions (incorporating micron-sized silver, dust, flake and massive forms, essentially metallic silver). Bulk forms can be further subdivided into:
        - massive silver (particle diameter ≥ 1 mm) and
        - silver powder (particle diameter > 100 nm < 1 mm).
- Silver compounds (substances other than elemental silver, with their own EC and CAS numbers) that have the potential to release silver ions (Ag+) into biological systems; these include

i. silver salts and

ii. a variety of low solubility silver compounds that act as slow-release matrices or ion exchangers (e.g. silver zinc zeolite).

The term, "colloidal silver" means a mixture of silver particles held in a liquid suspension without forming an ionic, or dissolved solution. Any reference to colloidal silver in this opinion therefore relates to suspensions of silver particles.

The DS included data from different silver salts and other silver compounds as well as from silver nanoparticles to support the classification proposals for silver in the CLH report, which was based on the assumption that free Ag^+ ions in biological systems mediate the toxicity of silver and the tested silver compounds. The DS considered all toxicities of the tested substances to arise from the released Ag^+ ion (noting however that silver nanoparticles could potentially also have direct effects on the target organs) and as all forms of silver which are placed on the market in forms in which it can reasonably be expected to be used have a potential to release Ag^+ ions in biological systems, it was considered justified to propose the same harmonised classification for all forms (i.e. combining all sizes and shapes) of silver. RAC agrees that free Ag^+ ions in biological systems can mediate the toxicity of silver. However, careful consideration must be applied to the results of individual studies using data from different silver compounds because the kinetics determining Ag^+ ion release can differ greatly depending on the nature of the source of silver ions. RAC acknowledges that read-across from a wide variety of silver compounds and their value to the hazard assessment of silver must be carefully assessed, noting that it may not always be the most appropriate approach to follow.

Silver nanoparticles represent the smallest manufactured particle size of marketed silver metal, thus data from studies using silver nanoparticles supporting classification for human health hazards should also be used to represent bulk forms of elemental silver such as massive and silver powder (including micron-sized silver dust). For practical reasons, silver nanoparticles are considered together, even though at an individual level, physicochemical characteristics such as size, shape, surface charge, surface functionalization, or core composition may be different and may influence their interactions with biological systems and affect their uptake, toxicokinetics, and toxicodynamics. Studies show that in biological media, silver nanoparticles may be transformed into different forms by aggregation, agglomeration, dissolution, interaction with biomolecules, or in their ability to generate reactive oxygen species (ROS), properties that may lead to the coexistence of nanoparticulate, ionic, metallic, and complex salt forms. Silver nanoparticles can oxidatively transform to release silver ions which in turn can also be reduced to regenerate a nanoparticulate form, depending on the biological environment they find themselves in (Pem *et al*. 2021).

Toxicokinetics and bioavailability

The toxicokinetics of silver and silver compounds have been described in the CLH report by the DS. When assessing the classification of silver metal for human health hazard classes such as skin sensitisation, mutagenicity and reproductive toxicity as proposed by the DS, consideration of the toxicokinetics of silver is needed to evaluate the applicability of data via read-across from other silver compounds.

The CLH report draws from a wide range of publications and summary reports, to assess the limited data available on the toxicokinetics of silver speciation. The oral absorption of silver ions (based on older, non GLP, non-guideline studies with $AgNO_3$) was estimated at 5%. Oral absorption is assumed to be similar for silver nanoparticles. Silver once absorbed becomes widely distributed across all organs and tissues. It can also bind to sulfhydryl groups on gluthathione and be eliminated in the bile. Results from a 28-day study in rats (Van der Zande *et al*. 2012) indicate a similar distribution pattern between silver nitrate and nanoparticles of silver following

oral exposure, with a higher uptake of silver in animals treated with the soluble salt $AgNO_3$. In addition, toxicokinetic modelling from *i.v.* administered ionic silver suggests that the majority of Ag^+ can be reduced and systemically distributed as *de novo* formed secondary nanoparticles. The presence of such particles have been directly observed by electron microscopy (Juling *et al*. 2016). The observation that silver ions form secondary particles, underlines the difficulties in distinguishing between particle- and ion-dependent effects of silver nanoparticles. Silver can bioaccumulate in human tissues leading to a condition known as argyria (described under "STOT RE").

RAC, in agreement with the DS, assumes that all forms of silver metal (i.e. all particle sizes and shapes) and several silver compounds (e.g silver salts and SCAS from which data was included in the CLH report) have the potential to release silver ions upon ingestion. However, little data for massive or powdered forms of silver (i.e. bulk silver) was available to clarify or compare the level of release of silver ions into a physiological compartment from elemental bulk silver (Ag^0) and different silver compounds.

Under the CLP Regulation, substances are classified in accordance with their intrinsic hazardous properties and not based on considerations of risk. The silver ion, Ag^+, is recognised as the toxicophore, but it was necessary to consider the potential toxicity of silver metal in reduced and aggregated form such as nanoparticles or massive forms compared to that from exposure to silver salts, such as $AgNO_3$, or ion exchange matrices such as silver zeolites. A specific evaluation and assessment of bioavailability was thus warranted for silver and different silver compounds from which read-across to silver was proposed in the CLH report. Oral exposure to different silver compounds (including soluble ionic silver salts) and to silver nanoparticles result in toxicological effects (e.g., developmental neurotoxicity, neurotoxicity, adverse effects on sexual function and fertility, and/or reductions in pup viability), but there are several difficulties associated with the current data. These include: a lack of guideline studies and a lack of several relevant parameters and properties investigated or reported on silver nanoparticles, as well as a deficiency of toxicological data for bulk silver (due to both a lack of animal studies investigating hazardous properties of bulk silver and lack of reports on observations from workers in different bulk silver-use industries). There is size-dependent dissolution that is lower for the massive and silver powder forms than for the silver nanoparticle forms. Dissolution increases at smaller particle sizes with silver nanoparticles exhibiting the greatest Ag^+ release.

Bioavailability is the rate and extent to which a substance (or its toxicophore or active moiety) can be taken up by an organism and is available for metabolism or interaction with biological targets. Bioavailability of an ion (biological availability) involves both release from a substance and absorption by an organism. According to Art. 12(b) of CLP, as a result of the evaluation carried out pursuant to Art. 9(5) (according to which available hazard information on the forms or physical states in which the substance is placed on the market and in which it can reasonably be expected to be used shall be considered for the purposes of classification), where conclusive scientific experimental data is identified that shows that the substance is not biologically available, then this property of the substance shall be taken into account for classification purposes. Also, under Art. 9(5) of CLP, hazard information on the physical states and forms of a particular substance placed on the market must be considered for the classification of a substance, and furthermore, under Art. 12(b), the lack of bioavailability of the substance, where identified, must also be taken into account for the purposes of classification. CLP states that bioavailability shall only be considered when conclusive data show that the substance is not biologically available, which is not the case here. However, it is not explained in CLP if/how different degrees of bioavailability for different forms of the same substance shall be considered for classification purposes and this is problematic for silver. It is noted that bioavailability *per se* is not considered an intrinsic property of a substance, it is however dependent on several factors, many of which

may be considered as specific intrinsic properties in their own right, e.g., solubility, silver ion surface release.

Comparative toxicokinetics of different forms of silver (nanoparticles and powder silver) and silver salts (silver acetate and silver nitrate)

The European Precious Metals Federation (EPMF) provided a comparative toxicokinetic study (in accordance with OECD TG 417 and GLP, Anon., 2021) in adult Crl:CD IGS (SD) rats (8-10 weeks of age at the start of treatment) by single and repeat administration of different doses of silver acetate, silver nitrate, micron-sized silver and silver nanoparticles, submitted during consultation. The tested top doses varied between substances and silver particle sizes, being 125, 175, 360 and 1000 mg/kg bw/day for silver nitrate, silver acetate, silver nanoparticles and micron-sized silver, respectively. The study included the measurement of silver levels in blood at different timepoints and in tissues (brain, bone marrow, small intestine, liver, spleen, ovaries, testes, uterus) at termination after different doses to facilitate a direct quantitative comparison of bioavailability, and the delivered dose to tissues occurring after administration of the two different silver particle sizes and silver salts.

Due to the significantly different dose spacing between test items and apparent non-linear dose responses, dose response curves are difficult to use for comparison. Therefore, effects at the same or similar doses are compared below. Silver was bioavailable from all test items and was distributed to tissues also across so-called protected compartments (brain and testes). The bioavailability of silver was highest from silver salts, followed by silver nanoparticles and it was lowest for micron-sized silver ($\approx$ 300nm). The following comparative values for peak blood silver concentrations were shown at day 15 after repeated oral dose administration of different Ag test items at similar doses (see also the table below):

i. 5x difference in blood concentrations of silver from micron-sized (~0.3 µm) particles and silver acetate at around 180 mg/kg bw/day.

ii. 3-5x difference in blood concentrations of silver from micron-sized particles and nanoparticles (15 nm) at 36 mg/kg bw/day.

iii. Similar bioavailability of silver from silver acetate and silver nitrate at 5 and 55 mg/kg bw/day.

Table: *The blood silver concentrations at predicted steady state (day 15) after repeated dose administration of the different silver test items (both a. and b. were corrected to show silver equivalent dosing in column 1). The maximum observed concentration was reported to occur between 3-9h post-dosing for repeat dose groups, the 6h values for the respective test substances were shown as an indicator of peak exposure. Coloured borders indicate comparable doses with respect to silver (salts converted to silver equivalents).*

(a) Silver acetate (AgAc)

Dose level mg Ag equiv/kg bw/d)	Mean 6h blood [Ag] ng/mL		Mean $[Ag]_{6h}$ / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ (n=3)	--	--
3.3	113 ± 15	204 ± 26	34.8	62.8
36	317 ± 93	423 ± 72	8.9	11.8
114	652 ± 171 (n=3)	918 ± 103 (n=3)	5.7	8.1

Results are expressed as the sub-group mean ± *SD*. Unless otherwise stated, samples were obtained from 4 animals per sub-group.

(b) Silver nitrate ($AgNO_3$)

Dose level mg Ag equiv/kg bw/d)	Mean 6h blood [Ag] ng/mL		Mean $[Ag]_{6h}$ / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ (n=3)	--	--
3.2	112 ± 12	170 ± 42	35.3	53.5
35	291 ± 28	377 ± 48 (n=3)	8.3	10.8
80	539 ± 113	636 ± 28	6.8	8.0

Results are expressed as the sub-group mean ± *SD*. Unless otherwise stated, samples were obtained from 4 animals per sub-group.

(c) Nanoparticulate silver (AgNP)

Dose level (mg/kg Test Item bw/d)	Mean 6h blood [Ag] ng/mL		Mean $[Ag]_{6h}$ / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ	--	--
3.6	117 ± 23	125 ± 52	32.5	34.8
36	174 ± 32	288 ± 36	4.8	8.0
360	263 ± 35	519 ± 101	0.73	1.44

Results are expressed as the sub-group mean ± *SD*. Samples were obtained from 4 animals per sub-group.

(d) Bulk elemental silver (AgMP)

Dose level (mg/kg Test Item bw/d)	Mean 6h blood [Ag] ng/mL		Mean $[Ag]_{6h}$ / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ	--	--
36	57 ± 17	55 ± 6	1.6	1.5
180	127 ± 25	190 ± 26	0.71	1.05
1000	154 ± 19	251 ± 71	0.15	0.25

Results are expressed as the sub-group mean ± *SD*. Samples were obtained from 4 animals per sub-group.

Measurements of silver in tissues and blood were repeated for AgMP, AgNP and $AgNO_3$ because of a technical issue affecting the GI tract and liver tissue measurements (this was the case even when other tissues were not affected by this technical issue). The silver blood concentrations in the second measurement were similar to those in the first set of measurements (mean concentrations (ng/mL) of silver in rat (m/f) blood following 15-day oral gavage administration of $AgNO_3$: 131/199 at 5 mg/kg bw/day (3.2 mg Ag eq.) and 449/471 at 55 mg/kg bw/day (35 mg Ag eq.); AgNP: 175/268 at 36 mg/kg bw/day; AgMP: 50.1/92.2 at 36 mg/kg bw/day and 74.4/138 at 180 mg/kg bw/day).

Selective comparative terminal tissue levels of silver following repeated oral dose administration of adult rats for 28 days of silver acetate, silver nitrate, nanoparticulate silver and bulk elemental silver were as follows (see also the table below):

- 9-12x difference in brain concentrations of silver from micron-sized particles (~300 nm) and silver acetate at around 180 mg/kg bw/day.
- 3-5x difference in brain concentrations of silver from micron-sized particles (~300 nm) and nanoparticles (15 nm) at 36 mg/kg bw/day.
- 6-7x difference in testis concentrations of silver from micron-sized particles (~300 nm) and nanoparticles (15 nm) at 36 mg/kg bw/day (silver concentration was

below the limit of quantification in the first measure of micron-sized particles at 36 mg/kg bw/day).

- 7-9x difference in testis concentrations of silver from micron-sized (~300 nm) particles and silver acetate at around 180 mg/kg bw/day.

Table: *Comparative terminal tissue silver levels following repeated oral dose administration. Coloured shading indicate comparable doses with respect to silver (salts converted to silver equivalents*).

	Test substance (dose in mg/kg bw/day)								
	AgAc (3.3)	AgAc (36)	AgAc (114)	AgNP (3.6)	AgNP (36)	AgNP (360)	AgMP (36)	AgMP (180)	AgMP (1000)
Brain 1st (m/f)	142/169 (ng/g)	637/805 (ng/g)	1458/1455 (ng/g)	124/91 (ng/g)	261/291 (ng/g)	568/975 (ng/g)	58/56 (ng/g)	119/128 (ng/g)	171/128 (ng/g)
Brain 2nd (m/f)				77.5/64.8 (ng/g)	226/221 (ng/g)	575/604 (ng/g)	75.6/76 (ng/g)	117/166 (ng/g)	159/187 (ng/g)
Testis 1st	167 (ng/g)	1508 (ng/g)	1531 (ng/g)	179 (ng/g)	570 (ng/g)	1053 (ng/g)	<LLOQ	160 (ng/g)	332 (ng/g)
Testis 2nd				66.1 (ng/g)	452 (ng/g)	1310 (ng/g)	76.8 (ng/g)	213 (ng/g)	296 (ng/g)

Read-Across

The DS referred to Articles 5 and 9 of CLP in justifying the use of data on silver compounds with the potential to release silver ions for classification of all forms of silver (i.e. massive, powder and nanoparticle forms of elemental silver). They also provided several lines of evidence from various studies in support of the use of results obtained with silver nanoparticles being relevant for the assessment of the intrinsic properties of silver in massive and powdered form. Care must be taken when weighting the relevance of studies with silver compounds (including silver salts) to classification of silver metal (massive, powder and nanoparticles of silver).

For the hazard assessment of silver metal (bulk forms and nanoparticles) relevant factors include the extent to which silver ions are released *in vivo* from the source substances and different forms of the target substance, the interaction of these with components of the physiological environment, the resulting speciation of silver (oxidation state, charge on the ion, oxidised ion or reduced atom, etc.) and finally the potential bioavailability of the silver ion (as determined by in-vivo toxicokinetic data). The *key data* for the hazard assessment of silver metal (bulk forms and nanoparticle forms) are from studies using silver nanoparticles, i.e. metallic silver, as the test item. Data generated with other silver compounds (silver salts and silver release matrices such as silver acetate or nitrate and silver zeolites, respectively) are considered to provide supporting information for classification of silver. However, this is not equivalent to agreeing with read-across from one substance such as a soluble silver salt to another very different substance such as silver metal. In such cases there is no conflict with rejecting read-across from silver salts and other silver compounds when evaluating the hazard potential of silver metal. The data from other silver compounds is not considered by RAC to be representative of the metal in either nanoparticles or bulk forms. Accepting read-across in this instance would indicate such substances to be surrogates for the hazard potential of silver when the silver levels in blood and tissues vary after exposure to each substance (see section 1.4.1) or when it is not known to what extent Ag^+ ion is released *in vivo.* This was only estimated for some substances based on silver release experiments in phosphate buffer at pH 4 simulating conditions in rat stomach after oral exposure and it is not always known how comparable the bioavailability of silver is from the different forms of metal (including nanoparticles and bulk forms) and silver-containing substances. The impact of contributing factors such as differences in surface chemistry and

coatings amongst different silver nanoparticles is also unknown. It is important to note that this does not negate the findings of studies with silver salts and silver release matrices, the results from which can be supportive of effects observed with the silver nanoforms. Where there is no co-existing reliable key data for any of the silver forms of the metal (such as developmental (neuro) toxicity), no comparison can be made with the silver salts or silver release matrices. Their chemical characteristics are so dissimilar that it is difficult to relate findings directly from one substance to the other. On this basis the read-across proposed by the DS from several different silver compounds is not supported by RAC.

To support the REACH registrants to comply with REACH information requirements, the concept of applying read-across to a worst case source material or "worst case approach" is given in the Read-Across Assessment Framework (RAAF)-document by ECHA, and it indicates that read-across may be applied even if the strength of the (potential) effect(s) of the target substance can be expected to be lower than the strength of the effects of the source substance. It is important that if the REACH registrant applies read-across from another substance to fulfil the data requirements under REACH, this does not lead to an underestimation or in a gross overestimation of the effect(s) that would be observed in a study with the target substance. In general, when assessing classification and labelling, all the available relevant data are considered and also read-across from another substance may be considered in the weight of evidence hazard assessment. RAC notes the difficulty in deciding what weighting the positive data on different silver compounds should have if implementing the read-across proposal for the classification of silver when there is no (reliable) toxicological data for the metallic forms of silver (bulk or nanoparticle form). The use of such data under a read-across approach in the absence of co-existing key data on metallic silver could lead to an overly conservative and unnecessary severe classification of silver that would not necessarily be justified if there was reliable data available on the substance itself (silver as a metal).

In the absence of conclusive data on the substance itself, the classification of silver should ideally rely on extrapolations of toxicological properties from surrogate substances with sufficiently similar bioavailability, physical, chemical and toxicological properties. This is not the case between silver metal (all forms; massive, powder, nanoparticle) and soluble silver salts and silver-containing active substances (SCAS) etc. The heterogeneity and variability of SCAS with respect to at least certain properties raise unique challenges for each toxicological endpoint.

The degree to which silver metal atoms and ions are available to cause toxicity to mammalian systems may be determined by site specific, i.e. local conditions controlling the speciation/precipitation and/or complexation of the metal. For example, relevant biological fluids such as gastric fluid, intestinal fluid, lung interstitial/alveolar fluid, sweat and blood all present different environments that may impact on the ultimate bioavailability of silver ions. The bioavailability of silver ions (and counter ions) may be key to the toxicities observed with different silver salts (of which only a few are soluble in aqueous systems) and other silver-containing active substances. The main problem is extrapolating these toxicities to the different forms of silver metal (the target substance), when the physical and chemical properties of the source substances are hugely variable and not comparable and when there is a lack of specific data for the target substance itself. Bioavailability is then determined by the initial degree of dissolution of silver combined with the final nature of the resultant silver species that is presented to a biological membrane for absorption. Comparing silver ion bioavailability from the silver forms with that from silver salts and other silver-containing active substances is difficult simply because of how silver compounds interact differently with the relevant biological environments as a consequence of their specific physicochemical characteristics. Soluble salts such as $AgNO_3$ and $Ag^{+}_2[CH3COO^{-}]_2$ (also reported as AgOAc) will release more silver ions than the different silver metal forms in such environments. On this basis the read-across proposed by the DS from several

different silver compounds is not supported by RAC when there is a lack of co-existing key data on silver.

In accordance with the RAAF (ECHA, 2017) and the decision tree for selection of the most appropriate read-across hypothesis, the read-across approach used by Industry follows *Scenario 3*. In which, a category approach is applied, in which the read-across hypothesis is based on transformation to (a) common compound(s). In this specific case, the common compound released is the silver ion. This is similar to the approach taken by the DS in the CLH report. The DS considers all toxicities to arise from the Ag^+ ion and thus considers all data from the different physical and chemical forms that have a potential to release Ag^+ ions in biological systems to be meaningful for the harmonised classification of elemental silver. The inclusion of data from different silver salts and other silver compounds was to support classification proposals for silver metal on the basis of free Ag^+ ions *in vivo*.

The classification via a read-across approach may be based on the rate and extent to which metals and metal compounds can produce soluble available toxic ionic and other toxic metal-bearing species. The hypothesis for silver relies on the same toxic metal ion being (bio)available from silver and silver compounds and not on the feature that describes chemical structural similarity, and therefore the approach proposed for silver could fall under the read-across hypothesis "transformation to common compounds", i.e. scenarios 1-3-5 of RAAF. Similarly, transformation as presented in the current RAAF (hydrolysis, dissociation, (bio)degradation) could be better expanded / elaborated or renamed by consideration of changes in the chemical speciation of metals/metal compounds. The RAAF does not present any detailed framework for an acceptable read-across approach for classification and labelling, e.g. by defining the threshold for an acceptable difference in the amount of common toxic metabolite transformed from the target and source substance. A read-across from positive studies on silver compounds to classify silver is not supported by RAC. RAC places emphasis only on the data from silver metal and its physical forms where available (mostly on silver nanoparticles, only a few data on massive silver). RAC recognises the limited data available describing differences in Ag^+ ion release from silver metal versus silver compounds such as soluble silver salts.

On page 19 of the CLH report the DS justifies the use of data on different silver compounds for the hazard assessment and classification of silver (all forms) via read-across as data is often lacking for silver metal. The DS also provided a justification why the results obtained with silver nanoparticles were considered relevant for the assessment of the intrinsic properties of silver in massive and powdered form. Caution needs to be exercised with the use of the term "forms" as it includes only different forms of one substance (i.e. massive, powder and nanoparticles of silver) and does not include other substances that release silver ions (i.e. silver compounds). The scope of the CLH report (regarding classification for human health hazards) is defined by the DS to only address silver metal (massive, powder and nanoparticles).

Another aspect regarding the available Ag^+ ion from nanoparticles is noteworthy. Industry states that in many nanoform preparations there is a significant level of free Ag^+ ion already present along with the particulate Ag^0 metal species. RAC is unable to determine the contribution to toxicity this fraction may potentially make in any of the studies carried out with nanoparticles. It poses the question that if toxicity is observed with a particular preparation of nanoparticles, is that toxicity a consequence of the nanoparticles themselves or the fraction of free Ag^+ ions present in the preparation or the fraction of free Ag^+ ions that are released from silver nanoparticles before or after absorption from GI tract, possibly only locally at the target organ, and is this fraction a dynamic one and subject to chemical equilibrium? Published studies with nanoparticles rarely have the detailed information required to assess these questions.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

The Dossier Submitter (DS) did not propose classification of elemental silver for physical hazards. Since the melting point is > 960 °C, the flash point was not considered applicable. According to the DS, silver is not flammable, auto flammable nor explosive and has no oxidising properties. The DS also stated that there was a lack of data for several physical hazard endpoints which prevented classification in a conclusive manner. In summary, the DS concluded no classification for the following physical endpoints:

- Silver was not considered a flammable substance. This was based on a negative screening test in EEC A.10 available for silver powder (Möller, 2009), which is valid for CLP.
- Silver was not considered an explosive substance based on structural properties (it has no chemical structures commonly associated with explosive or self-reactive properties), experience in use and because it is known to be thermally stable.
- No information on self-reactivity for silver was provided. Silver is an element and therefore devoid of any reactive functional groups associated with self-reactive properties.
- No data on pyrophoric potential was provided. Testing was not considered to be required, since silver metal is stable at ambient temperature. It is thermally stable under ambient conditions (melting point ca. 960°C, boiling point ca. 2200°C) and has been shown to be non-flammable in direct contact with a flame. Long-term industrial experience in use shows that it is not a pyrophoric solid.
- The DS presented a waiving argument stating that it was not necessary to test for self-heating as silver is thermally stable up to its melting and boiling point.
- Silver does not react with water to emit flammable gases based on structural properties and experience in use.
- Silver does not possess oxidizing properties based on structural properties. No classification was proposed.
- Silver is not an organic peroxide.
- There is no data indicating silver is corrosive to other metals. Testing for this hazard class was technically not feasible since silver has a high melting point and it is practically insoluble. The DS did not propose classification.

Silver is an elemental metal and many of the tests for the evaluation of physical hazards were not considered to be relevant. Significant industrial experience has also shown a lack of reactivity for many of these endpoints.

Comments received during consultation

One Member State commented that they believed particle size and the specific surface area affects the outcome of the physical hazard test results. Tests should also be provided for the nanoparticles of silver in the absence of any surface treatment, only then could a conclusion on classification be possible.

This line of thinking is unsupported, there is no requirement for testing silver nanoparticles for physical hazards when applicable tests have been performed on macroscale or bulk silver metal. The question ignores the fact that surface treatment, capping and stabilisation are required to engineer silver nanoparticles of a discreet size. Without such treatment silver atoms aggregate and agglomerate into physical species on a scale larger than the nanoscale. Even if it were theoretically possible to engineer pure silver particles on the nanoscale, these structures, in the

absence of stabilising/capping agents and other components that typically make up a colloidal mixture, would be composed of Ag^0 atoms and therefore expected to have the same physical properties as pure silver metal in all its bulk forms. This is due to the atomic arrangement of the silver atoms into a well-defined crystalline lattice described as a face-centred cubic (FCC) unit cell. This naturally occurring atomic arrangement confers a very close packing order on silver atoms, more so than in other types of crystalline structure. This arrangement is responsible for some common properties amongst metals such as aluminium, platinum, silver, gold and copper, i.e. these metals are typically softer and more ductile than metals exhibiting other forms of atomic arrangement.

In line with the DS, RAC notes that the proposal for no classification of silver (including nanoparticles of silver) is mostly based on waivers due to the intrinsic properties of silver metal itself – in many cases the hazard classes are either not applicable, or silver conforms to the exclusion criteria for a particular test under the UN recommendations on the transport of dangerous goods, manual of tests and criteria.

Assessment and comparison with the classification criteria

The DS did not propose classification for physical hazards. RAC has summarised the available information and conclusions regarding all physical hazards (table below) and agrees with the DS. **RAC does not propose classification for physical hazards**.

***Table**: Summary of physical hazard data and classification conclusions*

Hazard Class	Chapter in CLP criteria Guidance	Comments	Conclusion
Explosives	2.1	Conforms to exclusion criteria. No functional groups associated with explosive properties.	No classification.
Flammable gases	2.2	Not applicable.	Not applicable.
Flammable aerosols	2.3	Not applicable.	Not applicable.
Oxidising gases	2.4	Not applicable.	Not applicable.
Gases under pressure	2.5	Not applicable.	Not applicable.
Flammable liquids	2.6	Not applicable.	Not applicable.
Flammable solids	2.7	Negative screening test in EEC A.10, (equivalent to UN N.1 test).	No classification.
Self-reactive substance/mixture	2.8	Conforms to exclusion criteria. No functional groups associated with explosive or self-reactive properties.	No classification.
Pyrophoric liquids	2.9	Not applicable.	Not applicable.

Pyrophoric solids	2.10	No reports of self-ignition from handling experience.	No classification.
Self-heating substance/mixture	2.11	No data on self-ignition.	No classification due to lack of data.
Water-reactive - emits flammable gases	2.12	No reports of violent reaction and emission of gas on contact with water from handling experience.	No classification.
Oxidising liquids	2.13	Not applicable.	Not applicable.
Oxidising solids	2.14	Conforms to exclusion criteria: no oxygen or halogen atoms in the structure.	No classification.
Organic peroxides	2.15	Not applicable.	Not applicable.
Corrosive to metals	2.16	Melting point is above 55°C, no test is needed.	No classification.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Acute Oral Toxicity

The DS did not propose classification, noting several sources of information including the biocides review programme, REACH registration dossiers and published studies identified in the open literature. The majority of data available included studies with nanosilver and silver containing active substances (SCAS, e.g. different silver compounds such as colloidal silver, $AgNO_3$, AgF, $KAg(CN)_2$, AgCN and Ag_2O), in which the toxicity of the silver ion (Ag^+) has been indirectly investigated as it is presumed to be released from the SCAS tested. The DS noted that the use of data obtained with SCAS is complicated by the content of additional constituents as well as by the content and degree of release of silver (and other metals) ions from the active substance investigated. On this basis the DS questioned the relevance of this data for massive or powder forms of silver and instead placed more reliance on studies performed with nanosilver and on the results from studies in the REACH registration dossier.

Information on acute toxicity was available for different silver forms and compounds (colloidal silver, $AgNO_3$, AgF, $KAg(CN)_2$, AgCN and Ag_2O) and indicated LD_{50} and MLD (median lethal dose) values between 21-800 mg/kg bw. Very little direct data was available in the CLH report to evaluate oral toxicity. Many of the values tabulated were taken from other reference sources or textbook descriptions so it was not possible to deduce the reliability of the data. The DS rejected this data in assessing classification for acute oral toxicity.

The DS focused on study summaries in the open literature with nanosilver and the registration dossier for silver submitted under REACH. Acute toxicity data for nanoparticles of silver and some

silver salts predominantly reported LD_{50} values above the limit for classification (i.e. >2000 mg/kg bw). There only key study, Kim *et al*. (2012), was GLP and guideline compliant and used a nanosilver preparation. The DS considered this data relevant for silver massive and powder forms and consequently proposed no classification for acute oral toxicity.

Acute Dermal Toxicity

The DS proposed no classification. An OECD TG 402 (1987), GLP compliant study (Kim *et al*., 2012) was available using a nano-sized colloidal silver suspension dispersed in 1% citric acid tested in Sprague-Dawley rats. Groups of five males and five females were administered 0 or 2000 mg/kg bw by a single topical administration to shaved dorsal skin, constituting approximately 10% of the body surface. All rats survived treatment and there were no clinical signs, no indications of dermal irritation, no effects on bodyweight except for a small weight loss on Day 1 and no macroscopic changes. The acute dermal LD_{50} in the study was greater than 2000 mg/kg bw. An additional study was noted in the REACH registration dossier, no deaths or adverse effects were observed in that study, but the top dose was only 348 mg/kg bw (no detail provided). A published study with guinea pigs was also available (Wahlberg, 1965; investigated several metal salts including $AgNO_3$ with an LD50 > 217 mg Ag eq./kg bw) but this was not considered of reliable value in the Silver Zinc Zeolite Competent Authority Report (CAR, 2012, IIIA 6.1.2 (05).

The DS stated the LD_{50} in rats was above 2000 mg/kg bw and that the criteria for classification were not fulfilled.

Acute Inhalation Toxicity

The DS proposed no classification for acute toxicity. There were two study summaries in the REACH registration dossier which were considered relevant and were both GLP and guideline-compliant. The original study reports were unavailable to the DS.

In the first study, rats were exposed to nanoparticles of silver in a whole body-exposure chamber. All animals survived treatment and there were no test-substance related clinical signs. The LC_{50} was considered to be greater than the highest dose tested, i.e. 0.75 mg/m^3.

In the second study, male and female rats were exposed nose-only to an actual concentration of micron-sized silver at 5.16 ±0.01 mg/L air and terminated after a 14 day post-exposure period. Detailed histopathological investigations of the respiratory tract revealed some evidence of inflammation in some animals.

The acute inhalation LC_{50} (dusts and mists) for male and female rats in the study was considered to be > 5.16 mg/L air and thus above the ATE triggering classification.

Comments received during consultation

Comments 192-205 from company-manufacturer/industry-trade groups addressed acute toxicity and were mainly of a general nature or supported no classification. There was one comment from a member state with support for no classification.

Assessment and comparison with the classification criteria

Acute Oral Toxicity

According to the CLP criteria, classification for acute oral toxicity is warranted if the LD_{50} (or ATE) of a substance is ≤ 2000 mg/kg bw.

The data package for acute oral toxicity consisted of several studies and/or summaries from diverse sources and reported in the original dossier under the BPR. Appropriate and detailed data was not available, and it is unclear in most cases how much silver ion was potentially bioavailable. In many cases it is not clear how the dose in actual silver ion equivalents administered is estimated. RAC agrees with the DS that the relevance of this data for elemental silver cannot be ascertained.

Most of the LD_{50} values were reported as being greater than the maximum tested estimates of the silver content of the silver containing active substances (SCAS) investigated, values typically ranged from 100 to >1000 mg Ag eq. /kg. There were no studies described in the CLH report with robust data for metallic silver in macro- or bulk form or silver ion.

In the CLH report there was only one key, reliable study, that by Kim *et al.* (2012); it was described as being GLP and guideline compliant, dosed up to 2000 mg/kg bw in rats with nanoparticulate silver colloid (which at 20.5% silver would be approximately 410 mg Ag^0 eq./kg) – this study had no deaths. RAC notes that the CLH report provides no further information to substantiate that (nearly) "all LD_{50} values reported therein are above the limit for classification (i.e. >2000 mg/kg bw)".

A study that was originally included in the REACH dossier for silver was made available by industry representation following discussions at RAC-59 CLH WG. The full study report is considered reliable and is guideline compliant and performed according to GLP. The tested material was micron-sized elemental silver at 2000 mg/kg bw. There was no lethality. The acute median lethal dose (LD_{50}) was found to be greater than 2000 mg elemental silver/kg bw.

RAC supports the DS proposal for **no classification for acute oral toxicity**. A meaningful ATE value for elemental silver cannot be determined.

Acute Dermal Toxicity

According to the CLP criteria, classification for acute dermal toxicity is warranted if the LD_{50} (or ATE) of a substance is ≤ 2000 mg/kg bw.

The DS proposed no classification based on a published OECD TG 402 (1987), GLP compliant study (Kim *et al*., 2012) where there were no deaths after application of 2000 mg/kg bw of the nanoparticulate silver product. The silver content was approximately 410 mg Ag eq./kg so that the true conclusion from this study is an LD_{50} (ATE) > 410 mg Ag eq./kg.

Further evidence was minimal. A published study by Wahlberg (1965) investigated several metal salts including $AgNO_3$ in guinea pigs with an LD_{50} > 217 mg Ag eq./kg bw. The Biocides CAR described a few studies with silver containing zeolites and other matrices at limit doses and with no deaths. However, detail on silver availability was not apparent.

RAC agrees with the DS proposal for **no classification for acute dermal toxicity based on inconclusive data** for dermal toxicity for silver (all forms).

Acute Inhalation Toxicity

In the key inhalation study, the Mass Median Aerodynamic diameter (MMAD) was 2.27±3.03 µm. A respirable fraction was technically achieved. According to the CLP criteria, classification for acute inhalation toxicity is warranted if the LC_{50} (or ATE) of a substance is ≤ 5 mg/L.

In the key acute inhalation study from the REACH registration dossier, all animals survived treatment when tested at an actual silver concentration of 5.16 ±0.01 mg/L air. There were some transient clinical signs, but these had abated by 3 hours post exposure in all animals. The result of this study is above the ATE triggering classification. RAC agrees with the DS that **no classification for acute inhalation toxicity is warranted**.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The DS considered there was no adequate data available to assess effects on specific target organ toxicity following a single exposure to silver in massive or powder forms. Based on results from acute toxicity and repeated dose toxicity studies available for a variety of silver containing active substances, the silver ion causes significant effects following repeated exposure rather than from a single exposure.

The DS did not propose classification.

Comments received during consultation

Comments #281-290 addressed specific target organ toxicity-single exposure and were mainly of a general nature or stating support for no classification from company-manufacturer/industry-trade groups.

The European Precious Metals Federation made available an original report of an acute toxicity study on micron-sized metallic silver (Anon., 1993). The only recorded sign of systemic toxicity was a decrease in body weight gain in the second week following administration of the silver suspension. Females (mean 43% reduction, $p<0.01$) were more effected than males (mean 12% reduction, non-significant). All animals showed an overall gain in bodyweight and no abnormalities were noted at necropsy.

Assessment and comparison with the classification criteria

STOT SE was not proposed by the DS.

STOT SE should be considered where there is clear evidence of toxicity to a specific organ after a single exposure event. In the present case there is some data available to assess effects following single exposure to silver in massive or powder forms. The Anon. (1993) study with micron-sized silver supports no classification.

RAC concludes that **no classification is warranted for STOT-SE.**

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

Two skin irritation studies performed in New Zealand White rabbits according to guideline OECD TG 404 (2002) were reported by the DS. One was from a paper encompassing a range of studies and available from the published literature (Kim *et al.,* 2012), the other was performed according

to GLP and submitted as part of the REACH registration dossier on silver. Both were acceptable to the DS.

Kim et al., 2012

The material tested was nanoparticulate silver (Ag-NP) in citrate solution. Limited details were taken from the published report and the DS was not privy to the original study report data. The test substance was applied to a shaved skin surface area and covered by a semi-occlusive dressing for 4 hours. The results indicated no erythema, eschar or oedema reactions (mean grades: 0.0) at any of the observation timepoints. Therefore, nanoparticles of silver were not considered to have irritating properties on the skin.

Anon., 1993

The material tested was silver powder CAP9 (a chemically precipitated silver powder of high tap density). Slight erythema was noted and all treated skin sites appeared normal at the 72-hour observation period. The results were below the CLP criteria for classification and the DS used this study in support of the Kim *et al.* (2012) findings to propose no classification for skin irritation potential.

Results

In both studies mean gradings for severity of damage, i.e. erythema/eschar or for oedema in 3 tested animals at the 24, 48- and 72-hour time points (after patch removal) were not sufficient to support a classification by the DS for skin irritancy.

Comments received during consultation

There were several comments submitted from a variety of sectors including company/downstream users; industry or trade association; individuals and manufacturers. Comments specific to skin irritancy/corrosion (#209 to #214) supported no classification. EU regulatory bodies or member states did not comment.

Assessment and comparison with the classification criteria

Both studies assessed by the DS for skin irritation were negative with respect to the criteria for classification. The Kim *et al.* (2012) study investigated nano-silver while the REACH registration dossier study used silver powder. Very slight to no effects were noted, the silver forms tested did not elicit a dermal reaction sufficient to satisfy the classification criteria.

RAC agrees with the DS and considers that **no classification for skin corrosion/irritation is warranted**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS did not propose to classify silver as an eye irritant based on information from three guideline studies detailed in table 37 of the CLH report. Two of the studies used rabbits, one used guinea pigs. All animals survived treatment and there were no clinical signs observed. The mean grades for effects on the iris and conjunctiva (redness, oedema, discharge) were negligible at all timepoints.

Comments received during consultation

There was a single comment from an MSCA supporting no classification for eye irritation but there was also concern that only a 100mg colloidal solution of 20.4% silver was tested in the Kim *et al.* (2021) study. Several generic comments were noted from other sectors including company/downstream users; industry or trade association; and manufacturers.

Assessment and comparison with the classification criteria

All studies assessed by the DS for eye irritation were negative with respect to the criteria for classification. Both nanoparticles (silver concentration in stock solution 20.48%) and powder were investigated. RAC agrees with the DS that **classification for serious eye damage/irritation is not warranted for silver.**

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

There were no studies available on respiratory sensitisation. There was limited information available on two documented cases in a textbook, "Silver in healthcare" (A. B. G. Lansdown, 2010), featuring a compilation of allergic reactions in humans. Two historical human case studies of allergic reactions to colloidal silver involving the respiratory system were summarised by the DS; an allergic reaction to nasal drops (Howard, 1930) and a case of an allergic reaction to a nasal spray (Criep, 1943). The information was considered to be of little value, therefore the DS proposed no classification due to lack of data.

Comments received during the consultation

A few comments were received and all were in line with the DS proposal.

Assessment and comparison with the classification criteria

RAC agrees with this assessment by the DS that **no classification for respiratory sensitisation is warranted due to a lack data.**

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

There were no guideline GLP studies available in which the sensitising potential of silver in ionic form was adequately investigated.

The DS reported data from both animal and human studies.

Data from animal studies

Four studies were referenced in total. One Guinea pig maximisation test (GPMT) and two Buehler studies along with a textbook description of a study by Hultman (1994) providing evidence for autoantibodies.

Study 01: Kim et al., (2012), Doc IIIA 6.1.5-11

In this study the skin sensitising potential of nanoparticulate silver dispersed in 1% citric acid was investigated in a GPMT. This was published in a peer reviewed paper as part of a series of toxicity tests carried out on a specific silver nanoparticle (AgNP) formulation consisting of 20.48% (w/v) silver. The study was stated as being performed in accordance with OECD TG 406 (1992) and GLP. At 24 and 48 h after removing the challenge patch, one animal (1/20) exhibited discrete and patchy erythema, respectively. Meanwhile, no visible changes were observed in the negative controls. Details on the test substance concentration used for intradermal injections of colloidal Ag-NPs were not explicitly reported in the original publication but were assumed to be 0.1ml of the 20.48% w/v. The DS interpreted the result as evidence for no sensitisation according to the CLP criteria.

Study 02: Anon., (1999), Doc IIIA 6.1.5-02 (data protected)

In this study the skin sensitising potential of a stabilised silver complex (Axenohl, 0.72% silver dihydrogen citrate stabilised in 22.4% citric acid, providing 0.24% Ag^+ or 2438 ppm Ag^+ ions) was investigated in a Buehler test. The study was performed in accordance with OPPTS. 870.2600 and GLP. Animals were challenged at concentrations inducing dermal reactions of grade 0.5 on the Magnusson & Kligman score for erythema in 2/4 of the animals during the preliminary irritation tests which is not in compliance with the guideline where the concentration used for the challenge should be the highest non-irritating dose. Of note, there was no sham treatment of controls. The results of the Buehler test indicated a net positive reaction in 20% of the test animals (i.e. 20% higher in animals treated with Axenohl compared to controls). There was some discussion over the severity of the response (0.5 for all positive animals) as this was not considered a positive result in the original study report by the performing laboratory and this grade is not recognised in the near equivalent OECD guideline (TG 406). However, the DS considered reactions of grade 0.5 to be valid and therefore indicative of a positive result supporting classification of Axenohl as a dermal sensitiser.

Study 03: Hultman, (1994), from "Silver in healthcare" by Lansdown (2010)

A brief description of a mouse study provided evidence for the generation of serum IgG anti-nucleolar antibodies following 5- and 10-week treatments with 0.05% silver nitrate in drinking water. The study was non-guideline and non-GLP and is not relevant for dermal sensitisation potential.

Study 04: Anon., (2006), Doc IIIA 6.1.5-08 (data protected)

In this study the skin sensitising potential of a silver zeolite compound (composed of 22% silver particles) was investigated in a Buehler test. The study was performed in accordance with OPPTS. 870.2600 and GLP. The test substance was used at a topical dermal concentration of 55%. The results of the Buehler test indicated a net positive reaction in 15% of the test animals (i.e. 15% higher in animals treated with silver zeolite compared to controls), with a score of 0.5 for erythema. This also satisfied the criterial for skin sensitisation according to the DS.

Data from human studies

There were several individual case reports describing silver (mainly as the nitrate salt but also colloidal silver) allergy, compiled in the textbook "Silver in healthcare" by A. B. G. Lansdown (2010). Half were of historical interest (1930-1948). All the studies presented had little detail or evidence for sensitisation.

The dossier submitter presented evidence of sensitising reactions to silver nitrate from human case studies and two different Buehler studies performed with silver zeolite and a formulation

containing 2438 ppm silver ions. Nanoparticles of silver tested in an M&K OECD TG 406 study did not show any sensitising properties. On this basis and with some caution as to the availability of silver ions in the M&K study, the dossier submitter proposed classification with Skin Sens 1, no sub-categorisation.

