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The applicability of the GHS classification criteria to nanomaterials

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The applicability of the GHS classification criteria to nanomaterials

Nordic Chemical Group,
Nordic Council of Ministers

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Prepared for Nordic Chemical Group,
Nordic Council of Ministers

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Executive summary

The current GHS classification criteria have been developed for conventional chemicals, however, the physicochemical and biological properties of nanomaterials may be different from conventional chemicals. Therefore, the aim of this project was to review the applicability of the GHS to manufactured nanomaterials taking into account the progress of international scientific work. In the recent years much data on nanomaterials have been generated and compiled in the nanomaterial testing program under the OECD Working Party on Manufactured Nanomaterials (OECD/WPMN). In this project these data were further assessed for some pre-selected nanomaterials. Additionally, the appropriateness of the GHS classification criteria for the generated data was evaluated for five health hazard classes for which an initial screening had shown a need for classification. Finally, if applicable, relevant classifications of the nanomaterials were assessed.

The project focused on four pre-selected nanomaterials. The criterion for the selection was that the four nanomaterials should represent differences with respect to chemical composition, shapes, water solubility, specific surface area, density. Based on an initial screening of the available data it was agreed to focus on specific hazard classes for each nanomaterial. The nanomaterials and the selected hazard classes were:

SWCNT:	*Acute toxicity, Eye irritation, STOT RE, Germ cell mutagenicity*
Nano silicon dioxide:	*Acute toxicity, STOT RE*
Nano silver:	*Acute toxicity, Skin sensitisation; STOT RE*
Nano zinc oxide:	*Acute toxicity, STOT RE*

The data from the OECD/WPMN dossiers were compiled together with other available data from the NanoReg project (EU 7th framework), the NanoSafety Cluster projects, relevant REACH registrations of the substances or available new information on these nanomaterials obtained from a focused web-based literature search.

For each of the relevant hazard classes the available test data of the nanomaterials were summarised and evaluated with respect to:

- *Applicability of the test methods*
- *Applicability of the GHS criteria and proposed classification*
- *Identified data gaps and uncertainties*
- *Need for revision of GHS criteria or further guidance*

Based on these evaluations it could be concluded that in general the GHS classification criteria is considered applicable for the data on the selected nanomaterials as indicated in the following table:

Applicability of the GHS classification citeria				
Hazard class	**SWCNT**	**Nano Silicon dioxide**	**Nano Silver**	**Nano zinc oxide**
Acute oral toxicity	++ Testing limitations	++	++	++
Acute dermal toxicity	++ Testing limitations	++	++	++
Acute inhalation toxicity	0	++ Testing limitations	0	++ Testing limitations
Eye damage/irritation	- *in vivo* test data +/++ *in vitro* test data	NA	NA	NA
Skin sensitisation	NA	NA	++ *in vivo* 0 *in vitro*	NA
STOT RE oral exposure	++ Testing limitations	++	++	0
STOT RE dermal exposure	NA	NA	NA	++
STOT RE inhalation	++ Testing limitations	++	++	++
Germ cell mutagenicity	++	NA	NA	NA

- : *not applicable* ***+:*** *applicable with limitations* ***++:*** *fully applicable* ***NA:*** *not assessed* ***0****: no assessment due to lack of data*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

However, it is noted that for voluminous nanomaterials (i.e. with relatively high specific surface areas and low pour densities) it may not be technically feasible to test up to dose levels that correspond to the less severe hazard categories for acute toxicity and STOT RE.

Based on evaluation of the data of each selected nanomaterial the following classifications were concluded as relevant for at least some type/qualities of the nanomaterials:

SWCNT: *Eye Irrit.2 H319; STOT RE 1 H372 (inhalation, lung); Muta. 2 H341*
Nano silicon dioxide: *STOT RE 1 H372 (inhalation, lung) or RE 2 H373 (inhalation, lung);*
Nano silver: *STOT RE 1 H372 (inhalation, lung)* may be considered
Nano zinc oxide: *not sufficient data for classification*

From the experience gained by this project some overall general findings/aspects can be highlighted:

a. In general, the current GHS classification criteria for the five evaluated hazard classes were found to be applicable to the generated data on SWCNT, nano silicon dioxide, nano silver and nano zinc oxide.

b. Differences in toxicity exist between the various types/qualities (e.g. related to production methods (e.g. silicon dioxide) or impurity profile (e.g. SWCNT) of the same nanomaterials which may result in different classifications of the various types/qualities.

c. STOT RE is considered a highly relevant hazard class to examine for all the nanomaterials especially considering the lung as the target organ.

d. For the voluminous nanomaterials (i.e. with a relatively high specifc surface area and low density) testing at high dose levels may not be technically achievable. Hence, testing in accordance with OECD TG method covering all relevant dose levels for acute toxicity classification and STOT RE classification according to the GHS criteria values may not be possible. This is especially relevant for testing via inhalation route.

e. For acute toxicity and STOT RE the GHS criteria based on a mass-based dose metric can be applied for voluminous nanomaterials, however, the dose levels corresponding to the less severe hazard categories cannot be technically achieved. It may be examined whether another dose metric (e.g. specifc surface area or particle number concentrations) would be a better metric for enabling differentiation in toxicity and the classification of nanomaterials.

f. It is noted that most testing regarding repeated inhalation exposure has focused on identification of NOAEC/LOAEC levels and the examination of early signs of toxicity (e.g. various inflammatory markers) rather than establishing data for STOT RE classification. So mostly very low exposure levels compared to the STOT RE criteria have been used. Thus, there are data gaps for assessing the proper STOT RE classification of the nanomaterials.

g. As support for a STOT RE classification it should be considered how to use an AOP or MOA approach for inflammatory signs/ markers or mild/ moderate histopathological effects induced in target organs at very low exposure levels for classification purpose.

h. Also, it may be examined how and under which circumstances data from e.g. intratracheal instillation or pharyngeal aspiration may be used as support for STOT RE classification if data from inhalation testing are limited or do not cover the relevant dose ranges for classification.

1. Background and objective

The United Nations' Globally Harmonised System of Classification and Labelling of Chemicals (GHS) provides a harmonised basis for globally uniform physical, environmental, and health and safety information on hazardous substances and mixtures. It sets up criteria for the classification of substances and mixtures for physical, health, and environmental hazards. GHS was adopted by the United Nations in 2002 and is periodically updated. The GHS has been implemented in the EU by Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (the 'CLP Regulation').

The current GHS classification criteria have been developed for conventional chemicals, however, the physicochemical and biological properties of nanomaterials may be different from their corresponding bulk chemicals. At the UN level, the GHS Sub-Committee therefore agreed to include "Nanomaterials" to its programme of work since 2013 to review the applicability of the GHS classification criteria to manufactured nanomaterials taking into account the progress of international scientific work.

In the recent years much data on nanomaterials have been generated and compiled in the nanomaterial testing programme under the OECD Working Party on Manufactured Nanomaterials. Thus, the aim of the current project was to further assess these data for some pre-selected nanomaterials and to evaluate the appropriateness of the GHS classification criteria for the generated data and to evaluate relevant classifications of the nanomaterials. The outcome of the project may be a relevant contribution to the ongoing regulatory work and further development of the legislation at UN and EU level.

This project was initiated by the Nordic Classification Group and funded by the Nordic Council of Ministers and co-founded by TUKES (Finnish safety and chemicals agency).
The project has been performed by Department of Environment and Toxicology, DHI A/S, Denmark. DHI A/S is fully responsible for the assessement of data and the conclusions made in this project report, and thus the views expressed cannot be taken as the views of the competent authorities in the Nordic Countries.

2. Methodology

Together with the Nordic Classification Group the project group agreed to focus on four pre-selected nanomaterials. The criterion for the selection was that the four nanomaterials should represent differences with respect to chemical composition, shapes, water solubility, specific surface area and density. Thus, the following nanomaterials were chosen:

- *Single wall carbon nanotubes (SWCNTs);*
  biopersistent nanofibres with high specific surface area

- *Nano silicon dioxide*
  nanoparticles, metal oxide with a relatively high water solubility and high specific surface area

- *Nano silver*
  nanoparticles, pure elemental metallic substance with high density

- *Nano zinc oxide*
  nanoparticles, metal oxide with a relatively low specific surface area

It was agreed only to focus on hazard identification for human health and to identify and select the most relevant hazard classes for classification of each of the nanomaterials, before a more in-depth evaluation of the data was undertaken.

In recent years a large amount of data has been generated on nanomaterials, not least in connection with the OECD testing programme of manufactured nanomaterials under the OECD Working Party on Manufactured Nanomaterials (WPMN). Therefore, the data on the pre-selected nanomaterials are preferably from the OECD/WPMN dossiers of the substances, supplemented with further data from the NanoReg project (EU 7th framework), the NanoSafety Cluster projects, relevant REACH registrations of the substances or available new information.