Comments received during consultation

There were approximately 40 comments from industry and trade organisations, companies large and small, including manufacturers, clothing, and jewellery businesses. In addition, three Member States also submitted comments.

All the comments from business and trade sources supported no classification regarding skin sensitisation. Several points were made including; the need to consider nano-, powder and massive forms of silver separately; reliable human evidence showing that silver or silver containing alloys cause skin sensitisation in a substantial number of people is lacking; downstream concerns with regarding the use of silver in several industries including the medical field; several sources claimed no sensitisation based on several decades of experience despite extensive silver exposure (medical, dental, jewellery applications etc.); a large number of animal studies with a variety of chemical forms of ionic silver illustrate the non-sensitizing potential of silver; the animal dataset in the CLH report is incomplete; there are a limited number of reported skin irritations which were mostly caused by silver nitrate and not pure metallic silver.

Three comments were received from Member States; one supported the classification proposal by the DS with no further comment and the other two disagreed with any skin sensitisation classification for silver.

In support of no classification, the two Member States noted several points of concern:

- the level of positive response compared to the controls were very limited and unconvincing when considering the study limitations,
- the human case reports referred to in the CLH report offered little reliable detail and did not constitute firm evidence for a sensitising effect,
- no data or case reports that indicate (solid) silver causes dermal sensitization reactions,
- no robust argument that skin contact with solid silver can lead to sufficient local exposure to the ion (which may have some skin sensitization potential),
- the relatively high rates in the negative controls, the low score of the graded reactions and the presence of citrate/laureate or zeolite limit the significance of the positive results in two "positive" Buehler assays (OECD TG 406 or US-EPA guideline).

Additional information from existing, or alternative studies were submitted during the consultation in addition to detailed comments from the European Precious Metals Federation (EPMF). Additional sources of information (in addition to a reinterpretation of existing data from a full study report, Moore 1999) are summarised below, in order to provide a more balanced weight of evidence approach to the assessment of skin sensitisation in the case of silver. These data have been added to the Additional Key elements section of the Background Document (BD).

Assessment and comparison with the classification criteria

As indicated in the CLH report, there is a paucity of reliable testing information covering elemental silver on the micron and larger scales.

Human evidence for skin sensitisation

Sporadic human case reports of dermal sensitisation were presented by the DS but human experience, (over a considerable period and various exposure contexts), has not demonstrated significant contact sensitisation from either elemental silver or ionic silver. The human case reports were not substantiated with robust data and presented uncertainties with respect to the selection of an appropriate patch test challenge; the exclusion of confounding due to cross-reactions from irritating substances like $AgNO_3$; or in identifying the actual causative agent where multi-metallic occupational exposure arises.

Elemental silver (including nanoparticles) and its compounds have been widely used in medical, industrial and consumer applications where skin contact is common; for instance, in the clinical use of topical antimicrobials or wound dressings containing silver, or as constituents of jewellery. Safety evaluations for skin sensitisation potential as part of the safety assessment process for medical and dental applications are noted for having an absence of skin sensitisation. In general, silver has rarely been implicated in contact hypersensitivity.

In conclusion, reliable evidence that silver has caused skin sensitisation in a substantial number of humans is lacking.

Animal evidence for skin sensitisation

Three acceptable animal studies were presented by the DS for assessment of skin sensitisation.

The first was a Guinea pig maximisation test performed using a silver nanoparticle (AgNP) formulation consisting of 20.48% (w/v) silver (Kim *et al*., 2012). OECD TG 406 (1992) and GLP compliant, the study in a peer-reviewed paper was considered acceptable and results were negative for skin sensitisation.

Two further studies according to the Buehler method were performed with (1) Axenohl, silver in ionic form at 0.24% Ag^+, stabilised in 22.4% citric acid and (2) silver zeolite, composed of 22% silver in a rigid aluminosilicate framework (Agion Antimicrobial type AD) functioning as an ion-exchanger. Both studies recorded minimal gradings in excess of similar results in the controls. The DS took a conservative position and treated these minimal gradings as positive for skin sensitisation. According to Buehler and the original study authors, these gradings (score of 0.5 per animal) indicate a doubtful/negligible erythema rather than a true positive response for skin sensitisation. High background levels of negligible erythema in controls do not support a specific sensitising effect for silver. There is no framework for harmonising the 0.5 grade observations in the two Buehler studies with those as specified in the OECD guidelines. There is no conclusion of a sensitising effect by silver according to the author of each report. Assuming this grading is indicative of a negligible erythema then these studies are negative. If, on the other hand, experts take a conservative approach then the study results may be described as equivocal for skin sensitisation (while there is no evidence for skin sensitisation a rechallenge should have been performed as a confirmatory step).

In addition, further evidence was available from the Silver REACH dossier and also arising from the public consultation period. In the REACH dossier there was a GPMT conducted in accordance with OECD TG 406 (1992) and GLP on sodium silver thiosulphate, this was acceptable and was negative for skin sensitisation, Prinsen (1995).

An LLNA was performed on a low concentration colloidal silver nano-form preparation (AgPURE W, 0.008%, 80 ppm Ag^0, report from 2007) according to OECD TG 429 (2002) and GLP. This was also negative. A summary of another LLNA test was described in the SCCP Opinion on citric acid (and) silver citrate, (2009) SCCP/1196/08. This test was also negative for skin sensitisation and used a silver-citrate formulation with a higher concentration of silver. There has been debate in the literature regarding the suitability of the LLNA for metals and their salts. There is no

definitive conclusion on this point, some publications show acceptable application of the assay, but one feature of note is that the sensitivity of the assay in these cases may be reduced necessitating the use of higher concentrations of test substance.

The European Precious Metals Federation (EPMF) supplied a report with a summary table of further silver substance studies that may be regarded as consistent and supportive of a non-sensitising potential for silver.

Comparison with the CLP criteria

Substances are classified as Category 1 skin sensitisers where data are not sufficient for sub-categorisation; if there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons; or if there are positive results from an appropriate animal test.

Substances are classified as sub-category 1A skin sensitisers where there is evidence of a high frequency of occurrence in humans and/or a high potency in animals. Similarly, substances are classified as sub-category 1B skin sensitisers where there is evidence of a low to moderate frequency of occurrence in humans and/or moderate potency in animals. There are no positive results from an animal test. Evidence in humans that silver can lead to sensitisation by skin contact in a substantial number of persons is lacking.

RAC disagrees with the DS on the weight of the evidence, in particular on the interpretation of the results from the two Buehler studies. RAC considers that **no classification for skin sensitization is warranted**.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS assessed the potential of silver to cause a variety of effects that could potentially lead to a classification with STOT RE. A wide variety of studies were available, the majority were publicly available, journal peer-reviewed articles investigating silver nanoparticles, studies summarised in the silver REACH registration dossier but lacking detail and a few OECD test guideline and GLP compliant studies that had been included in the BPR assessment report for silver zeolite. There was extensive human data from a variety of public review reports describing incidences of generalised argyria which may be regarded as a well-established series of effects caused by excess silver exposure.

The DS concluded that there was insufficient evidence to fulfil the criteria for classification for STOT RE. There were no studies assessed for bulk silver, but studies utilising silver nanoparticles were available and assessed. Several confidential studies from the original BPR assessment report were not included in Annex I to the CLH report and were not evaluated under STOT RE, however, they were performed with other silver compounds that included additional chemical components such as zinc or involved complex ion-exchange matrices where either the biological effects were difficult to ascribe to silver alone or it was not possible to establish quantitative dosing with regards to silver content.

Tables 65, 66 and 67 in the CLH report detail the most relevant studies used for assessing STOT RE. Table 65 covers the animal data from all routes of exposure, studies are predominantly from peer reviewed journals with a few adhering to OECD test guideline and GLP compliance. These studies used silver nanoparticles and some soluble silver salts such as silver nitrate and silver

acetate. A summary table is presented in the "assessment and comparison with the classification criteria" section later in this document.

Table 66 in the CLH report presented human data from several studies, case reports and reviews of silver exposure in industrial and medical settings. Table 67 in the CLH report referenced other relevant studies such as the rat and mouse carcinogenicity studies.

ASSESSMENT BY THE DS

Silver REACH registration dossier (2015)

The DS summarised a number of studies from the 2015 silver REACH registration dossier (see below). There was little information available to the DS to conclude on the reliability or robustness of the results described. Therefore, this section was considered to provide little supportive data for a STOT RE classification proposal.

i. Williams, *et al.* (2014); rat, 90-day oral gavage, silver nanoparticles and silver acetate, severe gastrointestinal inflammation and irritation with the soluble silver acetate salt at doses above the cut-off criteria for classification.

ii. Ebabe, *et al.* (2013); rat, 81-day oral gavage, silver nanoparticles (20nm, 0 and 500 mg/kg bw/day), indications of oxidative stress and inflammation at a dose above the cut-off criteria for classification.

iii. Heydrnejad, *et al.* (2015); oral gavage study in mice using 40nm silver nanoparticles, unclear dose information, effects on liver function noted.

iv. Adeyemi *et al.* (2015); rat 7-21-day oral gavage, silver nanoparticles at doses of 100, 1000 and 5000 mg/kg bw/day. LOAEL = 100 mg/kg bw/day (increased serum creatinine).

v. Rungby, (1986); Silver lactate and silver nitrate, administered in water or via ip in rats, study on the tissues of the eye. Confirmation of induction of argyrosis of the eye.

vi. Belyaeva, *et al.* (2014); 1-6 month dietary exposure study to silver nanoparticles and silver sulfate. Described as unreliable.

vii. Espinosa-Cristobal *et al.* (2013); rat 55-day oral dietary study, stated as OECD TG 407 and GLP compliant. Silver nanoparticles (14nm and 36nm) in drinking water. Dose levels not documented, no clinical signs or reductions in body weight.

viii. Rathore *et al.* (2014); rat 21-day oral study with silver nanoparticles (25nm) at 3mg/kg bw/day and gold nanoparticles at (20 µg/kg). Gold nanoparticles were more toxic than silver towards the liver and kidney. Described as unacceptable by registrant.

ix. Shahare *et al.* (2013); mouse 21-day oral gavage study, 5, 10, 15, and 20 mg/kg bw/day of silver nanoparticles (10nm). This was an exclusive histopathology investigation of the small intestine. Only qualitative results were presented indicating destruction of the microvilli and mucosal lining.

x. Shrivastava *et al.* (2016); mouse 14-day oral gavage study, dose not well described, silver and gold nanoparticles (20nm) compared. Mechanistic study, silver effects greater than gold effects, evidence for oxidative damage in several tissues and increased inflammatory markers and formation of 8-OHdG DNA adducts.

xi. Patlolla *et al.* (2015); rat 5-day oral gavage study, 5, 25, 50, and 100 mg/kg bw/day of silver nanoparticles (10nm). Mechanistic study, blood and liver collected. Positive dose response in all tests. Hepatotoxicity demonstrated through oxidative stress

and histopathology with increased serum hepatic enzymes. There was induction of reactive oxygen species in liver homogenates with increasing silver nanoparticle dose. Positive Comet assay.

xii. Walker (1971); rat 81-week oral dietary study, 12mM $AgNO_3$ in drinking water, no consumption data, qualitative mechanistic study with electron microscopy investigation of various tissues including kidney and liver. Demonstration of argyria. Treatment with 24mM $AgNO_3$ in drinking water discontinued, lethalities and severe clinical signs.

Subacute and subchronic animal studies with silver nanoparticles and silver salts

The most robust data was obtained from those animal studies investigating silver nanoparticles, silver nitrate and silver acetate by various routes of exposure. These are tabulated in the assessment and comparison with the classification criteria section. The bulk of these studies were from peer-reviewed journals. The overall picture outlined by the DS described silver as having a wide distribution, affecting many organ systems with evidence of alterations in clinical chemistry and histopathology. These were indicative of multiorgan adverse effects and increased oxidative stress without substantial effect on the animals' general health as indicated by a low occurrence of clinical signs though there was often an effect on body weight in treated groups. In general, the majority of substantial but moderate effects occurred at doses of silver above the guidance values for STOT RE 1 and 2. In the study by Charehsaz, *et al.,* (2016), hippocampal sclerosis was observed even at the lowest dose level of 0.2 mg Ag/kg bw/day (Doc IIIA, 6.8.2-10) in dams dosed orally once daily from Day 7 to Day 20 of gestation with silver nanoparticles. The same effect was also observed with $AgNO_3$ at a test dose of 20 mg Ag/kg bw/day. The DS noted that the neuronal loss is permanent and thus considered to be a significant effect fulfilling criterion CLP 3.9.2.3 (g) as it occurred within the guidance value range for STOT RE 1. However, the DS concluded that these effects, which were only noted in a single publication, were not sufficient evidence to fulfil the criteria for classification.

Subchronic and chronic/carcinogenicity animal studies with other silver compounds

The DS also described a few studies with dogs, rats and mice investigating either silver zinc zeolite or silver sodium zirconium hydrogenphosphate ion-exchange matrices. The difficulty with using the the zeolites to address the potential hazardous properties of silver is that the content of zinc was frequently higher than that of the silver so that any adverse reactions were difficult to assign specifically to silver. The amount of available silver from these matrices is not simply defined by the total content of silver in such compounds, and the degree (in mg silver ion equivalents /kg bw/day) to which silver or silver ion was released in stomach from the test substance at each dose is mainly unknown. However, this was estimated based on silver release data from certain silver substances into 150 mM phosphate buffer at pH 4 and 37°C simulating rat stomach conditions (see CLH report table 6).

<u>Silver zinc zeolites</u>

Doc IIIA 6.4.1-07: The Anon. (2003) 90-day dog study (0.2, 1.0 and 5.1 mg silver ion equivalents /kg bw/day estimated to be released in rat stomach conditions at the tested doses of the test substance) that utilised silver zinc zeolite (AgION Antimicrobial Type AK10D) had clinical signs at the highest dose (occasional salivation, shaking of head and vomiting). These types of effects are commonly noted in dogs following capsule administration and could also be due to local effects in the gastro-intestinal tract arising from the route of administration. This was a guideline and GLP compliant study. Silver equivalents were calculated based on information in the confidential CAR where it is stated that 42% of the silver content may be released from the zeolite at pH 4.0 (assumed to mimic physiological conditions in the rat stomach). The only

substantial effect was that the level of haemoglobin was 20% lower in high dose males compared to controls and is considered a stand-alone criterion for haemolytic anaemia. This effect falls under the criterion for classification because an effect level of 5.1 mg silver ion/kg bw/d is within the guidance value for STOT RE category 1 (i.e. ≤ 10 mg/kg bw/d). Complicating this assessment is the composition of the zeolite. AgION Type AK10D is composed of 4.9% silver and 13% zinc. Zinc toxicity may include inhibition of haem synthesis and there is more zinc than silver potentially in the high dose animals (250 mg/kg bw/day zeolite in gelatine capsules). The DS could not exclude a toxicological impact of other constituents in silver zinc zeolite and therefore did not propose classification based on this study.

Doc IIIA 6.4.1-06: Similarly, for a number of effects noted in the guideline and GLP compliant Anon. (2001) rat 90-day oral study (also performed with AgION Antimicrobial Type AK10D at equivalent doses of 0.65, 2.0 and 6.0 mg silver ion/kg bw/d estimated to be released in rat stomach conditions at the tested doses of the test substance) alterations in erythrocytic parameters suggested zinc toxicity rather than silver toxicity. There were many minor effects noted regarding clinical chemistry and body weight gain and no significant effects were observed in the neurobehavior, functional observational battery (FOB) or motor activity evaluations performed.

Doc IIIA 6.5-05: The Anon. (1992) 2-year combined chronic toxicity/carcinogenicity study of AgION Zeomic AJ 10N in mice utilised (estimated) equivalent doses of 0.65, 2.0 and 6.0 mg silver ion/kg bw/d estimated to be released in rat stomach conditions at the tested doses of the test substance). The study was guideline compliant but there was no information on GLP status. Effects on haematological parameters (decrease in HCT, Hb, MCV and increase in MCHC) were observed along with a number of histopathological changes in numerous tissues and pigmentation of skin and organs. There was no clinical chemistry performed and no other major effects of note.

Doc IIIA 6.5-06: The Anon. (1992) 2-year combined chronic toxicity/carcinogenicity study of AgION Zeomic AJ 10N in rats utilised (estimated) equivalent doses of 0.03, 0.09 and 0.84 mg silver ion/kg bw/d estimated to be released in rat stomach conditions at the tested doses of the test substance. Pathological examination revealed pigmentation of liver, kidneys, pancreas, stomach, lymph nodes and the choroid plexus in high-dose rats. There were no other indicators in support of STOT RE classification.

<u>Silver sodium zirconium hydrogenphosphate</u>

Doc IIIA 6.4.1-04: The Anon. (1995); 90-day rat study (estimated doses of 0.29, 2.9 and 9.5 mg silver ion equivalents /kg bw/d to be released in rat stomach conditions at the tested doses of the test substance) utilised AlphaSan RC5000, a silver ion exchange matrix (Novaron AG-300, 3.8% silver). This study was guideline and GLP compliant. A slightly increased heart weight without accompanying histological change was noted in the high dose group. There were no deaths and no clinical signs were reported. Several minor histopathology changes were seen, but no changes in haematological values indicative of a similar effect as that seen with the zeolites. The study provided insufficient evidence to support STOT RE classification.

Doc IIIA 6.4.1-05: The Anon. (2002) 90-day dog study (estimated doses of 5, 10 and 18/20 mg silver ion equivalents /kg bw/d to be released in rat stomach conditions at the tested doses of the test substance) utilised AlphaSan RC2000, a silver ion exchange matrix (Novaron AG-300, 10.1% silver) different to that used in the rat 90-day study. This study was guideline and GLP compliant. Numerous effects were noted. One male and one female in the high dose group were found dead or sacrificed. Body weight gain was impacted in the high dose group. Haematology was unremarkable, numerous small changes in clinical chemistry and histopathology were noted indicative of liver inflammation. The DS did not comment on the significance of any of these effects for classification.

Human data

The DS described an extensive database of human experience with silver and silver compounds. All routes of exposure were assessed and a number of effects characteristic to silver were noted. These included:

1. Argyria (blue-grey pigmentation to the skin and mucous membranes),
2. Irritation of the respiratory tract and lungs from inhalation of silver salt/oxide dusts,
3. Ocular argyrosis, staining of ocular tissues, sometimes with decreased night vision.

DS conclusion on silver

The DS did not consider the inflammation or histopathology noted in the two 90-day dermal toxicity studies to fulfil the criteria for STOT RE as outlined in section 3.9.2.7.3 in Annex I of CLP.

The DS did not consider the liver effects including bile duct proliferation noted in the inhalation studies as sufficient evidence to fulfil criteria for classification as outlined in section 3.9.2.7.3 in Annex I of CLP.

The DS did not specifically comment on the human data. A description of review reports, industrial exposure incidents and several case studies was presented and the most notable effects were the three presented above, i.e. generalised argyria, ocular tissue staining and in cases of inhalation exposure, respiratory irritation. The main conclusion from the human data substantiates the animal data with respect to the ability of silver to widely distribute in the body and to cause discoloration of tissues, organs and skin.

The oral studies in animals presented the most relevant data for consideration of STOT RE classification. Complicating the overall picture was the multi-organ and tissue involvement caused by exposure to silver nanoparticles and soluble silver salts. Studies with other silver compounds involving a silver ion exchange matrix presented problems with respect to determining actual silver exposure or if toxicity was also due to other metals such as zinc. Generalised toxicity and histopathology were a feature of silver exposure, however, there was no evidence to suggest functional impairment and clinical signs were mainly unremarkable. In many cases the adversity of the histological changes was not considered sufficient to fulfil criteria for classification.

A well-known effect of silver ions is the pigmentation of organs and tissues. Many of the reports presented by the DS substantiate this effect. The DS referred to previous deliberations by the ECHA RAC (RAC 35, Dec 2015) where pigmentation by silver zinc zeolite was discussed in the context of classification and labelling. The conclusion by RAC did not consider this effect to warrant classification and this was also the position taken by the DS.

The DS elaborated on the findings of hippocampal neuronal cell loss in dams after a 14-day exposure during pregnancy at a low dose level of 0.2 mg/kg (silver nanoparticles) and 20 mg/kg (silver nitrate). The effect was considered permanent and substantial and importantly occurred within the guidance value range for STOT RE 1. However, there were no other studies to robustly corroborate these findings and the DS was unconvinced the effects noted in a single publication were sufficient evidence to fulfil the criteria for classification.

Overall, the DS did not propose classification for STOT RE.

Comments received during consultation

One MSCA commented (#294) raising concern for neurotoxicity but agreed with the DS that the criteria for classification were not satisfied.

Comments #291-301 from industry or companies were very generic in nature briefly stating no evidence for this hazard.

Following the RAC-60 CLH Working Group meeting on 27 January 2022, additional data was made available that could impact on the STOT RE assessment:

1. A review by Mota & Dinis-Oliveira (2021) provided an update on Argyria and reviewed the state of the art regarding pathophysiology, diagnosis, treatment, and relevant clinical and forensic features of argyria. It refers to argyria as an inert silver deposition in tissues. Discolouration develops following *uv* exposure since silver ions undergo photoreduction to atomic silver (Ag^0), which can be oxidised to low-solubility and chemically stable compounds such as silver sulphide (Ag_2S) and silver selenide (Ag_2Se). In the eye, silver deposits exhibit a clear preference for Descemet's membrane along with many other ocular structures. There was no firm consensus that ocular argyrosis resulted in a functional disturbance of the eye, silver deposition would seem to be innocuous in most cases though the reviewers acknowledged that more data is required to fully substantiate this claim.

1. Anon. *et al.* (2021). A 90-day Study of Silver Acetate by Dietary Administration in Wistar Han Rats. This was a GLP and guideline compliant study incorporating a TK subgroup, 10 animals/dose/sex were administered AgOAc in the diet at nominal levels of 0, 40 (41-42, M-F), 120 (122-126, M-F) and 320 (287-319, M-F) mg/kg bw/day. Necropsy showed staining assumed to be silver deposition in several organs including the brain. There were no treatment related deaths, an FOB (functional observational battery) indicated no treatment-related effects, clinical signs were unremarkable and there was no pathology of brain structures indicating e.g. hippocampal involvement. Histopathology findings in the brain were confined to extracellular pigmentation of the area postrema in all treated animals. This brain area is located in the medulla oblongata of the brainstem. Pigmentation in a nearby structure (the subfornical organ), did not show a dose response relationship. As regards the brain areas investigated in the histopathological examination, there was no reference to the hippocampal area, and other standard brain sections normally examined according to OECD TG 408 including the cerebellum, cerebrum, and midbrain were also unremarked.
2. Anon. *et al.* (2021). Silver Acetate: Preliminary Reproductive Performance Study in the Sprague Dawley Rat by Dietary Administration. There was no claim to GLP or guideline compliance. This study served to assist in dose level selection for an Extended One Generation Reproductive Toxicity Study (EOGRTS) according to OECD TG 443. Dosing was similar to the 90-day 2021 dietary study, 12 animals/dose/sex were administered AgOAc in the diet at nominal levels of 0, 4 (4-5, M-F), 40 (39-50, M-F), 80 (76-92, M-F), 160 (152-188, M-F) and 320 (308-358, M-F) mg/kg bw/day. There was excessive lethality in the two highest doses. At littering, females at 160 or 320 mg/kg bw/day were observed to have high litter loss which necessitated termination of these dose groups for welfare reasons. All females of the P0 parental generation were sacrificed prior to scheduled termination (i.e., between GD20 and LD4 and not the scheduled date corresponding to LD21). Clinical signs in all dose groups were unremarkable, there was no evidence/no reporting of behavioural disturbances or lethargy. Detailed brain histopathology of F1 offspring at LD21 did not reveal any pathological changes or developmental abnormalities. As confirmed by the study pathologist, there was no neurohistopathological examination performed in parental animals in this dose range finder to the main EOGRTS study.

3. Anon. *et al.* (2022). Silver Acetate: Extended One Generation Reproductive Toxicity Study in the Sprague Dawley Rat by Dietary Administration. This was a GLP and guideline compliant study (OECD TG 443) including cohorts 2A and 2B for developmental neurotoxicity and cohort 3 for developmental immunotoxicity. The P0 generation was administered AgOAc in the diet at nominal levels of 0, 40, 80 and 120 mg/kg bw/day. The F1 generation was exposed prenatally via mothers and postnatally in accordance with the OECD TG 443 for each cohort. Developmental neurotoxicity was evident at 80 or 120 mg/kg/day in the cohort 1A and 2A animals: effects noted included reduced activity and rearing of males and females in the arena, reduced reactivity, abnormal motor movement/gait, intra-myelinic oedema and dose related neuronal necrosis in the hippocampus, and neuronal/glial cell necrosis in the thalamus (both sexes). Brain morphometry measurements revealed statistically significantly low hippocampus measurement for males that received 80 or 120 mg/kg/day. No neurohistopathological effects were observed in the P0 generation, and the neuropathologist of the EOGRTS study confirmed that standard brain sections investigated in the parental P0 generation, including cerebellum, cerebrum and midbrain routinely include hippocampus and thalamus.

An analysis of the available reproductive toxicity studies in the CLH report offered little evidence for neurodevelopmental toxicity or neurotoxicity for other silver compounds. There were some indications from silver zinc zeolite in the form of decreased activity in pups but there was no histopathology data available.

Additional evidence from the published peer reviewed scientific literature

A number of studies from a brief investigation by RAC of the published scientific literature suggest that the size, shape and surface coating, as well as rates of silver ion release and interactions with proteins are some of the key factors determining the neurotoxicity of AgNPs. AgNPs potentially target endothelial cells forming the blood-brain barrier, neurons and glial cells leading to oxidative stress-related cell death (see the Background Document for further details).

Assessment and comparison with the classification criteria

The DS reviewed an extensive set of data from OECD TG and GLP compliant studies as well as peer reviewed journal articles. There were no studies where silver in bulk form was investigated. The majority of relevant studies for consideration of STOT RE utilised silver as nanoparticles or as soluble salts in the form of silver nitrate, silver acetate and or silver lactate. Some 90-day studies and chronic toxicity/carcinogenicity studies utilised silver zinc zeolites or silver sodium zirconium hydrogenphosphate. RAC also performed a general survey of the published literature regarding silver-mediated neurotoxicity. A summary of the most relevant studies for assessment of STOT RE are presented in the table below followed by a table presenting the available human data.

***Table**: Summary of the most relevant animal studies with silver*

Study	Substance	STOT-RE 1	STOT-RE 2	Ref.
Oral Route:				
1. Rat 28d, OECD TG 407/GLP	60nm AgNP 30, 300 or 1000 mg/kg/d	None	None	Reliable. Kim, et al. (2008) Doc IIIA 6.3.1-06
2. Rat 28d, OECD TG 407/GLP	AgNO3 oral gavage, 13, 32	No mortalities	None	Reliable. Barraclough

	and 63.5 mg Ag/kg bw/d	and no clinical signs. No effects on the FOB. NOAEL = 63.5 mg Ag/kg bw/d		(2017) Doc IIIA 6.3.1-07? Page 518, annex to CLH report.
3. Rat 42/52d, OECD TG 422/GLP	8nm AgNP 62.5, 125, 250 mg/kg/d	None	None	Reliable. Hong *et al.* (2014) Doc IIIA 6.3.1-07
4. Rat 90d, OECD TG 408/GLP	56nm AgNP 30, 125 and 500 mg/kg/d	None	None	Reliable. Kim, *et al.* (2010) Doc IIIA 6.4.1-08
5. Rat 28d, non-TG /non GLP.	Ag-acetate & 14nm AgNP at 9 mg Ag/kg/d	None	None	Supplemental. Hadrup, *et al.* (2012a) REACH dossier
6. Rat 30d, non-TG /non GLP.	0.5 % $AgNO_3$ systemic dose not known	None	None	**Not acceptable.** Unusual route of dosing, mouth washing? Tamimi, *et al.* (1998) REACh dossier
7. Rat 14d, non-TG /non GLP.	55nm AgNP & $AgNO_3$ 0.2-20 mg Ag/kg/d	**Yes**. Hippocampal neuronal cell loss in dams.	None	Reliable. Charehsaz, *et al.* (2016) Doc IIIA, 6.8.2-10
8. Rat, 14d, low-dose (1 mg/kg bw) or high-dose (10 mg/kg bw) intragastric admin.	AgNPs	**Yes.** Neuronal degeneration and astrocyte swelling at 1 mg/kg bw or 10 mg/kg bw		Reliable. *Xu et al.* (2015). Literature survey by RAC.
9. Rat EOGRTS, OECD TG 443 / GLP, dietary admin.	AgOAc	None in P0 (neurohistopathological investigation including hippocampus and thalamus performed), DNT in cohort 2A and in cohort 1A	None in P0 (neurohistopathological investigation including hippocampus and thalamus performed), DNT in cohort 2A and in cohort 1A	Reliable. Anon. *et al.* (2022). Provided during the CLH process.
10. Rat 90-day study, OECD TG 408 /GLP, dietary armin.	AgOAc	None (neurohistopathological investigation including hippocampu	None (neurohistopathological investigation including hippocampus	Reliable. Anon. *et al.* (2022). Provided during the CLH process

		s and thalamus performed)	and thalamus performed)	
11. 28d oral rat, 0.5 mg/kg bw	Coated AgNPs and silver ions	**Yes.** Impairment of cognitive functions and behavioural disturbances.		Reliable. Dziendzikowska *et al*. (2021). Literature survey by RAC.
12. Rat 28d, non-TG /non GLP.	14nm AgNP & Ag-acetate at 9 mg Ag/kg/d	None. Changes in neurotransmitter levels (increased dopamine and noradrenaline). No functional deficit. No info on clinical signs or bw.	None	Supplemental. Hadrup, *et al.* (2012b) REACH dossier
13. Dog 90d, OECD TG 409/GLP	Silver zinc zeolite (AgION AK10D) 0.2 – 5.1 mg Ag/kg/d	**Yes.** Reduced Hb 20% relative to controls. Effect of Zn?	None	Reliable. Anon. (2003) Doc IIIA 6.4.1-07
14. Rat 90d, US EPA OPPTS 870.3100 /GLP	Silver zinc zeolite (AgION AK10D) 0.7 – 6 mg Ag/kg/d	Some haematological effects: ↓[Hb (m/f, 15/10%); MCV (m/f 18%/11%); MCH (m/f, 23%/15%)]	None	Reliable. Anon. (2001) Doc IIIA 6.4.1-06
15. Mouse, 2year, OECD TG 453/GLP?	Silver zinc zeolite (AgION AJ10N) 0.7 – 6 mg Ag/kg/d	↓[Hb (m/f, 18/18%); HCT (m/f 18%/22%); RBC (m/f, 15%/14%)]	None	Reliable. Anon. (1992) Doc IIIA 6.5-05
16. Rat, 2year, OECD TG 453/GLP?	Silver zinc zeolite (AgION AJ10N) 0.03 – 0.84 mg Ag/kg/d	None	None	Supplemental. Anon. (1992) Doc IIIA 6.5-06
17. Rat 90d, OECD TG 408/GLP	AlphaSan RC5000, doses 0.3 – 9.5 mg Ag/kg/d	None	None	Reliable. Anon. (1995) Doc IIIA 6.4.1-04

18. Dog 90d, US EPA OPPTS 870.3100 /GLP	AlphaSan RC2000, doses 5 – 20 mg Ag/kg/d	None	None	Reliable. Anon. (2002) Doc IIIA 6.4.1-05
Inhalation Exposure:				
1. Rat 90d, OECD TG 413/GLP	18nm AgNP 49-515 µg/m^3 /6H/d	None. Minor histopath.	None	Acceptable. Sung, *et al.* (2009) Tox Sciences, vol. 108, 2, p452-461. REACH dossier
2. Rat 90d, non-TG /non GLP.	18nm AgNP 49-515 µg/m^3 /6H/d - 5d/week	None. Reductions in lung function tests, increase in alveolar inflammation.	None	Acceptable. Sung, *et al.* (2008) Inhal. Toxicol., vol. 20 p567-74. REACH dossier
3. Rat 28d, OECD TG 412/GLP	12-15nm AgNP 0.5-61 µg/m^3	None	None	Acceptable. Ji*, et al.* (2007). Inhal. Toxicol., vol. 19 p857-71. REACH dossier
4. Mouse 10d, non-TG /non GLP.	5nm AgNP 3.3mg/m^3 /4H/d	None. Minimal inflammatory response.	None	Supplemental. Stebounova, *et al.* (2011). Part. Fibre Toxicol. 8: 5. REACH dossier
Dermal Route:				
1. Guinea pig 90d, non-TG /non GLP.	<100nm AgNP 100, 1000 or 10000 µg/mL, $AgNO_3$ 100 µg/mL/ 5d/week (0.1 mg/kg).	None. Inflammation noted with histopath in skin liver and spleen. No deaths, no clinical signs.	None	Supplemental. Korani *et al.* (2011). Int Jour of Nanomed, 6: p855-862. Doc. IIIA 6.4.2-04
2. Guinea pig 90d, OECD TG 411 /unknown GLP.	<100nm AgNP 100, 1000 or 10000 µg/mL, $AgNO_3$ 100 µg/mL/ 5d/week (0.1 mg/kg).	None. Inflammation in multiple organs. Various histopath. No lethalities, no clinical signs, no bw change.	None	Supplemental. Korani *et al.* (2013). Iranian Journal of Pharmaceutical Research 12 (3): 511-519.
Other Routes:				
1. Rat 14d, nasal admin., **adult** male Wistar	AgNP at 3, 30 mg/kg bw/2d	Supportive for STOT RE. Learning ability / memory		Reliable. Liu, *et al.*, (2012).

		impaired. Hippocampal pathology evident	Literature survey by RAC.
2. Mice, oral admin. and i.p. (Ag lactate)	Silver lactate, $AgNO_3$	Supportive for STOT RE. Indications of impaired activity.	Reliable. Rungby & Danscher, (1984). Literature survey by RAC.
3. Single/multiple dose (7d), mice (M), intranasal.	AgNPs	Supportive for STOT RE. ↑ Expression of Hmox1 in hippocampus, effects on learning and memory.	Reliable. Davenport *et al.* (2015). Literature survey by RAC.

Assessment of study data presented in the CLH report in support of STOT-RE. All studies were considered acceptable and reliable unless otherwise noted.

***Table**: Summary of Human data*

Study	Substance	Support for STOT-RE 1/2	Ref.
Medical data: direct observation, e.g. clinical cases & poisoning incidents where available.			
1. US EPA R.E.D. FACTS for Silver. Pesticide re-registration document (1992).	Dusts containing silver nitrate and/or silver oxide. No specific detail.	Generalised respiratory irritation and inflammation affecting several systems. Abdominal pain, and decreased night vision in workers.	Supplemental. Silver Zinc Zeolite Doc IIIA 6.12.2-02.
2. US EPA (1998) Integrated Risk Information System. Silver EPA IRIS system.	Silver arsphenamine, critical paper reported 70 cases of generalized argyria.	Generalised argyria. Lowest chronic oral LOAEL estimated at 0.014 mg Ag/kg/day.	Supplemental. Gaul and Staud (1935). Silver Zinc Zeolite Doc IIIA 6.12.2-03.
3. Single case report.	Ag-acetate, dose unknown.	Generalised argyria. Total body burden = 6.4g Ag. Estimated dose 0.12 mg/kg bw/day.	Supplemental. East *et al*. (1980). Silver Zinc Zeolite Doc IIIA 6.12.2-04.
4. Single case report.	0.53 g $AgNO_3$ daily over 9 years	Generalised argyria. Estimated dose 0.63 mg/kg bw/day.	Supplemental. Westhofen & Schafer (1986). Silver Zinc Zeolite Doc IIIA 6.12.2-05
5. Single case report.	Colloidal silver, unknown concentration.	Generalised argyria.	Supplemental. Brandt *et al*. (2005). Silver Zinc Zeolite Doc IIIA 6.12.2-06
6. Single case report.	$AgNO_3$ Unknown amount.	Acute renal and hepatic failure following treatment for chyluria.	Supplemental. Kulkarni, *et al*. (2005) Silver Zinc Zeolite Doc IIIA, 6.12.2-07
7. Review article of adverse events from a variety of silver-containing products.	Various.	No. Generalised argyria, between 1802 and 1951 there were 365 reported incidents.	Supplemental. Fung & Bowen, (1996) Silver Zinc Zeolite Doc IIIA, 6.12.5-01

Key aspects to consider

There are basically three areas of concern regarding silver exposure:

1. *Generalised Argyria.* Silver becomes widely distributed throughout the body regardless of the route of exposure. Multiorgan effects are manifested by histopathological features that indicate oxidative insult. All compartments (including brain and testes) are open to exposure and there is deposition of silver in complexes with sulphide or selenium. The result is a blue-grey or grey pigmentation to internal organs and skin and mucous membranes; however, it is generally accepted that this in itself is not predictive or associated with any other toxic effect of silver. Both the animal data and human data confirm the development of Argyria in response to silver exposure.
2. *Ocular argyrosis.* Chronic exposure to silver compounds through ingestion, inhalation or skin contact results in the accumulation of silver in the eyes and in the adjacent tissues. This condition is defined as ocular argyrosis, the most common type of localized argyria. In workers, argyrosis of the cornea may be accompanied by turbidity of the anterior lens capsule and disturbance of the dark adaptation, not resulting in loss of vision. There is currently little evidence to support a functional defect of the eye following silver exposure. Drake & Hazelwood (2005) described four studies where evaluations of the eye were carried out in workers exposed to silver nitrate, silver oxide, silver and other metal powders (silver chloride, and silver cadmium). While silver exposure was correlated with corneal and conjunctival argyrosis, no functional deficits were found. In a recent publication by Mota & Dinis-Oliveira (2021), there was no firm consensus that ocular argyrosis resulted in a functional disturbance of the eye.
3. *Neurotoxicity.* Charehsaz, *et al.* (2016) Doc IIIA, 6.8.2-10, described hippocampal neuronal cell loss in dams dosed with silver nanoparticles and silver nitrate by gavage from gestation day 7 – 20. Histopathological examination of brain tissue revealed a high incidence of hippocampal sclerosis in the treated dams. There are additional publications available supporting adverse effects on the hippocampal neurons. Liu *et al*. (2012) used nasal drops (Ag-np 3 mg/kg and Ag-np 30 mg/kg) in adult male specific-pathogen free (SPF) Wistar rats once every two days for 14 consecutive days and after two-week exposure, Morris water maze (MWM) test was performed for the spatial cognition, followed by the long-term potentiation (LTP) recording and reactive oxygen species (ROS) detection in hippocampal homogenate. Results showed that compared with the control group, both LTP and MWM were abnormal in the low-dose group and the high-dose group. The quantity of ROS in hippocampal homogenate was increased significantly in low-dose group and high-dose group. Rungby & Danscher (1984) performed crude behavioural studies in which they showed that silver nitrate and silver lactate treated mice (age of animals not given) were significantly hypoactive relative to controls. In Dziendzikowska *et al.* (2021) AgNPs coated with BSA, polyethylene glycol or citrate or silver ions (Ag^+) were orally administered at a dose of 0.5 mg/kg bw to 11.5-week-old male Wistar rats for a period of 28 days. Impairment of cognitive functions and behavioral disturbances which differed depending on the form of silver were reported. Davenport *et al.* (2015) applied a single dose (10–500 mg/kg) or repeated dose (50 mg/kg/d for 7 d, followed by a 7d wait period) exposure of male C57BL/6 mice (age not given) to silver nanoparticles via intranasal instillation. Silver nanoparticle deposition was systemic, including in the brain. Expression of the oxidative stress-responsive gene Hmox1 was elevated in the hippocampus, but not in the cortex of

treated mice. There was only limited evidence for effects on learning- and memory-related behaviours. However, effects on gene and protein expression and spatial reference memory indicate that some changes took place in the hippocampus. In Xu *et al.* (2015) silver nanoparticles were examined in rats after intragastric administration. After a two-week exposure to low-dose (1 mg/kg bw) or high-dose (10 mg/kg bw) AgNPs, neuronal shrinkage, cytoplasmic or foot swelling of astrocytes, and extra-vascular lymphocytes were observed in silver nanoparticle exposure groups. The authors concluded that silver nanoparticles can induce neuronal degeneration and astrocyte swelling via proinflammatory mechanisms.

Developmental neurotoxicity (assessed under developmental toxicity). There were also indications of neurotoxicity in offspring when exposure occurred via mothers in utero or during the early postnatal developmental period. Wu *et al.* (2015) prenatally dosed offspring via mothers every two days from GD10 to GD18 ip with uncoated AgNPs and showed that AgNPs increased ROS in the hippocampus of the offspring rat brain and induced histopathological changes with hippocampal neuronal cell loss along with impaired spatial learning and memory ability tested in MWM test in rat offspring at postnatal day 35. A study by Ganjuri *et al.,* (2015) showed that maternal exposure to AgNPs via drinking water from day 1 to the end of pregnancy significantly increased expression levels of Procaspase-3 nRNA in the brains of pups at birth (Doc IIIB 6.8.2-23, page 514). Liu *et al*., (2022) showed that nasal sprays with silver in either ionic or nanoform leads to the accumulation and transformation of Ag-containing particles in the neonatal rat brain (exposed from the second day after birth for 2 weeks and then recovered for 4 weeks until week 6). The developing brain in the rat is quite susceptible to neurotoxicity caused by silver (Yin *et al*., 2015, intranasal application). This study (performed on neonatal SD rats exposed to citrate stabilised AgNPs for 14 weeks) also shows a dose-response relationship. AgNPs caused cerebellar ataxia -like symptoms in rats, evidenced by dysfunction of motor coordination and impairment of locomotor activity. Observation of cerebellum sections revealed destruction of the cerebellum granular layer. In the EOGRTS on silver acetate (Anonymous, 2022), intramyelinic oedema in the thalamus, caudate putamen and/or the corpus callosum and dose related neuronal necrosis in the hippocampus and neuronal/glial cell necrosis in the thalamus in the DNT cohort 2A and cohort 1A were observed. A diminution of the size of the hippocampus was observed in the morphometric measurement along with deficits in motor function (rearing, ambulation, abnormal gait, movement and activity) and other neurobehavioral parameters such as mean auditory startle peak amplitude, latency to peak values and habituation of acoustic startle response in the DNT cohort 2A. The absolute brain weight was also reduced by 8 and 12% in males and females, respectively, in offspring sacrificed on PND 22. The DS had included also Ghaderi *et al*. (2015) and Fatemi *et al*. (2013) for the assessment of developmental toxicity. In Ghaderi *et al*. (2015) NMRI mice had been treated subcutaneously once every three days from gestation day 3 until delivery, by 0, 0.2 and 2 mg/kg of bodyweight of silver nanoparticles. Spatial memory, passive avoidance learning, stress, anxiety-like behaviour and locomotor activities were assessed in adult offspring. Prenatal exposure to silver nanoparticles significantly impaired the cognitive behaviour in the Morris water maze. Also, the number of defecations and leanings in the open field assay and number of passages in the light-dark box were greater in groups prenatally exposed to silver nanoparticles. Fatemi *et al*. (2013) tested the effects of prenatal exposure via pregnant dams with silver nanoparticles (20 ± 4 nm, sodium citrate buffer) on the developing brain in neonatal Wistar rats at 25 mg/kg bw/d from GD9 until parturition via intragastric administration. The offspring were

sacrificed the day after birth. The effects in offspring consisted of an increase of silver and the number of microvacuolar structures in brain (612 vs 159 in controls), reduced antioxidant activity and increased peroxidation, statistically significant decrease in bw on PND0 and of a statistically significant decrease in the ratio of brain/body weight. No significant differences in maternal weight gain were reported. The DS also referred to a human case study in Robkin *et al*. (1973). According to this publication the concentration of silver in the human foetal liver of 12 anencephalic human foetuses was higher (0.75±0.15 mg/kg) than the values from 12 foetuses obtained either through therapeutic abortions (0.23±0.05 mg/kg), or in 14 spontaneously aborted foetuses (0.21±0.05 mg/kg). The concentration in 9 premature infants was 0.68±0.22 mg/kg. The authors could not determine if the malformation was associated with higher concentration of silver in anencephalic foetuses or with foetal age.