It is to be noted that focus is exclusively on collecting and evaluating data on the nanoforms of the substances. Thus, it is not the intention with this project to collect and evaluate test data and information on the bulk substances and to compare data on the non-nanoform to data on the nanoform.

3. Screening for identification of relevant data for further assessment

The project was initiated with a screening phase to select the most relevant hazard classes for classification of the selected nanomaterials, i.e. hazard classes for which the existing data indicated a cause for concern based on the initial screening.

A very important basis for this screening was the Lee *et al.* (2017) report published by WHO: *"Which hazard category should specific nanomaterials or groups of nanomaterials be assigned to and how?"*. In the report by Lee *et al.* (2017) the OECD/WPMN dossiers for eleven nanomaterials from the Testing Programme of Manufactured Nanomaterials were systematically reviewed in order to obtain an overview of the availability of data with respect to amount and quality for the various human health hazard classes. Further, the data were assessed and GHS classification was proposed. However, the level of details was limited with respect to the actual data and arguments for classification using the GHS classification criteria.

Thus, the report by Lee *et al.* (2017) serves as a screening for relevant data and relevant hazard classes for the four nanomaterials covered by this project.

In Table 1 an overview of the data, the assessment of the data and the proposed classification from the Lee *et al.* (2017) report is given for the four pre-selected nanomaterials for this project.

Table 1 Overview of relevant classification hazard classes for the four selected nanomaterials. Data and interpretations compiled from Lee et al. (2017).

Hazard class	SWCNT	Silicon dioxide	Silver	Zinc oxide
Database for hazard assessment	Classification of SWCNTs is based on the pooled data from 14 different SWCNTs from 14 different manufacturers.	Classification of SiO_2 nanoparticles is based on the pooled data from 5 different SiO_2 nanoparticles (number of manufacturers not indicated).	Classification of Ag nanoparticles is based on the pooled data from 3 different Ag nanoparticles from 3 different manufacturers.	Classification of ZnO nanoparticles is based on the pooled data from 4 different ZnO nanoparticles from a single manufacturer.
Acute toxicity, oral/dermal/inhalation	No classification for oral exposure with moderate-strong evidence. No classification for inhalation with weak-moderate evidence. No data for acute dermal toxicity.	No classification for oral and inhalation exposure. Strong evidence. No data for dermal acute exposure.	No classification for oral/ dermal/inhalation exposure. Strong evidence.	No classification for oral exposure. Moderate evidence. No classification for dermal exposure. Strong evidence. No data on acute inhalation.
Skin damage/ irritation	No classification. Strong evidence.	No classification. Strong evidence.	No classification. Strong evidence.	No classification. Strong evidence.
Eye damage/ irritation	No classification. Strong evidence.	No classification. Strong evidence.	No classification. Strong evidence.	No classification. Moderate evidence.
Skin sensitisation	No classification. Moderate -strong evidence.	No classification. Strong evidence.	**Skin Sens. 1B Moderate evidence**.	No data
STOT SE, Oral, dermal, inhalation	No data	No data	No data	No data
STOT RE	**STOT RE 1 (inhalation, lung). Weak evidence** Oral repeated exposure only up to 12.5 mg/kg bw/d No classification, oral. Moderate evidence	**STOT RE 2 (inhalation, lung). Strong evidence**	**STOT RE 1 (inhalation, lung/ liver). Strong evidence** **STOT RE 2 (oral, liver). Strong evidence**	**STOT RE 1 (inhalation, lung). Moderate evidence**
Germ cell mutagenicity	**Muta. 2 Weak evidence**	No classification. Weak evidence.	No classification. Strong evidence.	No classification. Strong evidence.
Reproductive toxicity	No classification. Weak evidence	No classification. Strong evidence.	No classification. Strong evidence.	No classification. Strong evidence.
Carcinogenicity	No data	No data	No data	No data

In Table 2, the classifications from Lee *et al* (2017, Table 1) are compared with the classifications indicated in the relevant REACH registrations of the substances.

Table 2 Comparison of classifications as indicated in Lee *et al*. (2017) versus REACH registrations for the four selected nanomaterials.

	SWCNT	Silicon dioxide	Silver	Zinc oxide
Lee *et al.* (2017) Classification	Muta. 2 STOT RE 1 H372 (resp. tract/ inhalation)	STOT RE 2 H373 (resp. tract/ inhalation)	Skin Sens. 1 H317 STOT RE2 H373 (liver/oral) STOT RE 1 H372 (resp. tract/ inhalation)	STOT RE 1 H372 (resp. tract/ inhalation)
REACH registrations Classifications	Eye Irrit. 2 H319 (EC 943-098-9)	No human health classification (EC number: 231-545-4; CAS number: 7631-86-9, 112926-00-8; (non-crystalline)	No human health classification (both nano and bulk) (EC number: 231-131-3; CAS number: 7440-22-4)	No human health classification (both nano and standard ZnO, bulk) (EC number: 215-222-5; CAS number: 1314-13-2, 7440-66-6)

Thus, the focus for further evaluation is placed on data regarding the following hazard classes:

SWCNT: *Acute toxicity*, Eye irritation, STOT RE, Germ cell mutagenicity*

Nano silicon dioxide: *Acute toxicity*, STOT RE*

Nano silver: *Acute toxicity*, Skin sensitisation; STOT RE*

Nano zinc oxide: *Acute toxicity*, STOT RE*

*In addition, data on acute toxicity from single exposure were further included. Such data (including data from single exposue via alternative exposure routes e.g. from intratrahceal instillation or pharyngeal aspiration) may provide additional information regarding type of toxic effects and possible target organs and also provide information on aspects/difficulties in relation to dose formulations and testing of the nanomaterials.

4. Evaluation of hazard information on selected nanomaterials

For each of the four nanomaterials, data on the prioritised hazard classes were compiled from the OECD/WPMN dossiers of the substances supplemented with further data from the NanoReg project (EU 7th framework), the NanoSafety Cluster projects, relevant REACH registrations of the substances or available new information from focused web-based search (using e.g. substance name, toxicological end-points and exposure route as relevant search terms) .

The available data for each of the relevant hazard classes are collected and described in Appendices A, B, C, D for each of the four nanomaterials.

In the next sections short summaries of the most relevant data included in the appendices are given together with an evaluation of the data, a discussion regarding applicability of the used test methods, a discussion regarding applicability of the GHS criteria and classification of the substance, an indication concerning data gaps and uncertainties, and - if possible – any needs for revison of the GHS criteria or guidance in relation to CLP.

4.1 SWCNT

Most detailed data on the various qualities of SWCNT in the OECD/WPMN dossier was available for the two qualities: *Nikkiso SWCNT* and *Super Growth SWCNT*.

Nikkiso SWCNT contains 4% of iron and very small amounts of other metallic impurities and is characterised with a tube diameter of 3.03 nm, a particle size diameter of 2.7 µm and a specific surface area of 878 m^2/g.

Super Growth SWCNT contains > 99% carbon and very small amounts of other metallic impurities and is characterised with a tube diameter of 1.86 nm, a particle size diameter of 8.2 nm, a length of 0.23 µm, a pour density of 0.0192 g/cm^3, and a specific surface area of 1064 m^2/g. Both qualities are indicated as insoluble in water (OECD/WPMN 2016, SWCNT summary).

4.1.1 Overview of data availability for relevant health hazard classification

The toxicity data on SWCNT concerning the prioritised hazard classes *acute toxicity, eye damage/irritation, STOT RE* and *germ cell mutagenicity* were retrieved from the OECD/WPMN dossiers (OECD/WPMN (2016, SWCNT summary) and OECD/WPMN (2015, SWCNT part 2). With respect to eye irritation, further data were obtained from the publicly available data in the REACH registration of SWCNT.

Table 3 Number of studies/ test data for the selected hazard classes.

Hazard class	Number of studies (OECD-dossier)	Further studies retrieved for this project
Acute toxicity	*Oral 1 (+2 other in vivo studies)*	*0*
	*Inhalation 0**	*0*
	Dermal 0	*0*
Serious eye damage/eye irritation	*2*	*1 (REACH-reg. data)*
Germ cell mutagenicity	*In vitro 17*	*1 (REACH-reg. data)*
	In vivo 7	*0*
Specific target organ toxicity repeated exposure	*Oral 1*	*0*
	Inhalation 3 *Studies using intrathraceal or pharyngeal administration 10*	*0*

***In the OECD dossier, one study is indicated, however, the study used 4 days of repeated exposure and thus is in the table above included under STOT RE (inhalation).*

Further descriptions of the studies are given in Appendix A.

In the next sections the test data and observations are discussed from those studies considered most relevant for assessment of GHS classification.

4.1.2 Acute toxicity

4.1.2.1 Oral exposure

Short summary of most relevant data

Acute oral toxicity in rats was investigated in a OECD TG 423 study (acute toxic class method), using an oral dose level of 50 mg Nikkiso SWCNT/kg bw.

No deaths or abnormal findings occurred in the study. In the description of the OECD TG 423 study it was mentioned that a dose level of 2000 mg Nikkiso SWCNT/kg bw could not be achieved due to the very high specific volume of the nanomaterial (OECD/WPMN 2015, SWCNT part 2).

Also, in the REACH registration of SWCNT it was noted that relevant oral acute toxicity testing could not be performed *"as the test item was found to be impossible to formulate satisfactorily in a suitable vehicle for oral dosing."*.

Applicability of test methods

Oral acute toxicity test methods seem only to be applicable for SWCNT at low dose levels as a higher dose level *was "impracticable because of very high specific volume of SWCNT"* (OECD/WPMN 2016, SWCNT summary report).

Applicability of GHS criteria and classification

The GHS criteria can in principle be applied, however, the criteria can only be used for the most severe acute toxicity categories (Acute Tox 1 ≤ 5 mg/kg bw; Acute Tox 2 ≤ 50 mg/kg bw and possibly Acute Tox 3 ≤ 300 mg/kg bw) as testing at higher dose levels does not seem possible.

Thus, the classification criteria has limited applicability for available test data on the nanomaterial and it is not possible to adequately assess the classification.

The only available acute oral toxicity study do not indicate any lethal or acute toxic signs at the tested dose level. For the Nikkiso SWCNT "no classification" for acute oral toxicity is concluded based on insufficient data.

Data gaps and uncertainties

Only oral acute toxicity data according to OECD TG 423 are available for Nikkiso SWCNT. Data on additional and different qualitites of SWCNT would be needed for a proper assessment of the potential for oral acute toxicity of SWCNTs.

Data are too limited for a conclusion on acute oral toxicity of SWCNTs.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria is possible based on available information. Guidance on acute oral toxicity testing of voluminous nanomaterials may be needed.

4.1.2.2 Dermal exposure

Short summary of most relevant data

No dermal acute toxicity studies were reported in the OECD/WPMN dossiers. However, two dermal irritation studies according to OECD TG 404 in rabbits on acute dermal irritation/

corrosion were conducted. In these tests, the highest attainable exposure allowing a uniform mixture in olive oil was 0.5 ml of 1% Nikkiso SWCNT and Super Growth SWCNT, respectively. This corresponds to a dose level of 5 mg. No local or systemic toxicity was reported.

Thus, similar dosing problems could be foreseen if conducting *in vivo* test for acute dermal toxicity.

Applicability of test methods

In vivo testing for acute dermal toxicity could probably only be conducted at very low dose levels due to the very high specific volume of SWCNTs.

Applicability of GHS criteria and classification

The GHS criteria can in principle be applied, however, the criteria can probably only be used for the most severe acute toxicity categories as testing at higher dose levels does not seem possible.

Due to the physical chemical properties of SWCNT as an insoluble substance, dermal absorption is considered very low/negligible, and thus a potential for acute dermal toxicity seems unlikely.

Data gaps and uncertainties

There is no data available from OECD TG studies for acute dermal toxicity.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria is possible based on available information.

Guidance on acute dermal toxicity testing of voluminous nanomaterials may be needed.

4.1.2.3 Inhalation

Short summary of most relevant data

No OECD TG study for acute inhalation toxicity has been conducted for SWCNTs.

For asssessing acute toxicity OECD/WPMN (2016, SWCNT summary) and OECD (2015, SWCNT part 2) referred to an inhalation study in which mice were exposed to SWCNT (CNI, HiPco) for 4 days, 5h/day at a concentration level of 5.52 ±1.37 mg/m^3. Although clear signs of pulmonary toxicity in this study occurred, no lethal outcome was noted.

Further, the OECD/WPMN dossiers provide descriptions and observations from a series of single dose exposure to SWCNT by intratracheal instillation and pharyngeal aspiration. Although clear signs of pulmonary toxicity in these studies occurred down to an exposure level of 1 mg SWCNT/kg bw in rats (persistent pulmonary inflammatory response for up to 6 months) and 20 µg in mice (collagen disposition and fibrosis in lung tissue), no lethal outcome was described.

Applicability of test methods

No data from OECD test guideline studies of acute inhalation toxicity are available. Consequently, no assessment of the relevance of the test methods for acute inhalation toxicity can be made. However, technical problems in generating high exposure levels in air of voluminous SWCNT can be foreseen.

Applicability of GHS criteria and classification

No data from testing for acute inhalation toxicity are available. Consequently, no assessment of the GHS criteria for acute inhalation toxicity can be made.

Data from intratracheal instillation and pharyngeal aspiration may be used in a weight of evidence approach but cannot in itself be used for classification of acute inhalation toxicity as no criteria are given for these administration methods.

Data gaps and uncertainties

Data from OECD TG studies for acute inhalation toxicity of SWCNT are missing. Thus, there is a lack of information (data) for hazard assessment and classification.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is possible based on available information.

Guidance on acute inhalation toxicity testing of voluminous nanomaterials may be needed.

4.1.2.4 Eye irritation

Short summary of most relevant data

In vivo data

The OECD/ WPMN (2016, SWCNT summary) dossier reported an OECD TG 405 study in rabbits on Nikkiso SWCNT and on Super Growth SWCNT, respectively. In the tests 0.1 mL test sample containing 0.1 wt% of SWCNTs in olive oil (maximum achievable concentration of test material) was applied to the eye. None of the studies indicated a potential for eye irritation.

In vitro data

From the publicly available data from the REACH registration on SWCNT an OECD TG 492 study (Reconstructed Human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage) was available. About 50 mg of the SWCNT (tube diameter 1.0 – 2.2 nm; length 1 - 10 µm) and 50 µL of each of the control formulations, respectively, were applied to each of duplicate EpiOcular™tissue for 6 hours. Based on the test result, classification as at least Eye Irrit. 2 was concluded by the registrant.

Applicability of test methods

In vivo

According to the OECD TG 405 guideline, a volume of 0.1 mL or up to 100 mg of the test substance should be used when testing solids and particulate substances. However, it should be noted that in the case of testing of SWCNTs, the concentration of test item in the 0.1 mL test sample applied to the eye was only 0.1 wt% corresponding to only 0.1 mg of SWCNT, i.e. 1/1000 of the highest dose to be used in the test. This limits the interpretation of the *in vivo* testing of the SWCNTs.

In vitro

The OECD TG 492 (Reconstructed Human Cornea-like Epithelium test method) seems to be applicable to SWCNTs.

Applicability of GHS criteria and classification

In vivo

The GHS criteria for *in vivo* testing may not be relevant for SWCNT as testing with SWCNT probably only can be performed at very low dose levels.

In vitro

At this time there are no GHS classification criteria for test results from *in vitro* test methods for serious eye damage/irritation. However, results from validated *in vitro* tests should be considered in an weight of evidence assessment. Adaptation of the classification criteria to incorporate *in vitro* test methods for this hazard class is forseen in the coming years. Nevertheless, the classification criteria as specified in the OECD TG 492 seems to be applicable as testing of SWCNT could be performed at relevant dose levels.It is to be noted that no *in vitro* test can currently discriminate between classification as Eye Irrit. 2 or Eye Damage 1 and the applicability of classification criteria can therefore not be fully assessed.

Based on the availble *in vitro* data, eye irritation/ damage is to be considered a relevant hazard class for assessment of SWCNT. Data on a specific quality of SWCNT indicate that at least a classification as Eye Irrit. 2 is warranted for this quality.

Data gaps and uncertainties

Further data and experience with *in vitro* testing of various SWCNT qualities are missing to confirm the applicability of these test methods.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria is possible based on available information.

Guidance may be needed on the relevance of *in vivo* testing for eye damage/ irritation of insoluble nanomaterials with high specific volume.

4.1.3 STOT RE

4.1.3.1 STOT RE, oral exposure

Short summary of most relevant data

In an OECD TG 407 28-day subacute repeated oral dose study, Crl:CD rats (5 or 10 animals/sex/dose) were administered Nikkiso SWCNT (suspended in 5% guam acacia) by gavage at dose levels of 0, 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). A few minor changes with statistical significance in white blood cells composition, organ weights and urine volume were detected. No relevant pathological changes were observed. A NOAEL of 12.5 mg/kg bw/day was concluded. Due to the very high specific volume of the SWCNT, higher dose levels were not achievable.

Applicability of test methods

When testing SWCNT with a high specific volume, only low dose levels far below the upper GHS criteria of 600 mg/kg bw/day for STOT RE 2 classification from a 28-day study can be achieved.

Applicability of GHS criteria and classification

The GHS criteria can in principle be applied, however, testing with SWCNTs can only be conducted at low dose levels and thus only the GHS criteria for STOT RE1 classification (i.e. below 60 mg/kg bw/day for a 28-day study) would be applicable.

Based on available information from an OECD TG 407 28-day subacute repeated oral dose toxicity study of Nikkiso SWCNT tested up to 12.5 mg/kg bw/day, no classification in STOT RE is warranted.