Neuronal cell loss is permanent and thus considered to be a significant effect. It also occurred within the Guidance value range for STOT RE 1 in Charehsaz *et al*. (2016) and Xu *et al*. (2015) and fulfils one of the STOT RE classification criteria [1](g) in section 3.9.2.7.3 of Annex I to CLP. Also, an impairment of cognitive functions and behavioural disturbances were observed in Charehsaz *et al*. (2016) within GV range for STOT RE 1. Such neurofunctional effects fulfil another STOT RE classification criteria [2](b) in CLP 3.9.2.7.3. Impaired learning and memory were reported also in Liu, *et al*., (2012) and Davenport *et al*. (2015) using intranasal route of exposure to silver nanoparticles. In Liu *et al*. (2012) also hippocampal pathology was reported. Clinical signs indicative of neurotoxicity were noted in rats treated with silver zinc zeolite but the higher level of zinc ions complicates drawing conclusions from this scenario. The DS only considered the Charehsaz*, et al.* (2016) study in isolation and on that basis found insufficient evidence to warrant a proposal for classification. RAC notes additional studies that suggest the rat hippocampal neurons may be susceptible to oxidative damage from silver but the available data is still limited and neurofunctional effects such as potential perturbations to motor activity or learning deficit were seldom studied and did not comprise a prominent set of tests in the available animal studies. The neurotoxicity section of the silver zeolite CAR (Doc IIIA 6.9) includes some further detail on the publication by *Rungby & Danscher* (1984) in which indications of impaired activity were reported by silver nitrate and silver lactate using oral and i.p. (for silver lactate) routes of exposure. The evaluation of that report acknowledged that "*accumulations of silver may have influenced the function of the mammalian brain but recognised the methods used to test the hypothesis were crude and insufficiently specific*".

Three recently available studies (2021-2022) include a 90-day rat dietary study and both a dose range-finding study and a full Extended One Generation Reproductive Toxicity Study in the rat exposed to AgOAc in the diet. The 90-day study and the dose-range finding study did not indicate behavioural or brain histopathological abnormalities, however only limited neurobehavioral tests were performed in the 90-day study (clinical observations hearing ability, pupillary reflex, static righting reflex, fore- and hind-limb grip strength and locomotor activity), and in the dose-range finding study neurohistopathology and neurobehavioral tests were not performed. In the Extended One Generation Reproductive Toxicity Study showed no effects were seen in the P0 generation in the neurohistopathological investigation (according to the study neuropathologist

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1 "..*evidence of appreciable cell death (including cell degeneration and reduced cell number) in vital organs incapable of regeneration."*

2 "*…significant functional changes in the central or peripheral nervous systems or other organ systems, including signs of central nervous system depression and effects on special senses (e.g. sight, hearing and sense of smell);"*

the investigated brain structures in the P0 generation included both the hippocampus and thalamus), but neurodevelopmental effects were observed in the F1 generation. The neurodevelopmental effects seen amongst F1 offspring however further support the published non-guideline studies reporting hippocampal neuronal cell loss also after exposure of mature rats.

A general survey of the published literature indicates an ongoing concern for silver-mediated neurotoxicity. Many newer studies recognise the potential for silver to (1) localise to neural tissues in the central nervous system from different exposure routes and (2) somehow be involved in oxidative insult and mitochondrial damage in regions of the brain associated with cognitive (e.g. learning and memory), motor and other neurobehavioural functions. As noted above, in some studies where immature rats were exposed pre- and/or postnatally, effects were seen in offspring when tested a day after birth (an increase of silver content and the number of microvacuolar structures in the brain), around weaning (reduced brain weight) or as juveniles (neurohistopathological and neurobehavioral effects) and that these effects shall be addressed under developmental toxicity. In Wu *et al*. (2015) the offspring were exposed only prenatally to uncoated AgNPs and this resulted in histopathological changes with hippocampal neuronal cell loss along with impaired spatial learning and memory ability tested in MWM test in rat offspring at postnatal day 35. In Ghaderi (2015) NMRI female mice had been treated subcutaneously once every three days from gestation day 3 until delivery, by 0, 0.2 and 2 mg/kg of bodyweight of silver nanoparticles. Prenatal exposure to silver nanoparticles significantly impaired the cognitive behaviour in the Morris water maze in offspring when tested as adults. Also, the number of defecations and leanings in the open field assay and number of passages in the light-dark box were greater in groups prenatally exposed to silver nanoparticles. In Fatemi *et al*. (2013) prenatal exposure of offspring to silver nanoparticles (20 ± 4 nm, sodium citrate buffer) via pregnant dams showed an increase of silver and the number of microvacuolar structures in brain (612 vs 159 in controls) in pups the day after birth. It was considered that microvacuolar structures in brain may have indicated developmental neurotoxicity but that researchers would need a recovery group to assess if this was a permanent effect along with FOB and memory assessment. In Robkin *et al*. (1973) it could not be determined if the increased number of human anencephalic foetuses was associated with the increased silver concentrations in these foetuses or with foetal age. In unselected offspring of F1 generation terminated on PND 22 in the EOGRTS on silver acetate, there was a decrease in absolute brain weight. However, even if classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity, developmental toxicity covers in its widest sense, any effect interfering with normal development of the conceptus resulting from exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. Effects can be manifested at any time point of the life span of the organism (see CLP 3.7.1.4). Importantly, the nervous system continues to develop after lactation and even after sexual maturation. It is generally not possible to distinguish the precise origin or timing of the toxicological insult if an adverse neuropathological, neurofunctional, or neurobehavioural outcome is observed in the offspring that has been exposed prenatally and/ or postnatally until and after sexual maturation. Thereby also effects seen in Yin *et al*. (2015) cohort 1A, 2A of EOGRTS are relevant for the assessment of developmental toxicity. STOT RE does not cover effects that are specifically addressed under reproductive toxicity or other hazard classes (CLP 3.9.1.1) and therefore data leading to classification for developmental toxicity would not also lead to classification for STOT RE, but if data for both hazard classes are available and criteria met, classification for both hazard classes should apply. RAC concluded that both classifications were to be considered because silver neurotoxicity was induced in animals exposed at any life stage including prenatal and later developmental periods, but considered the read-across from the EOGRTS on silver acetate was not justified and the published studies on silver nanoparticles (Wu *et al*., 2015, Ghaderi *et al.,* 2015 and Yin *et al*., 2015) were not sufficiently reliable to alone justify the classification for developmental toxicity. The toxicological significance of the increased

number of microvacuolar structures in offspring brain that had been exposed prenatally to silver nanoparticles (Fatemi *et al*., 2013) was considered unclear and it was not possible to conclude whether the increased number of human anencephalic foetuses was associated with the increased silver concentrations in these foetuses or with foetal age. Therefore, RAC concluded that the evidence on DNT were not sufficient for classification for developmental toxicity.

In summary, the neurotoxic effects demonstrated by several studies consisting of effects in the rat hippocampal neurons and associated neurofunctional effects, especially on learning and memory, are considered relevant to humans and present adverse effects of concern. While there is a lack of a coherent set of tests to investigate all potential neurobehavioral outcomes across species, there is sufficient data in rats (with limited data in mice), that make it difficult to disregard these effects. Regarding this aspect of neurotoxicity, with additional information provided during the CLH process, there is now more evidence than was presented initially in the CLH report. Classification for STOT RE is considered appropriate and supported by RAC.

Overall Conclusion

The most concerning effect noted by the DS and RAC is the potential for neurotoxicity. Taking a weight of evidence approach which included a selection of different studies, mainly with silver NPs, which consistently show morphological and/or functional neurotoxic effects, it was concluded that classification for STOT RE was warranted. While some neurotoxic effects occurred within the guidance value range for STOT RE 1, that category was not proposed by RAC because the overall database on the lowest dose levels where hippocampus toxicity and neurofunctional deficits involving learning and memory started to occur was considered insufficiently robust or inconsistent to support the more severe category. Although neuronal effects were not always reported or seemed less obvious in other studies and published reports utilising different silver compounds, RAC noted that thorough examinations (e.g. on advanced histopathology and functional studies on memory and learning), that allow a proper observation of effects in the hippocampus region of the brain, were only available in a few studies. In contrast to the DS proposal not to classify for STOT RE, RAC proposes to **classify silver as STOT RE 2 (nervous system).**

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS proposed to classify elemental silver (all forms) as Muta. 2 (H341) using evidence from silver nanoparticles and silver containing compounds including salts from both *in vitro* and *in vivo* studies. There were no studies that investigated silver metal in bulk form (either as dust, i.e., < 1 mm >100 nm or massive, i.e. > 1 mm). There were no *in vivo* heritable germ cell mutagenicity tests described in the CLH report. There is no data to determine if silver metal in bulk form is mutagenic. There is an abundance of data on silver substances in the open literature, but it is not possible to include every study or publication.

Most of the studies assessed by the DS (tables 46 and 47 from section 10.8 of the CLH report), are taken from peer-reviewed public literature investigating silver nanoparticles (median metal particle size <100 nm). In a few cases there were original study reports available (used to support the 2012 CAR or silver zinc zeolite assessment report under the biocides work programme, encompassing silver zinc zeolite, silver copper zeolite and silver sodium hydrogen zirconium phosphate). Several studies were also taken from the lead registration dossier submitted under Regulation (EC) No 1907/2006 (REACH)). Very few studies were available using silver salts, i.e., silver in ionic form. In addition, individual study results were taken, where possible, from a

literature review document submitted under the biocides programme (Doc IIIA 6.6.1-10, Jenkinson, P).

All bacterial mutation tests were negative for genotoxicity. This is unsurprising given that silver nanoparticles and silver containing substances are antimicrobial agents. Gene mutation tests in bacteria are unsuitable for assessing the genotoxicity of silver.

The remaining studies comprising assays that use mammalian cells or live animals gave mixed responses but on balance there are numerous *in vitro* and *in vivo* mutagenicity studies that indicate genotoxicity after exposure to silver nano-forms and zeolites. The DS acknowledged the limitations of several studies and confirmed that not all studies were GLP or guideline compliant. The DS described a few studies in more detail in the CLH report.

In vitro assays

- 16 × Ames tests (reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli*), all negative.
- 10 × mammalian cell gene mutation tests (split 2:8; negative / positive).
- 9 × mammalian chromosome aberration tests (split 5:4; negative / positive).
- 9 × mammalian micronucleus assays (split 3:1:5; negative / equivocal / positive).
- 10 × Comet assays (split 4:6; equivocal / positive).
- 1 × UDS test (DNA repair), positive.

In vivo assays

- 5 × chromosome aberration tests (split 2:3; negative / positive).
- 8 × micronucleus assays (split 6:2; negative / positive).
- 6 × Comet assays (split 2:1:3; negative / equivocal / positive).
- 1 × UDS test (DNA repair), negative.

Conclusion

The data presents a complicated situation for the assessment of elemental silver. As per CLP guidance (2017), where there are also negative or equivocal data, a weight of evidence approach using expert judgement can be applied. In summary, the DS noted sufficient concern for genotoxicity whilst remarking on the reliability and relevance of data for silver nanoparticles and specific silver substances. The uncertainty in the mutagenic database was adequately described by the DS and the data taken from *in vitro* mammalian cell test systems including the comet assay and micronucleus (MN) assay and *in vivo* tests such as the MN test in rodent blood or bone marrow erythrocytes, chromosome analysis in rodent bone marrow cells and the alkaline comet assay in target tissues was considered sufficient to propose classification in Category 2.

Comments received during consultation

Several general comments were received (#0-128 RCOM document), from a variety of sources including company/manufacturers; industry/trade associations; individuals and company/downstream users. The main points may be summarised as follows:

1. The concern with Ag^+ ions is an overly conservative and simplistic approach with respect to potential toxicity from elemental silver, particularly with regard to bulk forms of the metal (i.e. particles > 100 nm).
2. The proposed classification is based on low-reliability studies which are inadequate for classification purposes.

3. There is no direct or historical evidence that silver induces heritable genetic mutations in humans.
4. The criteria for classifying silver as a mutagen have not been conclusively met: a selective choice of data is included in the CLH report that does not reflect the full dataset.
5. The weight of evidence from a series of reliable studies support a non-classification for this endpoint.

In addition, the European Precious Metals Federation (EPMF) submitted a position paper which included extensive comments on the proposed Muta. 2 classification. Many of the public comments referred to this document, reiterating its main conclusions. The EPMF presented its main points as follows:

1. The EPMF does not support classification for this endpoint. The Muta. 2 classification proposal is based on conclusions from a number of low-reliability investigations and should be disregarded.
2. The CLH report does not properly take account of the weight of evidence from a series of reliable studies.
3. For bulk elemental silver, such as massive and powder forms, there are no *in vivo* mutagenicity studies and few reliable *in vitro* studies.
4. They recognise that a plethora of reports exist for silver nanomaterials.
5. No read-across uncertainties between AgNP and more massive Ag forms have been addressed.
6. In many cases the study reports used as sources of data in the CLH report are considered to be unreliable due to a lack of methodological details and descriptions of results.
7. The EMPF refers to several studies, not included in the CLH report, that it considers to be more reliable, the DS responded to these in the RCOM document having assessed the publications in detail. The DS indicated that there was no reason to believe the data was any more relevant or robust than that already described in the CLH report, see comment 95 of the RCOM document.
8. The overall dataset covering *in vivo* effects in somatic cell models yielded predominantly negative or equivocal results.
9. The outcomes of several investigations of moderate to good reliability status covering several SCAS variants have been uniformly negative.
10. SCAS also contain constituents other than silver which introduces some interpretative complexity, but the achieved Ag-equivalent treatment levels were moderately high indicating useful confirmatory information regarding an absence of mutagenic or DNA damaging effects.
11. Conventional bacterial mutagenicity systems are not recognised as a valid component of a genotoxicity test battery where nanomaterials are concerned. Bacterial test system results should be excluded from consideration altogether.
12. The mechanism of action of putative genotoxic effects due to AgNP are controversial. There is no evidence for direct interaction with intranuclear targets that support a primary genotoxic effect. The uptake of AgNP within intracellular cytoplasmic vesicles is of low relevance to bulk silver. Indirect genotoxicity linked to the

evolution of reactive oxygen species (ROS) is thought to be inversely proportional to particle size, but EPMF do not further expand on the relevance of this statement.

13. The EPMF tabulated studies where emphasis has been given to findings from higher quality mutagenicity/genotoxicity assays, most of which conform to OECD test guidelines.

There were three comments received from Member States:

1. All support Muta. 2, H341.
2. Support Muta. 2, H341 but indicated there was some data where silver was distributed to testis in rats and pigmentation in ovaries was observed following administration of silver in both nano and ionic form and that this could allow for a more stringent classification. The DS did not consider these studies sufficiently reliable or robust to support a category 1B classification.
3. Support Muta. 2, H341 based on a weight of evidence assessment. Suggest some distinction amongst the silver species and pose the question whether bulk silver forms should be exempted.

Many similarly phrased public comments were addressed by the DS, briefly summarised as follows:

1. In a WoE approach all the quoted studies, regardless of deficiencies, provide sufficient information on the substance to propose classification.
2. The DS acknowledges that most of the published studies, irrespective of being positive or negative, suffer from deficiencies compared to guideline-compliant studies.
3. It is appropriate to integrate testing information on ionic silver (Ag^+), including that for simple silver salts such as silver nitrate and silver acetate, as well as multi-constituent silver-containing active substances (SCAS), which are Ag^+-releasers.
4. Direct evidence that silver induces heritable genetic mutations in humans is not required to fulfil the criteria for classification in Category 2.

Following the RAC-60 CLH Working Group meeting on the 27 January 2022, the DS submitted further comments reiterating their view that the silver ion possess genotoxic properties. While it is true that oxidative stress is often reported as an effect specific for nanoparticles, it has also been observed with ionic silver. Charehsaz *et al.* (IIIA, 6.8.2-10) observed oxidative stress in the livers of rats treated with silver nitrate and *"…concluded that the oxidative response/damage of Ag-NPs reported in previous studies depends not only on the NPs, but also on the amount of Ag ions released from the surface of the NPs"*. The silver ion has also been shown to induce oxidative stress in other studies performed with silver nitrate that are available in the open literature (e.g. Cortese-Krott *et al., 2009*).

Although nanoparticles may have a higher potency with respect to the production of reactive oxygen species (ROS) due to their oxidative potential, there is insufficient data to conclude if these results are not applicable to other forms of silver than nano- since it seems to be an intrinsic property of the silver ion or its ability to be reduced to metallic particles in affected tissues.

Most of the *in vivo* studies on genotoxicity available for silver are performed with nanosilver, less interest is paid to ionic silver, thus *in vivo* data on the silver ion is limited. Consideration is also given to the low bioavailability of silver. The test systems used (bone marrow) may not be optimal to study the genotoxic potential of the silver ion since the doses tested may be very low.

The different results obtained with different types of nanoparticles of silver may thus be due to differences in nanoparticle properties such as coatings which affect the time, location and extent of silver ion release. Oxidative stress is also relevant for the assessment of some effects seen in studies investigating reproductive toxicity.

The DS finishes their comments by posing the question – is there sufficient evidence to exclude a genotoxic potential of the silver ion?

There were no *in vivo* heritable germ cell mutagenicity tests described in the CLH report. However, RAC noted two additional studies as presented in the Additional key elements section of the Background Document (BD): Sycheva et al., (2016) and Gromadzka-Ostrowska et al., (2012) which were added to the analysis.

Assessment and comparison with the classification criteria

Summary

The DS proposes classification as Category 2 based on the criterion of positive indications from *in vivo* somatic cell genotoxicity tests which are also supported by positive results from *in vitro* mutagenicity assays. The dataset assessed comprised studies utilising elemental silver (predominantly AgNP), some Ag^+-releasing SCAS and a small number of investigations using ionic silver salts. Unfortunately, no robust and relevant genotoxicity data for silver in massive or bulk form is available though some reports have investigated silver particles of 200nm in diameter which is technically outside the definition for nanoparticles.

Category 1

No human data are available for silver, therefore a classification with Muta. 1A is not supported. There is no direct evidence in humans that elemental silver, or ionic silver substances, can induce heritable genetic mutations in humans (nor is there useful data on somatic cell mutagenicity in humans). Data illustrating the induction of mutagenic effects in germ cells (a criterion for Category 1B) seem sparce and unconvincing. The study by Gromadzka-Ostrowska *et al.* (2012) indicates a potential for silver nanoparticles to induce reversible DNA damage, however, the mammalian dataset is not sufficient to support a Category 1B classification.

Category 2

The table below presents a summary of all the data included in the CLH report. Most of the table entries relate to publications in scientific journals. Full descriptions of methodology are uncommon in such reports, and results for individual test subjects are rarely provided. The underlying assumption is that all of these studies are considered to provide a minimum level of reliable data.

***Table**: Genotoxicity weight of evidence analysis: silver nanoparticles and silver-containing active substances (SCAS) from the published literature, REACH and biocide dossiers.*

Endpoint	Negative	Equivocal	Positive
in vitro:			
Reverse mutation bacteria	**16**		
Mammalian gene mutation	**2**		**8**
Mammalian chromosomal aberration	**5**		**4**
Mammalian micronucleus assay	**3**	**1**	**5**

DNA strand breaks (Comet)		**4**	**6**
DNA repair (UDS)			**1**
Total	**10***	**5**	**24**
in vivo:			
Chromosomal aberrations	**2**		**3**
Micronucleus assay	**9**		**2**
DNA strand breaks (Comet)	**3**	**1**	**4**
DNA repair (UDS)	**1**		
Total	**15**	**1**	**9**

* Value reported minus the bacterial reverse mutation studies. No weighting is placed on the *in vitro* bacterial reverse mutation studies. Silver is a known antimicrobial agent, the experimental system (i.e., bacteria) is incompatible with the substance tested (AgNPs).

In respect of *in vitro* tier investigations, the existing dataset for bulk elemental silver, e.g. micron-size and bulk massive, is essentially non-existent. As is the case for the *in vivo* testing tier, data relating to elemental silver nanoparticles is considered relevant but the availability of well performed and robust studies is generally lacking. The most robust and reliable *in vivo* studies (being guideline and GLP compliant) are those involving multi-constituent silver-containing active substances (SCAS), which are Ag^+-releasers. These include investigations in rodents based on DNA damage (Comet assay), chromosomal aberrations, and micronuclei induction (IIIA 6.6.5-02; IIIA 6.6.4-01; IIIA 6.6.4-05; IIIA 6.6.3-07; IIIA 6.6.4-04; IIIA 6.6.4-05; IIIA 6.6.4-02). These *in vivo* studies are broadly negative but their ability to deliver free Ag^+ ions into solution is not as well understood as that for silver nano-forms and ionisable salts. Several of the *in vivo* studies in the dataset relied on a parenteral route of administration (i.p. or i.v. bolus dose). CLP guidance allows for inclusion of evidence from such studies while recognising they are not normally a route of human exposure (in non-medical applications).

In vitro tests

Negative results were evident for all the bacterial reverse mutation assays. It is generally accepted that bacterial reverse mutation assays have limited applicability in the case of certain metal ions, including silver. Ag^+ possesses potent antimicrobial and bactericidal activity and therefore is significantly cytotoxic to the tester strains thereby potentially interfering with test fidelity. Therefore, this data series has limited value in the weight of evidence analysis for mutagenicity and are excluded from consideration.

Results obtained *in vitro* for various silver forms in mammalian cell gene mutation tests, micronucleus assays and chromosomal aberration tests provided a range of negative and equivocal results for mutagenic activity as well as positive findings. The majority of published studies were non-GLP and not performed according to any guideline so a weight of evidence approach was pursued. Furthermore, it is assumed that the majority of the studies considered in the CLH report are acceptable (having been published in peer-reviewed scientific journals, unless otherwise noted to be unsuitable by RAC), while recognising significant methodological or reporting limitations exist when compared with the robust studies (conforming closely to OECD test guidelines) normally available for specific substance characterisation under the EU regulatory frameworks for biocides and plant protection products.

The use of toxicological data on nanosilver as well as data on different silver salts for the hazard assessment of silver in different forms is based on the release of silver ions. Since all forms of elemental silver are expected to release silver ions in contact with biological fluids, all data investigating the toxicity of silver ions is considered potentially relevant. RAC notes that data from a study on the bioavailability of silver ions in solution from a variety of forms including micron-sized silver and nanoparticles as well as salts indicate that the release of silver ions from bulk forms is possible but quantitatively less than that released from nanoparticles and soluble salts. It is uncertain whether this *in-vivo* data could support a split classification as Category 2 for silver nanoparticles only (there is essentially no data on macro sized, metallic silver). Distinguishing different forms of silver on the basis of size, bioavailability and solubility only serve to illustrate differences in the release of silver ions, due to inherent characteristics of the silver forms themselves, since the hazard assessment recognises the silver ion or reduced silver atom as the ultimate toxicophore. Toxicity will of course be dependent on exposure regardless of the source but the degree of silver ion release is different and not well characterised for each silver-containing substance. Therefore, a split classification would seem to be untenable for silver for human health endpoints. The dataset is sparse and incomplete for ionic (soluble) salts and Ag^+-releasing SCAS and they are more ideally considered to supplement the data for silver and be weighed on a case-by-case basis as such.

The *in vitro* test results with respect to the substance tested are summarised below, each symbol (-, +, o) represents a distinct result:

1. Bacterial Reverse Mutation

AgNPs	Ag Zeolite (Zn)	Ag Zeolite (Cu)	Ag.Na.Zr.HPO_4	$AgNO_3$	AgCl	Ag sulfadiazine	Unknown
----	--	-	---	---	-	-	-

- negative; + positive; o equivocal

2. Mammalian Gene Mutation

AgNPs	Ag Zeolite (Zn)	Ag_2SO_4	Ag.Na.Zr.HPO_4	$AgNO_3$	Diammine[1]
- ++	++	+	++	+	-

- negative; + positive; o equivocal

[1] Diamine silver tetraborate

3. Mammalian Chromosome Aberration

AgNPs	Ag Zeolite (Zn)	Ag Zeolite (Cu)	Ag.Na.Zr.HPO_4	$AgClTi_2$
-- ++	+	-	-	- +

- negative; + positive; o equivocal

4. Mammalian Micronucleus Assay

AgNPs	Ag_2SO_4
-- +++++ o	-

- negative; + positive; o equivocal

5. Comet Assay

AgNPs
++++++ oooo

- negative; + positive; o equivocal

6. UDS Assay

$AgNO_3$
+

- negative; + positive; o equivocal

Positive as well as negative findings are reported in the *in-vitro* studies. Nanosilver is the most representative test substance and results indicate a clastogenic and gene mutation potential in mammalian cells. For bulk elemental silver, such as massive and powder forms, there were no *in vitro* mutagenicity studies (and therefore no data) presented in the CLH report.

It should be noted however, that Article 5(1) of CLP on the identification and examination of available information on substances states that "*The information shall relate to the forms or physical states in which the substance is placed on the market and in which it can reasonably be expected to be used*." According to the EPMF (2021), the total tonnage of nanosilver is below 3 tonnes/year (i.e. 0.03% of the total tonnage of silver metal). This information covers two forms of nanosilver that are currently registered under REACH and placed on the market. Also, Article 9(5) of CLP states that the forms or physical states in which the substance is placed on the market and in which it can reasonably be expected to be used shall be considered when evaluating the available information for the purposes of classification. Therefore, at a minimum, data on nanosilver are considered relevant for massive or powdered forms of silver under the human health hazard classes. This recognises that unlike other silver substances releasing silver ions, nanoparticles in their pure state are composed of silver metal with identical atomic packing and comparable physical properties to that of the bulk metal. Complicating this picture in comparison to bulk elemental silver forms, is that nanoparticles may be engineered to display different physicochemical properties that can impact on the biological responses of cells utilised in *in vitro* systems. This is due to an exceptionally high surface-to-volume ratio. At the nanoscale, this material exhibits unique electrical, optical, and catalytic properties. Factors such as nanoparticle size, shape, surface area, surface charge, surface functionalization, and particle dispersion state also affect cytotoxicity in mammalian cells. Silver nanoparticles and/or Ag^+ ions tend to induce size-, dose- and time-dependent toxicity by creating reactive oxygen species (ROS), oxidative stress, and DNA damage (Ferdous & Nemmar, 2020) . The release rate of silver ions from the nanoparticles is related to both the size and the surface characteristics of silver nanoparticles. The size of the nanoparticle influences the contact area and interaction of the

nanoparticle with the medium, while the charge and surface composition determine the stability of the nanoparticles (Sharma & Zboril, 2017) . Smaller nanoparticles have a higher potential for Ag^+ dissolution. This is due to their higher surface area-to-volume characteristics, and oxidative surface chemistry reactions which yield ionised Ag^+ into solution.

Presently it is not possible to say whether there is a threshold for silver nanoparticles in their ability to release silver ions. A comprehensive overview within the public literature is lacking, details from published studies are lacking, a comparative assessment of silver nanoparticles and their properties and the influence of different types of stabilising or capping agents is also lacking.

In common with several other metallic and non-metallic nanomaterials, the prevailing mode of action from published peer-review papers is that putative genotoxic responses (e.g. DNA damage) caused by nanosilver may be most consistent with genotoxicity induced via a secondary effect such as increased ROS activity. Other common principal effects observed after exposure to silver nanoparticles include a reduction in cell viability along with the generation of an inflammatory response. The responses are dose-dependent and influenced by nanoparticle properties such as size, shape, surface charge, concentration and colloidal state (Bruna *et al*., 2021). The toxicity of silver is probably due entirely to Ag^+ ions and the variability in toxic response (such as the mixed results from the silver *in vitro* toxicity database) is thus due to different concentrations of the Ag+ ions in the local environment, which may be dictated by the size and the surface characteristics of different silver nanoparticle preparations.

In conclusion, the results of the *in vitro* genotoxicity studies indicate concern for DNA damage/clastogenicity/gene mutation.

In vivo tests

In terms of *in vivo* testing, the dataset relates almost entirely to mammalian somatic cell systems (rodent models). For silver powder and massive forms, no *in vivo* studies are available for consideration. The *in vivo* test results with respect to the substance tested are summarised below, each symbol (-, +, o) represents a discreet result:

1. Chromosomal aberrations

AgNPs	Ag Zeolite (Zn)	Ag Zeolite (Cu)
+++[1]	-[2]	-[3]

- negative; + positive; o equivocal

[1.] Route: ip / oral / ip
[2.] Route: ip
[3.] Route: oral

2. Micronucleus assay

AgNPs	$Ag.Na.Zr.HPO_4$	$[AgCH_3OO]_2$
------ ++[1]	--[2]	-[3]

- negative; + positive; o equivocal

[1.] Route: oral / oral /oral / inhalation / iv / ip // oral / iv
[2.] Route: ip / oral
[3] Route: oral

3. DNA strand breaks (Comet)

AgNPs	Ag Zeolite (Zn)
-- ++++ o[1]	-[2]

- negative; + positive; o equivocal

[1.] Route: oral / iv / iv // oral / ip / oral // iv

[2.] Route: oral

4. DNA repair (UDS)

$Ag.Na.Zr.HPO_4$
-

- negative; + positive; o equivocal

Route: ip

Generally, *in vivo* data generated via physiological routes are weighted more heavily than evidence coming from *in vitro* tests, because they can more accurately indicate potential hazards in humans. Genotoxicity tests measuring unrepairable alterations, such as gene mutations and chromosomal damage, have greater weight than assays measuring DNA damage events (e.g. Comet), that can still be repaired (and therefore reversible).

The evidence for *in vivo* mutagenic effects is not particularly strong. The *in vivo* studies were conducted primarily with nanoparticulate silver, a few studies investigated other forms including silver zinc (or copper) zeolite and Alphasan (silver sodium hydrogen zirconium phosphate). These latter forms were all negative in respect of mutagenicity. The studies utilising silver nanoparticles gave both positive and negative results. On balance the available data indicates concern for both chromosomal aberrations and DNA damage. The available data is derived from studies with differing degrees of reliability and compliance with respect to GLP and established OECD technical guidelines. A substantial number of studies were not guideline compliant but were peer reviewed and published in the public domain and deemed to be acceptable for consideration of the mutagenicity endpoint.

RAC notes that the Kim *et al.* (2008) study (28-day oral rat) which included a micronucleus assay was considered by the authors of the study to be negative in contrast to comments by the DS. RAC considers that it does not meet the criteria for a positive result in OECD TG 474, i.e. is not statistically significant. The publication by Ordzhonikidze *et al.* (2009) (*in-vivo* mammalian comet assay) was considered to be unacceptable and was not considered further (the anionic surfactant used as a silver nanoparticle stabilizer was found to have equal DNA damaging properties to the silver nanoparticles, results for $AgNO_3$ were not reported).

Papers submitted by the European Precious Metals Federation (EPMF) also examined the mutagenicity data for silver and have referred to studies not considered by the Dossier Submitter, and therefore not discussed in the CLH Report. In contrast to the opinion of EPMF, the CLH report properly weighs the evidence from available studies while acknowledging that most of the published studies, irrespective of being positive or negative, suffer from deficiencies compared to guideline compliant studies.

It is worth mentioning one extra study that was not included in the original CLH report. The study by Boudreau *et al.* (2016) is relevant and well performed according to GLP and guidelines and should be given a greater weighting than the other studies. They investigated many different

endpoints, including kinetics and micronuclei after repeated oral dosing with AgNPs (of different sizes) and silver acetate (AgOAc). Genotoxic effects of AgNP exposure or AgOAc exposure in male and female rats were examined with a micronucleus assay, using flow cytometric analysis of peripheral blood. Bone marrow was exposed to Ag only in the acetate treated animals. Levels in blood were elevated after wk1 and wk12 with a weak dose response for AgNPs but a distinct one for AgOAc. The micronucleus assay was negative for each time point in the peripheral blood of rats treated with AgNP. Silver bioavailability differed between 10nm AgNPs and AgOAc (but not by much) and higher doses were administered and were bioavailable with the salt. The accumulation of silver in tissues and organs showed significant dose relationships, irrespective of silver form or particle size, suggesting that the uptake and deposition of silver was proportional to the administered amount of silver and the bioavailability from each form. There were no qualitative differences between the elemental composition of deposited particles or granules in any of the examined tissues of rats exposed to either AgNP or AgOAc. This study adds significantly to the body of negative *in vivo* mutagenicity studies for both silver nanoparticles and a soluble salt, silver acetate.

In addition, industry indicated there was another study available (Narciso *et al*., *2020*) that utilised unstabilised AgNP 20 nm. Both Comet and Micronucleus assays were performed in accordance with OECD TG 489 and OECD TG 474 under GLP conditions. Both assays were negative. Mono-dispersion of the silver nanoparticles was confirmed in solution by Dynamic Light Scattering (DLS) and transmission electron microscopy. CD-1 mice were treated once a day orally by gavage for 3 consecutive days at doses of 50, 150 and 300 mg/kg bw/day AgNPs. Two hours after the last treatment (day 3), mice were anaesthetised with a gaseous solution of isoflurane and subsequently sacrificed by CO_2 inhalation. No statistically significant increased DNA damage was observed in blood, liver, kidney, spleen and duodenum of male and female mice exposed to AgNPs in comparison to controls. The oral exposure to AgNPs didn't increase the percentage of micronuclei in lymphocytes of spleen in male and female mice, although a slight, dose-related increased frequency was present in Ag150-dose and Ag300-dose female mice. Methyl methanesulphonate (MMS) was used as the positive control for the genotoxicity assays. Tabulated data was not reported in the paper for the frequency of micronuclei in lymphocytes derived from murine spleen cells cultured for 44 h *ex vivo* of male and female mice (it was supplied for the Comet assay). The graph of the MN data showed a highly variable positive control response, while indicating a positive response trend it was not particularly convincing and could be argued to be lacking a true positive response, this is a serious limitation in the MN aspect of this study. The Comet data appears reliable. Single Particle-inductively coupled plasma mass spectrometry (SP-ICP-MS) confirmed exposure in several tissues including the spleen. TEM analysis showed the presence of AgNPs into the cells of liver and duodenum.

A further in-depth look at the available in vivo studies was required by RAC to apply a reliability weighting in order to decide if the majority of the evidence favoured the negative result studies or the positive result studies.

Table: *Analysis of in vivo genotoxicity studies – Chromosomal Aberrations*

Test / Study	Substance	Reliability & Relevance	Result	Ref:
1. Rat 28d, i.p., no GLP, no guideline	9nm AgNP	K3: Not reliable. Poor reporting.	+	El Mahdy *et al*., 2014
2. Mouse 18hr, i.p., no GLP, OECD 475.	<100 nm AgNP	K2: Reliable.	+	Ghosh *et al*., 2012
3. Mouse 5d, oral, no GLP, no guideline	34nm AgNP	K3: Not reliable. Deletion assay.	+	Kovvuru *et al*., 2015
4. Rat 1d, GLP, guideline	silver zinc zeolite	K2: Reliable but not relevant? Low Ag^+.	-	IIIA 6.6.4-01
5. Rat 1d, GLP, guideline	silver copper zeolite	K2: Reliable but not relevant? Low Ag^+.	-	IIIA 6.6.4-02

K3, K2 etc. Klimish score

Table*: Analysis of in vivo genotoxicity studies – Micronucleus formation*

Test / Study	Substance	Reliability & Relevance	Result	Ref:
1. Mouse 5d, oral, no GLP, no guideline	34nm AgNP	K3: Not reliable. Several issues.	+	Kovvuru *et al*., 2015
2. Mouse, single dose or 3d via i.v.	5nm & 15–100nm AgNP	K2: Reliable, well conducted.	-	Li *et al*., 2014
3. Rat, 90d oral, no GLP, OECD 474	10-110nm AgNP	K2: Reliable, well conducted.	-	Boudreau *et al*., 2016
4. Mouse, single i.p, no GLP, OECD 474	9nm AgNP	K3: Not reliable. low dose, tissue exposure?	-	Chen *et al*., 2015
5. Rat, single i.v., no GLP, OECD 474.	20 / 200nm AgNP	K2: Reliable, well conducted.	+	Dobrzyńska *et al*., 2014
6. Rat, 28d oral, GLP, OECD 474	60nm AgNP	K2: Reliable, well conducted.	-	Kim *et al*., 2008
7. Rat, 90d inhal., GLP, OECD 474	2 - 64nm AgNP	K2: Reliable with some restrictions.	-	Kim *et al*., 2011
8. Mouse, single i.p. dose, GLP & TG	Alphasan RC2000	K2: Not relevant, low dose, tissue exposure?	-	IIIA 6.6.4-04 Anon., 2000
9. Mouse, single oral dose, GLP & TG	Alphasan RC5000	K2: Not relevant, low dose, tissue exposure?	-	IIIA 6.6.4-05 Anon., 1994
10. Rat, 90d oral, no GLP, OECD 474	AgOAc	K2: Reliable, well conducted.	-	Boudreau *et al*., 2016
11. Mouse, 3 doses, oral gavage, GLP OECD TG 474	20nm AgNP	K3: Well conducted but problem with positive controls?	-	Narciso *et al*., 2020

K3, K2 etc. Klimish score

Table: *Analysis of in vivo genotoxicity studies – Comet Assay*

Test / Study	Substance	Reliability & Relevance	Result	Ref:
1. Mouse 5d, oral, no GLP, no guideline	34nm AgNP	K3: Not reliable. Several issues.	+	Kovvuru *et al*., 2015
2. Mouse, single dose i.v. no GLP, no guideline	15–100nm AgNP	K2: Not reliable, many questions.	0	Li *et al*., 2014
3. Mouse, single dose, i.p. no GLP, OECD 489	44nm AgNP	K2: Reliable	+	Al Gurabi *et al*., 2015
4. Rat 2 doses, oral, GLP, OECD 489	Silver zinc zeolite.	K2: Reliable.	-	IIIA 6.6.5-02
5. Mouse oral gavage, single dose / single dose once a week for 5 weeks.	5nm AgNP	K2: Reliable.	+	Awasthi *et al*., 2015
6. Rat, single i.v., no GLP, OECD 474	20 / 200nm AgNP	K2: Reliable, well conducted.	-	Dobrzyńska *et al*., 2014
7. Rat, single dose, i.v. no GLP no guideline.	20 / 200nm AgNP	K2/K3? Reliable but limited.	+	Gromadzka-Ostrowska *et al*., (2012)
8. Mouse, 3 doses, oral gavage, GLP OECD TG 474	20nm AgNP	K2: Reliable, well conducted.	-	Narciso *et al*., 2020

K3, K2 etc. Klimish score

When focussing on the most relevant and reliable in vivo studies the following can be concluded:

- Chromosomal aberrations: 1 x positive study.
- Micronucleus formation: 1 x positive, 6 x negative.
- Comet Assay: 3 x positives, 3 negatives.

The picture presents weak evidence for mutagenicity. Most of the results are determined from silver nanoparticles, only one well performed study has utilised a soluble salt of silver, AgOAc and this is negative with respect to mutagenicity.

In conclusion, the results of the *in-vivo* genotoxicity studies are mixed, data from silver nanoparticles indicate some concern for DNA damage and clastogenicity. However, the majority of reliable (i.e. acceptable) micronucleus assays were negative (five negative vs one positive test).

Classification Assessment - Muta 2 / No classification

The outcomes from the *in vivo* tier tests present a complex picture where Ag^+, or SCAS able to release Ag^+ present a mixed set of results for mutagenic hazard. SCAS such as zeolites and other ion exchangers may not be relevant because they release only small amounts of silver ion that cannot be properly tested and are likely to give a negative result because insufficient silver ions are available to the testing environment. Some testing data are available for bulk elemental forms of silver (e.g. micron-sized or powders with silver particle sizes >100nm) but the majority is tested in the form of nanoparticles. The *in vivo* assays conducted on silver nanoparticles have shown some genotoxic potential. Results obtained *in vitro* for various ionic silver substances in

mammalian cell gene mutation tests, micronucleus assays and chromosomal aberration tests provided a range of negative and equivocal results for mutagenic activity as well as positive findings. The majority of published studies were non-GLP and not performed according to any guideline so a weight of evidence approach was pursued. The applicability of bacterial reverse mutation assays in the case of silver nanoparticles is questionable; therefore no weight was applied to these results.

The DS presented a snapshot of the available published studies regarding genotoxicity testing for several species of silver. There are several features to note:

1. There is no direct evidence in humans that elemental silver, or ionic silver substances, are able to induce heritable genetic mutations in humans (nor is there useful data on somatic cell mutagenicity in humans).
2. The selection of studies presented in the CLH report are representative of what is publicly available.
3. It is unlikely that the incorporation of more published studies would clarify the situation further.
4. It is acknowledged that most of the published studies, irrespective of whether they are positive or negative, suffer from deficiencies compared to guideline compliant studies. In this case it is not feasible nor is it advisable to forensically analyse each and every single publication. It is appropriate to judge whether a given study is acceptable or not and regardless of deficiencies, provide sufficient information on the genotoxic potential of silver, silver nanoparticles or silver ions.
5. A weight of evidence approach is the most pragmatic for the assessment of silver.
6. The *in vitro* data indicates a concern for DNA damage / gene mutation and clastogenicity for silver nanoparticles and other silver complexes and salts requiring further verification with *in vivo* studies.
7. It is reasonable to assume that *in vitro* studies would be most susceptible to the various different properties presented by different silver nanoform preparations. Because of the high surface area/volume ratio and surface-active properties, silver nanoparticles are often more effective than their salt and bulk metal counterparts in their biological effects. Silver behaviour and speciation in *in vitro* assays is driven by environmental composition (like presence of chlorides) rather than an intrinsic property such as solubility. *In vivo* studies are considered with greater weighting. Bioavailability is a multifactorial process made even more complex by the interaction of silver and its speciation *in vivo* where a variety of chemical and biochemical processes, and environmental conditions influence the absorption characteristics of different silver forms. In contrast to the *in vitro* environment, other components used to stabilise and coat silver nano particulates tend to be stripped away in *in vivo* test systems to expose biological compartments to the pure silver cores allowing for dissolution to Ag^0 and oxidation to Ag^+ under physiological conditions to proceed.
8. Extrapolating via read-across from silver nanoparticles to bulk forms of the metal allows RAC to apply the principle of a worst-case scenario and is understood by RAC to reflect a more conservative approach. Under such an assumption, a single classification can be applied equally to silver nanoparticles and massive forms of silver since the atomic properties of both core elements are identical.
9. Applying data from the toxicokinetic studies and bioavailability it is noted that it is only the quantity of bioavailable silver from bulk silver, ionic species and

nanosilver that differs. That is, although the degree of risk (systemic exposure) is different for the different forms of silver, the silver ion (Ag^+) is ultimately common to all and assumed to mediate the various chemical interactions between silver and tissue components and cellular macromolecules and organelles. Bioavailability is simply a measure of risk, the hazard remains unchanged for all types of silver metal because they all release silver ions, it is just the extent of release that differs from one form of the metal to another.