Data gaps and uncertainties

Information on repeated oral dose toxicity of other types (than Nikkiso SWCNT) of SWCNT is missing.

The classification criteria has limited applicability for available test data on the nanomaterial and it is not possible to adequately assess classification.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria possible based on available information.

Guidance for relevant repeated oral dose toxicity testing of voluminous nanomaterials may be needed.

4.1.3.2 STOT RE, inhalation

Short summary of most relevant data

An OECD TG 412 (28-day inhalation study) was conducted in rats that were exposed to *Nikkiso SWCNTs* 6 hours/day, 5 days/week during 4 weeks at exposure levels of 0; 0.08 and 0.40 mg/m^3. The particle number concentrations in the two groups were 5.0 ± 0.7 x 10^4 and 6.6 ±2.1 x 10^4 particles/cm^3, respectively.

Further, an OECD TG 412 (28-day inhalation study) was conducted in rats that were exposed to *Super Growth SWCNTs* 6 hours/day, 5 days/week during 4 weeks at exposure levels of 0 mg/m^3; 0.03 and 0.13 mg/m^3. The particle number concentrations in the two groups were 5.0 ± 0.7 x 10^4 and 6.6 ±2.1 x 10^4 particles/cm^3, respectively.

In both studies, observations and examinations were performed 3 days, 1 month and 3 months after exposure; however, no adverse pulmonary effects or signs of neutrophil inflammation were noted in the studies.

In a non-OECD guideline study mice were exposed to 5.52 ±1.37 *(CNI, HiPco) SWCNTs* mg/m^3, 5 hours daily during 4 days. LDH accumulation in BAL fluid of mice that inhaled SWCNT revealed a statistically significant increase (118%, 80%, and 71%) over control groups throughout recovery time (1, 7, and 28 days post-exposure). Regarding the histopathological observations, four mice at 28 days post-exposure had bronchiolar epithelial cell hypertrophy with one mouse having both hypertrophy and hyperplasia, one mouse having peribronchiolar bronchiolisation accompanying bronchiolar epithelial cell hypertrophy, and two mice having bronchiolar epithelial cell hypertrophy without other bronchiolar alterations. Further, foci of

granulomatous inflammation were noted with fibrosis seen. Thus, inhalation of SWCNT resulted in an inflammatory response, oxidative stress, collagen deposition, and fibrosis in the lung 28 days post-exposure (Shvedova et al. 2008).

It should be noted, however, that the SWCNT used was non-purified and as produced, having a diameter of 0.8-1.2 nm, a length of 100–1000 nm and a content of 82% elemental carbon, 17.7% iron, 0.16% copper, 0.049% chromium, and 0.046% nickel. Shvedova et al. (2008) noted the importance of the content of transition metals (especially the high content iron) as these transition metals can act with a prooxidant potential. Thereby a combination of inflammatory response with catalytically metal-containing carbon nanotubes would synergistically enhance damage to cells and tissue.

Pharyngeal aspiration / intrathraceal instillation

In one non-guideline study, ApoE-/- mice were repeatedly dosed by pharyngeal aspiration to 20 µg SWCNT/mouse once every second week for 8 weeks. Effects in relation to accelerated plaque formation in the aorta were noted (Li *et al.* 2007).

For non-soluble persistent substances also, single dose exposure data may be relevant to consider in connection with STOT RE assessment, as a single dose applied by pharyngeal aspiration/ intrathraceal instillation may mimic a dose accumulated in the lung from repeated inhalation exposure.

Thus, the OECD/WPMN (2016, SWCNT summary) reported three studies using single dose pharyngeal aspiration of SWCNT in either mice (up to 40 µg/mouse) or rats (2 mg/kg). In these studies lung inflammation, lung damage and fibrosis were observed in mice and histopathological signs of lung inflammation were found in rats.

Also, OECD/WPMN (2016, SWCNT summary) reported a total of six studies using single intratracheal instillation of SWCNT in either mice (up to 0.5 mg/mouse) or in rats (up to 2.25 mg/rat or 17.5 mg/kg). Consistently, inflammatory responses lasting for several months were noted in the studies. In mice the most severe effects were found and were reported as interstitial inflammation, peribronchial inflammation and necrosis extending into the alveolar septa at dose levels of 0.1mg and 0.5 mg SWCNT/mouse.

Applicability of test methods

The recent updated 2018-versions of OECD TG 412 28-day (subacute) inhalation toxicity study and OECD TG 413 90-day (subchronic) inhalation study specifically address the applicability and design of the tests methods for testing of nanomaterials.

Although not specifically stated in the descriptions of the repeated dose inhalation studies it can be foreseen that generation of test atmospheres with considerable higher concentrations would be difficult to achieve due to the high specfic volume of the SWCNTs. By reference to the dose/concentration guidance values (at or below which a significant toxic effect is observed) for STOT RE 2 classification according to GHS criteria, concentrations up to 600 mg/m^3 for a 28 day inhalation study and up to 200 mg/m^3 for a 90 days inhalation study would be needed to be tested.

Applicability of GHS criteria and classification

The GHS criteria can in principle be applied, however, the criteria can only be used for STOT RE1 as testing at higher dose levels relevant for STOT RE 2 classification does not seem possible.

The two OECD TG 412 studies using exposure levels up to 0.40 mg SWCNT/ m^3 in rats did not lead to any significant adverse toxic response that would warrant a STOT RE classification.

However, it should be noted that the highest concentrations used in these studies are considerable lower than the GHS STOT RE 2 guidance value for classification of 600 mg/m^3 which applies for 28 days inhalation exposure in rats. Thus, the rats in the studies have only been exposed to levels up to 1/1500 of the limit for classification.

In the non-guideline study, 4 days exposure to 5.52 mg SWCNT/m^3 5 hours/day resulted in a clear inflammatory response still present 28 days post exposure. In addition, histopathological changes were observed in the lung tissues showing foci of granulomatous inflammation and fibrosis. For a 28 day study a classification as STOT RE 1 would apply at exposure levels below 60 mg/m^3 according to the GHS-criteria. Due to accumulation of SWCNT in the lung, even more pronounced effects at 5.52 mg SWCNT/m^3 would be expected if the study had covered a 28 days exposure period instead of only 4 days. No general practice has been agreed on how to extrapolate from such a short term repeated dose toxicity study. However, the results of this study indicate a need for a STOT RE1 (inhalation, lung) classification considering the very low exposure level and the very short exposure duration that were used.

Thus, these data indicate that STOT RE is a hazard class of concern for SWCNTs.

Further, such a classification is supported by data from studies using single dose exposure to SWCNT by intratracheal instillation or pharyngeal aspiration. Particularly for persistent substances, these studies may mimic effects after repeated inhalational exposure and accumulation in the lung tissue. In connection with the study it was calculated that inhalation exposure to SWCNT in mice at 5.17 mg SWCNT/m^3, 5h/day for 4 days with an assumed deposition rate of 0.5% of the inhaled dose would correspond to a deposition of 5 µg SWCNT in the lung. Also, the study found very comparable lung response when comparing effects from inhalation exposure to SWCNT via pharyngeal aspiration exposure.

No specific GHS criteria exist for administration methods mimicking inhalation exposure such as intratracheal administration or pharyngeal aspiration.

According to section 3.9.2.4. of the GHS, a weight of evidence approach should be used for all data that may substantiate a STOT RE classification. Where weight of evidence according to 1.3.2.4.9.1 of the GHS-regulation *"means that all available information bearing on the determination of toxicity is considered together, including the results of valid in vitro tests, relevant animal data, and human experience….."*.

Consequently, also data not directly applicable to the criteria, but relevant for the overall weight of evidence, can be used as support for the conclusion with respect to classification.

Thus, data from intratracheal or pharyngeal exposure to SWCNT may be used as supportive data in the overall assessment of hazardous effects in relation to inhalation exposure as it provides information regarding effects in the target organ of interest, the lung, irrespective of the method of exposure.

Data gaps and uncertainties

The above assessment indicates that test data on SWCNTs specifically addressing the GHS criteria for STOT RE classification are lacking. Also, the classification criteria has limited applicability for available test data on the different qualities of the nanomaterial and it is not possible to adequately assess classification. It appears to be not technically feasible to test up to the highest dose/concentration guidance values for STOT RE classification via inhalation in 28- or 90-days repeated dose toxicity studies.

Further knowledge and assessment s needed to determine to which extent data from intratracheal instillation or pharyngeal aspiration can be used as supporting information for classification in a weight of evidence approach.

A discussion is needed whether subtle effects (e.g. inflammatory responses) seen at low exposure levels of inhalation can be regarded as markers for severe toxicity that would be assumed to be evident at higher dose levels and if such data may fit into an adverse outcome pathway (AOP) or mode of action (MOA) that may support a classification based on a weight of evidence approach.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nanospecific adaptions of GHS/CLP criteria is possible based on available information.