10. The evidence for mutagenicity is not conclusive, it relies on some positive study data for chromosomal aberrations and DNA strand breaks with silver nanoparticles in *in vivo* studies supported by the positive indicators from numerous *in vitro* studies. The data indicating DNA strand breaks (Comet assay results) is not as robust as that from other types of genotoxicity tests simply because the effect is potentially repairable and thus reversible relative to other more permanent effects such as gene mutations and chromosomal damage.
11. Bioavailability is not interpreted as a fundamental property of silver that determines hazard. In the case of metals, it may seem overly simplistic and highly conservative to ascribe all hazard in equal proportion to the free metal ion and/or reduced elemental species. However, the potential for toxic effects to be expressed by silver metal will depend on the concentration of silver ions and atoms in contact with a biological system, i.e. toxicity will be a function of exposure, regardless of the source.
12. The evidence, limited as it is and at times contradictory, is heavily dependent on silver nanoparticles. There is little data available for soluble salts of silver. However, Boudreau *et al., 2016* presents valuable information with silver acetate, showing that the release of copious amounts of silver ion does not give rise to a positive *in vivo* genotoxic response.
13. If the overall weight of evidence is inconclusive and presents sufficient concern for uncertainty then a classification proposal for silver may also not be warranted for mutagenicity.

RAC agrees with the DS that while the mutagenicity database for silver is extensive for several forms and compounds of silver, the data are inconclusive overall because of contradictory findings and in many cases a lack of sufficient information for each study report. Some concerns remain with respect to the *in vivo* findings for both chromosomal aberrations and DNA strand breaks but the negative results generally in this case outweigh the positive ones. RAC considers silver nanoparticles are representative of silver bulk forms. Applying read-across to a more conservative source material (silver nanoparticles) and applying supporting data from soluble silver salts reinforces the need for a single conclusion for silver metal.

RAC recommends **no classification for mutagenicity due to inconclusive data**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

On the basis of inconclusive data, the DS did not propose to classify silver. There was no data available that was considered appropriate to assess the intrinsic carcinogenic potential of silver in either bulk metallic form, nanoparticles or as the free ion. The DS presented some animal

studies of limited scope and in each case acknowledged the lack of useful data available for this endpoint regarding silver. The studies available from the open literature were generally poorly reported.

The most relevant and accessible data that was available from various sources and presented by the DS was comprised of the following:

1. Silver foil was reported to be carcinogenic if embedded under the skin of rodents, 30% of the rats tested developed fibrosarcomas at the site of implantation. There were no sham operated controls but animals tested with tin foil did not develop tumours (Oppenheimer *et al*. 1956).
2. Weekly subcutaneous injections of colloidal silver resulted in tumours in rats surviving longer than 14 months. Six of the eight tumours found among the 26 rats (23%) were located at the injection site. There were no vehicle controls included in the study and the spontaneous tumour frequency at any site was estimated to be 1-3% (Schmahl *et al*., 1960).
3. In a 2-year rat study, no fibrosarcomas developed at the injection sites in Fischer 344 rats intramuscularly injected with silver metal powder (Furst and Schlauder, 1978).
4. Silver zinc zeolite, mouse, 2-year dietary study, OECD TG 453/GLP unknown (Takizawa *et al*., 1992; Doc IIIA 6.5-05).
5. Silver zinc zeolite, rat, 2-year dietary study, OECD TG 453/GLP unknown (Takizawa *et al*., 1992; Doc IIIA 6.5-06).

Regarding the findings of fibrosarcomas at the site of application, the DS notes the review by Furst (1981). Herein the interpretation of these findings is dubious due to the phenomenon of solid-state carcinogenesis in which even insoluble solids such as plastic have been shown to result in local fibrosarcomas. He concluded that *i.p.* and *s.c.* implants were invalid as indicators of carcinogenicity. The DS also noted from several reviews that there is no published evidence of cancer caused by silver exposure in humans despite frequent therapeutic use and industrial exposure of silver compounds over many years.

The DS summarised two combined chronic and carcinogenicity studies with the silver zinc zeolite AgION type AJ, one conducted in mice and a second in rats. This zeolite is composed of 2.5% Ag^+ (2.4 – 2.6%) and 14.4% Zn^{2+} (13.7 – 15.1%) and is a powder for industrial use as an antibacterial biocide additive. Silver zinc zeolite is used to create hygienic fibres and coatings. Silver and zinc ions are reversibly bound to sites within the zeolite matrix and release of Ag^+ ions from silver zinc zeolite depends on solution chemistry with the highest release occurring under conditions that approximate those within the mammalian stomach, i.e. low pH and high ionic strength. It is stated by the Member State evaluator in the 2012 silver zinc zeolite CAR (Doc IIIA 6.5-05), that approximately 33.9 to 42.2% silver is released from silver zinc zeolites at pH4 (phosphate buffer). Assuming that this can be used to represent the chemical environment of the rodent stomach, the dose level for the silver ion would be [silver content of zeolite × dietary zeolite intake × proportion of silver ion release]. The theoretical Ag^+ ion dose was presented by the DS in the CLH report and is reported in the study descriptions below.

In a 2-year dietary study compliant with OECD TG 453 (GLP not stated), silver zinc zeolite was administered to B6C3F1 mice in daily doses of 0, 0.1, 0.3 and 0.9% corresponding to approximate intakes of 0, 0.67, 2.0 and 6.9 mg Ag^+/kg bw/day. The amount of silver actually released was not analysed but was estimated according to the above description. At study termination, the total number of tumours per animal was lower in high dose males (1.00) compared to controls (1.26) and comparable between high dose females and controls. With regard to non-neoplastic

effects, a NOAEL could not be determined due to statistically significant changes occurring at the lowest dose (increased ovarian cysts in females). There was no evidence of carcinogenicity due to exposure to silver zinc zeolite.

In a 2-year dietary study compliant with OECD TG 453 (GLP not stated), F344 rats received daily doses of 0, 0.01, 0.03, 0.1 and 0.3% silver zinc zeolite corresponding to approximate intakes of 0, 0.03, 0.09, 0.3, 0.9 mg Ag^+/kg bw/day. At study termination, the total number of tumours per animal was lower in high dose males (1.86) compared to controls (1.96). In contrast, a higher number of total tumours was observed in high dose females (2.11) compared to controls (1.37) but the difference was not statistically significant. Statistical trend analysis did however reveal a positive increase in the frequency of:

1. pituitary adenomas,
2. leukaemia,
3. endometrial polyps.

Each of these was dismissed by the DS for a variety of reasons including comparison with historical control data; recognition that some of these effects are of a high background in the F344 rat strain; citation of the Technical Meeting for Biocides in June 2013 where the positive trend for endometrial polyps was not considered strong enough to warrant concern and most importantly both studies have already been evaluated in depth following public consultation and discussions of the silver zinc zeolite CLH dossier during the 35th meeting of ECHA RAC. RAC concluded that the results of these studies did not meet the criteria for classification for carcinogenicity. Silver zinc zeolite has an existing entry in Annex VI to CLP and is not classified for carcinogenicity.

Comments received during consultation

Industry groups stated that there was no evidence and no historical context for carcinogenicity following silver exposure. Two Member States commented, both supported no classification on the basis of insufficient data and that read-across from silver zinc zeolites was not applicable.

Assessment and comparison with the classification criteria

There is no appropriate animal data to assess the carcinogenic potential of silver metal. No evidence of cancer in humans has been reported despite frequent therapeutic use over many years or in workers due to exposure from industrial use.

***Table**: The data presented by the DS in the CLH Report*

Study	Substance	Support for Carcinogenicity	Ref.
Reviews, published journal articles and non-public animal studies performed according to OECD TG/GLP.			
1. US EPA IRIS review of silver. Doc IIIA 6.5-07	(i) Silver metal. (ii) Colloidal silver. (iii) Silver dust.	(i) Silver foil placed subcutaneously in the abdominal wall of rats. Incidence of local fibrosarcomas at site of implantation 32% vs 0% for tin. No sham operated controls. Study period unknown, at least 625 days.	(i) Oppenheimer BS, Oppenheimer ET, Danishefsky I, Stout AP. Carcinogenic effect of metals in rodents. Cancer Res. 1956 Jun;16(5):439–441 (ii) Schmahl, D., and D. Steinhoff, Versuche zur krebserzeugung mit kolloidolen silberund

		(ii) Colloidal silver injected weekly both i.v. and s.c. into rats which survived longer than 14 months resulted in tumours in 8 out of 26 rats (31%). Gold gave no tumours. (iii) Silver powder injected monthly i.m. in F344 rats, positive control Cd; 60%, no tumours with Ag, 2-year study.	goldosungen an ratten. Z. Krebsforsch. 63; 586–591, 1960 (iii) Furst, A. and Schlauder, M.C. (1978) Inactivity of two noble metals as carcinogens. The Journal of Environmental Pathology, Toxicology and Oncology, 1(1), 51- 57. Doc IIIA 6.7-04
2. B6C3F1 Mouse, 2-year, OECD TG 453/GLP unknown	Silver zinc zeolite (AgION AJ10N) 0.7 – 6 mg Ag/kg/d.	No evidence of carcinogenicity.	Supplemental with respect to silver carcinogenicity. Reliable study. Anon, (1992) Doc IIIA 6.5-05.
3. F344 Rat, 2-year, OECD TG 453/GLP unknown.	Silver zinc zeolite (AgION AJ10N) 0.03 – 0.84 mg Ag/kg/d.	Statistically significant dose response relationship (trend) for endometrial polyps, pituitary adenomas in females and leukaemia in both sexes. Latter two tumours have a high spontaneous incidence in the F344 rat strain.	Supplemental with respect to silver carcinogenicity. Reliable study. Anon, (1992) Doc IIIA 6.5-06.

Evidence from peer-reviewed journals

Local fibrosarcomas have been induced after subcutaneous (s.c.) implantation of foils and discs of silver and other noble metals (Oppenheimer *et al*., 1956). Furst (1981), notes that so many substances are positive when tested by this technique that it is difficult to see how any extrapolations to humans can be made when a substance does induce a fibrosarcoma at the site of application. He concluded that i.p. and s.c. implants are invalid as indicators of carcinogenicity because a phenomenon called solid-state carcinogenesis may complicate the interpretation of the cause of these tumours.

Schmahl and Steinhoff (1960) reported, in a study of silver and of gold, that colloidal silver injected both *i.v.* and *s.c.* into rats resulted in tumours in 8 of 26 rats which survived longer than 14 months. In 6 of the 8, the tumour was at the site of the *s.c.* injection. In about 700 untreated rats the rate of spontaneous tumour formation of any site was 1 to 3%. No vehicle control was reported.

Furst and Schlauder (1978) evaluated silver and gold for carcinogenicity in a 2-year study designed to avoid solid-state carcinogenesis. Metal powder was suspended in trioctanoin (a triglyceride obtained by acylation of the three hydroxy groups of glycerol by octanoic acid), and injected monthly, *i.m.*, into 50 male and female Fischer 344 rats per group. The dose was 5 mg each for 5 treatments and 10 mg each for 5 more treatments for a total dose of 75 mg silver. The treatment regimen included a vehicle control and cadmium as a positive control. Injection site fibrosarcomas were found only in vehicle control (1/50), gold (1/50) and cadmium (30/50); no tumours (0/50) appeared at the site of injection in the silver-treated animals. The mean survival time for the cadmium injected group was 16.5 months, whereas for all other groups it was approximately 23.5 months. It was concluded that the noble metals, silver and gold, were not carcinogenic.

The only other available information came from a guideline compliant 2-year study that investigated chronic toxicity and carcinogenicity in both B6C3F1 mice and F344 rats fed with

silver zinc zeolite. The results from this study are not considered appropriate as it is questionable to perform read-across from silver zinc zeolite to silver metal as either silver nanoparticles or silver in bulk form – recognition is given to the fact that silver metal appears to have substantially different bioavailability of the silver ion. Nevertheless, there is no evidence for a substance related increase in tumours as indicated by the ECHA RAC following the assessment of the carcinogenic potential of silver zinc zeolite in December 2015. A summary of the RAC opinion is presented below for completeness.

Rat and mouse 2-year study with silver zinc zeolite

Mouse study – RAC noted three effects of concern; an increased dose-related frequency of renal cysts in males and females, a statistically significant dose-response in the enlargement of the islets of Langerhans (endocrine pancreas) in males, and a statistically significant increase in the incidence of ovarian cysts (with no clear dose-response). RAC did not consider any of these lesions as being relevant for a carcinogenicity classification.

Rat study – the carcinogenicity study in rats demonstrated significant positive trends for leukaemia in both males and females and benign pituitary adenomas in females. RAC put forward arguments in favour of no classification for carcinogenicity based on the following:

i. There was only a weak statistical significance for the reported pituitary adenomas, no indications of malignancy combined with the fact that this strain of rat is known to have a high spontaneous incidence of this tumour. In addition, the incidence of pituitary adenomas in all (control and treated) groups of the study was within the range observed in the historical control data;
ii. There was only a weak statistical significance of leukaemia cases in a very susceptible strain of rats and an absence of leukaemia in mice;
iii. The mean survival period in both rodent species amongst all groups was not significantly different;
iv. There was a comparable ratio of tumours/animal among control and exposed rats and mice at the termination of the study;
v. There are doubts on the human relevance of the leukaemia reported in rats.

Comparison with the classification criteria

As there is no epidemiological evidence regarding the carcinogenicity of silver in humans, a classification in Category 1A is not appropriate. RAC also considers that the animal evidence is insufficient for classification in category 1B.

RAC considers that a classification in category 2 is not appropriate, but based on the poor availability of any relevant and robust data, the information presented in the CLH dossier is considered inconclusive for the assessment of carcinogenicity. **No classification for carcinogenicity is proposed due to inconclusive data.**

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Sexual function and fertility

In order to assess whether silver can cause adverse effects on sexual function and fertility, the DS described studies that focused mainly on:

- silver nanoparticles (several published studies, one of which also tested $AgNO_3$),

- silver acetate (a one generation non-guideline study),
- silver zinc zeolite (OECD TG 416 study, GLP) and
- silver sodium zirconium hydrogenphosphate (OECD TG 416 study, GLP).

These studies are illustrated in the table below and summarised in the following text. There were no studies which assessed human data.

Table: *Summary of animal studies relevant for toxicity on sexual function and fertility*

Study	Substance	Comment	Ref.
Fertility & Sexual Function:			
1. SD Rat 1-gen drinking water study.	AgOAc	Non-GLP. 20/sex, top dose ≈25 mg Ag^+/kg bw/d. ↓ implantations (22%)	Sprando, *et al*., (2016) Doc IIIA 6.8.2-06
2. SD Rat 2-gen study. OECD 416 (2002) GLP	AgNaZrHPO4	Dietary admin. 28/sex, top dose ≈40 mg Ag^+/kg bw/d. Lots of small effects.	Anon., (2002). Doc IIIA 6.8.2-03
3. SD Rat 2-gen study. OECD 416 (2002) GLP	Silver zinc zeolite	Dietary admin. 28/sex, top dose ≈23 mg Ag^+/kg bw/d. Pup mortality, cardiomegaly.	Anon., (2002). Doc IIIA 6.8.2-04. RAC-35.
4. Review article, repro and dev tox of AgNPs.	AgNPs	Limited data indicating noteworthy repro tox in rodents.	Ema *et al*., (2017).
Published studies with nanosilver referred to by Ema, *et al*., (2017)[2]:			
1. SD Rat, oral gavage for 28 days.	AgNPs	Non-GLP/non TG. 20/dose, top dose 500 mg Ag^+/kg bw/d. Tissue persistence.	Lee *et al*. (2013) Doc IIIB 6.8.2-13
2. Wistar Rat (M), oral gavage for 48 days.	AgNPs	Non-GLP/non TG. 8/dose, up to 200 mg Ag^+/kg bw/12hr. ↓ Spermatogenesis.	Miresmaeili *et al*., (2013) IIIB 6.8.2-14
3. Wistar Rat (M), oral gavage for 45 days.	AgNPs	Non-GLP/non TG. 15/dose, top dose 200 mg Ag^+/kg bw/12hr. ↓ Leydig cells.	Baki *et al*., (2014) Doc IIIB 6.8.2-15
4. Wistar Rat (M), oral gavage for 35 days.	AgNPs	Non-GLP/non TG. 10/dose, top dose 30 µg Ag^+/kg bw/d. ↑ BPS.	Mathias *et al*., (2015) Doc IIIB 6.8.2-17
5. Wistar Rat (M), oral gavage for 90 days.	AgNPs	Non-GLP/non TG. 8/dose, top dose 20 µg Ag^+/kg bw/d. Apoptotic germ cells.	Thakur *et al*., (2014) Doc IIIB 6.8.2-18
6. SD Rat oral gavage for 90 days. OECD 408	AgNPs	Non-GLP/+ TG. 6/dose, top dose 200 mg Ag^+/kg bw/d. ↓ sperm viability.	Lafuente *et al*., Doc IIIB, 6.8.2-19
7. Wistar Rat (F), admin. up to 4 wk.	AgNPs	Non-GLP / non TG. Top dose 300 mg Ag^+/kg bw/d. Limited reporting.	Amr El-Nouri *et al*. (2013)
8. SD Rat, oral gavage exposure for 28 days.	AgNPs / $AgNO_3$	Non-GLP / non TG. Top dose 90 mg Ag^+/kg bw/d. Distribution and elimination.	Van der Zande, (2012) Doc IIIB 6.8.2-12
1. Wistar Rat (M), Single tail injection.	AgNPs	Non-GLP/non TG. 24/dose, 5-10 mg Ag^+/kg bw/d. ↑ DNA damage in testicular cells.	*Gromadzka-Ostrowska *et al*., (2012).
2. NZW Rabbit (M), single i.v. injection	AgNPs	Non-GLP/non TG. 8/dose, 0.6 mg AgNPs /kg. Found in testes. ↓ in sperm kinetic traits. No histopath.	*Castellini *et al*. (2014) Limited study.

2 *Ema M, Okuda H, Gamo M, Honda K. A review of reproductive and developmental toxicity of silver nanoparticles in laboratory animals. Reprod Toxicol. 2017 Jan; 67:149-164. doi: 10.1016/j.reprotox.2017.01.005. Epub 2017 Jan 11. PMID: 28088501.*

3. Rat (M), oral gavage exposure for 28 days.	AgNPs	Non-GLP/non TG. 8/dose, 200 mg Ag^+ /kg bw/d. ↓ testosterone in high dose group.	*Rezaei-Zarchi *et al*. (2012). Poor study.
4. Wistar Rat (M), oral gavage for 35 days.	AgNPs	Non-GLP/non TG. 15/dose, 200 mg Ag^+ /kg bw/12hr. ↓ mobility of sperm.	*Amraie *et al*. (2013) Poor study.

* REACH registration dossier

Sprando, *et al*., (2016) Doc IIIA 6.8.2-06

A limited one-generation oral (via drinking water) study on **silver acetate** in rats was performed with doses of 0, 0.4, 4.0 and 40.0 mg/kg bw/d **(0, 0.25, 2.5 and 25 mg estimated released Ag^+/kg bw/d)** in a published study (2016). According to the CLH report it was performed in accordance with the current protocols for testing foods and food additives (FDA CFSAN Redbook, 2000). The 90-day exposure period of parental P0 males covered a 10-week premating period and a mating period, and P0 females were exposed over the premating, mating, gestation and lactation periods. The F1 offspring were terminated on PND26. The study lacked GLP compliance, lacked haematological assessment, and lacked individual animal data and several parameters required in EOGRTS such as investigations on oestrus cycle, sperm parameters and histopathological analyses of reproductive tissues (other than the testis), but the DS considered the study was acceptable and results were considered reliable.

The only reported indications of general toxicity in parental P0 animals included occasionally reduced fluid consumption, reduced stomach weights and pigmentation of organs and tissues. There were no reports of any clinical signs that could indicate neurotoxicity. There was no report that the brain was examined microscopically.

Table: *Condensed fertility and litter parameters*

silver eq. mg Ag^+/kg bw/d	**Females**			
	0	**0.25**	**2.5**	**25**
Mated	20	20	20	20
Plug or sperm positive females	20	20	20	20
Producing litters	20	20	20	**16**
With implantations	20	20	20	18
Total resorption	-	-	-	2
Litters	20	20	20	16
Total litter loss	1	1	1	2
Non-viable pups only	-	-	1	-
Viable litters	19	19	18	14
Implantations	14.4	14.0	14.3	**11.3***
Litter size	13.1	12.4	13.4	**10.3***
Live pups	13.0	12.3	12.8	**10.5[a]**

* statistically significantly different to controls ($p \leq 0.05$); [a]$p \leq 0.1$

The number of litters at 25 mg Ag^+/kg bw/d was lower than in the other groups. Two females mated but were without implantations and two additional females had total resorption of implantations. An increase in early post-natal litter loss (two females) was also seen at the highest dose level compared to the other dose groups (one female in each group), but this is a developmental toxicity parameter.

Sexual function and fertility parameters: there were no effects on the mating index, but fertility index (calculated as "(number of females with implantations/number of sperm positive females) x 100" was 90% (18/20) at the top dose as compared to 100% (20/20) in other groups. In the original article "fertility index 1" and "fertility index 2" were calculated as "(No. prod litter/no prod plugs/sperm-positive) ×100" and "(No. prod litter/no exposed to mating) ×100", respectively. Both "fertility index 1" and "fertility index 2" can be influenced not only by effects on sexual function and fertility but also by total litter losses after implantation which is an effect on development. The 90% fertility index in this case appears to reflect effects on sexual function and fertility. In addition to the reduced fertility index at the top dose, there was a statistically significant reduction in the mean number of implantations at the top dose compared to other groups (11.3 at the top dose compared to 14.4 in control).

Summary of results:

1. No substantial maternal toxicity.
2. Fertility indices lowered by 10%
3. Pigmentation of tissues in treated animals.
4. Number of implantations reduced (-22%) relative to controls in the high dose group.
5. Reduced pup weights not statistically significant with no dose response.

Anon., (2002). Doc IIIA 6.8.2-03

An OECD TG 416 oral (via diet) study on **silver sodium zirconium hydrogenphosphate** applied doses of 0, 72.5/78.2, 363/400 and 1465/1612 mg/kg bw/day (calculated for P0 males and P0 females, respectively, during the premating period) to two generations of rats. These doses contributed approximately **1.9, 9.9 and 40 mg silver ion equivalents estimated to be released in stomach/kg bw/d** in females.

Parental general toxicity in P0 animals consisted of increased relative spleen weight in mid and high dose males, decreased absolute thymus weight in high dose males and pigmentation of pancreas in high and mid dose males and females. There were no treatment-related deaths or effects on bodyweight or food consumption. There was no report of any clinical signs that could be associated with neurotoxicity.

As regards the effects on sexual function and fertility in P0 animals, the absolute weight of seminal vesicles and coagulating gland was statistically significantly lower at the high (2.466 g) and mid (2.480 g) dose as compared to the control (2.881 g). Some semen parameters (lateral amplitude and straightness) had statistically significant changes in the high dose group. There were no effects on semen morphology, spermatid counts in testis or epididymis, on oestrous cycles, mating performance or number of pregnant females.

Of the parental F1 animals four high dose males (numbers 305, 308, 313 and 417) and two high dose females died/were killed *in extremis* (relative to no mortalities in the control animals). Male 308 had full body convulsions prior to death but there were no obvious macroscopic anomalies.

Two females (numbers 398 and 401) were killed *in extremis* during the course of the study. Female 398 was killed due to suspected dystocia. The macroscopic postmortem findings confirmed the presence of offspring *in utero*. Female 401 was killed *in extremis* with clinical signs of ataxia, hunched posture, dehydration and emaciation. At macroscopic examination there was some evidence of gastrointestinal changes. At microscopic examination there was evidence of gastric submucosal oedema.

At mid dose one selected female was killed *in extremis* due to suspected hermaphroditism although no female sex organs could be detected microscopically, the arrangement of sex organs at macroscopic examination did show some anomaly of sexual development.

At low dose and control there were no mortalities.

The bodyweights of male rats at the top dose were lower as compared to controls over the entire period before pairing and the bodyweights of female rats were statistically significantly lower as compared to controls for the first three weeks of the pre-mating period (study weeks 19-21) and during the entire gestation and lactation periods. Food consumption was reduced in males during the last weeks of maturation (study weeks 32-34) and during the first days of gestation and lactation in females (≤ 10%). Pigmentation of pancreas, lymph nodes and thymus were observed in high and mid dose animals.

Regarding effects on sexual function and fertility in F1 animals, at the top dose there was a reduced absolute weight of seminal vesicles/coagulating gland at the top dose (2.368 g vs 2.725 g in control; statistically significant) and right testis (1.747 g vs 1.888 g in control; statistically significant) and of absolute and relative prostate weight (abs/rel 33/25%), as well an increased relative epididymides weight (left/right 9.6/19%).

In high dose females there was a reduced absolute/relative weight of uterus (28/13%) and a longer pre-coital interval as compared to controls. Since this was considered not to affect fertility (the group mean total implantation counts for females were 16, 16.3, 15.9 and 14.7 at 0, 1.9, 9.9 and 40 mg silver ion equivalents/kg bw/d, respectively), it was not given further significance by the DS. The total number of follicles (small, medium and large) was lower in high dose animals (7.7/7.5/5.6 in (ovary 1/ ovary 2/ overall respectively) compared to controls (10.4/10.1/10.2 respectively) was in line with the reduction of the mean primordial follicle counts noted in F1 animals in the 2-generation study with silver zinc zeolite. However, in the absence of statistical significance and no effects on reproductive performance, the significance of these observations was considered unclear by the DS.

Summary of results:

1. Many small effects without any gross pathological changes or effects on fertility.
2. No clear clinical signs of neurotoxicity. No specific neurobehavioral tests performed in any generation.
3. Some semen parameters affected (lateral amplitude and straightness, mean sperm head area).
4. Reduced abs. wt. of seminal vesicles/coagulating gland at the top dose rel. to controls (-13%).
5. Pre-coital interval extended.
6. Number of ovarian follicles reduced.

Anon., (2002). Doc IIIA 6.8.2-04. RAC-35

OECD TG 416 oral (via diet) study on **silver zinc zeolite** (AgION Type AK) applied doses of 0, 72/87, 472/548, 984/1109 mg/kg bw/day (calculated for P0 males and females, respectively, during the premating period) to two generations of rats in accordance with the guideline. This corresponds to doses of approximately **0, 1.5/1.8, 9.8/11.3; and 20.3/22.9 mg silver ion equivalents estimated to be released in stomach /kg bw/d** in males and females respectively (assuming *circa* 2.4% silver).

As regards the general toxicity in parental P0 animals, three males at high dose and one male at mid dose died during the study. Bodyweight and bodyweight gains were lower relative to controls with reductions of ≤10 and 17%, respectively, in top dose males during the premating period, but after mating the bodyweight gain was comparable for all groups.

No deaths occurred among the treated P females. In high dose females the bodyweights were up to 11% lower relative to controls on gestation day 20 and lactation days 7, 14 and 21. Bodyweight gain was reduced during gestation, on days 0-20 by 16% and days 14-20 by 29% (the body weight and body weight gains during gestation day 20 were not corrected for the gravid uterine weight). The overall bodyweight gain during lactation (days 0-26) was not statistically

significantly different from controls. Food consumption was reduced between 12 and 27% in the high dose group during lactation and the changes were statistically significant.

High dose males and females had increased levels of erythrocytes, platelets and decreased levels of haemoglobin (Hb), haematocrit (HCT), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Some of these parameters were also slightly affected in mid dose males and females. These effects were considered to be caused by zinc. There were no clinical signs observed. Pigmentation was observed in several tissues of mid and high dose animals and mild pigmentation of pancreas and thymus was observed also in some females of the low dose group. Histopathological changes in the kidneys (including hydronephrosis) were noted in high and mid dose animals. Kidney weights were decreased in high dose male and females. The thymus was not weighed.

Sexual function and fertility parameters: In P0 animals the only effect on sexual function and fertility was a slightly increased gestation length in treated animals (22.3 in all treated groups compared to 21.9 days in controls) that was statistically significant at mid and high dose. At the top dose the weight of left and right epididymis and testis relative to body weight were statistically significantly increased (about 10 %) as compared to the controls, but there were no effects on the absolute weights of these organs. As the top dose also caused excessive mortality (10%) in males, the top dose level was not considered relevant for assessing effects on sexual function and fertility in males.

In the F1 pups from the P0 parental animals, excessive mortality was seen at the top dose (discussed further in developmental toxicity). In the F1 parental animals, excessive mortality was also seen at the top dose; 28/30 males and 23/30 females died prior to the end of the premating period. The group was therefore terminated prior to mating and there were consequently no data assessed on fertility parameters (such as delayed sexual maturation) or litter/pup data for the high dose group. This is in line with the CLP guidance criteria where adverse effects on fertility and reproductive performance seen only at dose levels causing marked systemic toxicity (e.g., as in this case, lethality) are not relevant for classification purposes. In the mid dose group, mortality rates were 23.3 and 3.3% for F1 males and females, respectively. Body weights of mid dose males were lower than controls at the start of and throughout the premating, pairing and post-pairing periods, but the body weight gain was comparable to controls over the entire premating period. Food consumption was also reduced in mid dose males during the entire study. Bodyweights of mid dose F1 females were statistically significantly lower during the first six weeks of premating and also at one time-point during lactation but there were no statistically significant effects on body weight gains during overall premating, gestation or lactation periods. Histopathological and weight effects as well as pigmentation were reported in different organs, some effects starting at the low dose level.

A dose-related significant delay in the day of vaginal opening and preputial separation was observed in mid- and top-dose treated animals. Large bodyweight reductions relative to controls from birth were accepted to be related to the delays in puberty milestones.

The DS concluded that there were no statistically significant or clear dose-related effects on fertility parameters (see the table below) by silver zinc zeolite tested up to a dose of 9.8/11.3 (M/F) mg silver equivalents/kg bw/day.

***Table**: Mating, fertility and fecundity indices in the F1 generation (mg/kg bw/day)*

Parameter	0	72/87	472/548
Silver Zinc Zeolite			
Ag^+ equivalents (M/F)	0	1.5/1.8	9.8/11.3
Female mating index.	90.0	83.3	93.1
Female fertility index.	83.3	63.3	79.3
Female fecundity index.	92.6	76.0	85.2
Male mating index	90.0	82.6	96.2
Male fertility index	83.3	62.1	84.6
Male fecundity index	92.6	75.0	88.0

The percentage of abnormal sperm was higher in treated animals as compared to controls but with no clear dose response (0.18, 1.41 and 0.50, for controls, low- and mid-dose respectively). The significance of this finding was considered unclear by the DS. Statistically significant effects on absolute weights of prostate, seminal vesicle, testes and uterus/oviducts/cervix as well as the delay in sexual maturation in males and females were considered by the DS secondary to the reduced bodyweight. The mean primordial follicle counts were reduced in treated females (group means of 83.1, 65.3 and 69 in control, low dose and high dose, respectively), but the significance of this observation was unclear to the DS because there were no effects on reproductive performance.

Other potentially fertility-related effects were reported in the chronic/carcinogenicity studies with silver zinc zeolite where a statistically significant increased incidence of ovarian cysts were reported in mice (6/49, 22/49, 19/50 and 16/49 at 0, 0.1, 0.3 and 0.9 mg/kg bw/day, respectively) and a statistically significant trend for increased endometrial polyps in rats (0/49, 2/50, 5/49, 9/50 and 7/49 at 0, 0.01, 0.03, 0.1 and 0.3, respectively).

Summary of results:

1. No maternal mortalities or other severe general maternal toxicity in P0 generation. 10% mortality in P0 males at the top dose.
2. Excessive mortality (93.3/76.7% in m/f, respectively) in F1 animals from the high-dose group resulted in complete termination of this dose group prior to scheduled mating.
3. The onset of sexual maturation landmarks (vaginal opening/preputial separation) were significantly delayed in animals from the mid dose group (days 39.8/47.4) and the top dose group (days 59.9/56.7) compared to controls (days 35.1/44.5). But these effects were related to significant reductions in bodyweight relative to controls from birth.
4. There were no clear clinical signs of neurotoxicity and no description of histopathology of the brain, except for a reduction in absolute brain weight relative to controls in the top dose group (24% (males) and 18% (females) lower than in controls at the top dose). No specific neurobehavioral tests performed in any generation.

Published literature

Several published articles (non-guideline and non GLP studies) on **silver nanoparticles** were included in the CLH report. These had been included either in the REACH registration dossier on silver or had been referred to in the review by Ema *et al.* (2017). The DS highlighted that it was not possible to present and evaluate all available data in the CLH report due to the large amount of data available:

Published articles included in the REACH registration dossier on silver:

1. Gromadzka-Ostrowska *et al*. (2012) examined the acute effects of intravenously administered single bolus dose of silver nanoparticles (20nm and 200nm at 5 and 10mg/kg bw) on spermatogenesis and morphology of seminiferous tubules in rats. Injection of silver nanoparticles increased the level of DNA damage in cells derived from whole testes, as measured by alkaline comet assay. Histological examination of the testes showed also a change in the morphometry of seminiferous tubules in rats treated with large silver particles (200 nm) but not with the smaller 20nm nanoparticles.
2. Castellini *et al*. (2014) evaluated the long-term effects of intravenously administered silver nanoparticles (0.6 mg/kg bw) on reproductive activity and sperm quality in rabbits. Silver nanoparticle-treated rabbits showed minor effects including higher seminal reactive oxygen species (ROS), less motile sperm, and lower curvilinear velocity and oxygen consumption than control animals whereas libido, serum testosterone, sperm concentration, and semen volume were hardly affected bysilver nanoparticles. Transmission electron microscopy analysis did not show any evident morphological damage in testes, but silver nanoparticles were visible in spermatids and ejaculated sperm.
3. Rezaei-Zarchi *et al*. (2012) investigated effects of orally administered silver nanoparticles (25, 50, 100 and 200 mg/kg bw/day for 28 days) on the blood concentrations of LH, FSH and testosterone in male rats. The only effect reported was a reduced testosterone concentration at the top dose (1.8±0.5 nmol/L vs. 2.5±0.6 nmol/L).
4. Amraie *et al*. (2013) studied the effects of 35-day oral exposure to silver nanoparticles at 25, 50, 100, 200 mg/kg on sperm parameters in young rats (4 weeks of age at the start of dosing). The exposure caused a significant reduction in sperm mobility and a change in their morphology.

Published articles referred to in the review of reproductive and developmental toxicity of silver nanoparticles in laboratory animals (Ema et al., 2017):

1. Lee *et al*. (2013) investigated biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats. 100 and 500 mg/kg bw/day silver nanoparticles (10 and 25 nm, purity: 99.98%) were administered once a day via oral gavage for 28 days. The silver concentrations in the testes and brain did not decrease to the control levels, even after the 4-month recovery period, indicating that silver clearance is difficult across biological barriers. Silver concentration clearance order was as follows: blood > liver = kidneys > spleen > ovaries > testes = brain. An increase of cholesterol, alkaline phosphatase, and aspartate aminotransferase (AST) was also reported.
2. Miresmaeili *et al*. (2013) evaluated the role of silver nanoparticles on the acrosomal reaction and spermatogenic cells in rat. Silver nanoparticles (70 nm) were administered once a day via oral gavage for 48 days at concentrations of 25, 50,

100, and 200 "mg/kg concentration". Silver nanoparticles had acute and significant effects on spermatogenesis and the number of spermatogenic cells as well as on the acrosome reaction in sperm cells. Microscopic studies showed a significant reduction in the number of primary spermatocytes (p=0.012), spermatids (p=0.03) and spermatozoa (p=0.03) compared to control group. General toxicity was not reported.

3. Baki *et al*. (2014) investigated the effects of silver nanoparticles on sperm parameters, number of Leydig cells and sex hormones in rats. They administered silver nanoparticles (70 nm) at 25, 50, 100, and 200 mg/kg/day every 12 hours via oral gavage. Significant effects reported included a reduction in sperm progression, increase in spermatozoa with non-progressive motility and immotile spermatozoa, a reduction in the percentage of normal spermatozoa, a reduction in the blood serum testosterone, an increase in blood serum luteinizing hormone (LH) and a reduction in the number of Leydig cells. General toxicity was not reported.
4. Mathias *et al*. (2015) exposed rats via oral gavage to silver nanoparticles (60 nm) suspended in aqueous solution at 0 µg/kg bw, 15 µg/kg bw, 30 µg/kg bw daily for 35 days (post-natal days (PND) 23-58). Post-exposure period lasted for 44 days (PND 58-102). The reported effects included delay in onset of puberty, decreases in sperm reserves in the caput, corpus, and cauda of the epididymis in both treatment groups at PND53 and PND90, and a significant reduction of sperm transit time through the segments of the epididymis at PND53. Sexual partner preference score in the group that received 15 µg/kg silver nanoparticles indicated a preference for the male sex. There were no significant differences in body weight between treated groups and the control. RAC notes this to be a poorly reported study, lacking detail, no tabulated data, and confusion over the actual dosing.
5. Thakur *et al*. (2014) exposed rats via oral gavage to citrate capped silver nanoparticles (5-20nm) via oral gavage at 20 µg/kg bw/day for 90 days. Severely impaired and apoptotic germ cells in the testis were reported. As regards general toxicity, there were no deaths, clinical signs or effects on body weights.
6. Lafuente *et al*. (2016) studied sperm effects of 90-day oral gavage exposure to PVP capped silver nanoparticles (20 – 30 nm suspended in a 0.9% saline solution) at 0, 50, 100 and 200 mg/kg in rats. RAC notes that the study was performed in accordance with OECD TG 408, but without a GLP status. A non-significant decrease in the number of spermatozoa and a decrease in sperm viability was noted at 200 mg/kg. An increased number of epididymal sperm morphological abnormalities were reported at 100 mg/kg but not at 200 mg/kg. No significant changes in tissue morphology, sperm count, or motility were observed. Conclusion: exposure to high doses of PVP-AgNPs induces slight adverse effects in rat sperm parameters.
7. M. Amr El-Nouri *et al*. (2013) investigated ovarian effects in rats after an exposure to 30 and 300 mg/kg silver nanoparticles for 2 and 4 weeks. No numerical data was available in the original article, but the DS summarised the reported effects. Congested blood vessels in stroma with inflammatory mononuclear cell infiltration was reported. The areas of congestion in the stroma with extravasations of blood were stated to be dose and duration dependent. Also, excess depositions of collagen fibres indicating fibrosis, positive reactions for Caspase3 indicating apoptosis and a relative increase in atretic and degenerated follicles were reported. General toxicity was reported in the form of statistically significantly reduced body weight gain.
8. Van der Zande *et al*. (2012) studied distribution, elimination, and toxicity of silver nanoparticles and silver ions in Rats after 28-day oral exposure to 90 mg/kg bw of non-coated silver nanoparticles (<20nm, matrix consisted of 4% polyoxyethylene

glycerol trioleate and 4% Tween 20 in H2O) and PVP-coated silver nanoparticles (<15nm, suspended in water) and to 9 mg/kg bw for the $AgNO_3$. The main target organs for silver nanoparticles and $AgNO_3$ were liver and spleen, followed by the testis, kidney, brain, and lungs, without differences in the distribution pattern between the two different silver nanoparticles or the $AgNO_3$. $AgNO_3$ exposed rats showed a higher uptake of silver in blood and organs. Elimination of silver occurred at an extremely slow rate in brain and testis, with high concentrations of silver remaining two months after the cessation of exposure. It was noted that the fraction of soluble silver was rather similar between the AgNPs < 20 nm and $AgNO_3$ treated animals in blood and in organs with the exception of testis and spleen in which the silver dose was higher for AgNPs (<20 nm).

Factors taken into consideration by the DS when performing the WoE assessment for classification of silver included silver content of the test substance, amount of silver released from the test substance, type of administration, potential influence of other constituents than silver to the toxicity of the test substance, OECD TG and GLP status of the study and relevance of the exposure duration.

The oral studies on silver nanoparticles were published studies and they were not performed in accordance with recognised guidelines or the principles of GLP. Even though the DS acknowledged the difficulties in assessing the robustness and reliability of such studies, the DS considered that the consistency of effects on germ cells (specifically the reduced number of sperm and alterations in sperm morphology) among these studies indicated a potential toxic effect of silver nanoparticles. It was noted however that statistically significant delays in the onset of puberty were also observed (Mathias *et al*., 2015). Furthermore, silver nanoparticles were detected in the testes of rats and in mice in several of the studies, and these levels were comparable or higher than in other tissues unless the size of nanoparticles were larger than approximately 70 nm. Following a four-month recovery period, the levels of silver nanoparticles gradually decreased in all investigated tissues but the testis. Thus, the clearance across the blood-testis barrier was delayed.

The DS also considered the relevance of data on silver nanoparticles for the assessment of bulk silver. The amount of silver ions released from nanoparticles depends on dose, size, surface coating and test conditions, i.e., a myriad of local environmental factors and intrinsic properties influence the bioavailability of silver ions. In the absence of such information in the public literature, the silver ion exposure in such studies with silver nanoparticles is uncertain. At least some of the effects noted in the silver nanoparticle studies could result from oxidative stress leading to apoptosis, and this could be caused by the nanoparticle itself or the local concentration arising from the release of silver ions. Silver ions have been shown to induce oxidative stress in studies performed with silver nitrate and thus it was considered reasonable to assume that it was an intrinsic feature of the silver ion in biological systems. Germ cells were considered highly sensitive to this mechanism, but it was recognised that there could be also other modes of action for the effects observed, and these have not been investigated with any rigor. The DS also considered whether nanoparticles of silver could be distributed to and penetrate germ cells differently from silver ions. With a reference to Van der Zande (2012), the DS concluded that silver from both silver nanoparticles and $AgNO_3$ were detected in testis indicating that the effects assessed under the sexual function and fertility were not specific to nanoparticles but also relevant for effects of ionic silver. Thus, the DS also considered that the reproductive toxicity of silver ions could also be assessed indirectly from the information in studies performed with different silver containing substances releasing silver ions. The DS also indicated an important point, acknowledging that these substances also contain additional elements of possible toxicological significance. The DS considered all silver compounds as potential sources of silver

ions and that the ultimate toxicophore was Ag^+ while not giving preference for data from a particular silver compound over another.

The studies on silver zinc zeolite and silver sodium zirconium hydrogenphosphate were performed in accordance with OECD TG 416 and GLP, and thereby these studies were considered by the DS as the most robust among the available data base. The observed effects on sexual function and fertility parameters in these studies included some changes in semen parameters, pre-coital interval and in the numbers of ovarian follicles and a slight increase of gestation length. The DS considered that these effects were of unknown significance.

The available 1-generation study on silver acetate was not performed according to OECD guidelines and thus important parameters were not analysed. Silver acetate is however chemically less complex compared to silver zinc zeolite and silver sodium zirconium hydrogenphosphate. The reduction of the fertility index (10%, not statistically analysed) and the statistically significant reduction in the number of implantations (22%, 11.3 compared to 14.4 in control) in dams observed in the study were considered to represent clear evidence of an adverse effect on sexual function and fertility.