Guidance on repeated dose inhalation toxicity testing of voluminous nanomaterials is needed.

In order to get more optimal use of data from experimental animal studies using very low exposure levels, guidance is needed on how to include other toxic parameters (e.g. markers for inflammatory responses and mild histopahtological changes) to be used as markers for severe effects. Also, guidance is needed on how data from studies using intratracheal instillation or pharyngeal aspiration may be used as support for classification purposes.

4.1.4 Germ cell mutagenicity

Short summary of most relevant data

In vitro
The OECD/WPMN (2016, SWCNT summary) reported results from bacterial mutation tests with Nikkiso SWCNT, Super Growth SWCNT and CNI,HiPco SWCNT which were all negative. Further, Nikkiso SWCNT and Super Growth SWCNT were tested negative in OECD TG 473 tests for chromosomal aberration in mammalian cells.

Five *in vitro* micronucleus tests in mammalian cells have been conducted with various qualities/ types of SWCNT of which four of the tests resulted in positive outcome for increased frequencies of micronuclei.(Nikkiso SWCNT and Super Growth SWCNT not included in these tests). The fifth study using CNI,HiPco SWCNT resulted in a negative result.

With respect to DNA damage and repair six *in vitro* Comet assays and two *in vitro* assays for identifying DNA double strand breaks in mammalian cells have been performed on various types of SWCNTs. Seven assays (including CNI, HiPco in a Comet assays) detected increased induction of DNA damage and only one type of SWCNT (indicated as SWCNT/Heji) was negative (Nikkiso SWCNT and Super Growth SWCNT were not included in any of these tests).

In vivo
Nikkiso SWCNT and Super Growth SWCNT were tested in chromosomal aberrations tests *in vivo* (OECD TG 474) in rat using oral gavage administration, both with negative outcome.

Nikkiso SWCNT was further tested negative in a comet assay in lung tissue from rats exposed via intratracheal administration.

However, positive findings have been reported in other non-guideline *in vivo* assays:

- Increased K-ras mutations were found in lung tissue of mice following inhalation of CNI,HiPco SWCNT while no such finding was observed after pharyngeal aspiration.
- Increased mitochondrial DNA damage was found in mice exposed to CNI,HiPco SWCNT by intrapharyngeal instillation
- Increased oxidative DNA damage was found in liver and lung tissue from rats exposed to another type of SWCNT by oral gavage.

Applicability of test methods

In “Appendix to Chapter R.7a for nanomaterials ((REACH Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint specific guidance, ECHA 2017) the applicability of the various mutagenicity assays for nanomaterials has been assessed. Bacterial mutagenicity testing is not recommended for nanomaterials as the nanomaterials may not be able to cross the bacterial wall. In vitro testing in mammalian cells and in vivo testing are overall considered applicable for determining the mutagenicity/ genotoxicity of nanomaterials. For *in vitro* testing using mammalian cells knowledge concerning uptake into the cells is important when interpreting the test results. When performing *tests in vivo* the distribution of the nanomaterial to the target tissue should be ensured. In the absence of toxicokinetic information it is therefore recommended to investigate the genotoxic effects in the tissue at the site of contact.
When looking on the overall mutagenicity data on SWCNT, all *in vitro* testing using bacteria resulted in negative results, which to some extent is to be expected considering the lack of penetration of the bacterial wall by persistent, non-soluble nanomaterials.
Also, for the *in vivo* OECD TG 474 micronucleus testing the negative results may be a consequence of lack of exposure to the target tissue i.e. distribution of the SWCNT to the bone marrow of the animals.

Applicability of GHS criteria and classification

The GHS criteria for classification for germ cell mutagenicity is considered applicable for nanomaterials including SWCNTs. This is supported by the assessment and recommendations given in Appendix R7-1 for nanomaterials applicable to Chapter R7b Endpoint specific guidance (ECHA 2017)

According to the GHS criteria the classification Muta. 2 is based on:

“Positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from: a) somatic cell mutagenicity tests in vivo, in mammals; or b) other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays”.

For classification assesssment only the data on Nikkiso SWCNT and Super Growth SWCNT as well as data on CNI,HiPco SWCNT will be evaluated as these types of SWCNT have been subject to the most thoroughly testing in vitro and in vivo. The negative results from testing of these types of SWCNT in bacteria will not be considered as these tests as indicated above are not considered relevant.

For Nikkiso SWCNT the relevant data pertain to OECD TG 473 *in vitro* testing for chromosomal aberration in mammalian cells, OECD TG 474 in vivo micronucleus testing in mice using oral exposure, and a Comet assay in lung tissue from rats exposed by intratracheal administration. All of these tests resulted in negative outcome, indicating lack of genotoxic potential.It is not known whether the negative result in the OECDTG 474 study is due to lack of distribution of the SWCNT to the bone marrow of the animals, however, site-of-contact tissue tested in a Comet assay indicate lack of a genotoxic potential. Thus, based on the available data classification for germ cell mutagenicity is not warranted for Nikkiso SWCNT.

For Super Growth SWCNT the relevant data pertain to OECD TG 473 in vitro testing for chromosomal aberration in mammalian cells and OECD TG 474 *in vivo* micronucleus testing in mice using oral exposure. Both of these tests resulted in negative outcome, indicating lack of genotoxic potential. However, it is not known whether the negative result in the OECDTG 474 study is due to lack of distribution of the SWCNT to the bone marrow of the animals. Based on the available data classification for germ cell mutagenicity is not warranted for Super Growth SWCNT.

CNI, HiPco SWCNT has been tested *in vitro* for micronucleus formation in CHL cells with negative outcome and in a Comet assay using CHL cells with positive outcome. *In vivo* the

substance was tested positive for inducing K-ras mutation in lung tissue following inhalation (but negative after aspiration exposure) and positive for mitochondrial DNA damage in mice exposed by intrapharyngeal instillation. Taken together, the induction of K-ras mutation in lung after inhalation in a non-guideline study in mice, and the evidence of genotoxicity in the in vitro Comet assay indicate a genotoxic potential of CNI, HiPco SWCNT and that a classification as Muta. 2 may be warranted for this type of SWCNT.

Further *in vivo* data (oxidative DNA damage in liver and lung tissue from rats orally exposed to another type of SWCNT) support concern for a possible genotoxic potential of SWCNTs.

Data gaps and uncertainties

More knowledge/ experience is needed in relation to applicability of the various OECD guideline mutagenicity/genotoxicity test systems for nanomaterials. Toxicokinetic data regarding *in vivo* distribution is important to conclude on wheter the nanomaterial has the ability to reach target organs and gonadal - /germ cells or not. Also, further experience using the OECD TG 489 (*in vivo* Mammalian Alkaline Comet Assay) for site-of-contact tissue may be relevant for the assessment of genotoxic potential of the various qualities of SWCNTs.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is possible based on available information.

4.1.5 Overview of findings for SWCNT

An overview of the findings for SWCNT is presented in Table 4.

Table 4 Overview of findings in the evaluation of the test data for SWCNTs of selected hazard classes.

SWCNT				
Hazard class	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
Acute oral toxicity	+ (only low dose level achievable for testing)	++ Testing limitations (?)	Yes (only test on one type of SWCNT available)	Guidance may be needed for testing voluminous NMs
Acute dermal toxicity	+ (only low dose level achievable for testing)	++ Testing limitations (?)	Yes (no guideline study available)	Guidance may be needed for testing voluminous NMs
Acute inhalation toxicity	0	0 (?)	Yes (no test data)	Guidance may be needed for testing voluminous NMs
Eye damage/ irritation	*In vivo* - (only low dose level achievable for testing) *In vitro* + / ++	+ (No GHS classification criteria adopted yet for test results from in vitro methods) (at least Eye Irrit. 2)	Yes (as only two non-adequate *in vivo* tests and one *in vitro* test available)	Guidance may be needed for testing voluminous NMs
Germ cell mutagenicity	*In vitro* ++ (Mammalian cells) In vivo ++	++ (as support) ++ (Muta. 2)	Yes (further testing on site-of-contact tissue for *in vivo* tests)	Guidance on NM testing available
STOT RE oral	+ (only low dose level achievable for testing)	++ Testing limitations (?)	Yes (only test on one type of SWNCT available)	Guidance may be needed for voluminous NMs
STOT RE inhalation	+ (only low dose level achievable for testing)	++ Testing limitations (STOT RE1)	Yes (only two 28-day studies available)	Guidance is needed for testing voluminous NMs. Also guidance on how to interpret mild toxic repsonse at low exposure levels and how to interpret data from instillation/ aspiration tests.

* ***0:*** *no assement due to lack of data.* ***- :*** *not applicable* ***+:*** *applicable with limitations*
++: *fully applicable* ***(?)*** *classification cannot be concluded due to lack of data* ***NM****: nanomaterial*

Testing limitations: *not technically feasible to test up to concentrations/ doses relevant for classification in the hazard category(-ies) representing the lowest potency (least severe) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of SWCNT but is related to one or several representatives of SWCNTs. However, a classification for a specific hazard class indicates that this may be a relevant classification for other SWCNTs as well and that testing/ information on this end-point is especially warranted.*

4.2 Nano silicon dioxide

Data covered in the OECD/WPMN testing programme pertain to synthetic amorphous nano silicon dioxide manufactured either by *precipitation* (three qualities indicated as NM-200, NM-201, NM-204 in the OECD dossiers) or *pyrolysis* (two qualities indicated as NM-202 and NM-203). All the substances characterised in the OECD dossier had a purity of SiO_2 ≥ 96% and an aluminium content ≤ 0.87%. Further, they were characterised with primary particle sizes in the range of 10 – 45 nm; aggregate medium diameters in the range of 31 – 53 nm; specific surface areas (BET) in the range of 137 – 204 m^2/g and pour densities in the range of 0.03 - 0.28 g/cm^3. Water solubilities according to data from the REACH registration are in the range of 76 – 166 mg/L at 37ºC.

4.2.1 Overview of data availability for relevant health hazard classification

Relevant data for this assessment has been found in the OECD/WPMN 2016, SiO_2 summary and the OECD/WPMN 2016, SiO part 1-6 as well as data obtained from the NanoReg project (EU 7th framework) and the NanoSafety Cluster projects.

Table 5 Number of studies/ test data for the selected hazard classes.

Hazard class	Number of studies (OECD/ WPMN)	Further studies retrieved for this project
Acute toxicity	Oral 10	
	Dermal 1	
	Inhalation 6	
Specific target organ toxicity repeated exposure	3 (oral, 90-day)	1 (from NanoCluster) 1 (from NanoReg)
Specific target organ toxicity repeated exposure	0, 6, 1 (inhalation, 28D, 90D, + 90D)	

Further descriptions of the studies are given in Appendix B.

In the following sections the test data and observations are described and discussed from those studies considered most relevant for GHS classification.

4.2.2 Acute toxicity

Only very short descriptions of the acute toxicity studies will be given as the overall data consistently pointed towards a very low acute toxicity potential of nano silicon dioxide.

4.2.2.1 Oral exposure

Short summary of most relevant data

The OECD/WPMN (2016, SiO_2 summary) reported OECD TG 401 acute oral toxicity testing in rats for five different qualities of nano silicon dioxide (NM-200, NM-201, NM-202, NM-203, NM-204) using maximum dose levels in the range of 3160 – 20 000 mg/kg bw in the testing. No mortality was found in any of the tests.

Applicability of test methods

The acute oral toxicity test (OECD TG 401) seems applicable for testing of nano silicon dioxide.

Applicability of GHS criteria and classification

The GHS criteria for acute oral toxicity are applicable. Based on the current data showing low acute toxicity potential, no classification of nano silicon dioxide is warranted.

Data gaps and uncertainties

Various nano silicon dioxide qualities have been tested according to OECD TG 401.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.2.2.2 Dermal exposure

Short summary of most relevant data

In a acute dermal toxicity test (from 1978), no systemic toxicity was noted in rabbits after dermal application of either NM-200, NM-201 or NM-203 under occlusive conditions at 5000 mg/kg bw (OECD/WPMN 2016, SiO2 summary).

Applicability of test methods

No difficulties with dosing of the test substance using the OECD TG 402 for acute dermal toxicity test was noted and the test method is considered applicable.

Applicability of GHS criteria and classification

The GHS criteria for acute dermal toxicity are applicable. Based on the current data showing very low acute toxicity potential, classification of nano silicon dioxide is not warranted.

Data gaps and uncertainties

No testing according to current OECD test guideline has been performed, however, such testing would not according to the data available be expected to warrant classification for acute dermal toxicity.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.2.2.3 Inhalation

Short summary of most relevant data

The OECD/WPMN (2016, SiO2 summary) reported six OECD TG 403 inhalation toxicity tests in rats performed in the period 1981-1983 covering four different qualities of nano silicon dioxide (NM-200, NM-201, NM-202, NM-203). No mortality was found in the tests at the maximum attainable concentrations of 0.14 - 2.08 mg/L (OECD/WPMN 2016, SiO2 summary).

It should be noted that higher exposure levels were not technically achievable.

Applicability of test methods

Acute inhalation toxicity test methods seem only to be technically applicable for nano silicon dioxide at concentration levels up to 0.14 – 2.08 mg/L most probably due to a high specific volume of nano silicon dioxide.

Applicability of GHS criteria and classification

The GHS criteria can in principle be applied, however, only testing in the range up to the GHS criteria for Acute Tox. 2 (0.05 – 0.5 mg/L) seems possible for nano silicon dioxide. As no mortality had occurred at the highest attainable concentration levels, no classfication for acute inhalation toxicity is warranted for nano silicon dioxide.

Data gaps and uncertainties

No testing data could be generated for exposure concentrations relevant for Acute Tox. 3/4/5 classification as such exposure levels seems not to be technically achievable.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.2.3 STOT RE

4.2.3.1 STOT RE, oral exposure

Short summary of most relevant data

The OECD/WPMN (2016, SiO2 summary) reported three identical OECD TG 408 90-day oral toxicity tests with NM-200, NM-201 and NM-202[1] conducted in 1981. In the studies Wistar rats were exposed during 13 weeks to 0.5, 2 and 6.7% nano silicon dioxide in the diet. A NOEL of 6.7% (corresponding to about 4000 mg/kg bw/day) was concluded in all three studies as no adverse effects were observed.

A recent repeated oral dose non-guideline study was conducted by van der Zande et al. (2014). In this study, groups of 5 male Sprague Dawley rats were fed with synthetic amorphous silica (SAS) at 0,100, 1000 and 2500 mg/kg bw/day or NM-202 at 100, 500, and 1000 mg/kg bw/day for 28 days. For two additional groups of five male rats dosed with the highest dose levels of SAS and NM-202 the exposure period was extended to 84 days. The synthetic amorphous silica was food-grade quality with a purity ≥ 99.8%, a primary particle size of 7 nm and specific a surface area of 380 m^2/g, whereas NM-202 had a purity ≥99.9%, a primary particle size of 10-25 nm, and a specific surface area of 200 m^2/g.After 84-days of exposure to SAS, but not to NM-202, silica accumulated in the spleen. Biochemical and immunological markers in blood and isolated cells did not indicate toxicity; however, at histopathological analysis, significantly increased incidence of liver fibrosis was found after 84-days of exposure to NM-202, whereas these findings did not reach the level of statistical significance for SAS.

The NanoReg project (NanoReg 2016) reported a OECD TG 408 90-day oral toxicity in which rats were exposed by oral gavage to NM-203 at dose levels of 0, 2, 5, 10, 20 and 50 mg/kg bw/ day. No signs of general toxicity were noted. The liver was found as the main target organ and histopathological findings in the hepatic tissue were noted at all dose levels. Overall, the authors

[1] **NM-202 manufactured by pyrolysis*

concluded that no NOAEL could be defined from the study, but also no clear dose-response-relationship was found.

It has to be noted that the current description of the findings by NanoReg(2016) is qualitative in nature and that quantitative data (e.g. the incidences of the findings) are lacking.

Applicability of test methods

The OECD TGs for repeated oral administration seem applicable for testing of nano silicon dioxide.

Applicability of GHS criteria and classification

The GHS criteria for STOT RE classification are considered to be applicable. In the study by van der Zande (2014), the observed fibrotic changes in the liver after 84 days of exposure are considered as relevant effects for a STOT RE classification. However, the effects were observed at 1000 mg/kg bw/day, i.e. a dose level far above the upper limit for STOT RE 2 classification (100 mg/kg bw/day from an oral 90-day study).

The dose levels used in the in the NanoReg study (in the range of 2- 50 mg/kg bw/day) are within the range for STOT RE classification. However, the histopathological hepatic findings from the study are currently not described in a manner that allows to draw conclusions from this study.

Thus, STOT RE classification for oral exposure may be relevant for some qualities of nano silicon oxide but currently the data do not warrant classification.

Data gaps and uncertainties

More detailed reporting and analysis of the data from the NanoReg study may further clarify the need for STOT RE classification in relation to oral exposure.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.2.3.2 STOT RE, inhalation

Short summary of most relevant data

From the OECD/WPMN (2016, SiO2 summary) six OECD TG 413 90-day inhalation studies are reported for the various qualities of nano silicon dioxide. Five of the studies have been performed at the same Dutch laboratory and the data is published in Reuzel et al. (1991) that tested two qualites of pyrogenic amorphous silica (Aerosol 200 and Aerosil R974) and one quality of precipitated amorphous silica (Sipernat 22S). For the sixth 90-day study no detailed results were reported in the OECD/WPMN dossier.