The DS concluded that the available data on silver acetate and silver nanoparticles indicate that the silver ion can cause adverse effects on sexual function and fertility possibly by a mechanism involving oxidative stress. The effects observed with silver acetate and/or silver nanoparticles (reduced fertility index, number of implantations, spermatogenesis and number of spermatogenic cells and delay in onset of puberty) were considered by the DS to clearly support classification. There was no marked general toxicity indicating that effects were of "a non-specific consequence of other toxic effects", and there was no reason to exclude the human relevance of these effects. The DS concluded that the CLP criteria for classification in category 1B, H360F were fulfilled.

Developmental toxicity

To assess whether silver can cause adverse effects on development, the DS included studies on:

- silver nanoparticles (several published non-guideline studies),
- silver acetate (a one generation non-guideline study and a published non-guideline study with exposure on GD6-19),
- silver zinc zeolite (OECD TG 416 study, GLP),
- silver sodium zirconium hydrogenphosphate also referred to as "Exp.add 9823-37" (OECD TG 416 study, GLP; OECD TG 414 study and its dose range finding study),
- silver copper zeolite (OECD TG 414 with a few deviations) and
- silver chloride (a published non-guideline study with exposure on GD1-20 or GD7-15).

As regards the evidence in humans, the DS referred to a summary prepared by the Agency for Toxic Substances and Disease Registry in which there was only one reference to a potential for human relevance but lacking firm evidence of causality, Robkin *et al*. (1973). All these studies are summarised below.

Sprando, *et al*. (2016), Doc IIIA 6.8.2-06

A one-generation oral (via drinking water) study on **silver acetate in rats with Ag^+ equivalent doses (estimated to be released in stomach) of 0, 0.25, 2.5 and 25.0 mg/kg bw/d** was performed in accordance with the current protocols for testing foods and food additives. Parental toxicity and effects on sexual function and fertility are summarised under the section on sexual function and fertility (see sexual function and fertility section 1.1).

The F1 litters were culled (5/sex where possible) on PND4 and offspring were further selected following weaning on PND21 (1/sex/litter) and remained untreated until termination on PND26. The DS concluded that there were severe developmental effects observed consisting of total

resorptions in 2/18 pregnant dams at the top dose (0/20 in other groups), total litter losses at birth/during lactation in 2/16 dams at the top dose and 2/20 at the mid dose (with 1/20 in other groups including the controls). These led to a reduced number of dams with viable litters on PND 21 at the top dose with a slight reduction also in the middle dose group (95 and 95, 95, 90 and 78% of pregnant dams at 0, 0.25, 2.5 and 25 mg Ag^+/kg bw/day, respectively). See the table under sexual function and fertility section 1.1.

A reduction in pup body weight (females, -12%) and an increase in the numbers of runts was reported in the 2.5 mg Ag^+/kg dose group. Such an effect was not as clear at the top dose group, and the DS considered that the foetal/pup mortality in the top dose group may be masking such effects. The developmental effects were considered not to be secondary non-specific consequences of maternal toxicity.

Price & George (2002), Doc IIIA 6.8.1-07, a published non-guideline, oral (gavage) study

Developmental toxicity of **silver acetate in CD albino rats during days 6-19 of gestation was investigated at doses of 10, 30, or 100 mg/kg/day**.

Maternal toxicity consisted of one mortality at the top dose on day 12 (another dam was excluded due to a misdirected dose). Clinical signs were generally minor. Single incidences of alopecia, red amniotic fluid in uterine horns, or small intestine/stomach full of gas were reported in top dose animals. There were no significant effects on maternal body weight gain, food or water consumption.

The incidence of litters with late foetal deaths was increased in the top dose group (incidences: 0/24, 0/23, 0/25 and 2/20) and this was above the historical control data range (0-4.35% and 0-1 litters). The percentage of late foetal deaths/litter was 1.22% in top dose group compared to 0% in other groups. A negative statistically significant trend on mean foetal bodyweight per litter (sexes combined) and mean male foetal bodyweight/litter was observed but there were no significant pairwise differences. The percentage of litters with skeletal variations and litters with any variation was increased in high dose animals compared to controls, but as there was no dose-response and the difference was not statistically significant, the observation was not given further toxicological significance by the DS.

The DS concluded that there were indications of adverse developmental effects (foetal deaths and a negative trend for foetal bodyweights/litter) that were not considered as secondary non-specific consequences of maternal toxicity.

Anon. (2002), 2-gen oral rat study, Doc IIIA 6.8.2-04. RAC-35

OECD TG 416 oral (via diet) study on **silver zinc zeolite (AgION Type AK) applied doses of approximately 0, 1.5/1.8, 9.8/11.3; and 20.3/22.9 mg silver ion equivalents** estimated to be released in stomach /kg bw/d in males and females respectively (assuming circa 2.4% silver). Parental toxicity and effects on sexual function and fertility are summarised under the section on sexual function and fertility.

The mortality rate in P0 males of the high dose group (10%) and F1 males at the top dose (93.3%) of the mid dose group (23%) was remarkable. However, maternal toxicity was considered acceptable in P0 dams up to the top dose (0% mortality) or in F1 dams up to the mid dose (3.3% mortality). In F1 generation top dose 76.7% of females died before the scheduled necropsy. The F1 offspring of the P top dose group died/was terminated before mating due to excessive toxicity, and thereby only the low and mid dose F2 offspring were available for the assessment of developmental toxicity. None of the clinical signs of maternal intoxication listed in the CLP Regulation or guidance (i.e. excessive mortality, coma, prostration, hyperactivity, loss of righting reflex, ataxia or laboured breathing) were observed among P or F1 dams during the gestation or lactation periods, and the DS concluded that the developmental toxicity could not

be a consequence of maternal neglect.

First indicator of developmental toxicity in F1: Developmental toxicity in the F1 neonates was confirmed in the high dose group as reduced mean number of live and total pups/litter at birth (27% and 15% less than in controls, respectively), reduced live birth index (85.5% vs 99.2% in control), increased number of stillborn pups/litter (1.5 vs 0.1 in control) and increased stillborn index (12.2% vs 0.8% in control). The reduced number of pups born/litter was considered to reflect an increase in post-implantation losses because there was only a slight decrease in the mean total implantation scars/dam.

Second indicator of developmental toxicity in F1: F1 offspring, early postnatal period - Day 0-4 pup survival index was also severely impacted in the high dose group (53.1% compared to 98.9% in controls) and 5/27 females that delivered litters with live pups failed to retain live pups to Day 4. There were no treatment related histopathological findings in the stillborn pups or in day 4 culled pups. Excessive F1 offspring mortality continued after PND 4 (see section 2.3.1 below).

Third indicator of developmental toxicity in F1: clinical signs in F1 pups before weaning included decreased activity in mid and high dose groups and discoloured skin (blue/pale) and difficulty in breathing amongst the high dose animals. The discoloration was mainly observed at day 26 whereas decreased activity and breathing difficulties were observed at day 0 or 4. RAC note: it is unclear whether this represents neurotoxicity or copper deficiency and effects on oxidative metabolism.

Fourth indicator of developmental toxicity in F1: Statistically significantly reduced bodyweights were observed at all measurement timepoints for F1 male and female pups in the top dose groups and at day 14, 21 and 26 in male and female pups in the mid dose group. Differences relative to controls increased as pups got older (top dose group: PND0 82/85%, PND7 76/79%, PND 28 49/54% of control values in males/females, respectively).

Fifth indicator of developmental toxicity in F1: Absolute brain weights were also affected, being 24% (males) and 18% (females) lower than in controls at the top dose. Spleen and thymus weights were also statistically significantly reduced in the top dose group. The changes also remained statistically significant when these organ weights (except for the spleen) were related to bodyweights. Cardiac changes were observed in both sexes of high and mid dose animals; mildly enlarged heart in 6/14 males and 6/18 females at top dose and 5/27 males and 4/26 females at mid dose compared to 0 in controls. Hydronephrosis was observed in adult animals of both generations.

F1 generation – continued sensitivity at the highest dose level:

F1 offspring that survived until premating were more sensitive to the lethal effects of the test substance compared to the P1 adults: in the top dose group, 93.3% (28/30) of males and 76.7% (23/30) of females died before mating. In the P0 generation 3/30 males and 0/30 females died. Considering that also the pre- and post-natal pup mortality rates were high in the top dose of the F1 generation, the DS assumed that the high mortality rates of both sexes at the top dose of the F1 generation after weaning also resulted from developmental toxicity. The cause of death of the F1 generation that died during the premating period was not clearly established.

F2 generation – developmental effects:

In the F2 generation developmental effects consisted of an increase in the percentage of females delivering litters with stillborn pups (5.4 vs 1.1% in controls) at the mid dose. This was also reflected in an increased stillborn index and decreased live birth index (93.1 vs 98.3% in ctrl). The number of live pups/litter was decreased in the low dose group at day 4, 14 and 21 due to the complete litter loss in two dams, but there was no effect at the mid dose. Pup body weights at birth were lower at mid dose as compared to controls and this difference increased further

throughout the pre-weaning period. Organ weight analysis showed reduced absolute/relative thymus and brain weights in males and females from the mid dose group. The macroscopic examinations of F2 pups at day 21 (weaning) revealed mildly to moderately decreased size of thymus, again mild cardiac enlargement (27/81 in males and 16/90 in females) was observed along with other minor effects.

Mechanistic explanation for the observed foetal toxicity:

An explanation for the foetal toxicity of silver ions was proposed where they may displace copper ions in ceruloplasmin which transports copper to the foetus. Ceruloplasmin is the main copper transporter in the blood and it seems to play a role in the cellular uptake of iron. The concentration is usually elevated during pregnancy and ceruloplasmin and copper are present in the amniotic fluid and in milk. Analysis of copper, silver and zinc in homogenates of three whole male and three female pups from control, low and mid-dose groups showed a general decrease of copper in the treated groups whereas the levels of silver and zinc were generally increased, which suggest that effects observed in pups are associated with a potential deficiency of copper, iron or both.

***Table**: Zinc, silver and copper levels (mg/kg bw) of F2 Day 4 culled pups*

	control		Low-dose group		Mid-dose group	
	males	females	males	females	males	females
Silver	<1	<1	1.04	1.06	1.68	2.2
	<1	<1	1.06	<1	1.1	<1
	<1	<1	<1	<1	1.07	1.84
Zinc	7.77	10	8.87	8.05	8.65	10.4
	6.44	6.31	11.8	6.88	7.32	7.56
	8.01	7.62	5.57	5.63	8.8/5	11.9
Copper	2.24	2.18	1.97	1.67	<1.5	1.86
	2.07	2.49	2.19	1.61	<1.5	<1.5
	2.15	2.72	1.61	1.76	1.96	1.52

According to Shavlovski *et al*., (1995, see section 2.6 below), the deaths of embryos and neonate rats could be a consequence of copper deficiency caused by silver inhibiting copper from binding to the transport protein ceruloplasmin (CP) as well as inhibiting copper-containing enzymes such as super oxide dismutase and reducing the ferroxidase activity ($Fe^{2+} \rightarrow Fe^{3+}$, assists in the binding of iron to transferrin) of the CP protein itself. Other effects noted by the authors included a dramatic increase in both the incidences of hydronephrosis (31%) and cryptorchidism (35%) compared to controls (5.3 and 1.3% respectively). An increase in hydronephrosis was also seen with silver zinc zeolite. The DS noted similar effects by several other silver releasing substances (silver chloride, silver acetate and to some extent by silver sodium zirconium hydrogen phosphate) and silver nanoparticles.

Anon. (2002), Doc IIIA 6.8.2-03

An OECD TG 416 oral (via diet) study on **silver sodium zirconium hydrogenphosphate applied doses of 0, 72.5/78.2, 363/400 and 1465/1612 mg/kg bw/day** (calculated for P0 males and P0 females, respectively, during the premating period) to two generations of rats. These doses contributed approximately **1.9, 9.9 and 40 mg silver ion equivalents estimated to be released in stomach/kg bw/d** in females. Maternal toxicity was uneventful according to the DS: there was no excessive mortality, coma, prostration, hyperactivity, loss of righting reflex, ataxia or laboured breathing in dams.

In F1 offspring there were no effects on litter parameters (litter size or viability). The mean litter weights and the mean pup weights were reduced by 8 and 9%, respectively, at the end of

lactation period (PND21). There were no effects on landmarks of development (pinna unfolding, tooth eruption or eye opening) or on reflexological responses (surface righting reflex, mid-air righting reflex, startle reflex, pupillary reflex). There were small effects on organ weight and the pathological examination showed some pigmentation in pancreas and the mesenteric lymph nodes in high and mid dose males and females.

After weaning four high dose males and two high dose females died whereas all control animals survived. The DS considered the increased mortality rates at the top dose of the F1 generation as compared to the P generation (0% mortality in both sexes) may have been a consequence of developmental exposure.

In the F2 generation the number born and the litter size at day 1 were statistically significantly reduced in high dose females compared to controls (13.4 vs 15.1 and 12.9 vs 14.8, respectively). The DS considered this as a developmental effect, but it is to be noted also that the mean implantation sites were lower at top dose as compared to controls (14.7 vs 16). The mean litter weights were reduced by 13% at day 1 of lactation and the mean pup weights were reduced by 13% at the end of lactation (day21). There were no effects on landmarks of development or on reflexological responses.

Other studies briefly mentioned

Preliminary study to OECD TG 414 study on silver sodium zirconium hydrogenphosphate applied doses of 0, 100, 300, 1000 mg/kg bw/day on GD6-15 to 8 Sprague Dawley female rats per group. No maternal or developmental effects were observed according to the CLH report, but no numerical data were provided. Absorption and bioavailability of the test substance was not investigated.

OECD TG 414 oral (gavage) study on silver sodium zirconium hydrogenphosphate applied doses of 0, 100, 300, 1000 mg/kg bw/day on GD6-15 to 25 Sprague Dawley female rats per group. No maternal or developmental effects were observed according to the CLH report, but no numerical data were provided. It was stated that "Further investigations to establish absorption and bioavailability of the test substance were not made".

OECD TG 414 study on silver copper zeolite applied doses of 200, 700, 2000 mg/kg bw/day on GD6-15 to 30 Sprague Dawley female rats per group. At the top dose one dam died, the body weight and body weight gain were 13% and 25% lower than in controls, and clinical signs consisted of sedation, void faeces, urogenital discharge and thinness. According to the CLH report no foetal effects were observed, but no numerical data was provided.

The OECD TG 414 studies on silver sodium zirconium hydrogenphosphate and on silver copper zeolite and the preliminary study on silver sodium zirconium hydrogenphosphate gave very limited information. No clear developmental effects were observed in these studies.

Shavlovski *et al*. (1995), Doc IIIA 6.8.1-03 Mechanistic Study:

A published, non-guideline, oral (via diet) study on silver chloride supplied 50mg of silver chloride per animal per day (estimated at 188 mg Ag/kg bw/d) to groups of inbred, pregnant albino female rats from GD1-20, (with scheduled sacrifice on GD20). Several other test groups were also incorporated into the study to investigate the role of copper homeostasis during embryogenesis. Silver chloride-treated rats received intraperitoneal injections of human ceruloplasmin (CP) at 48-hour intervals, covering two gestational time periods; one silver-dosed group with supplemented CP was treated from GD2-14 to cover the effects of silver chloride toxicity in the presence of functional CP during organogenesis; the second supplemented group was treated with functional CP from GD8-21. Further investigations were performed to study the excessive excretion of copper and iron from the dams by

specific chelators, namely penicillamine (for Cu) and bipyridyl (for Fe), as the animals were continuously dosed with AgCl.

Maternal toxicity was not in evidence in this study despite the fact that enzymatically active copper-containing ceruloplasmin (CP) was eliminated from the blood plasma. However, severe developmental toxicity was expressed through abnormalities of embryos, their prenatal death (increased post-implantation loss) and 100% mortality of neonates within the first 24 h of life.

Silver chloride treatment was established to be severely embryotoxic. In dams (n=20) that were exposed to silver chloride between gestation days 1-20, the incidence of post-implantation deaths (36%) was increased compared to control (9.6%, n=36) and historical controls (8.7%, n=237) and all new-born animals from the silver chloride treated group died within 24 hours. Moreover, the incidences of hydronephrosis (31%) and cryptorchidism (35%) increased substantially compared to controls (5.3 and 1.3% for hydronephrosis and cryptorchidism respectively) and historical controls (1.2 and 0.8% respectively).

Silver chloride causes a sharp decrease of the copper concentration in serum due to the inhibition of the enzymatically active form of CP and competition with Cu binding. Copper content in the placenta and embryonic tissues dropped to near zero. Active copper-containing CP supplementation between gestation day 2 and 14 caused partial normalization of embryonic development. Active CP supplementation via ip administration was found to ameliorate the developmental toxicity of silver chloride. The incidence of foetuses with hydronephrosis and cryptorchism declined sharply as did embryolethality. Postnatal survival also improved greatly and concurrent treatment with CP and daily dosing with silver chloride, especially between gestational days 8-21 saw mortality decline to within control levels. CP treatment was shown to increase the amount of copper in placenta and embryonic tissues.

The treatment regimens with the chelators penicillamine (for Cu) and bipyridyl (for Fe) were used in conjunction with daily dietary exposure to silver chloride. Both penicillamine and bipyridyl (introduced in subembryotoxic amounts) over the course of GD1-20 potentiated the toxicity of silver chloride, causing a substantial increase in post-implantation loss and reduction in live embryos (relative to silver chloride treatment alone). This was especially so for penicillamine. Additionally, the copper chelator penicillamine increased the frequency of hydronephrosis and cryptorchism.

The deaths of embryos and neonates were explained as a consequence of copper deficiency induced by silver inhibiting copper from binding to the transport protein ceruloplasmin. This theory was supported by the increased survival (and reduced frequency of teratogenic effects) in silver chloride-treated rats who received injections of human ceruloplasmin as well as by the lack of copper in placenta, embryos and blood serum of adult rats treated only with AgCl.

<u>Published articles (non-guideline and non GLP studies) on silver nanoparticles and silver salts</u>

Several papers from the published scientific literature that had been included either in the REACH registration dossier on silver or had been referred to in the review by Ema *et al.,* (2017) were addressed in the CLH report.

Published articles included in the REACH registration dossier on silver:

1. Austin *et al*. (2012) applied intravenous application of silver nanoparticles (50 nm) to pregnant CD-1 mice on gestation days 7, 8, and 9 at 0, 35, or 66 µg Ag/mouse. Silver nanoparticles distributed to most maternal organs, extra-embryonic tissues, and embryos, but did not accumulate significantly in embryos.
2. Mahabady *et al*. (2012) evaluated effects of silver nanoparticles on the skeletal system of

rat foetuses and on the placenta by dosing the test substance intraperitoneally to pregnant rats at 0.4 and 0.8 mg/kg at the 8th and 9th day of gestation, respectively. Foetuses were collected at GD20. The mean weight of foetuses from dams that received silver nanoparticles (0.4 and 0.8 mg/kg) at GD8 and weight and length (0.8 mg/kg) at GD9 were significantly decreased compared to control. The weight, volume and width of placentas in treated animals were lower than in the control group. No macroscopic anomalies were seen in any group.

3. Babu *et al*. (2016) studied effects of maternal silver acetate exposure on immune biomarkers of the offspring. They exposed male and female rats (26-day old) to 0.0, 0.4, 4 or 40 mg/kg body weight silver acetate in drinking water for 10 weeks prior to and during mating. Sperm positive females remained within their dose groups and were exposed to silver acetate during gestation and lactation. Maternal exposure to silver acetate was concluded to have a significant impact on rat splenic development during the early lactation period.
4. Hang *et al.* (2013) studied the effect of silver nanoparticle suspensions on rabbit reproduction. Sixteen rabbit does (10 months of age) were located in individual cages and allocated to a treatment with or without 1% silver nanoparticle suspension added to their drinking water. No adverse effects were reported following treatment.
5. Taylor *et al*. (2014) investigated the *in vitro* toxicity of gold and silver nanoparticles on mammalian preimplantation development by injecting nanoparticles into one blastomere of murine 2 cell-embryos, while the sister blastomere served as an internal control. After treatment, embryos were cultured and embryo development up to the blastocyst stage was assessed. The results did not indicate any detrimental effect of colloidal gold or silver nanoparticles on the development of murine embryos. An exposure to comparable silver ion concentrations resulted in an immediate arrest of embryo development.
6. Gao *et al*. (2015) exposed 26-day-old male and female rats to 0.0, 0.4, 4 or 40 mg/kg body weight of silver acetate in drinking water for 10 weeks prior to and during mating. Sperm-positive females remained within their dose groups and were exposed to silver acetate during gestation and lactation. At postnatal day 26, the effect of silver ions on the developing F1 generation rat thymus was evaluated at the transcriptional level using whole-genome microarrays. The study did not reveal an adverse effect on the developing thymus.
7. Ghaderi *et al*. (2015) treated subcutaneously, NMRI mice once every three days from gestation day 3 until delivery, by 0, 0.2 and 2 mg/kg of bodyweight of silver nanoparticles. Spatial memory, passive avoidance learning, stress, anxiety-like behaviours and locomotor activities were assessed in adult offspring. Prenatal exposure to silver nanoparticles significantly impaired the cognitive behaviour in the Morris water maze. Also, the number of defecations and leanings in the open field assay and number of passages in the light-dark box were greater in groups prenatally exposed to silver nanoparticles.

Published articles referred to in the review of reproductive and developmental toxicity of silver nanoparticles in laboratory animals (Ema et al., 2017):

1. Philbrook *et al*. (2011) applied a single oral (via gavage) dose of 20nm silver nanoparticles at 10, 100, or 1000 mg/kg on gestation day 9 to pregnant CD-1 mice in a 0.5% tragacanth gum solution in distilled water. Time resolved dynamic light scattering (TRDLS) indicated the AgNP size was 24-47nm in water and 144-260nm in tragacanth Gum. There were no obvious signs of maternal toxicity, including behavioural changes or weight loss throughout the 10 days post-exposure at any dose. The dams were euthanised on GD 19

and the uterus was examined. The number of litters examined were 18, 15, 12 and 13 for controls, low, mid and high dose groups respectively. The only reported effect was an increase in the percent of non-viable foetuses (3.3, **9.6**, 5.5 and 6.1% at control, low, mid and top dose, respectively). Only the low dose incidence was statistically significant ($p \leq 0.05$). The lack of effects at higher doses was postulated to be due to agglomeration of nanoparticles after administration resulting in reduced toxicity and clearance by the animals. There was no maternal toxicity. The authors cited a paper by Skebo *et al*., 2007 where cells *in vitro* only take up particles ≤ 130 nm. According to their own analysis about 50% of the silver nanoparticles in the study were visualized as individual 100–200 nm particles even prior to administration.

2. Lee *et al*. (2012) applied 250 mg/kg bw/d citrate-capped silver nanoparticles (7.9 ± 0.95nm) via oral (gavage) to rats (Sprague-Dawley). The study was not a guideline one and was not GLP compliant. It was a short communication publication that used 4 rats in controls and a single dose group of 4 rats. 1 pup from each dam was assessed for silver in liver, brain, kidney and lungs only. The exposure period in males was 14 days before and during mating and in females 14 days before and during mating, during gestation, and 4 days after parturition. The main finding reported was the observation of silver nanoparticles in 4 neonates (PND4) in livers, kidneys, brain and lungs. The authors "suggested a possible transfer of AgNPs from pregnant dams to offspring mainly through the placenta or minor pathway of milk".

3. Melnik *et al*. (2013) applied a single oral (gavage) dose of radio-labelled silver nanoparticles (34.9 ± 14.8 nm) at 1.69 mg/kg bw to 3 pregnant females (GD20), 2.21 mg/kg bw to 4 pregnant females (GD20) and 2.11 mg/kg to 9 lactating females (PND 10-11). This study was considered to provide evidence for the transfer of silver nanoparticles from a mother to offspring through the placenta and breast milk, the presence of silver nanoparticles in milk was inferred from the measured radioactivity in excess of the background. The average nanoparticle level in foetuses was 0.085–0.147% of the administered dose, and total inflow of nanoparticles into the milk was about 2% of the administered dose over a 48-hour period of which 25% was absorbed in the digestive tract of infant rats.

4. Charehsaz *et al*. (2016) applied citrate-capped silver nanoparticles (55nm) orally (gavage) to pregnant Sprague-Dawley rats daily on gestation day 7-20 with 0, 0.2, 2, 20 mg/kg bw/day or 20 mg Ag/kg bw/day as $AgNO_3$. The day after parturition, dams and pups were sacrificed and silver levels assessed in several maternal and pup organs. Also, hepatotoxicity and oxidative stress parameters and histopathology were evaluated. Prenatal exposure to silver in both ionic and nanoparticle forms increased the levels of silver in offspring tissues. Both silver nanoparticles and $AgNO_3$ induced oxidative stress in dams and pups, $AgNO_3$ being more potent. Oxidative stress in offspring brain tissue (indicated by an increase of glutathione peroxidase) was statistically significant for $AgNO_3$, statistically non-significant but dose-dependent with silver nanoparticles. In brains from exposed dams a high incidence of mild to moderate hippocampal pyramidal neuronal loss and mild gliosis were revealed. This was observed in 4/5, 3/5, 4/5 and 4/5 of the low, middle, and high silver nanoparticle dose groups and the $AgNO_3$ dams, respectively. Neuronal loss and mild gliosis (hippocampal sclerosis) was further classified according to the new International League against Epilepsy (ILAE) consensus (ILAE type 1: classical hippocampal sclerosis, ILAE type 2: CA1 sector sclerosis, ILAE type 3: CA4 sector sclerosis. Type 1 refers always to severe neuronal cell loss and gliosis predominantly in CA1 and CA4 sectors, compared to CA1 predominant neuronal cell loss and gliosis (type 2), CA4 predominant neuronal cell loss and gliosis (type 3). In this study, mild to moderate neuronal cell loss and gliosis event in animals exposed to Ag in nanoparticulate or ionic

forms was observed predominantly in CA1 sector, which can be categorized as a type 2 hippocampal sclerosis according to the ILAE classification. Normal morphology of pyramidal neurons in all regions of the hippocampus was observed in dams from the control group. Histopathological examination of brain, heart, liver, kidney and lung tissues of the offspring did not reveal changes related to treatment.

5. Van der Zande (2012) is summarised under the section on sexual function and fertility. There was no developmental toxicity.
6. Mathias *et al*. (2015) is summarised under the section on sexual function and fertility. There was no developmental toxicity.
7. Kovvuru *et al*. (2015) tested silver nanoparticles coated with 0.2% PVP at a dose of 500 mg/kg bw/day in pregnant mice treated orally (gavage) from 9.5 to 13.5 days post coitum. There was no developmental toxicity as the mutagenic effects in the offspring are addressed under germ cell mutagenicity hazard class. The embryonic effects consisted of DNA deletions affecting 70 kb repeats in the p^{un} locus (pink-eyed unstable allele), following deletion of one of the repeats, the p^{un} allele is reverted to the functional *p* gene, allowing black pigment accumulation in the cells of the retinal pigment epithelium (RPE) of the eye of developing p^{un}/p^{un} mice, these were assessed and visualised following sacrifice at PND20. Genome alterations were associated with DNA damage, increased double strand breaks and downregulation of DNA repair genes.
8. Yu *et al*. (2013) tested silver nanoparticles (7.5 ± 2.5 nm, suspended in 0.5% carboxymethylcellulose aqueous solution) via oral (gavage) dosing of Sprague-Dawley rats on GD6-20 at 0, 100, 300 and 1000 mg/kg bw/day in accordance with a modified OECD TG 414 (non-GLP) study. Oxidative stress in hepatic tissues was reported in dams at 100 mg/kg bw/day. No developmental toxicity was reported (pre-implantation losses were 2.4, 14.5, 3.8 and 25.5% at control, low, mid and high dose respectively, but note the relevance of these effects for sexual function and fertility; exposure started on GD6, after implantation).
9. Fatemi *et al*. (2013) tested the effects of prenatal exposure in dams with silver nanoparticles (20 ± 4 nm, sodium citrate buffer) on the developing brain in neonatal Wistar rats at 25 mg/kg bw/d from GD9 until parturition via intragastric administration. The offspring were sacrificed the day after birth. The effects in offspring consisted of an increase of silver and the number of microvacuolar structures in brain (612 vs 159 in controls), reduced antioxidant activity and increased peroxidation, statistically significant decrease in body weight on PND0 and of a statistically significant decrease in the ratio of brain/body weight. No significant differences in maternal weight gain were reported. Microvacuolar structures in brain may have indicated developmental neurotoxicity but researchers would need a recovery group to assess if this was a permanent effect along with FOB and memory assessment.

Evidence in humans

The DS referred to a summary prepared by the Agency for Toxic Substances and Disease Registry according to which it was not known whether silver causes developmental toxicity in humans. There were no such studies available other than Robkin *et al*. (1973). According to this publication the concentration of silver in the foetal liver of 12 anencephalic human foetuses was higher (0.75±0.15 mg/kg) than the values from 12 foetuses obtained either through therapeutic abortions (0.23±0.05 mg/kg), or in 14 spontaneously aborted foetuses (0.21±0.05 mg/kg). The concentration in 9 premature infants was 0.68±0.22 mg/kg. The authors could not determine if the higher concentration of silver in anencephalic foetuses was associated with the malformation or with foetal age.

Conclusions

Within the overall data there were no robust studies investigating the developmental toxicity of bulk silver.

The DS considered that the toxicity of silver ions that are potentially released from bulk silver can be indirectly investigated in a valid manner with other silver compounds and silver nanoparticles. The DS concluded that based on the available data, developmental toxic effects of silver were rarely observed if exposure was limited to the short period of organogenesis (i.e. gestation days 7-15). The DS acknowledges that the silver content of test substances and the degree of release from such test substances impact greatly on the severity of effects observed.

The DS considered that the one and two-generation studies with continuous exposure during pre-mating, gestation and lactation periods were the most appropriate to assess the developmental toxicity of silver. The forms of silver in these studies included silver acetate, silver zinc zeolite and silver sodium zirconium hydrogenphosphate.

The DS speculated that the reason for observing less obvious effects in the two-generation study performed with silver sodium hydrogen zirconium phosphate as compared to that with silver zinc zeolite and the one-generation study on silver acetate (despite a similar or even higher exposure to silver ions in the study on silver sodium hydrogen zirconium phosphate) could be due to the difference in administration routes (in drinking water and diet respectively) or due to normal variation between studies. In the case of silver zinc zeolite, the DS considered that zinc could possibly also replace copper in ceruloplasmin and that could hypothetically contribute to the observed effects if this was the mechanism or one of the mechanisms causing the developmental effects as proposed by Shavloski (1995). However, silver zinc zeolite was not the only silver-containing and releasing substance that caused developmental effects and therefore zinc was not expected to be the critical contributing factor to the observed developmental effects.

The DS concluded clear evidence of developmental toxicity from a variety of effects:

1. Increases in mortality rates of foetuses/pups by exposure to: silver chloride (Doc IIIA, 6.8.1-03, Shavlovski, *1995*), silver zinc zeolite (Doc IIIA 6.8.2-04, *Anon., 2002*), silver acetate (Doc IIIA 6.8.2-06, Sprando *et al., 2016*) and nanosilver (Doc IIIB 6.8.2-07, Philbrook *et al.,* 2011; RAC comment – reliable result? No dose response, evidence for no effect by micron sized silver particles) and to a weak extent by silver sodium zirconium hydrogen phosphate (Doc IIIA 6.8.2-03, Anon., 2002).
2. Increases in mortality rates of adolescents and young adults (possibly a consequence of developmental exposure) by silver zinc zeolite.
3. Significant brain damage by silver nanoparticles in offspring exposed during the developmental period including in utero exposure via mothers.
4. Increase in relative brain weight by silver zinc zeolite in the offspring (decrease in absolute brain weight by 24% (males) and 18% (females) as compared to controls at the top dose in F1).
5. Enlargement of heart/increase in heart weight in offspring by silver zinc zeolite.
6. Increase in chryptorchidism in offspring with silver chloride, (Doc IIIA, 6.8.1-03, Shavlovski, 1995).
7. Increase in hydronephrosis in offspring by silver zinc zeolite and silver chloride.
8. Increased number of runts and/or reduced pup bodyweight by silver chloride, silver acetate, silver nanoparticles, silver zinc zeolite and by silver sodium zirconium hydrogenphosphate.

The adverse effects on development were considered not to be secondary non-specific consequences of maternal toxicity. The DS considered it plausible that the mechanism for silver-induced developmental toxicity involved perturbations to ceruloplasmin ferroxidase activity and copper-binding potential resulting in reduced availability of copper, iron or perhaps both metals to the foetus. As ceruloplasmin has the same function in humans, this potential mechanism is also considered relevant for humans. The DS concluded that classification as Repr. 1B, H360D was warranted for silver.

Lactation

The DS assessed effects via lactation from:

1. The Lee *et al*., (2012): a short communication in which citrate-capped silver nanoparticles were dosed via oral gavage to dams 14 days before mating, during mating and gestation at 250 mg/kg bw/day. Pups (n=4) were subsequently necropsied on PND4 and the silver content in the offspring was investigated and confirmed in four organs only. (Note: RAC comment – transfer to milk was not analysed).
2. Melnik *et al*., (2013): a non-guideline study with radio-labelled silver nanoparticles in which lactating female rats were dosed once at 2.11 mg/kg bw via oral gavage and the transfer to offspring via milk was observed as radioactivity above background levels.
3. Charehsaz *et al*., (2016): a non-guideline study on citrate-capped silver nanoparticles in which pregnant female rats were dosed orally once daily from Day 7 to Day 20 of gestation with 0, 0.2, 2, 20 mg/kg silver nanoparticles or 20 mg Ag/kg as $AgNO_3$ and the subsequent effects and silver content in offspring tissues was investigated and confirmed. Silver content was also shown to be present in milk at levels greater than in controls (not statistically significant).
4. Mathias *et al*., (2015): a non-guideline study on silver nanoparticles in which male rats were dosed via oral gavage at 0 – 15 - or 30 µg/kg daily on PND23-53 and certain reproductive parameters were subsequently investigated.

According to the DS, Melnik *et al*. (2013) showed that nanoparticles releasing silver ions were detected in breast milk and in offspring following oral administration of ^{110}Ag-labeled nanoparticles to dams, although it is to be noted that according to the study remarks the presence of silver nanoparticles in milk was not directly investigated. In Charehsaz *et al.* (2016), silver was detected in the milk of suckling pups. The delay in onset of puberty in Mathias *et al*. (2015) was observed in pups exposed only via food after the lactational period and thereby the study was not investigating potential effects on or via lactation.

The DS concluded that the published studies indicate that nanoparticles of silver can be transferred in milk (and across the placenta) to offspring. Silver was also shown to accumulate in foetal and adult tissues and was associated with adverse effects. However, there were no studies clearly demonstrating that adverse effects in pups resulted from lactational exposure rather than exposure *in utero* and/or via food post weaning as the exposure was not limited to the lactation period in any of the studies. Therefore, the criteria for effects on or via lactation were considered not fulfilled by the DS.

The DS did not propose classification for effects on or via lactation.

Comments received during consultation

Sexual function and fertility

There were 44 comments from Industry/trade assoc./manufacturers, these were of a generic nature concluding in most cases that there was no evidence for reproductive toxicity. They did not support classification. In addition, 4 MSCA also commented: insufficient data with no evidence for classification (1 MSCA); one MSCA supported Repr. 2; H361f and two MSCA supported Repr. 1: H360F.

Development

There were 44 comments from industry/trade assoc./manufacturers, these were of a generic nature concluding in most cases there was no evidence for reproductive toxicity. They did not support classification. In addition, 3 MSCA also commented: insufficient data with no evidence for classification (1 MSCA); and two MSCA supported Repr. 1: H360D.

Lactation

There were no specific comments on lactation by industry. There were no comments by MSCAs.

New studies submitted in 2022 in support of the Silver CLH dossier can be found in the Additional Key elements section, see Background document (BD).

Assessment and comparison with the classification criteria

Sexual function and fertility

The CLH report is extensive in its description of effects of the silver ion on sexual function and fertility parameters arising from a limited database of available guideline and GLP compliant studies. Classification in category 1B, H360F was proposed based on these studies and on a variety of peer reviewed published papers investigating the effects of silver nanoparticles. However, no comparable *in vivo* toxicity data exist in the specific case of bulk elemental silver (e.g., micron-size or larger powders, or massive forms).

A newly available Extended One Generation Reproductive Toxicity Study (EOGRTS) in rats exposed to silver acetate (AgOAc) along with its preliminary dose range-finding study was also made available to RAC in early 2022. Both studies show a range of effects consistent with the ability of the silver ion to affect sexual function and fertility parameters.

RAC accepts that the fundamental assumption in this study is that the silver ion is responsible for all toxic effects. With due consideration of other studies that use non-metallic forms of silver, RAC agrees that the primary toxicophore is the Ag^+ ion, though there may be other contributions from other ions such as Zn^{2+} or localized effects due to surface stabilization treatments on silver nanoparticles. The approach taken in the EOGRTS study above (read-across from the soluble salt - silver acetate), actually represents the worst-case scenario with respect to the greatest bioavailability of Ag^+ ions. However, the bioavailability of Ag^+ ions from a soluble silver salt is not representative of the bioavailability of Ag^+ ions from silver metal. Silver metal, either in massive form or nanoparticles, is the subject of the classification proposal and the extent to which the effects seen with silver acetate are due to a greater Ag^+ ion release and are therefore representative of toxicity of silver metal is unknown.

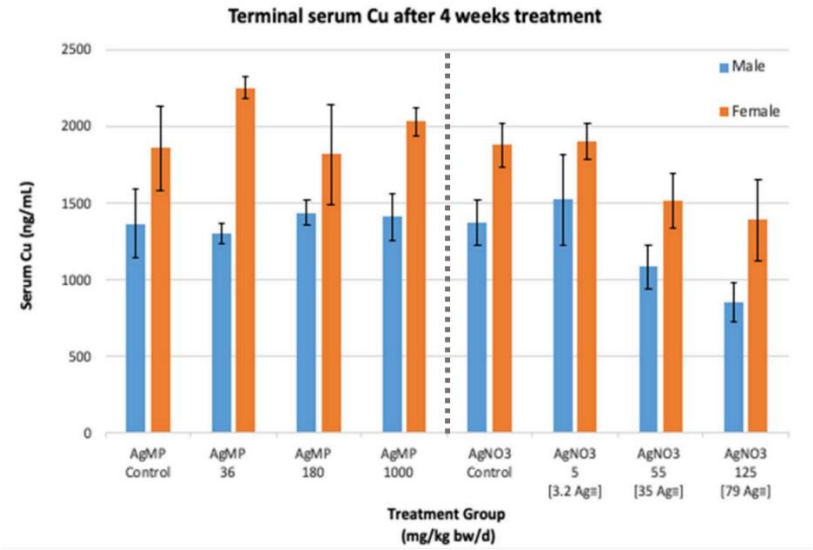
<u>Read across</u>

The ability of different forms of silver or silver compounds, be they metals, salts or more complex matrices, to release silver ions into an organism once ingested or otherwise introduced internally, differs fundamentally from one form and compound to another. The implication here being the

bioavailability of silver ions is a consequence of more fundamental properties (i.e., solubility, surface ion release, surface to volume ratio), of the silver-based material used in the studies investigating the reproductive toxicity of silver. Simple read-across amongst these different compounds is not ideal (though there are circumstances where some forms give supporting information), because the physicochemical characteristics are not comparable. This recognises inherent fundamental differences between silver metal in bulk form compared with a soluble salt such as AgOAc. The silver ion is common to both, but the ability to release silver ion in sufficient amounts to affect an organism is an inherent characteristic of each material and can differ significantly such that it is questionable if treatment with silver acetate for example can be representative of treatment with silver metal in either massive or nanoparticle forms. Nanoparticles themselves are not a homogeneous group, surface characteristics and the size distribution profile can differ substantially depending on the preparation being tested and the environmental conditions to which the material is subjected. In some cases, the smaller sized nanoparticles can behave in a similar manner to the soluble silver salts, i.e. bioavailability can be within the same order of magnitude, but it is unclear if that is because small nanoparticles release silver ions more readily or because the preparation of nanoparticles, the particular product being tested, contains a higher level of silver ion compared to others due to differences in manufacturing processes. There is no comparative data at this time to answer such a question.

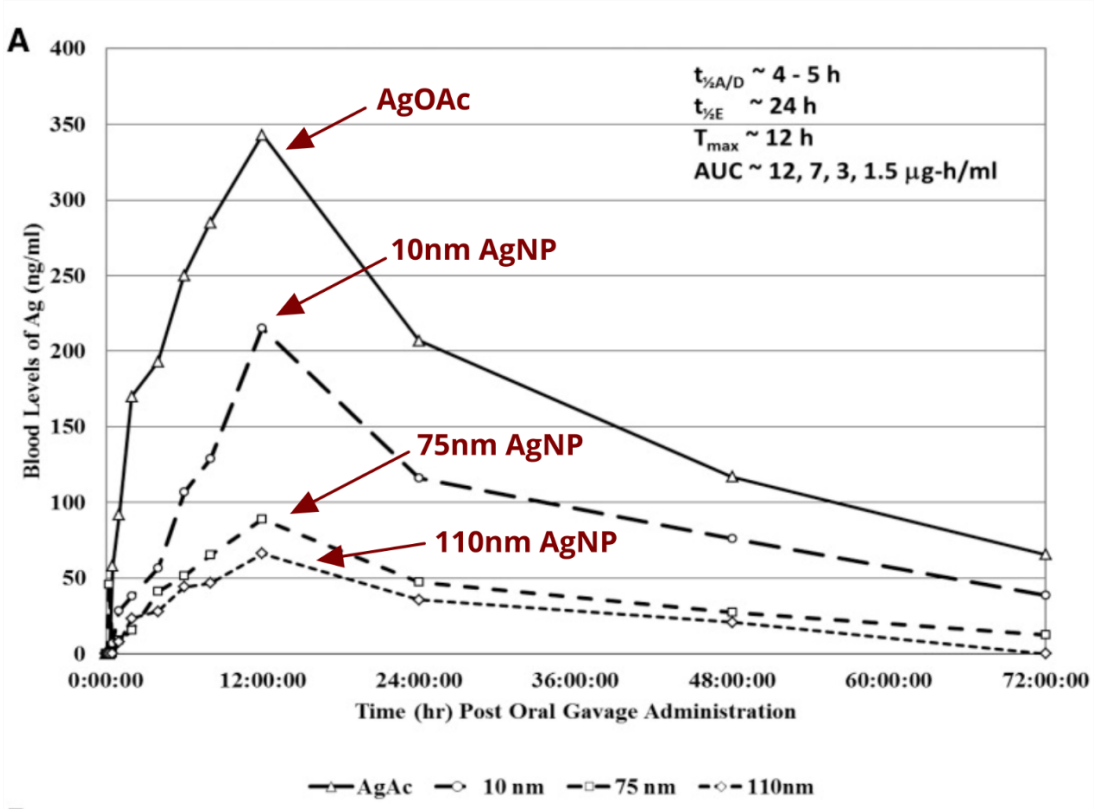
The recent comparative toxicokinetics (TK) study by Anon. *et al*. (2021) - an *in vivo* 28-day oral route investigation using adult CD Sprague Dawley rats, used two ionic silver salts (silver acetate (AgOAc) and silver nitrate ($AgNO_3$); a well-characterised silver nanoparticle (AgNP) reference material (surfactant stabilized Ag; d50 = 15 nm); and a sub-micron sized powder-form (AgMP; uncoated; d50 = ~300nm) of bulk elemental silver. Results indicate considerably lower systemic and tissue-level exposures to bioavailable Ag were evident for AgMP versus either ionic or nanosilver test materials. Massive silver forms can liberate substantially less Ag^+ than more bioavailable ionic or (some?) silver nanoparticles, and according to the prevailing mechanistic evidence, this is pertinent to their respective potentials for interference with Cu transporters *in vivo*, including ceruloplasmin and ultimately the induction of copper deficiency states that can impact the normal function of an organism. The Cu status of rats treated with a sub-micron silver metal powder (AgMP) was also investigated in the TK study. The findings show that even at a limit dose (1000 mg Ag/kg bw/d), AgMP did not cause Cu-deficiency in rats following 28 days treatment via the oral route, in contrast with the $AgNO_3$ treatment, where circulating Cu levels were already depressed (≈20% less than controls) from 35 mg Ag equivalents/kg bw/d (treatment with 55 mg $AgNO_3$/kg bw/d). The AgMP dose equates to an Ag equivalent dose level which was 12 times higher than that employed in the form of $AgNO_3$. The ability of soluble silver salts to promote a potential copper depletion state by lowering blood levels of copper is also substantiated in the recent Extended One-Generation Reproductive Toxicity Study (EOGRTS, Anon., 2022) and its preliminary dose range-finding study (Anon., 2021) where AgOAc at levels greater than 120 mg/kg bw/d (≈ 76 mg Ag equivalents/kg bw/d) significantly and steeply depresses copper levels in the blood and result in a high degree of systemic toxicity to parental animals resulting in death, particularly at doses ≥ 160 mg/kg bw/d (≈ 102 mg Ag equivalents/kg bw/d). In contrast, the ability of nanoparticles of silver to depress blood copper levels has not been well researched even though plenty of publications have routinely tested silver nanoparticles across a range of doses.

Figure: *Taken from the 2021 TK study data. Differences in serum Cu status of rats orally exposed to massive silver versus ionic Ag^+*



Dose level (mg/kg bw/d)	Serum Cu conc % of Control mean	
	Male	Female
AgMP 36	95	121
AgMP 180	105	98
AgMP 1000	103	109
$AgNO_3$ 5	111	101
$AgNO_3$ 55	79	81
$AgNO_3$ 125	62	74

Figure: *Courtesy of Boudreau et al., 2016. Blood concentration versus time curves for silver. Female rats administered single dose AgNP (10, 75, 110 nm) or AgOAc at 10 mg/kg bw by oral gavage.*



TK Conclusion:

Ag^+ ions are formed, released and found in blood from all forms of silver and silver salts. Only silver salts seem to achieve sufficient suppression of blood copper levels when used at low dose concentrations. Very high doses of massive silver have little impact on blood copper levels. Smaller sized silver nanoparticles can release more Ag^+ ions than larger ones. The comparative

effects of surface chemistry were not investigated and could impact these results. The level of free Ag^+ ion already in the nanoparticle preparation prior to dosing is not known.

The main question here is whether it is correct to assume that data generated from soluble silver species such as AgOAc and $AgNO_3$ are representative for elemental silver in bulk or nanoparticles. The results of the TK study (and those also published by Boudreau *et al*., 2016) clearly illustrate fundamental differences in the release of Ag^+ ions into a biological system. The results of any study investigating the reproductive potential of silver must consider these points.

Based on the physico-chemical dissimilarity (i.e. size, shape and surface coating, as well as rates of silver ion release) of the different silver compounds and differences in their plasma kinetics, distribution pattern and potential effects on serum copper concentrations it is considered justified by RAC that read-across from silver acetate to elemental silver for the assessment of reproductive toxicity is **not** supported.

Key studies relied upon in the CLH Report

The data on silver zinc zeolite and silver sodium zirconium hydrogen phosphate are considered the most robust but these substances also contain additional elements of possible toxicological significance and the amount of silver ions tested are limited by the actual silver content and potential for release. Silver acetate and nanosilver are chemically less complex but studies are not performed according to guidelines in the case of the silver nanoparticles and the 2016 one-generation study. The DS relied upon two key guideline- and GLP-compliant studies in its assessment of sexual function and fertility. A published 1-generation study with AgOAc was also available and provided supporting data. Newer studies with AgOAc were not available when drafting the CLH report. RAC considers the new studies with AgOAc to be well performed and acceptable, the data is reliable but read-across from the soluble species to metallic silver is not considered to be representative of the potential toxicity of metallic silver. The data reported for each of the respective substances clearly show concerns for sexual function and fertility with the following effects:

1. Reduced fertility index (-10% vs controls) and implantations (-22% vs controls) with AgOAc at a dose of 25 mg Ag equivalents/kg bw/d (Sprando, *et al*., (2016)).
2. A statistically significant decrease in cauda epididymis and testicular weight ($p<0.01$) and at all dose levels testicular and cauda epididymal total spermatid and sperm counts (millions) were low when compared with concurrent Control ($p<0.01$) (Anon., 2022, new EOGRTS study with AgOAc).
3. A consistency of effects on germ cells (specifically the reduced number of sperm and alterations in sperm morphology) among numerous oral studies on silver nanoparticles from the public literature.

RAC does not accept the read-across from the key guideline- and GLP-compliant studies noted by the DS and therefore does not support classification of silver with category 1B, H360F.

For nanoparticles there are no fully guideline-compliant studies but there are a few which were well conducted with protocols similar to guideline compliant studies. The 90-day study with PVP-coated silver nanoparticles by Lafuente *et al.* (2016) reported some indications of changes in sperm parameters and testicular histopathology (not statistically significant) at 200 mg/kg bw/d. The OECD TG 422 screening study with citrate-capped silver nanoparticles by Hong *et al.* (2014) reported no toxicity on sexual function a fertility up to 250 mg/kg bw/d; unfortunately, the top dose was a NOAEL.

RAC acknowledges that many non-standard publications with silver nanoparticles (which were not included in the CLH report) indicate various effects on the male reproductive system. This

raises a question whether the publicly available reports with silver nanoparticles sufficient to consider a category 2 classification for sexual function and fertility for silver metal. Many publications investigating silver nanoparticles appear to report on effects in the germline, typically involving alterations to normal morphology, gamete production and transport and effects on accessory sex glands (prostate, seminal vesicles). This presents a challenge due to a variety of issues; it is difficult to assess the reliability and relevance of the results due to insufficient reporting, they are not conducted in accordance with guidelines and there is a lack of data on silver ion exposure. The majority of effects from the silver nanoparticle studies describe an impact on sperm motility and numbers with some evidence for changes in morphology of the germ cells. The ability of silver nanoparticle-treated animals to reproduce was not tested but such evidence is not needed in order to classify other relevant effects for sexual function and fertility. RAC agreed that the published studies with silver nanoparticles could support classification with category 2; H361f, and further noted that a comprehensive literature search and a careful analysis of each study could help resolve some of the discrepancies seen amongst the different reports. However, a substantive review is beyond the RAC mandate and cannot be done at this point in time. It is important to note that from the point of view of effects on sexual function and fertility, spermatogenic parameters should be evaluated in conjunction with any available reproductive organ weight, histopathology and fertility data to best assess reproductive effects that may support classification. However, this information is not always available from the public literature. RAC agrees that many publications investigating silver nanoparticles do appear to consistently report effects on the germline. While none of the individual reports present sufficient robust evidence alone, and major limitations include the absence of data on silver ion exposure. Studies were not performed according to guidelines or the principles of GLP and hence used fewer animals and dose levels than required in guidelines were used in most of the studies. Nevertheless, the collective available literature does indicate silver nanoparticles have potential adverse effects on sexual function and fertility. RAC concludes there is some evidence for effects on testes and germ cells and concludes that classification with category 2, H361f for silver metal is warranted.

Comparison with the CLP criteria

Substances are classified in category 1A based on evidence from humans. Such data is not available for elemental silver thus the criteria for this category are not fulfilled.

Substances are classified in category 1B based on data from animal studies and according to CLP criteria such data shall provide clear evidence of an adverse effect on sexual function and fertility. The data available on silver acetate and nanosilver indicate that the silver ion can cause adverse effects on sexual function and fertility possibly by a mechanism involving oxidative stress and perturbations of Cu homeostasis. However, a classification of 1B is dependent on having sufficiently robust data to support the proposal. RAC does not support the proposed read-across from the silver compounds, the release of silver ions from such compounds is assumed to be too disproportionate (either too much or unknown or complicated with additional elements), relative to that arising from silver itself. The TK data support this view.

The only data remaining is that generated from nanoparticles and in a few cases micro-sized silver particles. A great deal of uncertainty remains regarding the overall robustness of data for adverse effects on fertility. Some studies were highlighted by the DS in the CLH report:

1. the studies referred to were published between 2012-2017,
2. the majority were *in vivo* studies and doses were applied via the oral route,
3. many of the studies were derived from a single reference source (Ema *et al.,* 2017)
4. quality of data and depth of reporting are unknown or lacking.

5. a variety of consistent effects are noted with respect to sperm parameters and testicular effects.

The overall data is not particularly strong, significant uncertainties remain in the absence of data for bulk forms or micron-sized silver particles. The study by Lafuente *et al*. (2016) studied sperm effects of 90-day oral gavage exposure to PVP-capped silver nanoparticles (20 – 30 nm suspended in a 0.9% saline solution) at 0, 50, 100 and 200 mg/kg in rats. RAC notes that the study was performed in accordance with OECD TG 408, but without any indication of GLP status. The overall conclusion was that exposure to high doses of PVP- capped silver nanoparticles induces slight adverse effects in rat sperm parameters. The study by Gromadzka-Ostrowska *et al*. (2012) examined the acute effects of an intravenously administered single bolus dose of silver nanoparticles and micron-sized silver particles. Injection with micron-sized particles was associated with a change in the morphometry of seminiferous tubules but this was not replicated with 20nm silver nanoparticles. Thakur *et al.* (2014) reported severe testicular atrophy at 20 µg/kg - this corresponds to a greater than several 1000-fold difference in potency compared to ionic silver and to other studies with nanoparticles. Such inconsistencies are noted by RAC and lead to a lack of robust data and insufficient evidence to conclude a classification with category 1B.

The published studies referred to in the CLH report indicate concern for adverse effects following exposure to silver nanoparticles. In common with nanoparticles of other substances (e.g., Au, TiO_2, ZnO, FeO, SiO_2), AgNPs have also been shown to cross the blood testes barrier and following accumulation in the testis, induce damage to the male reproductive system by affecting the seminiferous tubules and spermatogenesis. The majority of effects from the silver nanoparticle studies may be due to a combination of particulate AND substance specific effects and frequently describe an impact on sperm motility and numbers with some evidence for changes in morphology of the germ cells but with little investigations/data to show a reduction in the ability of treated animals to reproduce. However, according to Annex I: 3.7.1.3 of CLP, adverse effects on gamete production and transport constitute adverse effects on sexual function and fertility and may therefore be considered for classification of sexual function and fertility. There was very little evidence presented for effects in female animals (a decreasing trend was seen in total ovarian follicles and ovarian cysts for some silver compounds). The published literature also constitutes an investigation into possible modes of action and contribute to a better understanding of elements in a proposed adverse outcome pathway but are also short of determining the actual impact on fertility. The public literature as a whole can support classification of silver due to data on silver nanoparticles but because of limitations in the various studies such as lack of adherence to guidelines, GLP, report detail, uncertainties in results etc. a case for category 1 classification of silver is not sufficiently robust. RAC is of the opinion that the published studies with silver nanoparticles can support classification with category 2; H361f. On the basis of data from published studies, **RAC proposes classification for adverse effects on sexual function and fertility with category 2, H361f for silver.**

Development

<u>The overall data available for developmental toxicity</u>

There is no robust information specifically addressing the developmental toxicity of silver metal in different physical forms. RAC is of the opinion that clear developmental toxicity has been observed with silver salts such as silver chloride, silver acetate, silver zinc zeolite (e.g., foetal/pup mortality) and to some extent with silver sodium zirconium hydrogen phosphate. One plausible mechanism for these instances of developmental toxicity involves silver interfering with copper binding to ceruloplasmin and thereby reducing the availability of copper, iron or perhaps both metals to the foetus (supported by the copper analysis of F2 pups in the silver zinc zeolite study

and copper and ceruloplasmin analysis in both the EOGRTS dose range-finder study (Anon., 2021) and the main EOGRTS study (Anon., 2022)).

Foetal/pup mortality was also indicated only at the low dose in a study performed with silver nanoparticles (Philbrook *et al*., 2011). However due to the limited dosing period (a single dose on GD9) and the lack of surface coating possibly preventing higher exposures to silver nanoparticles and/or silver ions, the results from this study are not comparable. The availability of silver ions is presumed to be highly variable from all these substances and this makes comparisons amongst the different silver forms and compounds difficult. The support for classification may be warranted for these silver compounds in their own right but to consider them suitable for the read-across for silver is overly conservative.

Developmental effects included:

1. increases in mortality rates of foetuses/pups: AgCl (Shavlovski, 1995), silver zinc zeolite (Doc IIIA 6.8.2 (04)), AgOAc (Sprando, *et al*., 2016; Anon. *et al*., 2022 EOGRTS) and AgNPs (Philbrook *et al.,* 2011, low dose only) and to some extent by silver sodium zirconium hydrogen phosphate. RAC noted a dramatically reduced post-implantation survival index (79% of controls), and live birth index (38% of controls) in the 160 mg AgOAc/kg bw/d dose group and above (Anon., 2021, Dose range-finding study for the main EOGRT study).
2. increases in mortality rates of adolescences and young adults, AgOAc EOGRTS (2022) study and Silver Zinc Zeolite, 2-generation study.
3. Neurodevelopmental toxicity in the AgOAc EOGRTS study (intramyelinic oedema in the thalamus, caudate putamen and/or the corpus callosum and dose related neuronal necrosis in the hippocampus and neuronal/glial cell necrosis in the thalamus in the DNT cohort 2A and cohort 1A were observed. A diminution of the size of the hippocampus was observed in the morphometric measurement along with deficits in motor function (rearing, ambulation, abnormal gait, movement and activity) and other neurobehavioral parameters such as mean auditory startle peak amplitude, latency to peak values and habituation of acoustic startle response in the DNT cohort 2A. The absolute brain weight was also reduced by 8 and 12% in males and females, respectively, in offspring sacrificed on PND 22).
4. Neurodevelopmental toxicity in the published literature studies on silver nanoparticles: Wu *et al*. (2015) prenatally dosed offspring via mothers every two days from GD10 to GD18 ip to uncoated silver nanoparticles and showed histopathological changes with hippocampal neuronal cell loss along with impaired spatial learning and memory ability tested in MWM test in rat offspring at postnatal day 35. In Ghaderi *et al*. (2015) NMRI mice had been treated subcutaneously once every three days from gestation day 3 until delivery, by 0, 0.2 and 2 mg/kg of bodyweight of silver nanoparticles. Spatial memory, passive avoidance learning, stress, anxiety-like behaviours and locomotor activities were assessed in adult offspring. Prenatal exposure to silver nanoparticles significantly impaired the cognitive behaviour in the Morris water maze. Also, the number of defecations and leanings in the open field assay and number of passages in the light-dark box were greater in groups prenatally exposed to silver nanoparticles. Fatemi *et al*. (2013) tested the effects of prenatal exposure via pregnant dams with silver nanoparticles (20 ± 4 nm, sodium citrate buffer) on the developing brain in neonatal Wistar rats at 25 mg/kg bw/d from GD9 until parturition via intragastric administration. The offspring were sacrificed the day after birth. The effects in offspring consisted of an increase of silver and the number of microvacuolar structures in brain (612 vs 159 in controls), reduced antioxidant activity and increased peroxidation, statistically significant decrease in bw on PND0 and of a statistically significant decrease in the ratio of brain/body weight. In Yin *et al.* (2015), an intranasal

application of citrate stabilised silver nanoparticles to neonatal SD rats for 14 weeks showed that silver nanoparticles caused cerebellar ataxia like symptom in these rats, evidenced by dysfunction of motor coordination and impairment of locomotor activity. Observation of cerebellum sections revealed destruction of the cerebellum granular layer. There was also a human case study Robkin *et al*. (1973), according to which the concentration of silver in 12 anencephalic human foetuses was higher (0.75±0.15 mg/kg) than the values from 12 foetuses obtained either through therapeutic abortions (0.23±0.05 mg/kg), or in 14 spontaneously aborted foetuses (0.21±0.05 mg/kg). The concentration in 9 premature infants was 0.68±0.22 mg/kg. The authors could not however determine if the malformation was associated with the higher concentration of silver in anencephalic foetuses or with foetal age.

In the EOGRTS (Anonymous, 2022), treatment with AgOAc at dose levels of 80 or 120 mg/kg/day was associated with a dose related neuronal necrosis in the hippocampus, and neuronal/glial cell necrosis in the thalamus of F1 animals. Note: No adverse CNS histopathology findings were reported for the EOGRTS P0 generation. Intramyelinic oedema in the thalamus, caudate putamen and/or the corpus callosum was also observed in males at 80 or 120 mg/kg/day and in females at 120 mg/k/day. A diminution of the size of the hippocampus was observed in the morphometric measurement along with deficits in motor function (rearing, ambulation, abnormal gait, movement and activity) and other neurobehavioral parameters such as mean auditory startle peak amplitude, latency to peak values and habituation of acoustic startle response in the DNT cohort 2A. The absolute brain weight was also reduced by 8 and 12% in males and females, respectively, in offspring sacrificed on PND 22. Reactivity investigations showed some treatment related changes relative to controls (reduced approach response, failed pupil closure, high landing foot splay, reduced limb strength). Altogether these results show clear evidence for neurodevelopmental toxicity by silver acetate. This is supported by findings from several other published studies with silver nanoparticles showing impairment of cognitive functions and behavioural disturbances caused by developmental exposure (including exposure in utero only); see point 4 above. Also, adult exposure resulted in neurotoxicity and this is covered under STOT RE. RAC concludes that the read-across from the EOGRTS on silver acetate is not justified and that the available published studies on silver nanoparticles (Wu *et al*., 2015; Ghaderi *et al.,* 2015 and Yin *et al*., 2015) are not sufficiently reliable to support a classification of developmental toxicity for silver in massive and powder form. There remains a lack of comparable data between the different forms of silver and its compounds with robust data only available for individual compounds such as silver acetate. The toxicological significance of the increased number of microvacuolar structures in offspring brain that had been exposed prenatally to silver nanoparticles (Fatemi *et al*., 2013) was considered unclear and it was not possible to conclude whether the increased number of human anencephalic foetuses was associated with the increased silver concentrations in these foetuses or with foetal age. Therefore, RAC concluded that the evidence for DNT was not sufficient for classification for developmental toxicity.

<u>Comparison with the CLP criteria</u>

Substances are classified in category 1A based on evidence from humans. Such data is not available for elemental silver thus the criteria for this category are not fulfilled.

Substances are classified in category 1B based largely on data from animal studies and according to CLP criteria such data shall provide clear evidence of an adverse effect on development in the absence of other toxic effects or if occurring with other toxic effects the effects are considered not secondary non-specific consequences of other effects

Teratology studies performed according to technical guideline and GLP-compliance are not available for silver. The data in support of developmental toxicity comes mainly from studies investigating AgCl, silver zinc zeolite, AgOAc and silver nanoparticles. From a developmental

point of view there were unfortunately very few reports available that used silver nanoparticles (Wu *et al*. (2015), Ghaderi *et al*. (2015), Fatemi *et al*. (2013), Yin *et al*. (2015), Robkin *et al*. (1973)). The lack of robust developmental studies with silver nanoparticles to compare with the results from the EOGRTS study and AgOAc is a serious limitation and precludes classification with category 1B for developmental effects on the basis of insufficient data.

In the context of developmental toxicity there are clear indications for classification of AgOAc but without more robust data where AgNPs could be tested for developmental toxicity no meaningful comparison can be made.

The clear developmental effects observed by AgOAc, including lowered pup survival, high lethality in mature offspring of F1 generation as compared to P0 and histopathological changes to several areas of the brain in F1, including the hippocampus and other brain structures are not at this moment in time considered representative of silver metal via read-across. In the absence of well conducted, guideline and GLP-compliant silver nanoparticle investigations into developmental effects, RAC considers there is insufficient data for an assessment of silver developmental toxicity and therefore **does not propose classification for development for silver**.

Effects on or via lactation

Published studies indicate that nanoparticles of silver can be transferred in milk to the foetus. However, RAC considers that there is inconclusive evidence on adverse effects on development and therefore also on adverse effects via lactation. The dose range-finding study (Anon., 2021) clearly shows an increase in the silver content of tissues from neonates (PND4) and this is corroborated with similar data from the main EOGRTS study (Anon., *2022*).

In conclusion, data on nanosilver and silver nitrate demonstrate that silver is transferred in milk and accumulates in foetal (and adult) tissues. However, the existing data is not considered to provide sufficient evidence of adverse effects on the quality of milk or that lactational transfer results in adverse effects in pups.

RAC does not propose classification for effects on or via lactation.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

There is currently no entry in Annex VI for silver metal (Ag).

Summary of the Dossier Submitter's proposal

The Dossier Submitter (DS) proposed a single environmental classification for both silver massive (particle size ≥ 1 mm) and silver powder (particle size < 1 mm, accepting that silver powder is representative of silver massive):

- Aquatic Acute 1, M-factor = 10
- Aquatic Chronic 1, M-factor = 10

The classification is based on the solubility (transformation/dissolution) of silver powder and silver ERVs:

- acute toxicity - LC_{50} of 0.22 µg/L (Bianchini, 2002) for *Daphnia magna*
- chronic toxicity - E_rC_{10} of 0.1 µg/L for (Schlich *et al*., 2017) *Pseudokirchneriella subcapitata.*

The Dossier Submitter (DS) proposed to classify silver nanoparticles with a separate entry:

- Aquatic Acute 1, M-factor = 1000
- Aquatic Chronic 1, M-factor = 100

The classification is based on the conclusion that Ag nano particles behaved as a soluble silver salt and by using the same ERV values compared directly with the CLP criteria (CLP guidance table IV.1).

Silver exists on the market both as massive silver, silver powder (with a diameter < 1 mm) and silver nanoparticles. All forms have been tested in Transformation/Dissolution protocol (T/Dp) studies presented in the REACH registration dossier for silver. The DS evaluation of these studies is presented in the chapter regarding environmental fate in the present opinion.

Transformation to non-bioavailable forms

Silver is a chemical element and by definition not degradable. The DS presented a summary of information on environmental transformation of silver (see below under solubility of silver) and noted that results from the available T/Dp tests demonstrate an increase in dissolved silver concentrations with test duration. The DS concluded that these data clearly showed no evidence of rapid environmental transformation of silver from soluble to insoluble forms and concluded that soluble silver is not transformed in a way that would rapidly and permanently remove it from the water column.

Bioaccumulation

Bioconcentration: silver may be released into the water column or sediment pore water and taken up by organisms through ion transport channels.

The DS noted that standard bioconcentration tests such as OECD TG 305 are not always applicable for metals or other inorganics compounds. However, results presented in the published literature showed that silver uptake is species-specific and controlled by specific physiological mechanisms.

The DS concluded based on: (1) A review prepared by eCA Sweden under the Review Programme of the Biocide Directive 98/8/EC, Draft May 2012 and (2) A position paper submitted by the applicant under the Review Programme of the Biocide Directive 98/8/EC in 2011, Biomagnification of silver in aquatic environments (European Silver Task Force via TSGE): that it is not possible to draw a general conclusion based on the available information regarding bioaccumulation of silver. The conclusion for bioaccumulation was thus "inconclusive".

Aquatic toxicity

The Dossier Submitter has performed an extensive screening of available data concerning silver toxicity in the aquatic and marine environment in collaboration with Stockholm University, the Swedish Agency for Marine and Water Management and the Swedish Environmental Protection Agency. The data was mainly collected from a report published by RIVM on environmental risk limits for silver (Moermond and Herweijnen, 2012) and the REACH registration dossiers for silver and silver nitrate. Studies with reliability scores of 3 and 4 (Klimisch *et al*., 1997) were removed from the initial data set. Study results for total silver, unknown silver concentration or free ionic silver were also disregarded.

Freshwater species are more sensitive to silver than marine species. However, for completeness, the most conservative results for aquatic organisms in the marine environment are presented in separate tables.

Acute aquatic toxicity

***Table**: Summary of relevant information on acute aquatic hazard.*

Method, Guideline, GLP status, Reliability	**Species**	**Endpoint**	**Exposure**		**Results**	**Remarks**	**Reference**
			Design	**Dura-tion**	**LC/EC_{50}**		
ASTM E729-80; published peer-reviewed research Reliability. 2	*Oncorhynchus mykiss* Steelhead trout	Mortality	$AgNO_3$ Flow-through	96h	9.2 (3.8) µg/L#	Measured <u>total</u> silver concentration (Values in parentheses are estimates based on <u>dissolved</u> <0.45µm silver)#	IIIA 7.4.1.1-01 Nebeker (1983), published
	Oncorhynchus mykiss Rainbow trout	Mortality	$AgNO_3$ Static	96h	4 tests: 8.5 – 72.9 (3.5-29.9) µg/L#		
		Mortality	$AgNO_3$ Flow-through	96h	2 tests: 8.6, 9.7 (3.5, 4.0) µg/L#		
	Pimephales promelas Fathead minnow	Mortality	$AgNO_3$ Static	96h	2 tests: 9.4, 9.7 (3.9, 4.0) µg/L#		
		Mortality	$AgNO_3$ Flow-through	96h	2 tests: 5.6, 7.4 (2.3, 3.0) µg/L#		
No guideline, published peer-reviewed research Reliability. 3	*Pimephales promelas* Fathead minnow	Mortality	$AgNO_3$ Flow-through and static	96h	2.3 µg/L*	Estimated dissolved silver (< 0.45 µm) concentration	IIIA 7.4.1.1-02 Erickson (1998), published
No guideline, published peer-reviewed research Reliability. 2	*Daphnia magna*	Mortality	$AgNO_3$ Static renewal	48h	**0.22 µg/L**	Mean measured dissolved silver (0.45 µm/L.	IIIA 7.4.1.2-03, Bianchini *et al*. (2002)
ASTM E729-80; published peer-reviewed research Reliability. 2	*Daphnia magna*	Mortality	$AgNO_3$ Static	48h	0.6 µg Ag/L (0.25 µg/L)	Mean <u>total</u> silver concentration (Values in parentheses are eCA estimates based on dissolved < 0.45µm silver)#	IIIA 7.4.1.2-01, Nebeker (1983)
No guideline, published	*Daphnia magna*	Mortality	$AgNO_3$ Static	48h	0.58 µg Ag/L	Measured dissolved silver	IIIA 7.4.1.2-02,

Method, Guideline, GLP status, Reliability	Species	Endpoint	Exposure		Results	Remarks	Reference
			Design	Dura-tion	LC/EC_{50}		
peer-reviewed research Reliability. 3					(0.52 µg Ag/L)**	(< 0.45 µm) concentration	Erickson (1998)
OECD TG 201 (1984) Reliability. 1	*Pseudokirchneriella subcapitata (Selenastrum capricornutum)*	Growth rate	AgCl on titanium dioxide (Ag 15%) Static	72h	4.0 µg/L	Mean measured total silver concentration	IIIA 7.4.1.3-02 Manson, P.S. (2002)
OECD TG 201 Reliability: 2	*Pseudokirchneriella subcapitata*	$AgNO_3$ Growth rate	static	72h	0.96 µg/L (dissolved)	Based on mean measured conventional dissolved Ag concentrations (0.45 µm PES filter)	Reach registration dossier for silver: 008 key. Schlich *et al*. (2017)

Dissolved silver (<0.45µm) has been determined at least once. The article states that 59% of the silver was lost after well water was filtered. Therefore, in order to calculate the amount of dissolved silver in the test solution the value for total silver has been multiplied by 0.41.

* The lowest 96h LC_{50} value is approximately 3 µg Ag/L (at pH 7.17). The mean dissolved silver concentration was 78% of measured total silver concentration resulting in a corresponding 96h LC_{50} value of 2.34 µg dissolved Ag/L.

** Dissolved silver was reported to be 89 % of total silver based on a laboratory study

Acute toxicity to fish

Nebeker (1983)

The acute toxicity of silver nitrate to fathead minnow, steelhead and rainbow trout was studied in both flow-through and static systems and the results were based on the measured total silver concentrations in the test media. Dissolved silver concentration (<0.45 µm) has been determined at least once in the mentioned study. The article states that 59% of the silver was lost after well water was filtered. Using this information, an LC_{50} of 0.0023 mg/L was calculated for fathead minnow, and an LC_{50} of 0.0035 mg/L can be calculated for steelhead /rainbow trout.

Erickson (1998)

The effect of water hardness, pH, alkalinity, and organic carbon as well as addition of sodium sulphate and sodium chloride on the toxicity of silver was investigated in a series of studies conducted with fathead minnows. The effects of ageing the test media and use of natural versus laboratory test media were also studied. The acute toxicity of silver to juvenile fathead minnows was substantially reduced by increasing hardness and dissolved organic carbon. Toxicity was also inversely related with pH and alkalinity when these were jointly altered by the addition of a strong base or acid. The toxicity of silver was lower in natural river water (106 µg total Ag/L) compared to laboratory water (10.4 µg total Ag/L). The lowest result was obtained from the unfed, flow-through study and was a 96-hour LC_{50} of 2.3 µg dissolved Ag/L. The decrease in LC_{50} for aged test solutions was unexpected according to the authors of the study, since ageing was supposed to result in formation of complexes with lower bioavailability. The authors do not further discuss this finding. Comparative results for silver toxicity in flow through systems versus static systems with fresh test solution and aged solutions most probably indicate that complexed silver may,

indeed, be bioavailable, and/or ionic and complexed silver have different toxicologically relevant targets within the organism. The reliability score of this study is 3 but was included as supportive information.

Acute (short-term) toxicity to aquatic invertebrates

Bianchini et al. (2002)

Silver toxicity to *D. magna* was studied in the presence of sulphide (as ZnS). LC_{50} for sulphide-free exposure of **0.22 µg/L** is reduced in the presence of sulphide, but only when results are based on total measured silver concentrations. When measured, filtered silver was considered, the toxicity curves were virtually identical, indicating that daphnids took up silver by ingestion of particles and that the dissolved fraction was the source of available silver.

Results for silver toxicity in the same range were obtained in two studies: with *Ceriodaphnia dubia* giving an EC_{50} of 0.1 µg Ag/L and with *D. magna* giving an EC_{50} of 0.23 µg Ag/L. The studies were not considered as reliable as the study by Bianchini (2002) but was be used as supportive data.

Nebeker (1983)

The acute toxicity of silver to *D. magna was studied* with the addition of food (one test) and without food (three tests). Toxicity results are based on measured total silver concentrations, dissolved silver concentration (<0.45 µm) has been determined at least once in the mentioned study. The addition of food decreased toxicity based on measured total silver. Considering the determined LC_{50} of 12.5 µg/L and 89 % of silver lost when food was added, the estimated LC_{50} based on dissolved fraction is 1.4 µg Ag/L. Similarly, considering that 59 % of the silver was lost when no food was added, an LC_{50} of 0.25 µg dissolved Ag/L can be estimated. The study is assigned a reliability score of 2 and was used for classification.

Erickson (1998)

The effects of different test regimes such as feeding or no feeding and ageing or no ageing of test solutions on the acute toxicity of silver (as silver nitrate) to *D. magna* was investigated in a static test. A 48-hour LC_{50} value of 0.58 µg Ag/L for *D. magna* was obtained in non-aged laboratory water without feeding. Dissolved silver is reported to be 89 % in the tests with *D. magna* and hence a LC_{50} of 0.52 µg/L for dissolved silver can be calculated. The toxicity of silver in natural water was found to be much lower than in laboratory water, by a factor of 60. The major difference between the two waters is the concentration of organic matter, the organic content of the river water being more than an order of magnitude higher. The study is assigned a reliability score of 3 and used mostly to assess the effect of laboratory conditions on silver toxicity.

Acute (short-term) toxicity to algae or other aquatic plants

Reach registration dossier for silver, Schlich et al. (2017)

The toxicity of silver to unicellular green alga *P. subcapitata* is studied in 72-hour growth inhibition test in a static system in compliance with GLP and OECD TG 201. The alga was exposed to nominal concentrations of 0.316, 1.00, 3.16, 10.0 and 31.6 µg Ag/L. The medium was prepared with reduced EDTA concentrations and compounds including chloride were replaced by nitrate compounds. Silver is measured by ICP-MS at test initiation, after 24 h, 48 h and at the test termination of the growth test (LOQ = 0.001 µg/L). Three different types of measurements were conducted: total silver, conventional dissolved silver after filtration of a subsample through 0.45 µm PES filters and truly dissolved silver after filtration with centrifugal filters at 3000 x g. The

particle size and the zeta potential were measured from samples of an extra analytical vessel without algae to characterise the test item in test media at test initiation and test termination. The evaluation of the results was based on the geometric mean measured concentrations of total silver, conventional dissolved silver, and truly dissolved silver. A dose-response relationship was shown for both inhibition of yield as well as inhibition of growth rate. For conventionally dissolved silver (< 0.45 µm), an ErC_{50} of 0.96 µg/L was determined. The study is assigned a reliability of 2 and can be used for classification.

Chronic aquatic toxicity

***Table**: Summary of relevant information on chronic aquatic hazard.*

Method, Guideline, GLP status, Reliability (Klimisch *et al.*, 1997)	**Species**	**Test compound End point Type of test**	**Exposure**		**Results**	**Remarks**	**Reference**
			Design	**Duration**	**NOEC/ EC_{10}**		
ASTM 1241-98; GLP Reliability: 2	*Oncorhynchus mykiss*	$AgNO_3$, Mortality, hatching success, time to hatch, growth, deformations	Flow-through	73-77 (30d post swim-up)	NOEC growth 0.38 µg/L (total) 0.21 µg/L (dissolved) NOEC mortality 1.48 µg/L (total) 1.09 µg/L (dissolved)	Four replicates according to private communication with the authors in 2018.	IIIA 7.4.3.2-05, Dethloff *et al.* (2007), published
- Reliability: 3 Non-guideline	*Oncorhynchus mykiss*	$AgNO_3$, Mortality, time to swim-up, growth	Flow-through	60d	NOEC growth 0.05 (= ½ of LOEC 0.1) (total) 0.02* µg/L (dissolved) NOEC mortality 0.36 µg/L (total) 0.15 µg/L (dissolved)	No information about number of replicates. The nominal and measured dissolved concentrations are not specified. No dose-response curves presented.	IIIA 7.4.3.2-01 Nebeker *et al.* (1983), published
Reliability: 3 Non-guideline	*Oncorhynchus mykiss*	$AgNO_3$, Mortality, percent hatch, percent swim-up, degree of yolk sac absorption, growth	Flow-through	51d	NOEC growth 0.14 (total) 0.13** µg/L (dissolved) NOEC mortality 0.14 µg/L (total)	Number of replicated test chambers = 2. Insufficient information about substance concentration (are the reported values	IIIA 7.4.3.2-03 Brauner and Wood (2002a), published

Method, Guideline, GLP status, Reliability (Klimisch *et al.*, 1997)	**Species**	**Test compound End point Type of test**	**Exposure**		**Results NOEC/ EC_{10}**	**Remarks**	**Reference**
			Design	**Duration**			
					0.13 µg/L (dissolved)	mean values?)	
Reliability: 3 Non-guideline	*Oncorhynchus mykiss*	$AgNO_3$, Mortality, time to hatch, growth	Flow-through	37d	NOEC growth 0.1*** (total) NOEC mortality 0.1 µg/L (total)	Number of replicated test chambers = 3. Insufficient information about substance concentration (are the reported values mean values?)	IIIA 7.4.3.2-04, Brauner and Wood (2002b), published
Reliability: 3 Non-guideline	*Oncorhynchus mykiss*	$AgNO_3$, Mortality, time to hatch, growth, physiological parameters	Flow-through	58d	NOEC growth 0.09 (total) NOEC mortality (< 0.09)# µg/L (total)	Number of replicated test chambers = 2 (mortality) and 1 (growths)	IIIA 7.4.3.2-06, Brauner *et al.* (2003), published
Reliability: 2 Non-guideline	*Pimephales promelas*	$AgNO_3$, mortality, growth (weight), hatching success	Unknown	30d	NOEC growth 0.35 µg/L (dissolved) NOEC mortality 0.35 µg/L (dissolved)		Naddy *et al.* (2007) (Ref in IIIA 7.4.3.2-02; Moermond and van Herwijen (2012), published
ASTM E729-80; published peer-reviewed research Reliability: 2	*Daphnia magna*	$AgNO_3$, Survival and reproduction	Static renewal	21d	1.6 µg/L (total) 0.7 µg/L (dissolved)*	Mean measured silver concentration.	IIIA 7.4.3.4 Nebeker (1983)
No guideline, published peer-reviewed research Reliability: 3	*Ceriodaphnia dubia*	$AgNO_3$, Survival and reproduction	Static	10d	NOEC 0.53 µg/L (dissolved)	Not entirely clear whether results are based on mean measured "dissolved" silver	IIIA 7.4.1.2-03, Rodgers *et al.* (1997a) (Published peer-reviewed research, added by the eCA)
	Daphnia magna				NOEC 0.8 µg/L (dissolved)		
	Hyalella azteca	$AgNO_3$, Survival			NOEC 4.0 µg/L (dissolved)		
	Chironomus tentans				NOEC 125 µg/L (dissolved)		

Method, Guideline, GLP status, Reliability (Klimisch *et al*., 1997)	**Species**	**Test compound End point Type of test**	**Exposure**		**Results NOEC/ EC_{10}**	**Remarks**	**Reference**
			Design	**Duration**			
OECD TG 201 (1984) Reliability: 1	*Pseudokirchneriella subcapitata*	AgCl on titanium dioxide (Ag 15%) Growth rate	static	72h	NOEC 0.75 µg/L (total)		IIIA 7.4.1.3-02, Manson (2002)
OECD TG 201 Reliability: 2	*Pseudokirchneriella subcapitata*	$AgNO_3$ Growth rate	static	72 h	ErC_{10} **0.1 µg/L (dissolved)**	Based on mean measured conventional dissolved Ag concentrations (0.45 µm PES filter). This is the most sensitive endpoint for chronic aquatic toxicity.	Schlich *et al*. (2017)

* The article states that 59% of the silver was lost after well water was filtered

** Small (<10%) but significant weight gain was observed at 0.02 µg/L dissolved silver in the presence of a higher level of dissolved organic carbon. A small difference in length and weight was also observed at 0.13µg/L. However, since the difference is very small and due to the suspected flaw in statistics, it is doubtful whether these are real effects. See discussion in text.

*** Based on weight increase in newly hatched larvae. A slight (<10%) but significant increase in growth of newly hatched larvae was also observed at 0.1 µg. However, due to the suspected flaw in statistics, this is probably not a real effect. See discussion in text.

Visually assessed from graph

Chronic toxicity to fish

The DS compiled data for chronic toxicity of silver from studies reported between 1998 and 2012 and identified the lowest chronic fish endpoint of 0.1 µg Ag/L from Brauner *et al*. (2003).

However, a study conducted by Nebeker (1983) reported a NOEC of < 0.1 µg Ag/L, which was contained in the dossier.

Based on the information provided in the table above, growth can be considered the most sensitive endpoint with values ranging between 0.02 and 0.35 µg Ag/L (Nebeker, 1983 and Naddy, 2007). In this respect, *Oncorhynchus mykiss* appears to be more sensitive than *Pimephales promelas*. Four independent studies testing the same sensitive species are mentioned in the report, where fertilized *O. mykiss* eggs and larvae have been exposed to silver nitrate and dissolved silver, have been measured. During re-evaluation of the mentioned studies, the eCA found out that in one of the studies the dissolved concentration was actually not measured at the relevant concentration, thus ending up with three useable studies (see above table). The durations of the studies ranged between 58 and 77 days with the most conservative endpoint derived following 60 days of exposure and the least conservative following 77 days of exposure.

Two studies by Davies *et al*. (1998), are reported in the REACH registration dossier. The study performed with the fish species *O. mykiss* resulted in an LC_{10} of 0.17 µg/L and the study

performed with the fish species *Salmo trutta* resulted in an EC_{10} of 0.19 µg/L. The studies bring further support to the results presented. More information about the studies is presented in the summary table in Annex I to the present CLH report.

Brauner and Wood (2002a)

O. mykiss embryos were exposed to silver nitrate (nominal of 0, 0.1, and 10 mg/L total silver). Exposures were conducted in Hamilton hard water, in the presence or absence of dissolved organic carbon at a concentration of 12 mg/L in control and reference treatments. Each day, mortality, percent hatch, and percent swim-up were determined, and degree of yolk sac absorption was visually estimated. At 51 days post fertilization mortality, percentage hatch, percentage swim-up, ion regulation, and degree of yolk sac absorption were examined. Fish were sampled for the determination of whole embryo/arval Na^+, K^+ -ATPase activity levels, extractable protein, and Na+, Cl-, and total silver concentrations and whole embryo/larval unidirectional Na^+ uptake. Total and dissolved silver concentrations were analysed. It was not clear whether the results presented were mean values or how they were derived. Throughout development, there was a large increase in percentage daily mortality at 10 µg/L total silver. The protective effects of DOC (in the form of humic acid) during chronic silver exposure appear to be less than that observed during acute exposure. Exposure to 0.13 µg/L total silver (filtered/dissolved, in the absence of DOC) resulted in a small reduction in growth at day 51 compared to the corresponding control. The reduction was reported to be statistically significant. When the day 51 weight data are compared, for the exposures including DOC, a statistically significant decrease in weight is observed at 10 µg/L total silver. However, a statistically significant increase in weight is observed at 0.11 µg/L total silver (unfiltered), 0.02 µg/L total silver (filtered/dissolved) when compared to the control. This conclusion is supported by the results of extractable protein that indicate no statistically significant difference at 0.1 µg silver/L in the presence of DOC, when compared to the corresponding control. However, it appears that the statistical method was not appropriate for weight and length data. Only two test chambers were applied per concentration. The presentation of results indicates pseudoreplication when ANOVA and Dunnett's post-hoc test was applied (i.e., ±-values, not specified whether standard deviation or standard error and small differences in growths appear to be significantly different) and comparisons were based on single fish as statistical units. This will lead to an artificially high statistical power, i.e., falsely identifying small differences as significant, whereas true differences related to treatment may remain undetected. Since the differences are small (< 10%) and due to the suspected flaw in the statistics, it is doubtful whether these are real effects. It is therefore suggested that a NOEC of 0.13 µg/L filtered, dissolved silver is used as supportive data for the purpose of classification. The study is assigned a reliability of 3.