For the precipitated quality Sipernat 22S (corresponding to NM-200, NM-201 and NM-204) rats were exposed by inhalation to 35 mg/m^3 6h/day, 5 days/week for 90 days and the animals were followed in a post exposure recovery period up to one year. The main findings in relation to aderse effects were according to OECD/WPMN (2016, SiO2, summary):

" The relative mean of lungs weighs slightly increased (≈ x 1.3). Thymus weight increased as well. Swollen lungs and enlarged mediastinal lymph nodes were noted. The effects gradually subsided after the exposure period. Lung weights were normalised after 13 weeks recovery in males and females. In the lung, accumulation of alveolar macrophage, intra-alveolar polymorphonuclear leukocytes and increased septal cellurarity in males and females were

noted. Treatment related microscopic changes in the nasal region were found at the end of the exposure, such as very slight local necrosis and slight atrophy of the olfactory epithelium, intracystoplasmic proteinaeceous droplets. Collagen content in the lungs slightly increased at the end of exposure. During the recovery period, all changes disappeared mostly within 13 – 26 weeks post exposure"

For the pyrogenic qualities Aerosil 200 and Aerosil R974 (corresponding to NM-202 and NM-203) rats were exposed by inhalation to 1,3,5,9 and 31 mg/m^3 6h/day, 5 days/week for 90 days and the animals wee followed in a post exposure recovery period up to one year. The main findings in relation to aderse effects were according to OECD/WPMN (2016, SiO2, summary):

"Swollen lungs and enlarged mediastinal lymph nodes at the end of recovery was found (treatment related degrees of severity). No lung weight effect was found for 1 mg/m^3 group, but an increase was observed for the 6 (1.7 x for males and 1.4 x for females) and 30 mg/m^3 (2.3 x for males and 2.0 x for females) groups. For the 6 and 30 mg/m^3 groups, collagen content in the lungs was clearly increased, most pronounced in males. The above mentioned effects gradually subsided after the exposure period. But in males exposed to 6 to 30 mg/m^3, the collagen content was still above control values at the end of the study. Granuloma like lesions were seen in animals at the end of exposure period and after the 13 weeks of recovery. They did not show fibroblastic activity and hyanilisation and regressed during recovery. Accumulation of macrophages was seen in the mediational lymph nodes (disappeared week 39). Treatment related microscopic changes in the nasal region were occasionally found at the end of exposure period such as focal necrosis and slight atrophy of the olfactory epithelium.

Interstitial fibrosis was not noted directly after the exposure period, but appeared with a delay. It was observed for the first time after 13 weeks post exposure, increasing incidence especially for 30 mg/m^3 and less for 6 mg/m^3. It decreased in severity and frequency until the end of the study. All types of pulmonary lesions were more marked in males than in females. The level of 1.3 mg/m^3 induced only slight changes after 13 weeks post exposure which generally recovered quickly. Morphological changes after 13 weeks exposure are considered statistically significant at 1.3 mg/m^3. Silica could be detected in lungs only in relatively small amounts at the end of exposure period. Only one male exposed to 30 mg/m^3 showed a small amount of silica in the regional lymph nodes. 90 days after termination of exposure, no silica could be recovered from any animal."

Further, a non-guideline 12 months inhalation study has been conducted in which female rats were exposed to 50-55 mg/m^3 of pyrogenic nano silicon dioxide 5h/day, 5 days/week. The frequency of exposure had to be reduced from daily exposure to exposure 2-3 times weekly because of losses of animals because of severe bronchitis and inflammation indicating very severe toxic effects from the daily exposure. Other findings were according to OECD/WPMN (2016, SiO2, summary):

"Microscopically visible small dust foci could be observed under the pulmonary pleura, mediastinal lymph nodes were moderately enlarged. In the interior of alveoles, numerous macrophages accumulated, partially normal, partially destroyed, associated with deposition of cell debris ("desquamation catarrh"). Perivascular and peribronchiolar small dust foci of macrophages were associated with mild and moderate formation of connective tissue (ranked as grade I to II, based on a ranking system according to Belt & King). In the alveolar septa the collagen formation was increased.

In some cases, collagenic fibrosis was detected, partially showing decay. There were no signs of typical silicosis. In the mediastinal lymph nodes, foci and clusters of phagocytes, partially normal, partially showing decay, were observed"

Overall, it can be seen that the pyrogenic derived nano silicon dioxide qualities (NM-202 and NM-203) seem to be of highest toxicological concern. At an exposure level of 1.3 mg/m^3 pulmonary morphological changes after 13 weeks exposure statistically significant increased incidence of granuloma-like lesions were seen in animals at the end of exposure period and also

after the 13 weeks of recovery at the dose level of 30 mg/m^3. Further, pulmonary interstitial fibrosis was observed after 13 weeks post exposure with significant increasing incidence at 30 mg/m^3 and less for 6 mg/m^3. Treatment related degrees of severity of swollen lungs and enlarged mediastinal lymph nodes were observed at the end of the recovery period.

Also, it should be noted that the daily exposure to 50 - 55 mg/m^3 in a 12 months inhalation study had to be reduced to an exposure frequency of 2-3 times per week due to mortality of the animals caused by severe bronchitis and inflammation.

For the precipitated nano silicon dioxide quality the most prominent findings were swollen lungs and enlarged mediastinal lymph nodes that gradually subsided after the exposure period. Lung weights were normalised after 13 weeks recovery in males and females. In the lung accumulation of alveolar macrophage, intra-alveolar polymorphonuclear leukocytes and increased septal cellularity in males and females were noted.

For both pyrogenic and precipitated nano silicon dioxide, treatment related focal necrosis and slight atrophy of the olfactory epithelium were found at the end of the exposure.

Applicability of test methods

The recent updated 2018-versions of OECD TG 412 28-day (subacute) inhalation toxicity study and OECD TG 413 90-day (subchronic) inhalation study specifically address the applicability and design of the tests methods for testing of nanomaterials.

From the testing of nano silicon dioxide the test methods described above are considered applicable.

Applicability of GHS criteria and classification

Nano silicon dioxide has been tested at relatively low dose levels compared to the GHS classification criteria for STOT RE as only levels up to 55 mg/m^3 in a 90-day inhalation test has been used.

Administration of 55 mg/m^3 of a pyrogenic derived nano silicon oxide resulted in severe toxicity and losses of animals during testing because of severe bronchitis and pulmonary inflammation. In another 90-day inhalation study exposure levels up to 30 mg/m^3 lead to interstitial fibrosis, most severely at 30 mg/m^3 but also present at 6 mg/m^3.

As the GHS criteria for STOT RE 2 classification is exposure concentration levels in the range of 20 – 200 mg/m^3 at least a STOT RE 2 classifcation is warranted for pyrogenic derived qualities of nano silicon dioxide.

For the precipitated nano silicon oxide qualities the data is inconclusive, and currently data do not fullfil the criteria for severity for a STOT RE classification. However, testing to the upper criteria level of 200 mg/m^3 would clarify whether a classification as STOT RE 2 would be warranted.

Data gaps and uncertainties

Data is needed to further clarify whether classification in STOT RE 1 would be warranted for pyrogenic derived nano silicon dioxide and data is needed to further clarify whether a STOT RE 2 classification would be warranted for precipitated pyrogenic derived nano silicon dioxide.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.2.4 Overview of findings for nano silicon dioxide

An overview of the findings for nano silicon dioxide is presented in Table 6.

Table 6 Overview of the findings in the evaluation of the test data for nano silicon dioxide for selected hazard classes.

Nano silicon dioxide				
Hazard class	**Appl. Test methods***	**Appl. GHS criteria* (Classification**)**	**Data gaps**	**Suggestions for Criteria/ guidance**
Acute oral toxicity	++	++ (No classification)	No	No
Acute dermal toxicity	++	++ (No classification)	No	No
Acute inhalation toxicity	+ (high exposure levels not achievable in testing)	++ Testing limitations (No classification)	No	No
STOT RE oral	++	++ (No classification/ ?)	Yes (new study suggests effects at low concentrations)	No
STOT RE inhalation	++	++ (STOT RE 2, maybe RE1)	Yes (Data on precipitated qualities lacking, testing at higher dose levels)	No

* ***0:*** *no assement due to lack of data* **-** *: not applicable* **+***: applicable with limitations*
++*: fully applicable* ***(?)*** *classification cannot be concluded due to lack of data*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of nano silicon dioxide as test data is related to one or several representatives of nano silicon dioxide. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities/types of nano silicon dioxide as well and that testing/ information on this end-point is especially warranted.*

4.3 Nano silver

In the OECD/WPMN dossier (2017, AgNP summary) nano silver (AgNP) is characterized with particle sizes in the range of 6 – 55 nm and a density of ca. 10.4 g/cm^3. From the REACH registration of silver a water solubility of 3.6 µg/L after 28 days at pH6 in relation to with a Ag particle size of 1.9 µm.