Dethloff et al. (2007)

The toxicity study to *O. mykiss* embryos and larvae was conducted following ASTM Method 1241-98 (complying with US EPA principles) with a nominal silver nitrate exposure range of 0.12-2.0 µg/L silver. Exposure in unmodified dilution water continued for 73 days and in dilution water amended with chloride (nominal 30 mg/L) continued for 77 days (30 days beyond the mean day to swim-up of the control for each). The parameters examined in the test were hatching, post hatch survival, and growth during the test. Fish were analysed for whole-body sodium and silver concentrations. Total and dissolved silver concentrations were also determined. Weight gain was decreased at dissolved silver concentrations of 0.21 µg/L and above in chloride-amended water. No effect on weight was observed in unmodified water at the tested concentrations up to 1.25 mg/L dissolved silver. The lowest-observed-effect concentrations were greater than 1.25 µg/L of dissolved silver for survival, mean day to hatch, mean day to swim-up and whole-body sodium content for both unmodified dilution waters and waters amended with NaCl. Irrespective of some limitations such as inappropriate statistical methodology for weight and length data, only one

test chamber applied per concentration, pseudo-replication, comparisons were based on single fish as statistical unit, the clear dose-response, the tight concentration intervals, and the fact that no effects were observed in the test without chloride amendment, which could be considered as a replicate (dissolved concentrations up to 1 µg/L tested), the results are considered sufficiently reliable. The NOEC based on mean dry weight was 0.5 µg/L of nominal silver (0.21 µg/L of dissolved Silver) and will be used as supportive data for the purpose of classification.

Chronic toxicity to aquatic invertebrates

Nebeker (1983) (Published peer-reviewed research)

The effect of water hardness is investigated in three *D. magna* reproduction studies conducted with silver nitrate. Survival and reproductive success (as young/survived adult) were equally sensitive endpoints. The dissolved silver concentration (< 0.45µm) has been determined at least once in the study and it was found that 59% of the silver was lost after filtration. Based on this information, values can be estimated for survival and reproduction at 0.7 µg Ag/L. The statement made that water hardness did not affect the survival or reproduction of *D. magna* has not been statistically verified. The study is assigned a reliability of 2 due to some shortcomings such as high mortality in the controls as well as lacking or inconclusive information about silver concentrations, and purity of the test substance.

Two studies (Kolts *et al*., 2009 and Diamond *et al*., 1990) that were part of the screening of available data concerning silver toxicity in the aquatic environment, show results in the same range as the study by Nebeker *et al*. (1983) and Rodgers *et al*. (1997). In the study by Kolts *et al*. (2009), a NOEC of 0.37 µg Ag/L was obtained (test organism: *C. dubia*) and in the study by Diamond *et al*. (1990), a NOEC of 0.58 µg Ag/L was observed (test organism: *Hyalella azteca*). These results further support the outcome of the two studies discussed above.

Chronic toxicity to algae or other aquatic plants

Study referenced as 008 key, Schlich et al. (2017)

The toxicity of silver to uni-cellular green alga *P. subcapitata* is studied in a 72-hour growth inhibition test in a static system following GLP and OECD TG 201. The alga was exposed to nominal concentrations of 0.316, 1.00, 3.16, 10.0 and 31.6 µg Ag/L. The medium was prepared with reduced EDTA concentrations and compounds including chloride were replaced by nitrate compounds. Silver was measured by ICP-MS at test initiation, after 24 h, 48 h and at the test termination of the growth test (LOQ = 0.001 µg/L). Three different types of measurements were conducted: total silver, conventional dissolved silver after filtration of a subsample through 0.45 µm PES filters, and truly dissolved silver after filtration with centrifugal filters at 3000 x g. The particle size and the zeta potential[3] were measured from samples of an extra analytical vessel without algae to characterise the test item in test media at test initiation and termination. The evaluation of the results was based on the geometric mean measured concentrations of total silver, conventional dissolved silver, and truly dissolved silver. A dose-response was shown for both inhibition of yield as well as inhibition of growth rate. For conventionally dissolved silver, an E_rC_{10} of 0.10 µg/L was determined for growth rate which is the most conservative, and sufficiently reliable (reliability score of 2), value. Compared to the study by Manson (2000), where the same

[3] *Zeta potential is an electrokinetic potential at the shear plane (the boundary between the compact layer and the diffuse layer) near a solid-liquid interface where the liquid velocity is zero. From Interface Science and Technology (2004), Vol 2, 617-640*.

test species (*P. subcapitata*) is used, the results in the present study are considered more relevant as they are based on dissolved silver (< 0.45 µm) rather than total silver.

Solubility of silver

Solubility of massive silver

Data from two transformation/Dissolution (T/D) studies are available for silver metal massive.

ECTX 2010, study number X10-01-53, silver wire, pH = 6

In ECTX 2010 (X10-01-53), the silver dissolution from a silver wire in pH 6 buffer (prepared as in OECD TG 203 reconstituted water and further diluted 10 times) was studied at 1 mg/L, 10 mg/L, and 100 mg/L, corresponding to a surface area loading of 0.67 mm^2/L, 6.7 mm^2/L, and 67 mm^2/L, respectively. The test at 1 mg/L was performed for 28 days whereas for the other two loadings the tests were conducted for 7 days. Dissolved silver was measured using ICP-MS (reporting limit 0.01 µg/L) on filtered and acidified solutions (0.2 µm syringe filters). For the 10 mg/L and 100 mg/L loadings the results showed an initial rapid increase in dissolved silver concentration until 48 h, whereby it decreased towards the end of the testing period reaching average concentrations of 0.07 µg/L and 0.17 µg/L, respectively. For the 1 mg/L loading the pattern was similar with silver levels as high as 0.9 µg/L after 2 hours followed by average concentrations of 0.05 µg/L and 0.03 µg/L after 7 and 28 days, respectively. Throughout the study, there were quite large variations between the replicates, in particular for the 1 mg/L loading. The DS accepted the limitations of this study (reliability 3) connected with the eventual sorption of silver ions on the surface of epoxy resin or possibility during the preparation of the test item minute particles of silver may become embedded in the epoxy vehicle increasing the exposed surface area and changing solubility.

ECTX 2013, study number X-01-115, silver wire, pH = 8

An analogous study on massive silver, as reported above (ECTX, 2013; X-01-115) was conducted in pH 8 buffer (prepared as in OECD TG 203). Loadings of 3, 9, and 27 mg/L were used, corresponding to surface area loadings of 1.7 mm^2/L, 5.1 mm^2/L, and 15.5 mm^2/L, respectively. The area of the particles in each loading corresponds to the area of spherical particles of 1 mm diameter (i.e., recommended particle size for testing of massive metal). Testing was done under 28 days for all three loadings. Quartz vehicles and fluorinated ethylene propylene vessels were used in this case. Although experimental results showed that pH 8 maximize silver dissolution at all sampling points, except for one replicate at 27 mg/L loading, the silver concentration was below the limit of detection of the analytical method (ICP-MS 0.02 µg/L). The DS assigned a reliability score of 1 for this study.

In order to obtain silver release from massive silver, the DS combined data for silver powder for pH 8 at 1 mg/L loading over 28 days (CIMM, 2009) with the data for massive silver (ECTX, 2013) at 27 mg/L loading (corresponding to a surface area loading of 15.5 mm2/L). Based on the relationship between silver release and surface area loading ($\log10(C_{Silver})$ = 0.9963 x log10 (surface area loading) – 2.7077) (equation 1) a concentration of 0.0011 µg/L for Silver at 1 mg/L loading (corresponding to 0.572 mm2/L surface area loading) was calculated.

***Table**: Relevant loadings and resulting concentrations of dissolved silver relevant for the classification procedure for silver massive.*

Massive silver				
Study: ECTX 2010, study number X10-01-53, silver wire, pH = 6				
Days	**Loading (mg/L)**	**SAL (mm^2/L)**	**Dissolved silver conc (µg/L)**	**ERV (µg/L)**
7	1	0.67	0.05	Acute 0.22
28	1	0.67	0.03	Chronic 0.1
Study: ECTX 2013, study number X-01-115, silver wire, pH = 8				
7	3, 9, and 27	1.7, 5.1, 15.5	[-]	Acute 0.22
28	(3, 9) and 27	(1.7, 5.1) 15.5	0.03 for 27 mg/L loading. (No results obtained for the other loadings.)	Chronic 0.1
Extrapolation through the relationship log10(CAg) = 0.9963 x log (SAL) – 2.7077				
28	1	0.572	0.0011	Chronic 0.1

Solubility of silver powder

Data from two transformation/Dissolution (T/Dp) studies are available for powdered silver, both accepted by the DS with reliability of 2.

CIMM 2009, silver powder, pH = 6 and 8

The dissolution of silver from a silver powder (D_{90}=11.2 µm, D_{50}=1.9 µm, and D_{10}=0.8 µm) in pH 6 and pH 8 buffers was tested at 1 mg/L, 10 mg/L, and 100 mg/L, corresponding to a surface area loading of 3 x 10^3 mm^2/L, 3 x 10^4 mm^2/L, and 3 x 10^5 mm^2/L, respectively. The test at 1 mg/L was performed for 28 days whereas for the other two loadings the tests were only conducted for 7 days. Silver was measured by ICP-MS after filtration (0.2 µm syringe filters). The similar release patterns were observed with an initial rapid increase in silver concentration followed by a slower increase towards the end of the testing period. At pH 6 silver dissolution ranged between 1.25-37.4 µg/L at 1-100 mg/L loading and 7 days. At 1 mg/L over 28 days the silver dissolution was 3.55 µg/L. At pH 8 the silver dissolution range was 2.55-26.03 at 1-100 mg/L loadings and 7 days. At 1 mg/L over 28 days the silver dissolution was 5.71 µg/L. The results indicated similar silver dissolution at both pHs with mostly slightly higher levels at pH 8, except at the highest loading.

ECTX 2010, study number X10-01-052, silver flakes, pH = 6

The silver dissolution from silver flakes (D_{90}=7.83 µm, D_{50}=2.61 µm, and D_{10}=1.07 µm) in pH 6 buffer was tested at 1 mg/L, 10 mg/L, and 100 mg/L corresponding to a surface area loading of

1.17 x 10^3 mm^2/L, 1.17 x 10^4 mm^2/L, and 1.17 x 10^5 mm^2/L, respectively. The earlier study (CIMM, 2009) did not show significantly different results between pH 6 and pH 8. The conditions of the test are similar to those reported above, pH 6 was chosen due to the non-significant difference observed with pH 8. Results are also similar to those obtained in the previous study. An initial rapid increase in silver concentration followed by a slower increase towards the end of the testing period was observed. The silver dissolution ranged between 1.79-38.1 µg/L at 1-100 mg/L loadings over 7 days. At 1 mg/L and 28 days the silver dissolution was 3.60 µg/L.

The DS used the following derived equation for massive silver: (log10(C_{Ag}) = 0.9963 x log10 (surface area loading) – 2.7077), to calculate silver dissolution over 28 days at 0.1 mg/L for the chronic classification.

***Table**: Relevant loadings and resulting concentrations of dissolved silver relevant for the classification procedure for silver powder.*

Silver powder					
Study: CIMM 2009, silver powder, pH = 6 and 8					
Days	**Loading (mg/L)**	**SAL (mm^2/L)**	**Dissolved silver conc (µg/L)**	**ERV (µg/L)**	**Dissolved silver/ERV** **10 ≤ Ratio < 100 gives M-factor = 10**
7	1	3x10^3	1.25 (pH = 6) 2.55 (pH = 8)	Acute 0.22	Acute 1.25/0,22 = 5.7 Acute 2.55/0,22 = 12
28	1	3x10^3	3.55 (pH = 6) 5.71 (pH = 8)	Chronic 0.1	Chronic 3.55/0.1 = 36 Chronic 5.71/0.1 = 57
Study: ECTX 2010, study number X10-01-052, sliver flakes, pH = 6					
7	1	1.17x10^3	1.79	Acute 0.22	Acute 1.79/0.22 = 8.1
28	1	1.17x10^3	3.60	Chronic 0.1	Chronic 3.60/0.1 = 36
Extrapolation through the relationship log10(CAg) = 0.9963 x log (SAL) – 2.7077					
28	0.1	300	0.58	Chronic 0.1	N/A

<u>DS final note on T/Dp data for massive silver and silver powder</u>

The DS noted that the media used for the T/Dp studies contain a high concentration of chloride. It can thus be suspected that any silver ions released will form poorly soluble silver chloride that would precipitate and render them unavailable for the chemical analysis after filtration. However, it is noted that from the T/Dp studies available in the REACH-dossier on silver powder indicate a log-log linear increase in analysed silver concentration with surface area loading which indicates that saturation concentrations of silver chloride were not reached. The solubility of silver chloride in water at room temperature is quoted as 1.9 mg/L (Merck Index, 13th Ed.), which corresponds to 1.4 mg/L silver. The analysed concentration of silver in the T/D-studies was max ~37 µg/L, which means that the solubility of silver chloride was not the limiting factor in the studies.

<u>Dissolution silver-nano</u>

VITO NV 2017, coated silver nanoparticles

One T/D study is available for silver ions release from silver NPs. The dissolution of coated silver nanoparticles (D_{75}=9 nm, D_{50}=8 nm, and D_{25}=7 nm) were tested at 1153 and 1230 µg Ag/L in Daphnia and algae medium respectively corresponding to surface area loading of 153 x 10^3

mm^2/L and 163 mm^2/L respectively. The daphnia and algae media were chloride free Elhendt and AAPmedium respectively. They were prepared as described in OECD TGs 211 (daphnia) and 201 (algae) and modified by omitting chloride (replaced by NO_3^-) and omitting EDTA (daphnia) or reducing it by 50% (algae) to avoid precipitation of AgCl and complexation of silver ions by EDTA. The modification was in agreement with the criteria given by ECHA for the testing of silver nanomaterial. The composition of the testing media is reported in the relevant tables. Results for silver dissolution (measured by ICP-MS) were presented as conventional dissolved silver (after 0.45 µm filtration) and truly dissolved silver (after 3 x 1 kDa ultrafiltration). The total silver concentration in the aqueous phase without filtration was also determined.

In the *Daphnia* media, the conventional dissolved silver ranged between 99-1074 µg/L, with a dissolution pattern of almost full initial dissolution followed by a rapid decline over the first 24 hours and a more stable silver-concentration for the rest of the study period. The conventional dissolved silver was 99 µg/L and 127 µg/L after 7 and 28 days, respectively. It should be noted that total silver in the aqueous phase also showed the same pattern with a silver content of 473 µg/L after 28 days. The truly dissolved silver was 88 µg/L and 146 µg/L after 7 and 28 days, respectively. The pH of the solutions varied between 7.9 (freshly prepared) and 8.2 (after 28 days).

In the algae media, the dissolution pattern was different with an almost full initial dissolution and only a slow decline towards the end of the study. The conventional dissolved silver ranged between 912-1230 µg/L, with 974 and 912 µg/L after 7 and 28 days, respectively. The truly dissolved silver was 125 and 214 µg/L after 7 and 28 days, respectively, indicating that a major part of the conventional dissolved silver was in particulate/colloidal form. The pH of the solutions varied between 7.2 (freshly prepared) and 7.5 (after 28 days).

The DS considered this study reliable with a score of 2, although the study was not performed in accordance with GLP.

The DS considered the potentially higher likelihood for interactions of silver ions with complex daphnia and algae media. Results obtained suggested precipitation/adsorption of Ag NPs in the daphnia media but not in algae media.

The DS agreed with the conclusion in the REACH dossier for silver nanoparticles that in the absence of standard methodology for conducting T/Dp studies for nanomaterials using data for soluble silver substances is appropriate for the self-classification. Accordingly, silver nanoparticles are classified as Acute 1 with M-factor of 1000 and Chronic 1 with M factor of 100 using acute and chronic ERV's of 0.22 and 0.16 µg/L, respectively. DS also noted that analogous classification Acute 1 with M factor 1000 or 100 will be proposed for silver nanoparticles, based on data for the measured silver concentration after 7 days (974 and 125 µg/L expressed as conventional and truly dissolved).

The DS used the derived equation: ($\log10(C_{Ag})$ = 0.9963 x log10 (surface area loading) – 2.7077), to calculate silver nanoparticles dissolution after 28 days and 0.1 mg/L loading for the chronic classification. A 0.1 mg/L silver loading corresponds to a surface area loading of 13300 mm^2/L for the coated silver nanoparticles used in the study. Using the formula above, this results in a silver dissolution of 25.2 µg/L after 28 days. This is far above the chronic ERV of 0.1 µg/L. A classification as chronic 1, is thus warranted. In addition, when the surrogate approach is followed the material should also be classified as Chronic 1, since the dissolution after 7 days is far above the acute ERV.

M-factors are estimated from the ratio of the soluble metal ions concentrations obtained from T/D studies and the ERV (derived from ecotoxicity results).

The DS calculated the M-factors, based on the maximum dissolution after 28 days (considering both algae and daphnia media) of 912 and 214 µg/L expressed as conventional and truly

dissolved silver respectively and achieved values of 1000 for both conventional dissolved silver and truly dissolved silver. However, the DS decided that if coated silver nanoparticles behave as soluble silver salt, it is more appropriate to apply the same classification strategy as for soluble metal compounds. Results are quite similar only potential difference being the M-factor for the chronic classification (1000 for T/D data on the silver nanoparticles instead of 100 for the silver ion from soluble silver salts).

Conclusions of the DS

Silver is a metal and falls under the classification scheme for metals and metal compounds in the CLP Guidance Annex IV, (ECHA, 2017).

The most sensitive species in the acute toxicity data set is *D. magna* with a LC_{50} of 0.22 µg/L (Bianchini, 2002). In the chronic aquatic dataset, the most sensitive species is an alga (*P. subcapitata*) with an ErC_{10} of 0.1 µg/L.

Acute ERV = 0.22 µg/L

Chronic ERV = 0.1 µg/L

A total of five transformation/Dissolution-studies are available: two for massive silver; two for silver powder and one for silver nanoparticles.

Based on these data, the DS proposed a single classification for silver > 100 nm (covering massive and powder), due to a lack of evidence that particles < 1 mm are not generated from use of massive silver, and a separate classification for silver nanoparticles (≥1 nm ≤ 100 nm). The DS proposed that silver powder is representative for silver metal in massive form and classification for silver metal (including massive and powder) will be based on the solubility (transformation/dissolution) from silver powder.

The DS noted that it is possible to compare results from the ecotoxicity tests and the T/D-test results at the same pH (including the highest test result from the T/D-tests with silver powder) since the key ecotoxicity tests in the data set for silver were performed at approximately pH = 8 and this is also the pH that showed the highest T/D-test result for silver powder.

<u>Silver (> 100 nm):</u>

Classification for short-term aquatic hazards: Aquatic Acute 1 (H400), M-factor = 10

Classification for long-term aquatic hazards: Aquatic Chronic 1 (H410), M-factor = 10

<u>Silver nano (≥1 nm ≤ 100 nm):</u>

Silver nanoparticles have the same crystal structure as silver metal, however the behaviour of silver nanoparticles in T/D-tests was similar to the soluble silver salts. DS concluded that silver nanoparticles were not representative for the aquatic hazard of silver in general and proposed a separate environmental classification for silver nanoparticles.

Classification for short-term aquatic hazards: Category Acute 1, M-factor = 1000

Classification for long-term aquatic hazards: Category Chronic 1, M-factor = 100

Comments received during consultation

Three Member States (MS) and 45 Company-Manufacturers and Industry or Trade Associations submitted comments on the DS's proposal during the public consultation.

All commenting MSs agreed with the proposed separate classification for silver nanoparticles. One MS requested clarification for the proposed M-factor of 100 for Aquatic Chronic 1 classification of silver nanoparticles, whilst supporting the acute M-factor of 1000. The DS agreed with this comment.

The Industry Associations disagreed with the DS proposal for silver massive metal classification. Comments received could be summarised as follow:

- In all 45 comments Industry Associations disagreed with a single classification covering silver massive and silver powder. In their opinion a separate entry for the massive form is justified based on T/D data and based on the fact that silver powder is produced by a special process and is not generally generated from the massive metal. The Industry Associations declared that the massive form does not produce powders under foreseeable uses and consequently they should not be classified for environmental hazards based on the results from T/D test.
- Most of commenting Industry Associations agreed with separate classification entry for silver nanoparticles and silver powder.
- In addition, Industry Associations commented that evidence for rapid removal of silver from the water column is not taken into account and not all available data for silver acute and long-term aquatic hazard have been used for classification. The effect of pH on silver toxicity as well as the application of the Biotic Ligand Model (BLM) for data normalisation should be considered for classification.

The DS responded that the proposals followed the CLP guidance and previous metal classifications by RAC.

Regarding the proposal that silver massive and silver powder should be considered separately, the DS noted that the Industry Associations did not present evidence that particles < 1 mm were not generated in sufficient quantities from the massive metal during reasonably expected use. The DS considered this point as crucial for a final conclusion on classification. The DS doubted whether the arguments that silver powder is produced by atomization and reduction process and silver is malleable and ductile material are sufficient.

Regarding the comment that not all available acute and chronic data has been taken into account, the DS pointed out all reliable and relevant aquatic toxicity data based on dissolved silver available to DS at the time of CLH dossier submission are listed and assessed. In addition, the DS indicated the most updated draft of the dossier, recently prepared by the JRC with the purpose of deriving environmental water quality standards for silver under the Water Framework Directive. Values for the most sensitive endpoints in the JRC report agree with ERV values used by the DS for silver metal classification.

Regarding the comment on pH, the DS pointed out that the key aquatic toxicity tests for silver in the data set were performed at approximately pH = 8 and this is also the pH that showed the highest T/D-test result for silver powder. As the T/D-data and aquatic toxicity data should preferably be compared at the same pH in a conservative manner a pH normalisation is not considered necessary in this case. Regarding BLM application and data normalization, the DS pointed out that only an acute BLM model is available for silver and that normalisation models are not yet available for silver.

Regarding the comment on silver removal from the water column, the DS cited the CLP guidance: "*However, partitioning of the metal ion from the water column to other environmental media*

does not necessarily mean that it is no longer bioavailable, nor does it necessarily mean that the metal has been made permanently unavailable." The DS noted that there is still no harmonised approach or guidance available on the concept of "rapid removal" for inorganic substances based on the conclusions from "Rapid Removal Workshop" that was organised by ECHA in Helsinki (11 June 2019. Consequently, it is not possible to conclude that silver is transformed in a way that would rapidly and permanently remove it from the water column.

Regarding the comment that the study by Schlich *et al*. (2017) should not be used for classification due to the reduced EDTA concentration and replacement of chloride salts with nitrate, the DS considered that as far as silver toxicity is based on dissolved measured concentration changes in aquatic media would not affect the conservativeness of the results with regards to bioavailability.

Assessment and comparison with the classification criteria

Transformation to non-bioavailable forms

In surface fresh waters, silver may be found as the monovalent ion; in combination with sulphide, bicarbonate, or sulphate; as part of more complex ions with chlorides ($AgCl_2^-$, $AgCl_3^{2-}$, $AgCl_4^{3-}$) and sulphates; and adsorbed onto particulate matter. In river water, one study showed silver presented as the monovalent ion at 53–71% of the total silver, as silver chloride at 28–45%, and as silver chloride ion ($AgCl^-$) at 0.6–2.0% (Whitlow and Rice, 1985). The monovalent ion does not hydrolyse appreciably in solution and is considered to be a mild oxidizing agent. Silver ions bind strongly with sulphide ions in dissolved inorganic and organic matter (DOM), resulting in nanogram per litre aqueous dissolved concentrations. The most important and crucial aspect of silver thiolate chemistry is the rapid exchange of silver ion among thiolates, whereby silver ion can transfer onto, or off of, particulate materials or the cells of an organism. When complexed to these strong-affinity ligands, Ag(I) is protected from further reduction to zero-valent.

Increasing salinity of brackish and marine waters increases concentrations of silver-chloro complexes (AgCl, $AgCl_2^-$, $AgCl_3^{2-}$, $AgCl_4^{3-}$); these chloro complexes retain some silver in dissolved form and relatively small anthropogenic quantities can substantially enrich the environment (Luoma, 1994). In the open ocean, the principal dissolved form of silver is $AgCl_2^-$, but the most bioavailable form is maybe the neutral monochloro complex silver chloride. Silver sorbs readily to phytoplankton and to suspended sediments. Nearly 80% of silver sorbed to suspended sediments at low salinities desorbs at higher salinities, but desorption is a bit slower when silver is associated with phytoplankton. Data presented in the open literature showed that silver at environmentally relevant concentrations exists in aquatic environment mostly as a complex with various ions. In this way processes of reduction or sorption would not account for the transformation of silver ions to non-bioavailable species. Laboratory experiments showed that silver reduction might be expected at high, environmentally, irrelevant concentrations.

Industry expressed the view that silver could be rapidly transformed to non-bioavailable forms, presenting a paper (Annex 2_Ag Env Transformation _final_212021) and a study (Nijs, 2021) for silver speciation in the presence of NOMs with sulphur containing functional groups. Calculations were performed using speciation code Visual Minteq 3.1, Silver complexation was modelled by the Stockholm Humic Model (SHM) (Gustafsson, 2001). In this study, the concentration of Chromium Reducible Sulphides (CRS) was calculated taking into account average values of DOM and relationships derived by Kramer *et al*. (2007) between DOM and CRS. Results showed values for CRS between 10 and 50 nM and full precipitation of Ag as Ag_2S. RAC agrees that silver forms strong complexes with sulphur containing functional groups. However, CRS in pristine waters is below 1 nM and calculations with this concentration do not support silver precipitation. RAC notes that silver transformation to nonbioavailable species would not be expected at environmentally

relevant silver and CRS concentrations. Furthermore, additional meso- and microcosm studies presented (Jiang *et al*., 2017; Colman *et al*., 2014) were performed at very high silver concentrations. Overall, results from these studies do not support the conclusion for silver transformation to non-bioavailable form under environmentally relevant conditions.

In conclusion, RAC considers that silver is not rapidly transformed to non-bioavailable forms.

Bioaccumulation

Data for bioaccumulation of silver ions and silver nanoparticles are available in the CLH report, with additional information bring submitted by Industry during RAC's discussions (EPMF cover letter to ECHA secretariat RAC_ENV 201117 and one-pager bioaccumulation_final). The bioaccumulation of metals according to the classification criteria should be evaluated on a case-by-case basis using expert judgement as the mechanisms for uptake and depuration rates of metals are very complex and variable and there is at present no general model to describe bioaccumulation. Silver is a non-essential element and the ability of dissolved silver to accumulate varies widely between species, depending on silver chemical species and external conditions (specific active transport systems, ligand binding and competitive interactions at the receptor site) (Ratte *et al*., 1999).

BCF values for fish varied from 0.4 to 327 L/kg ww (data compiled in addition by Industry in submitted documents referenced above). Accumulation of silver in fish was shown to occur primarily at the gill surfaces and in the gastrointestinal tract for juvenile rainbow trout exposed to 12 µg/L of ^{110}Ag.

In available field studies, it was demonstrated that silver does not accumulate through the food chain from lower organisms such as plankton to higher organisms such as fish.

Invertebrates can accumulate silver, but the degree of accumulation is species dependent and varied in widely from 2.5 to 27500 L/kg ww (data compiled from Ratte *et al.* (1999) and by Industry Associations). Even at the highest tissue concentration, toxicity effects are not observed due to efficient sequestration mechanisms, which involves binding to metallothionein or solid metallic phosphate or metallic thiosulphate granules. RAC notes that very high values reported for BCFs in invertebrates might not be valid from an experimental point of view. For example, values for *D. magna* varied between 9 and 2200 L/kg ww. A value of 2200 L/kg was obtained from Terhaar *et al*. (1977) in the presence of $NaAgS_2O_3$, where authors reported that this value is due to the transformation of compound to Ag_2S which is further adsorbed on the surface of the organism. A very high value of 24000 L/kg for tissues of *Crangon crangon* (Bertine *et al.*, 1972) was calculated using an old analytical method and using the system detection limit instead of real exposure concentrations for silver. Values for kinetic BCFs presented by Industry for *D. magna*, varied between 1520-3600 L/kg, however these values are for dry weight (wrongly presented by Industry as wet weight) and a recalculation (taking into account the relationship dry weight/wet weight = 0.1 for *D. magna*) resulted in 10 times lower values below the threshold of 500 L/kg.
In conclusion, aquatic organisms have evolved mechanisms to regulate silver as a consequence of exposure to natural sources. Industry submitted further information during the RAC discussions by way of position papers (EPMF cover letter to ECHA secretariat RAC_ENV 201117 and one-pager bioaccumulation_final), which supports the conclusion that silver is unlikely to bioaccumulate through aquatic food chains.

In conclusion, based on valid experimental results for fish, supported by data for invertebrates, RAC does not agree with the DS and considers that for classification purposes, silver has a low potential for bioaccumulation.

ERV derivation

Influence of pH and pH banding

As a general rule, silver toxicity decreases at higher pH values. Laboratory experiments (e.g., Whitlow and Rice, 1985) have shown that the acute toxicity of soluble silver (silver nitrate) to fathead minnows decreased as pH and alkalinity were increased (Fig. below). The reasons for such a trend are not obvious. Toxicity might be affected as a result of complexation of silver by a hydroxide or carbonate ions or by a competition between the toxic metal and hydrogen ion for binding sites. For silver, complexation by a hydroxide is not expected to be significant even at the highest pH, although complexation by carbonate is uncertain. Competition between hydrogen ion and silver for sorption sites at the gill should result in reduced toxicity at lower pH rather than at higher pH. Effects of pH on the complexation of silver by organic ligands in the test water could be also a factor. It is also not clear to RAC that the trend seen in Whitlow and Rice (1985) is consistent across species and taxa. Therefore, resolution of this issue requires better characterization of silver speciation and chemical interactions at the gill surface. Overall, the effect of pH appears relatively minor and given that a majority of the aquatic toxicity data, including the key data for classification, is generated at pH 8, pH banding is not considered warranted.

Dissolved Organic Carbon (DOC) and hardness

Based on information available to RAC, hardness does not appear to have a significant effect on silver toxicity although the influence of DOC is much more pronounced. For all aquatic organisms for which data is available silver toxicity is lowered significantly in the presence of high concentrations of DOM especially in the presence of sulphur containing groups.

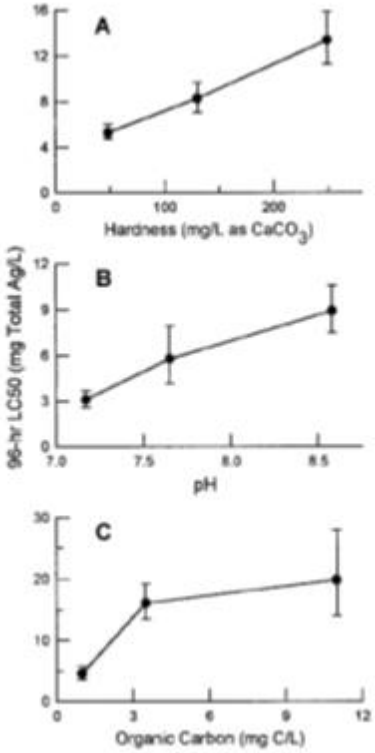


***Fig**: Effects of added hardness, acid and base titration, and added organic carbon on acute toxicity of silver nitrate to juvenile fathead minnows (from Whitlow and Rice (1985).*

Conclusions regarding pH and water quality parameters

Although there may be stronger pH effects for individual species, RAC does not consider that there is strong influence of pH on silver toxicity in any general sense. Furthermore, of the water quality parameters, only DOC has any notable generic effect on silver toxicity in the aquatic environment. Therefore, based on the relatively minor influence of pH on silver toxicity and a limited dataset across the pH range (a majority of the aquatic toxicity data are from approximately pH 8), aquatic toxicity data will not be banded for pH and the lowest ERV values will be compared with T/Dp data at the pH that gives the highest dissolution.

Biotic Ligand Model (BLM) for silver

A BLM is a thermodynamic, mathematical model that uses information on water chemistry, e.g., pH, calcium concentration, hardness, alkalinity, dissolved organic carbon (DOC) to predict metal toxicity. A BLM accounts for both the biological component (including ion competition at the biotic ligand) and chemical complexation interactions in the water column. A BLM is derived from a structured programme of ecotoxicity testing (numerous tests conducted under different physico-chemical conditions) using a single species of aquatic organism. Therefore, several discrete BLMs are usually required to describe the bioavailability of a metal across several trophic levels *i.e.*, fish, invertebrates, and algae. Whilst BLMs are derived for a particular species, it is possible to perform cross species validation and extrapolation of a BLM model to allow all available ecotoxicity data for a metal to be "normalised" to a specific water physico-chemistry (as long as sufficient information on water physico-chemistry parameters are reported alongside ecotoxicity endpoints). An acute BLM for dissolved silver has been developed by Di Toro & Paquin, (2008), and updated in 2010 to account for sulphide complex formation. Furthermore, a BLM does not intrinsically include a dietary exposure which is a relevant route of silver exposure and the acute BLM model for silver is validated only for fish. The relationship between chronic toxicity and the physico-chemical parameters of water is still less understood and the chronic BLM is still under development. In addition, normalisation models are not yet available for silver, thus no evidence is available to RAC that the silver BLM SSD (Species Sensitivity Distribution) normalisation tool is validated for more generic hazard assessment, *i.e.*, under CLP. RAC notes that the CLP guidance IV.2.1.1 only refers to the BLM with reference to pH and not to other water quality parameters like DOC, hardness, and thiols. In the case of silver, several investigations of the available aquatic toxicity data set show that the thiol group more significantly influences silver toxicity than pH. Consequently, from a scientific (statistical) point of view it is very likely that the ERV values as generated under experimental conditions, without further modification, best represent the aquatic hazards posed by silver. An identical conclusion was also reached in the present draft EQS dossier.

RAC concludes that the BLM would not provide more reliable data on silver toxicity and will not be used for data normalization.

Aquatic toxicity

Acute aquatic toxicity

A total of 120 effect values for 16 species are available. The dataset includes reliable data for all three trophic levels: fish, crustacea and algae.

***Table**: Lowest acute effect values for three trophic levels*

Method/ Test material	Species	Substance	Results	Reference
Fish Reliability 2	*Pimephales promelas*	$AgNO_3$	96h LC_{50}: 0.0031 mg/L (mortality).	Van Genderen *et al*. (2003)
Crustacea Reliability 1-2	Water flea (*Daphnia magna)*	$AgNO_3$	**48h LC_{50}: 0.00022 mg/L (mortality).**	Bianchini *et al*. (2002)
Algae Reliability 2	*Pseudokirchneriella subcapitata*	$AgNO_3$	72h EC_{50}: 0.00024 mg/L (yield)	Schlich *et al*. (2017)

– Deterministic approach

Based on the available acute data, crustacea is the most sensitive taxon. Bianchini *et al*. (2002) investigated acute toxicity to *D. magna* resulting in an LC_{50} of 0.00022 mg/L. The DS used this ERV value for classification. The result from this *D. magna* study was supported by a further *D. magna* LC_{50} of 0.00023 mg/L conducted with a similar set-up (DOC < 0.4 mg/L^{-1} and without food added) (Glover *et al*., 2005).

– Probabilistic approach

The SSD method was not considered applicable for two reasons: (i) the lack of information on major taxonomic groups (e.g., mollusca, amphibian, and insects) according to the requirements in European Communities (2018), (ii) it was not possible to compile consistent datasets because of different experimental set-ups affecting the bioavailability of silver, thus the datasets would not reflect the distribution of sensitivity among the species.

Chronic aquatic toxicity

A total of 94 effect values for 19 species were available at the end of 2020. The data set represents 10 taxonomic groups, including reliable data for fish, crustacea, and alga.

***Table**: Lowest chronic effect values for three trophic levels.*

Method/ Test material	Species		Substance	Results	Reference
Fish Reliability 2-4	*Oncorhynchus mykiss*		$AgNO_3$	196d LC_{10}: 0.00017 mg/L (mortality).	Davis *et al*. (1998) (in REACH dossier)
Fish Reliability 2	*Oncorhynchus mykiss*		$AgNO_3$	73-77d NOEC growth 0.00038 mg/L (total) 0.00021 mg/L (dissolved) NOEC mortality 0.00148 mg/L (total) 1.09 µg/L (dissolved)	IIIA 7.4.3.2-05 Dethloff, Naddy *et al*. (2007), published

Method/ Test material	Species		Substance	Results	Reference
Crustacea Reliability 1-2	Water flea (*Daphnia magna)*		$AgNO_3$	21d EC_{10}: 0.00214 mg/L (growth).	Bianchini and Wood (2008)
Algae Reliability 1	*Pseudokirchneriella subcapitata*		$AgNO_3$	**72h EC_{10}: 0.00010 mg/L (growth/yield)**	Schlich *et al*. (2017)

- Deterministic approach

The study by Mertens *et al*. (2019) (published version of Schlich *et al*., 2017) on the toxicity of silver to *P. subcapitata* with an EC_{10} of 0.1 µg/L for growth inhibition was used as the key data in the deterministic approach. The same value was proposed by the DS as the ERV value for chronic toxicity. In this study the concentration of EDTA in the culture medium was lowered twice and chloride salts were replaced by nitrates. Industry commented that due to these non-standard conditions irrelevant to natural conditions (no chloride) this study should be replaced with a more reliable one and the chronic ERV reassessed. Several studies have explored the impacts of Cl^--concentration on silver toxicity (e.g., Ratte *et al*., 1999; Dethloff *et al*., 2007; Matson *et al*., 2016). Here, results for some species showed that silver nitrate is less toxic in seawater than in fresh water and this is probably due to the high levels of chloride combined with the low concentration of free silver ion in seawater, and the predominance of negatively charged silver-chloro complexes. However, high levels of silver nitrate are toxic to marine invertebrates despite the absence of silver ions and this is attributed to the bioavailability of stable silver-chloro complexes. Experimental results showed that significant Cl^- protection from silver toxicity on zebrafish (*Danio rerio*) or fathead minnow (*P. promelas*) was not observed. Partial protection in the presence of increased Cl^- concentrations has been reported for larval rainbow trout (*O. mykiss*), although chronic silver toxicity was not predictably reduced by Cl^-. Some protection from Cl^- concentrations was observed but not to the extent expected against silver toxicity in rainbow trout, and similar protection was not observed with European eel (*Anguilla anguilla*) (Bielmyer, 2008). As a result of the differing toxicity of silver in different species under varying conditions, silver toxicity appears to depend on the species physiology and on the bioavailability of kinetically labile silver-chloro complexes, as well as on the local aquatic chemistry. Taking into account the variable influence of chloride on silver toxicity and that some types of fresh waters might be characterised by relatively low chloride concentrations, RAC is of the opinion that the absence of chloride would not result in overestimation of silver toxicity. Additionally, RAC is of the opinion that the lower EDTA content would not change silver speciation and bioavailabilty. RAC notes that silver forms complex with EDTA with the lowest stability among all other metals e.g., lower than EDTA complex of Ca and Mg (major cations in culture medium) so at any concentration EDTA would not influence silver speciation and complex formation. In conclusion RAC, does not accept the argument that the ERV from Schlich *et al*. (2017) is unreliable and agrees with the DS that this should be used for classification.

It was also suggested by Industry during RAC's assessment that the chronic ERV generated using *Isonychia bicolor* (0.16 µg/L) (Diamond *et al*., 1990; included in Arijs *et al*., 2021) would be a more appropriate value than Schlich *et al*. (2017). RAC considers this value reliable with restrictions but as the lowest ERV value is to be compared with the CLP criteria, the chronic ERV for *I. bicolor* (0.16 µg/L) will not be used for classification.

- Note on the approach of the biocidal active substance applicant

Chronic aquatic toxicity studies are available for fish, invertebrates, and algae for soluble silver substances. The lowest reliable chronic value is an EC_{10} of 0.16 µg Ag/L for silver chloride toxicity to the algae *Nostoc muscorum* (Rai *et al*., 1990, non-GLP, non-guideline, test the 15 d EC_{10} (yield)

of AgCl to *N. muscorum* was determined to be 0.16 µg Ag/L.). This result confirms the chronic classification of this substance under the CLP Regulation. RAC agrees with the DS that this study warrants a reliability score of 3. Optical density was measured in cultures grown in control and test solutions after 15 day's incubation, static test ser up was used and only nominal silver concentrations are available, no data for dissolved silver is available.

- Probabilistic approach

In order to support the ERV values derived from the DS, RAC will present a brief summary of results from JRC dossier (draft) with Environmental Quality Standards (EQS) for silver. The acute and chronic freshwater datasets compiled in the JRC report include all available and reliable data until 2020 (the reliability evaluated using the CRED evaluation method and/or the in-house developed JRC Literature Evaluation Tool (LET) based on the CRED evaluation method). In case of multiple effect data for different water chemistries, the most sensitive data was used to derive reasonable worst-case AA (Annual Average) and MAC (Maximum Allowable Concentration) values. Finally, 12 species representing 10 taxonomic groups (salmonid fish, cyprinid fish, crustacean, two orders of insects (Diptera and Ephemeroptera), molluscs, higher plants, rotifer, cyanobacteria, and algae) were considered and RAC considers the dataset fulfils the requirements to perform a chronic SSD. Endpoint data were not normalized as a chronic BLM for silver is not available. The calculated HC_5 was 0.064 µg/L.

Due to differing study assessments (discussed below) in the study of Diamond *et al*. (1990), a second SSD evaluation was also performed in the JRC dossier, including the lowest chronic toxicity values derived for the three additional species, *H. azteca*, *Corbicula fluminea*, and *I. bicolor* available in Diamond *et al*. (1990). The calculated HC_5 was 0.084 µg/L. The study of Diamond *et al*. (1990) has been assessed several times prior to RAC, briefly: the in-house developed JRC Literature Evaluation Tool (LET) evaluated the sublethal toxicity values for *H. azteca* as *Reliable without restrictions* by both the European Precious Metals Federation (EPMF) and JRC while the studies for *C. fluminea* and *I. bicolor* were assessed as *Reliable with restrictions* by the JRC, and *Reliable without restrictions* by EPMF. In a former RIVM evaluation, the three values were assessed as *Reliable with restrictions* (RIVM, 2012). Based on the CRED evaluation, all studies were instead assessed as *Reliable with restrictions* by the JRC in a second re-evaluation.

According to the DS, the three sublethal toxicity tests could be classified as either *Reliable with restrictions* or *Not assignable*, due to the poor reporting. In particular, the following main shortcomings were identified:

- Silver concentrations were poorly reported and the dissolved NOEC values were extrapolated from mean % recovery rates, thus increasing the uncertainty in the results;
- A low number of replicates (n=2) was used in the *C. fluminea* and *I. bicolor* sublethal toxicity tests. The statistical power of the analyses is therefore expected to be low.
- The statistical analysis, and in particular the suitability of the pairwise comparison, was also questioned. Due to the lack of raw data, or additional information such as which of the tests mentioned in the paper were used for which species and endpoint, monotony of the response curve (except two graphs), homogeneity of variance, natural distribution etc., the appropriateness of the statistical analysis cannot be assessed.

In contrast, the general reporting and information in the publication has been considered sufficient for PNEC derivation purposes, despite a few main uncertainties being recognised, such as the low statistical power and extrapolation of NOECs from mean % recovery rates.

During RAC's assessment, Industry submitted a study by Arijs *et al*. (2021) which presents an HC_5 calculated using a new data set. RAC compared both data sets and found some differences:

- Rodgers *et al*. (1997a) *for D. magna* is accepted as reliable and included in data set of Arijs *et al*. (2021) with a value of 0.8 µg/L.
- Rodgers *et al*. (1997a) was ranked as unreliable in the JRC dataset and instead a value of 2.14 µg/L from Bianchini and Wood (2008) was used.
- Diamond *et al*. (1990) is accepted as reliable and included in data set of Arijs *et al*. (2021) – taxonomic group 12 with 15 species
- The SSD of JRC was calculated with and without the study of Diamond *et al*. (1990).
- Dethloff *et al*. (2007) was accepted as reliable by Arijs *et al*. (2021) and used in the calculation of the geometric mean (0.46 µg/L) for *O. mykiss*.
- The same study although accepted as reliable was not used in the JRC data set and instead a value of 0.17 µg/L from Davis *et al*. (1998) (REACH dossier) was used.

RAC considers Diamond *et al*. (1990) reliable and RAC does not consider the differences substantial and accepts the JRC report HC_5 value of 0.084 µg/L as reliable and suitable for classification, as the inclusion of the Diamond *et al*. (1990) study increases the number of taxonomic groups and robust data points included in SSD data set. This value is close to the same order as this obtained by deterministic approach – 0.1 µg/L.

RAC notes that the value from Schlich *et al*. (2017) (referenced as Mertens *et al*., 2019, the published version) is the lowest chronic value available in the Arijs *et al*. (2021) dataset as well as the JRC dataset.

Conclusion on ERV values for comparison with classification criteria

As RAC agrees with the DS regarding the approach using the lowest ERV values compared with highest dissolution, the lowest ERVs (highest toxicity) are selected for classification. RAC agrees with the DS regarding the particular ERVs to be used (below). The calculated HC_5 values, if normal distribution is accepted by using the ETx 2.3 model, are almost identical for both datasets: 0.084 µg/L (0.026–0.176 µg/L for JRC and 0.088 µg/L (0.029–0.18 µg/L) for Arijs *et al*. (2021). Industry argued that the best-fitting distribution (Rayleigh) resulted in HC_5 0.116 µg/L (0.065–0.23 µg/L) and this value should be accepted as ERV for chronic toxicity. However, as described above, RAC considers the JRC HC5 value as the most suitable, although only as supportive information given the conclusion to use the deterministic approach and the lowest ERV.

In conclusion, RAC agrees with the DS that the following ERV should be used for classification, and adds the HC5 in support if the chronic ERV:

- Acute aquatic toxicity: 0.00022 mg/L *D. magna*
- Chronic aquatic toxicity:
    - Deterministic : 0.00010 mg/L *P. subcapitata*
    - Probabilistic: 0.000084 mg/L, used as supportive data.

Forms of silver

The information on silver usage is taken from the REACH registration dossier and the Industry clarification document of July 2021 (silver metal CLH – clarifications to ECHA Dossier Manager (prepared by EPMF, 1 July 2021)) and October 2021 (Manufacturing and use of silver metal). Silver exists on the market as silver massive, silver powder and silver nanoparticles with all these forms being structurally identical and produced via dedicated procedures: atomisation /reduction. However, it needs to be established whether silver massive (≥ 1 mm) and silver powder (< 1 mm) warrant separate consideration for hazard assessment. According to information received from Industry, the total tonnage of Silver is approx. 10,000 tonnes/year, based on figures from 2017-2019. A general breakdown of silver uses in the EU is given in the table.

Table: *Uses of silver in EEA + Switzerland 2017-2019. From: clarifications to ECHA Dossier Manager (prepared by EPMF, 1 July 2021*

Use	**Description**
Electronics and electrical equipment	Electronics and electrical equipment used in consumer applications, industrial applications, automotive use, green energy (including solar and wind) and brazing and soldering applications
Aerospace and defence	Aerospace and defence industry, including uses that feed into aeroplanes, satellites, and defence applications (e.g., missiles and torpedoes). Some of these uses relate to electronics and electrical equipment used in the sector.
Medical devices	Medical devices and in-vitro diagnostic (IVD) medical devices. This too includes electronics uses, but these are not accounted for in the EEE.
Jewellery	Watches, necklaces, rings, and other ornamental objects that are intended to be worn
Tableware/silverware	Table service and tasting (e.g., cutlery) and decorative objects for use on the table and in homes (e.g., candle holders).
Cosmetics	Personal care products, which includes products often found in health and beauty shops and/or departments.
Other industrial uses	Biocides, diamond tools, investments, manufacture of other chemical substances (including manufacture of ethylene oxide), mirrors, surface treatment etc
Other consumer uses	Batteries, adhesive, silver threads (e.g., in conducting gloves) and toys (Note: no explicit evidence was noted of these uses with the EEA and Switzerland through the industry survey and interviews!)

Based on the information provided, it can be concluded that for silver metal manufactured in the EEA + Switzerland:

- 40.5% is exported (i.e., 10310 tonnes/year – 5942 tonnes/year),
- 15.5% is used for electronics & electricals, brazing alloys, and solder,
- 13.5% is used for investment,
- 10.5% is used for jewellery and tableware,
- 10.5% is used for photography, and
- 9.5% is used for other industrial fabrication (like cosmetics, medical devices, mirrors, surface treatment).

Based on a recent questionnaire to the EPMF membership and downstream users (DUs) of silver metal (including the major silver manufacturers in the EU), the reported amount of silver powder manufactured in the EU is 625 tonnes/year. It can be assumed that the total EU tonnage for silver powder is below 1000 tonnes/year (i.e., max 10% of the total tonnage of silver metal). Additionally, the total production of silver nanoparticles is below 3 tonnes/year. Consequently, the total tonnage of massive silver is approx. 9000 tonnes/year.

***Table**: Use of silver or silver-based alloys in the different use categories with an indication of powder release potential. From: clarifications to ECHA Dossier Manager (prepared by EPMF, 1 July 2021.*

Use	% of EU market	Expected form of silver used	Potential for release of particles > 1 mm of pure silver
Electronics, and electricals, brazing alloys, and solder	15.5	Silver (massive or powder) and Silver-based alloys	No release
Other industrial fabrication	9.5	Silver (massive or powder) and Silver-based alloys	No release of pure silver
Investment	13.5	Silver (massive)	No release
Jewellery and tableware	10.5	Silver-based alloys mainly, minor amount of Silver massive	No release pf pure silver. Any material is considered a UVCB
Photography	10.5	Silver massive	No release

Information from an Ind document of October 2021 (Annex 1_Ag manuf use vs powder_final_211021) indicates that particles < 1 mm might be released from the manufacturing/finishing of jewellery and tableware.

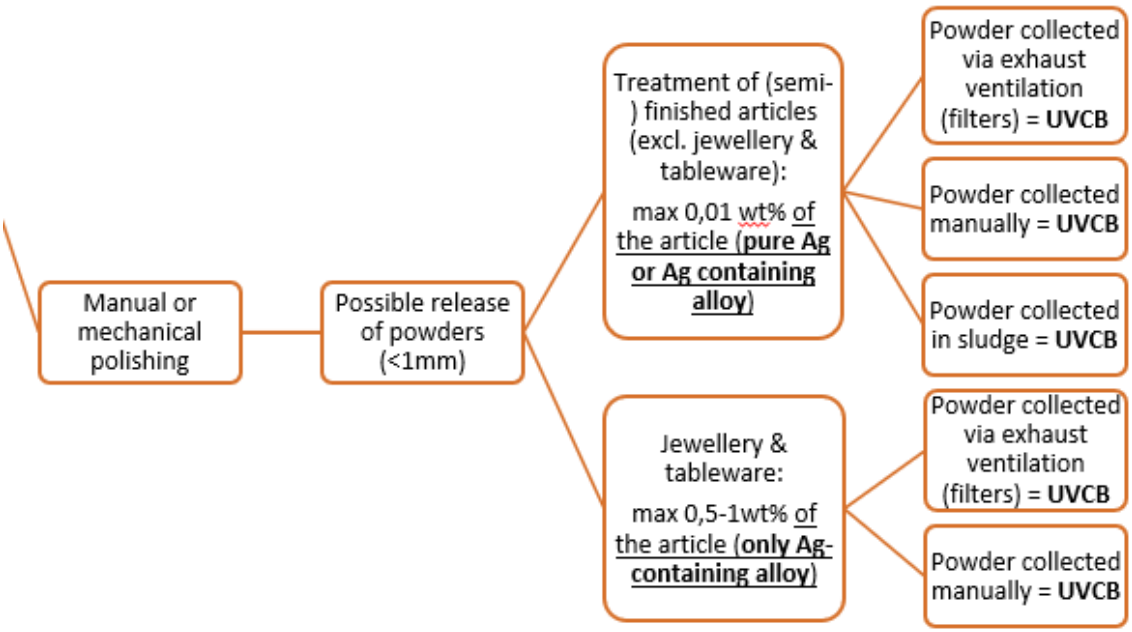


***Fig**: Possible release of particles <1 mm. From: Annex 1_Ag manuf use vs powder_final_211021.*

The only possible treatment with a potential for generation of silver particles < 1 mm, is polishing of (semi-) finished articles - processes of polishing (dry or wet), sanding or brushing might generate particles <1 mm. As declared by Industry Associations - losses via these processes are intentionally kept minimal, and material loss from these surface treatments are <0.01 wt% of the article. The collected material (either from the dry or wet processes) is never pure silver metal but always of unspecified and variable composition including e.g., sand, alloying elements, carrier material or polishing cloth next to silver metal. This implies that all these released and collected fractions are registered as UVCBs and not pure silver metal. These UVCBs subsequently undergo a stepwise treatment and refinement to recover the silver again. Most of the jewellery/tableware are being manufactured using silver-containing alloys (often silver-copper alloys (like Sterling silver) or silver-gold alloys) and not pure silver metal. Industry Associations

reported that based on the input received from representative DUs, maximally 10% of the jewellery/tableware is made of pure silver metal with the remaining >90% thus being Silver containing alloys. Pure silver metal is only applied via electrodeposition on a carrier as a thin layer with a thickness of 10-30 µm (plating use). As a general rule, silver plated articles do not need a polishing step after the electrodeposition. Polishing is only needed for jewellery/tableware made of silver-containing alloys, and that the released particles are thus never pure silver metal. Polishing is performed in (closed) chambers with exhaust ventilation and released particles are collected in the filters or by collecting the material manually.

Assessment of silver forms for classification and labelling

Following the decision scheme developed to interpret Section IV.5.5 of the CLP guidance (Fig. below), RAC considers the following options possible for massive metals.

Information submitted by Industry indicates that particles < 1 mm are not released from industrial processes and from the manufacturing/finishing of jewellery and tableware in any appreciable quantities. This appears to be < 0.01% of the articles being finished as a maximum. The available information indicates that this is exclusively in the form of silver alloys and that the particles < 1 mm produced form part of a UVCB type material. There is no documented process that indicates that (pure) silver particles < 1 mm are produced from any use or process, or that there is any reasonably expected use that would do so.

Following the scheme derived for the classification of massive forms (Fig. below), RAC considers the following general conclusions can be derived for a metal:

Option 1:

Particles < 1 mm are generally generated from a documented reasonable handling and use of massive silver are relevant for the classification and labelling of massive silver.

Option 1(a): Based on Annex IV.5.5 of the CLP guidance, any quantity of particles generated < 1 mm is relevant for hazard assessment of the massive form. Furthermore, it has been concluded that as particles < 1 mm fall into the same range as metal powders in Annex VI to CLP (i.e., < 1 mm diameter) both with regards to particle size and surface area, where such particles are generated basing classification of the massive metal on power is justified. Therefore, silver powder is suitable for classifying massive silver.

Option 1(b): As generated particles < 1 mm are relevant for hazard assessment of massive silver, data on the generated particles would be suitable for such an assessment.

Option 2:

Particles < 1 mm are not generated through any documented use or process, or from any documented reasonably expected handling and use of massive silver. Consequently, hazard assessment should be based on 1 mm particles or equivalent surface area.

Conclusion

RAC concludes that option 2 is appropriate for silver as although massive silver and silver powder are structurally identical materials, there is no evidence of particles < 1 mm being generated from any documented reasonable handling and uses of pure massive silver. Furthermore, as is shown below, silver powder has a higher release of silver ions leading to a more stringent classification. The consequent route to classification of the massive form is indicated in the figure below.

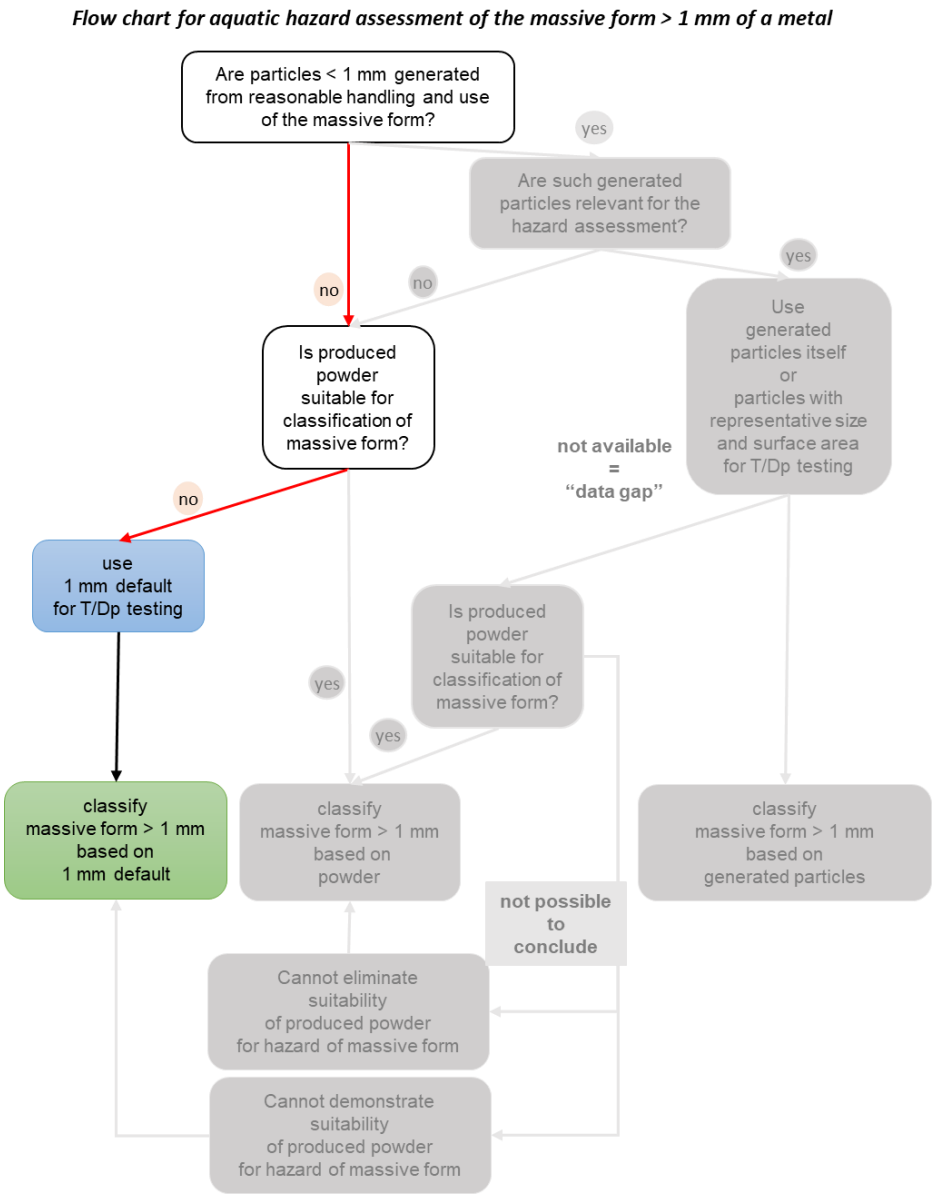


Figure: Decision tree for classification of massive metals indicating classification route when particles < 1 mm are not generally generated from documented reasonable handling and use of the massive metal.

Solubility of silver

Massive silver

Reliable data from transformation/Dissolution (T/Dp) test are available from one study.

Data for silver dissolution above the detection limit were obtained for 27 mg/L loading after 28 days. RAC accepts as a worse case highest result of 0.00011 mg/L from one replica vessel instead of average result of 0.00003±0.00007 mg/L from three replicate vessels. The derived equation in this case is: log10(CAg) = 0.7497 x log (SAL) – 1.8502. RAC considers that solubility is higher at pH 8 and, therefore, only T/Dp data at pH 8 is presented in the opinion. Data at other pH values can be found in the background document.

***Table**: Results from T/D test for massive silver at pH 8.*

Days	Loading (mg/L)	Surface area (mm^2/L)	Dissolved Silver conc (mg/L)
7	3, 9, and 27	1.7, 5.1, 15.5	<LOQ
28	(3, 9) and 27	(1.7, 5.1) 15.5	0.00011 for 27 mg/L loading. (No results obtained for the other loadings)
28	0.1 (calculated)*	0.572	$1.1x10^{-6}$

* - log10(CAg) = 0.7497 x log (SAL) – 1.8502

Silver powder/silver flakes

Reliable solubility data for silver powder are available from two studies: silver powder (D_{90}=11.2 µm, D_{50}=1.9 µm, and D_{10}=0.8 µm) and silver flakes (D_{90}=7.83 µm, D_{50}=2.61 µm, and D_{10}=1.07 µm).

***Table**: Results for dissolved Silver from dissolution studies (mg/L).*

Days	Silver powder, loading 1 mg/L, surface area $3x10^3$ mm^2/L		Silver flakes, loading 1 mg/L, surface area $1.17x10^3$ mm^2/L
	pH 6	pH 8	pH 6
7	0.00125	0.00255	0.00179
28	0.00355	0.00571	0.00360

Silver nanoparticles

One T/D study is available for silver ions release from silver nanoparticles. Solubility of silver nanoparticles was tested in one study = dissolution of coated silver nanoparticles (D_{75}=9 nm, D_{50}=8 nm, and D_{25}=7 nm). The tested silver nanoparticles are in the form of aqueous suspension, the composition of the coating is unknown.

Table: *Dissolution of silver from silver nano particles.*

Days	Conventional dissolved (450 nm pore size) – silver nanoparticles + soluble silver species	Truly dissolved (3x1kDa) 1.3 nm pore size soluble silver species (mg/L)
Daphnia magna, OECD TG 211; chloride free, no EDTA, Initial 1.153 mg/L, pH 8.1; silver nanoparticles size (8±1) nm		
7	Results difficult to interpreted. Strong aggregation of nanoparticles due to reactions with medium components, almost in the beginning of T/D study. The particles of reduced elemental silver precipitated and adsorbed on the walls of the vessel.	
28		
Algae, OECD TG 201; chloride free, 50% EDTA; pH 7.4 Initial 1.230 mg/L, pH 7.4; silver nanoparticles size (8±1) nm		
7	0.974	0.125
28	0.912	0.214

Silver nanoparticles are characterized with high chemical activity, reactions with components of testing media are expectable. These reactions would be more pronounced for *D. magna* medium – so far reactions of silver nanoparticles with thiamine, vitamin B12 are described in the open literature. In alga medium silver nanoparticles exist until the end of T/D study, the aggregated fraction is about 38% from total silver until the end of study (sorbed on the walls of vessel). DLS data for algae medium showed main particle sizes between 2.2 and 8.1 nm (mean 3.6 nm ± 1; median 3.5 nm) for the <10 nm fraction and between 16-36.8 nm (mean 24.7 nm ± 3.2; median 25.3) for the 10-40 nm fraction. About 90% of the particles was present in the latter fraction. RAC noted that after filtration through 0.45 µm all silver nanoparticles will pass in the resulting solution.

The conventional dissolved fraction is mixture of silver nanoparticles and soluble silver species, filtration through 1 kDa membrane ensures real separation between silver nanoparticles and ionic silver species.

From the viewpoint of aquatic toxicity studies, it is already clear that silver nanoparticles are bioavailable and may enter into cells by passive transport and may even be transferred across generations. The conventional dissolved fraction (operationally defined as filtered through 0.45 µm filter) is accepted in all aquatic toxicity studies. In the case of silver nanoparticles, this fraction contains nanoparticles + silver ions. True dissolved fraction contains only silver ions. It is accepted that silver ions are bioavailable and responsible for silver toxicity, but silver nanoparticles are bioavailable as they entered into the body of aquatic organisms and in RAC's opinion the conventional dissolved fraction should be used to derive final classification. RAC agrees with the DS that silver nanoparticles behave as soluble silver salts and should be classified as in the same manner (i.e., by comparing the ERVs directly with the criteria in the CLP guidance table IV.1). It is worth mentioning that in the open literature several studies have indicated higher toxicity of silver nanoparticles toward some organisms (Abramenko *et al*., 2018; Li *et al*., 2015; Pakrashi *et al*., 2017).

***Table**: Summary of data from Transformation/Dissolution studies.*

Days	Loading (mg/L)	Surface area (mm^2/L)	Dissolved Ag conc (mg/L)
Massive			
7	1		<LOQ
28	1	5.71	0.000011
28	0.1 (calculated)	0.571	0.0000011
Powder - pH 8			
7	1	3.10^3	0.00255
28	1	3.10^3	0.00571
28	0.1 (calculated)	3.10^2	0.00106
Silver nanoparticles			
7	1	accept as soluble salt and classify based on ERV value.	
28	0.1		
7 (alga)	1		0.974 (conventional dissolved)
28 (alga)	0.1 (calculated)	13300	0.017

Note: Loadings at 0.1 mg/L are calculated using the derived equation: log10(CAg) = 0.7497 x log (SAL) – 1.8502

Classification outcomes

Silver exists on the market as silver massive, silver powder and silver nanoparticles. All these forms are structurally identical but are produced via dedicated process/procedures:

Massive - smelting

Powder - atomisation/reduction.

Nano – reduction of silver salts

The ERV values to be used for deriving the classification are:

- Acute aquatic toxicity: 0.00022 mg/L *D. magna*
- Chronic aquatic toxicity: 0.00010 mg/L *P. subcapitata*

Note: Although pH banding has not been used and the lowest ERV value is compared with the highest T/Dp values, the ERV values were derived at approximately pH 8, which coincides with the pH of highest dissolution (pH 8).

Massive silver

Information submitted by Industry indicates that particles < 1 mm of pure silver are not released from industrial processes and from the manufacturing/finishing of jewellery and tableware in any appreciable quantities. This appears to be 0.01% of the article being finished as a maximum. According to the available information, RAC understand this to be in the form of silver alloys.

As particles < 1 mm are not generally generated from any documented reasonable use of pure massive silver, from the available options for making a hazard assessment of a massive metal presented above, RAC concludes that option 2 should be followed. Furthermore, although silver massive and powder are structurally the same material, the powder gives rise to higher dissolution and a more stringent classification. Consequently, RAC disagrees with the DS and concludes that massive silver should be assessed independently. As such, T/Dp data on the default 1 mm particle (or equivalent specific surface area) should be used for the classification of massive silver.

- *Acute Aquatic Hazard*

As the T/Dp value after 7 days at 1 mg/L loading (< LOQ) is below the acute ERV (0.00022 mg/L), no classification is warranted.

- *Chronic Aquatic Hazard*

As the T/Dp value after 28 days at 1 mg/L loading (0.000011 mg/L) is below the Chronic ERV (0.00010 mg/L), no classification is warranted.

Silver powder

RAC accepts that the silver power data is representative of the smallest form available on the market, besides nanoforms of silver which are considered separately.

- *Acute Aquatic Hazard*

In this case, conditions for ideal comparison are met as the pH values at which maximum solubility of silver powder/silver flakes was reached coincide with pH values at which most sensitive endpoint is obtained. The ERV value is much below the determined solubility concentration silver powder/silver flakes are classified as Aquatic Acute 1 with an M - factor of 10, as 0.00255/0.00022 = 11.59.

- *Chronic Aquatic Hazard*

Data for loading 0.1 mg/L are calculated using the derived equation. For a worst-case surface area for the silver particles used of 300 mm2/L, silver dissolution of 0.00106 mg/L at pH 8. As this T/Dp value is above the chronic ERV (0.00010 mg/L), classification as Aquatic Chronic 1 is warranted. Furthermore, as 0.00106/0.0001 = 10.6, an M-factor of 10 is warranted. This classification is in agreement with the DS albeit based on recalculated T/Dp values.

In conclusion, RAC agrees with the DS that silver powder (> 100 nm < 1 mm) warrants classification as:

Aquatic Acute 1 (H400), M = 10

Aquatic Chronic 1 (H410), M = 10

Silver nanoparticles

RAC concludes that due to the physical properties that distinguish nano forms from other forms of silver, nano silver should be considered separately from massive and powder for hazard assessment. Following RAC's conclusion that that silver nanoparticles behave as soluble salts and are fully bioavailable, the ERV values for silver should be compared directly with the criteria in the CLP guidance Annex IV (the same as the CLP reg. part 4).

- *Acute aquatic toxicity*

As the ERV is below 1 mg/L, classification as Aquatic Acute 1 is warranted. As this value is in the range $0.0001 < L(E)_{50} \leq 0.001$, M = 1000.

- *Aquatic Chronic toxicity*

As silver is not rapidly transformed to non-bioavailable forms and as the chronic ERV is below 0.1 mg/L, classification as Aquatic Acute 1 is warranted. As this value is in the range $0.00001 < E_rC_{10} \leq 0.0001$[4], M = 1000.

In conclusion, nano silver (≥ *1 nm* ≤ *100 nm*) warrants classification as:

Aquatic Acute 1 (H400), M = 1000

Aquatic Chronic 1 (H410), M = 1000

This is essentially in agreement with the DS, although the chronic M-factor is modified due to the clarification on the derivation of M-factors in Table IV.1 of the CLP guidance (see footnote 2).

In the absence of any specific guidance for nano forms, the classification based on the T/D study using algae medium is also presented for information, but this is not supported by RAC.

Aquatic Acute 1, M = 1000

Aquatic Chronic 1, M = 100

(Acute and Chornic ERVs below dissolution at 1 and 0.1 mg/L, respectively. Acute M-factor = 0.974/0.00022 = 4427, so M=1000. Chronic M-factor = 0.017/0.0001 = 170, so M=100).

In conclusion, RAC considers that **silver warrants classification as:**

Silver massive: [particle diameter ⩾ 1 mm]

- **Aquatic Acute - No classification**
- **Aquatic Chronic – No classification**

[4] *RAC notes that Table IV.1 in Annex IV of the Guidance on the Application of the CLP criteria (V.5 2017) contains an error in that the upper limits of each concentration band should contain an '≤' instead of '<', resembling table 4.1.3 of CLP. This has been corrected in GHS and will be corrected in the CLP guidance.*

Silver powder: [particle diameter > 100 nm < 1 mm]

- **Aquatic Acute 1 (H400), M = 10**
- **Aquatic Chronic 1 (H410), M = 10**

Silver nano: [particle diameter ⩾ 1 nm ⩽ 100 nm]

- **Aquatic Acute 1 (H400), M = 1000**
- **Aquatic Chronic 1 (H410), M = 1000**

Alternative classification outcomes

If particles > 1 mm were considered to be generated from reasonably expected use of massive silver, then following the derived scheme for classification of massive forms, RAC notes that different classification conclusions for massive and powder would apply. However, as no such particle are considered to be generated, these outcomes are not supported by RAC and **are included for information only**.

Option 1(a)

RAC could conclude that particles < 1 mm generated from the reasonable handling and use of massive silver are relevant for the hazard assessment of massive silver. Furthermore, as the generated particles are < 1 mm and will have a surface area great than 1 mm particles, classifying massive silver using data on silver powder is justified.

The most sensitive species in the acute toxicity data set is *D. magna* with a LC_{50} of 0.22 µg/L for mortality; test performed at pH 8.2 (Bianchini, 2002).

In this case, conditions for ideal comparison are met. The pH values at which maximum solubility of silver powder/silver flakes was reached coincide with pH values at which most sensitive endpoint is obtained. The ERV value is much below the determined solubility concentration silver powder/silver flakes are classified as Aquatic Acute 1 with an M - factor of 10, as 2.55/0.22 = 11.59.

Data for loading 0.1 mg/L are calculated using the derived equation. For a worst-case surface area for the silver particles used of 300 mm2/L, silver dissolution of 0.58 µg/L at pH 8. In the chronic aquatic data set the most sensitive species is an alga (*P. subcapitata*) with an E_rC_{10} of 0.1 µg/L achieved at the pH values 7.94 and 9.37. As the T/Dp value is above the chronic ERV (0.1 ug/L) classification as Aquatic Chronic 1 is warranted. As 1.06/0.1 = 10.6, an M-factor of 10 is warranted.

In conclusion silver (> 100 nm) warrants classification as:

Aquatic Acute 1 (H400), M = 10

Aquatic Chronic 1 (H410), M = 10

Option 1(b)

Massive silver

RAC could conclude that particles < 1 mm generated from the reasonable handling and use of massive silver are relevant for the hazard assessment of massive silver. Therefore, massive and silver powder are considered separately for hazard assessment. However, data on generated particles is not available.

Additional references

HUMAN HEALTH

Anon (1993). Silver powder CAP 9: Acute oral toxicity (limit test) in the rat. Project No. 52/40

Anon., *et al*., (2021) Silver Acetate, Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Single and Repeat Administration. Labcorp Study Number: CC71MP.

Boudreau *et al.* (2016). Differential Effects of Silver Nanoparticles and Silver Ions on Tissue Accumulation, Distribution, and Toxicity in the Sprague Dawley Rat Following Daily Oral Gavage Administration for 13 Weeks. Tox. Sciences, 150(1); 131-160.

Bruna T, Maldonado-Bravo F, Jara P, Caro N. (2021). Silver Nanoparticles and Their Antibacterial Applications. International journal of molecular sciences, 22(13), 7202.

Buehler EV. (1994). Occlusive patch method for skin sensitization in guinea pigs: the Buehler method. Food Chem Toxicol. 32(2):97-101.

Charehsaz, M. *et al*., (2016). Effects of developmental exposure to silver in ionic and nanoparticle form: A study in rats. Journal of Pharmaceutical Sciences 24:24.

Davenport LL, Hsieh H, Eppert BL *et al.* (2015). Systemic and behavioural effects of intranasal administration of silver nanoparticles. Neurotoxicol Teratol 51:68–76.

Dietl HW, Anzil AP, Mehraein P. (1984). Brain involvement in generalized argyria. Clin Neuropathol. 3(1):32-6. PMID: 6705320.

Drake & Hazelwood (2005). Exposure-Related Health Effects of Silver and Silver Compounds: A Review. Ann. occup. Hyg., Vol. 49, No. 7, pp. 575–585.

Dziendzikowska K, Węsierska M, Gromadzka-Ostrowska J, Wilczak J, Oczkowski M, Męczyńska-Wielgosz S, Kruszewski M. (2021). Silver Nanoparticles Impair Cognitive Functions and Modify the Hippocampal Level of Neurotransmitters in a Coating-Dependent Manner. Int J Mol Sci. 22(23):12706. doi: 10.3390/ijms222312706. PMID: 34884506.

Dziendzikowska K, Wilczak J, Grodzicki W, Gromadzka-Ostrowska J, Węsierska M, Kruszewski M. (2022). Coating-Dependent Neurotoxicity of Silver Nanoparticles -An In Vivo Study on Hippocampal Oxidative Stress and Neurosteroids. Int J Mol Sci. 23(3):1365. doi: 10.3390/ijms23031365. PMID: 35163290.

Ferdous & Nemmar (2020). Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure. *Int. J. Mol. Sci*., 21, 2375. http://dx.doi.org/10.3390/ijms21072375.

Furst A. (1981). Bioassay of metals for carcinogenesis: whole animals. Environmental health perspectives, 40, 83–91.

Ganjuri M, Moshtaghian J, Ghaedi K. (2015): Effect of Nanosilver Particles on Procaspase-3 Expression in Newborn Rat Brain. Cell J. 17(3): 489-493

Giampietro *et al*. (2018). The Pivotal Role of Copper in Neurodegeneration: A New Strategy for the Therapy of Neurodegenerative Disorders. Mol. Pharmaceutics, 15, 3, 808–820

Gromadzka-Ostrowska *et al*. (2012). Silver nanoparticles effects on epididymal sperm in rats. Toxicology Letters 214: 251– 258. http://dx.doi.org/10.1016/j.toxlet.2012.08.028.

Hadrup N, Loeschner K, Mortensen A, Sharma AK, Qvortrup K, Larsen EH, Lam HR. (2012). The similar neurotoxic effects of nanoparticulate and ionic silver in vivo and in vitro. Neurotoxicology. 33(3):416-23. doi: 10.1016/j.neuro.2012.04.008. Epub 2012 Apr 15. PMID: 22531227.

Juling S, *et al*. (2016). In vivo distribution of nanosilver in the rat: The role of ions and de novo-formed secondary particles, Food and Chemical Toxicology, 97: 327-335.

Khan AM, Korzeniowska B, Gorshkov V, Tahir M, Schrøder H, Skytte L, Rasmussen KL, Khandige S, Møller-Jensen J, Kjeldsen F. (2019). Silver nanoparticle-induced expression of proteins related to oxidative stress and neurodegeneration in an in vitro human blood-brain barrier model. Nanotoxicology. 13(2):221-239. doi: 10.1080/17435390.2018.1540728. Epub 2019 Jan 9. PMID: 30623748.

Lansdown, A B G. (2007). Critical observations on the neurotoxicity of silver. Crit Rev Toxicol. 37(3):237-50. doi: 10.1080/10408440601177665.

Lison D, Ambroise J, Leinardi R. *et al*. (2021). Systemic effects and impact on the gut microbiota upon subacute oral exposure to silver acetate in rats. Arch Toxicol 95, 1251–1266. https://doi.org/10.1007/s00204-021-02998-1

Liu Y, Guan W, Ren G, Yang Z. (2012). The possible mechanism of silver nanoparticle impact on hippocampal synaptic plasticity and spatial cognition in rats. Toxicology Letters 209; 227-231.

Liu *et al*., (2022) Administration of Silver Nasal Spray Leads to Nanoparticle Accumulation in Rat Brain Tissues. Environmental Science & Technology 56(1), 403-413.Mota & Dinis-Oliveira (2021). Clinical and Forensic Aspects of the Different Subtypes of Argyria. J. Clin. Med. 10, 2086. https://doi.org/10.3390/jcm10102086 .

Narciso *et al*., (2020) Genotoxicity, biodistribution and toxic effects of silver nanoparticles after in vivo acute oral administration. NanoImpact. 18. 100221.10.1016/j.impact.2020.100221.

Oppenheimer BS, Oppenheimer ET, Danishefsky I, Stout AP. (1956). Carcinogenic effect of metals in rodents. Cancer Res. Jun;16(5):439–441

Pem B. *et al*., (2021). Fate and transformation of silver nanoparticles in different biological conditions. Beilstein journal of nanotechnology, 12, 665–679. https://doi.org/10.3762/ bjnano.12.53

Price CJ and George JD. (2002). Final study report on the developmental toxicity evaluation for silver acetate770 (CAS No. 563-63-3) administered by gavage to Sprague-Dawley (CDN) rats on gestational days 6 through 19. NTIS Technical Report (NTIS/PB2002-109208).

Ritz HL. and Buehler EA. (1980) Planning, conduct, and interpretation of guinea pig contact sensitization. In: Drill VA, Lazar P (eds) Current concepts in cutaneous toxicity. Academic Press, New York, pp 25–40.

Rungby J and Danscher G. (1984) Hypoactivity in silver exposed mice. Acta Pharmacol Toxicol, 55(5): 398-401.

Sharma A, Muresanu DF, Patnaik R *et al*. (2013). Size- and age-dependent neurotoxicity of engineered metal nanoparticles in rats. Mol Neurobiol 48:386–396.

Shavlovski *et al*. (1995). Embryotoxicity of silver ions is diminished by ceruloplasmin: further evidence for its role in copper transport. BioMetals (8): 122-128.

Skalska J, Strużyńska L. (2015). Toxic effects of silver nanoparticles in mammals--does a risk of neurotoxicity exist? Folia Neuropathol. 53(4):281-300. doi: 10.5114/fn.2015.56543. PMID: 26785363.

Skalska J, Dąbrowska-Bouta B, Frontczak-Baniewicz M, Sulkowski G, Strużyńska L. (2020). A Low Dose of Nanoparticulate Silver Induces Mitochondrial Dysfunction and Autophagy in Adult Rat Brain. Neurotox Res. 38(3):650-664. doi: 10.1007/s12640-020-00239-4. Epub 2020 Jun 25. PMID: 32588355.

Skebo JE, Grabinski CM, Schrand AM, Schlager JJ, and Hussain SM. (2007). Assessment of metal nanoparticle agglomeration, uptake, and interaction using high-illuminating system. Int J Toxicol 26(2):135–141. https://doi.org/10.1080/10915810701226248

Spisni *et al*. (2009). Effect of copper on extracellular levels of key pro-inflammatory molecules in hypothalamic GN11and primary neurons. Neurotoxicology;30(4):605–12.

Sprando RL, Black T, Keltner Z, *et al*. (2017). Silver acetate exposure: Effects on reproduction and postnatal development. Food & Chemical Toxicology 106:547-557.

Strużyńska L, Skalska J. (2018). Mechanisms Underlying Neurotoxicity of Silver Nanoparticles. Adv Exp Med Biol. 1048:227-250. doi: 10.1007/978-3-319-72041-8_14.

Sycheva LP, Murav'eva LV, Zhurkov VS. *et al.* (2016). Study of cytogenetic and cytotoxic effects of nanosilver and silver sulfate in germ cells of mice in vivo. Nanotechnol Russia 11, 256–262. https://doi.org/10.1134/S1995078016020191.

Van der Zande *et al*. (2012). Distribution, Elimination, and Toxicity of Silver Nanoparticles and Silver Ions in Rats after 28-Day Oral Exposure. ACS Nano vol 6, no. 8, 7427-7442.

Wu J. *et al*., (2015). Effects of prenatal exposure to silver nanoparticles on spatial cognition and hippocampal neurodevelopment in rats. Environmental Research 138; 67-73.

Xu L, Shao A, Zhao Y *et al*. (2015). Neurotoxicity of silver nanoparticles in rat brain after intragastric exposure. J Nanosci Nanotechnol 15:4215–4223.Yin N, Zhang Y, Yun Z, Liu Q, Qu G, Zhou Q, Hu L, Jiang G. (2015). Silver nanoparticle exposure induces rat motor dysfunction through decrease in expression of calcium channel protein in cerebellum. Toxicol Lett. 237:112–120.

Zhang *et al*. (2016). Silver nanoparticle-mediated cellular responses in various cell lines: An in vitro model. Int. J. Mol. Sci. 17: 1603.

ENVIRONMENT

Abramenko NB, Demidova TB, Abkhalimov EV, Ershov BG, Krysanov EY and Kustov LM. (2018). Ecotoxicity of different-shaped silver nanoparticles: case of zebrafish embryos. *J. Hazard. Mater.* 347, 89–94. doi: 10.1016/j.jhazmat.2017.12.060.

Annex 1_Ag manuf use vs powder_final_211021

Annex 2_Ag Env Transformation _final_212021

Arijs K, Nys C, Van Sprang P, De Schamphelaere K, and Mertens J. (2021). Setting a protective threshold value for silver toward freshwater organisms. Environmental Toxicology and Chemistry—Volume 40, Number 6—pp. 1678–1693

Bertine KK, Goldberg ED. Trace elements in clams, mussels and shrimp. (1972), Limnology and oceanography, 17, 877-884 https://doi.org/10.4319/lo.1972.17.6.0877

Bielmyer GK, Brix KV, Grosell M. (2008). Is Cl− protection against silver toxicity due to chemical speciation? Aquatic Toxicology 87 81–87

Colman BP, Espinasse B, Richardson CJ, Matson CW, Lowry GV, Hunt DE, Wiesner MR and Bernhardt ES. (2014). Emerging contaminant or an old toxin in disguise? Silver nanoparticle impacts on ecosystems. Environ Sci Technol 48, 9, 5229-5236.

Di Toro DM and Paquin PR. (2008). Silver Biotic Ligand Model (BLM): Refinement of an Acute BLM for Silver, Phase 2. The International Water Association (IWA) publishing. WERF Report 99-ECO-1-2T

EPMF cover letter to ECHA secretariat. RAC_ENV 201117

Erickson RJ, Larry T, Brooke LT, Kahl MD, Venter FV, Harting SI, Markee TP, Spehar PR. (1998). Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. Environmental Toxicology and Chemistry 17, 572–578.

Jiang HS, Yin L, Ren NN, Xian L, Zhao S, Li W and Gontero B. (2017). The effect of chronic silver nanoparticles on aquatic system in microcosms. Environmental Pollution 223, 395-402.

Kramer JR, Bell RA, Smith DS. (2007). Determination of sulfide ligands and association with natural organic matter. Applied Geochemistry 22, 1606-1611.

Li L, Wu H, Ji C, Van Gestel CA, Allen HE, and Peijnenburg WJ. (2015). A metabolomic study on the responses of daphnia magna exposed to silver nitrate and coated silver nanoparticles. *Ecotoxicol. Environ. Saf.* 119, 66–73. doi: 10.1016/j.ecoenv.2015.05.005

Luoma S. (1994). Fate, bioavailability and toxicity of silver in estuarine environments. In: Andren A, Bober T, eds. Transport, fate and effects of silver in the environment. Proceedings of the 2nd international conference. 11–14 September 1994. Madison, WI, University of Wisconsin Sea Grant Institute, pp. 151–155

Matson CW, Bone AJ, Auffan M, Lindberg TT., Arnold MC, Hsu-Kim H, Wiesner M R, Di Giulio RT. (2016). Silver toxicity across salinity gradients: the role of dissolved silver chloride species (AgClx) in Atlantic killifish (*Fundulus heteroclitus*) and medaka (*Oryzias latipes*) early life-stage toxicity, Ecotoxicology, 25,1105–1118

Mertens J, Oorts K, Leverett D, and Arijs K. (2019) Effects of silver nitrate are a conservative estimate for the effects of silver nanoparticles on algae growth and Daphnia magna reproduction. Environ Toxicol Chem 38:1701–1713. Published version of Schlich *et al*. (2017).

Nijs (2021). Influence of Environmental relevant concentrations of DOC and sulfide on Ag bioavailability and toxicity, Arche Consulting. Unpublished report.

one-pager bioaccumulation_final

Pakrashi S, Tan C, and Wang WX. (2017). Bioaccumulation-based silver nanoparticle toxicity in *Daphnia magna* and maternal impacts. *Environ. Toxicol. Chem*. 36, 3359–3366. doi: 10.1002/etc.3917

Terhaar C, Ewell WS, Dziuba WS, White WW, Murphy PJ. (1977). A laboratory model for evaluating the behavior of heavy metals in an aquatic environment. Water Research, 11, 101-110, https://doi.org/10.1016/0043-1354(77)90190-7

Whitlow S. and Rice D. (1985). Silver complexation in river waters of central New York. Water Research, 19, 619-626. https://doi.org/10.1016/0043-1354(85)90068-5

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).

Annex 3 Records of the targeted consultation following the submission of a preliminary summary of an in-vivo comparative Toxicokinetic Study on four Silver (Ag) forms

Annex 4 Records of the targeted consultation following the submission of a final comparative toxicokinetic study as well as a 90-day repeat dose toxicity study, a EOGRTS and its preliminary study on silver acetate