4.3.1 Overview of data availability for relevant health hazard classification

The toxicity data on nano silver (AgNP) on the prioritised health hazard classes acute toxicity, sensitisation, and STOT RE were retrieved from the OECD/WPMN (20165, AgNP part 7) and OECD/WPMN (2017, AgNP summary) dossiers, the publicly available data in the REACH registration dossier of silver, and from recent scientific publications regarding inhalational/ intratracheal exposure to AgNP.

Table 7 Number of studies on AgNP for the prioritised hazard classes.

Hazard class	Number of studies (OECD-dossier)	Further studies retrieved for this project
Acute toxicity	Oral 2	0
	Inhalation 0	0
	Dermal 1	0
Skin Sensitisation	2	0
Specific target organ toxicity repeated exposure	Oral 5	5 key studies from REACH reg
	Inhalation 3	1 key study from REACH reg Sung et al. (2009) Sung et al. (2008)

Further descriptions of studies are given in Appendix C.

In the following sections the test data and observations are described and discussed from those studies considered most relevant for GHS classification.

4.3.2 Acute toxicity

Only very short descriptions of acute toxicity studies will be given as the overall data on AgNP point in the direction of low acute toxicity.

4.3.2.1 Oral exposure

Short summary of most relevant data

OECD/WPMN (2016, AgNP part 7) reported two Acute oral toxicity studies for citrate-AgNP (cAgNP) according to OECD TG 423 and using oral gavage of 300 and 2,000 mg cAgNP/kg bw in 6 animals/group (female or male rats). No symptoms were observed at a starting dose of 300 mg/kg bw, therefore the dose was increased up to 2,000 mg/kg bw. No deaths or abnormal findings were observed at the maximum concentration for 14 days. LD50 of cAgNPs was considered to be higher than 2,000 mg/kg bw in both female and male rats.

Applicability of test methods

The OECD test methods for acute oral toxicity are considered applicable to AgNP.

Applicability of GHS criteria and classification

The GHS criteria for acute oral toxicity are applicable to the test data on AgNP. As the oral LD50 was found to be above 2000 mg/kg bw for AgNP no classification is warranted.

Data gaps and uncertainties

Test data is only available for one type of AgNP: citrate stabilised AgNP. Therefore it cannot be ruled out that other types of AgNPs may have a different toxic potential. However, due to the lack of any toxic potential acute oral toxicity is not considered an end-point of concern for AgNPs.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.3.2.2 Dermal exposure

Short summary of most relevant data

Acute dermal toxicity study of cAgNP was conducted according to OECD TG 402 in female and male rats. No deaths or abnormal findings were observed at the maximum concentration, 2,000 mg/kg bw, after 14 days. Although the dose tested was specified as 2,000 mg/kg bw, 2,400 mg/kg bw was administered because the specific gravity of 1.2 was not considered in preparation for the test material. LD50 of cAgNPs was considered to be >2,000 mg/kg bw in rats.

Applicability of test methods

The OECD TG 402 test method for acute dermal toxicity is considered applicable to AgNP.

Applicability of GHS criteria and classification

The GHS criteria for acute dermal toxicity is applicable to the test data on AgNP. No toxicity was observed at a dose level of 2000 mg AgNP/kg bw and thus, no classification is warranted.

Data gaps and uncertainties

Test data is only available for one type of AgNP: citrate stabilised AgNP. Therefore, it cannot be ruled out that other types of AgNPs may have a different toxicity potential. However, due to the lack of any toxic potential acute dermal tocixity is not considered an end-point of concern for AgNPs

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.3.2.3 Inhalation exposure

No acute inhalation toxicity studies were available for inhalation exposure.

4.3.3 Skin sensitisation

Short summary of most relevant data

Two skin sensitisation studies on cAgNP were conducted according to the OECD TG 406 and in compliance with GLP (OECD/WPMN 2016, AgNP part 7).

In a Buehler test (not performed according to GLP) , guinea pigs (20 males) were induced dermally (day 7 and 14) with 0.4 mL of cAgNP (20.48%) in an occlusive patch. Challenge was performed day 21 with a 0.1% (w/v) 1-chloro-2,4-dinitrobenzene(DNCB) and the test substance. The negative control was distilled water and the positive control 1% (w/v) DNCBc (10 males/group). Skin reaction was graded according to "Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions" in OECD TG No. 406. No skin reaction was observed in any of the treated groups at 24 and 48 hours after the challenge. Positive control induced skin sensitization.

In a GPMT test (not performed according to GLP), guinea pigs (20 males) were at the first induction induced by 3 intradermal injection with 0.1 mL of cAgNPs (20.48%) or 1.0% citrate solution for negative control. At the second induction (1 week later) and the challenge test (two weeks later) 0.5 mL of the test substance was applied by occlusive patch. For negative control (10 males), 1.0% citrate solution was applied instead of the test material. Skin reaction was graded according to "Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions" in OECD TG 406. One out of 20 test animals (5%) exhibited grade 1 erythema at 24 or 48 hours after challenge, but no other skin reaction was observed in other animals. No data regarding any positive control group.The authors concluded that cAgNPs were a weak sensitizer in Guinea pig in this GPMT test.

Applicability of test methods

The OECD TG 406 test method for skin sensitisation is considered applicable to AgNP.

Applicability of GHS criteria and classification

The GHS criteria for skin sensitisation are applicable to the test data on AgNP. In the studies in the OECD/WPMN dossiers, AgNP was concluded to be a weak skin sensitiser and should be classified in category 1 according to the GHS criteria.

However, only the GPMT test (the second study) recorded a positive reaction in one animal following the challenge exposure at 24 and 48 hours (i.e. a response of 5%). This is considerably lower than the response indicated in the GHS classification criteria for category 1B described in paragraph 3.4.2.2.3.1:

"When an adjuvant type guinea pig test method for skin sensitisation is used, a response of at least 30% of the animals is considered as positive. For a non-adjuvant guinea pig test method a response of at least 15% of the animals is considered positive".

Also, a classification in category 1B based on results from a GPMT test would require ≥ 30 % of the animals responding at > 1 % intradermal induction dose according to GHS paragraph 3.4.2.2.3.2.

Therefore, based on the data on skin sensitising effects of cAgNP provided in the OECD/WPMN dossiers, classification for skin sensitisation of AgNP is not warranted according to the CLP and GHS criteria.

Data gaps and uncertainties

Test data are only available for one type of AgNP: citrate stabilised AgNP. Therefore, it cannot be ruled out that other types of AgNPs have a slightly different sensitising potential. However, due to the very low sensitising potential to skin at a very high induction concentration, skin sensitisation cannot be considered an end-point of concern for AgNPs.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.3.4 STOT RE

4.3.4.1 Oral exposure

Short summary of most relevant data

Three OECD TG 408 90-day repeated dose oral toxicity studies were available of which two were included in the OECD/WPMN (2015 part 7 and 2017 summary) dossiers and further one was included in the REACH registration of silver (CAS: 7440-22-4).

In the first OECD TG 408 study, female and male Sprague-Dawley rats (10/sex/dose) were exposed to cAgNPs (0, 25, 100, 400 mg/kg bw/day) via the drinking water, once daily for 90 days. Study examination included mortality, toxic effects, body weight, clinical biochemistry and macroscopic examination. No significant toxicity or mortality were observed in any of the groups. However, Serum triglyceride (TG) was decreased while total bilirubin levels were increased in treated groups in a non-dose-dependent manner. Thus, the overall conclusion was that cAgNPs did not exhibit any toxicity in the 90-day repeated dose oral toxicity study in rodents and a NOAEL > 400 mg/kg bw/day was suggested (OECD/WPMN 2017, AgNP summary).

In the second OECD TG 408 study, female and male Fisher 344 rats (10/sex/dose) were exposed to nanosized silver powder (0, 30, 125, 500 mg/kg bw/day) via oral gavage, once daily for 90 days. Examinations during the study included mortality, clinical signs, body weight, food and water consumption, haematology, clinical chemistry, ophthalmoscope, organ weights, gross and histopathological examinations. No mortality or clinical signs were observed. Changes in body weight gain was observed in male rats treated with 500 mg/kg bw/day with no changes observed on water and food consumption for any group. For blood chemistry parameters, total cholesterol and total bilirubin were elevated in male rats treated with 125 mg/kg bw/day and decreases in magnesium, total protein, and inorganic phosphorus were detected in female rats treated with 125 mg/kg bw/day. No significant changes in organ weights were observed in female and male rats except major increased testis weight in males treated with 500 mg/kg bw/day and decreased weight of the right kidney were observed in female rats exposed to 30 and 125 mg/kg bw/day. Moreover, effects in the liver such as bile duct hyperplasia and focal inflammation were prominent, although dose-response relationship was not detected. In conclusion the authors suggested a LOAEL of 125 mg/kg bw/day and NOAEL < 30 mg/kg bw/day for repeated nano silver powder exposure in rat (OECD/WPMN 2017, AgNP summary).

In the third OECD TG 408 study, female and male Sprague-Dawley rats (12/sex/dose) were exposed to cAgNPs (0, 257.6, 515.3, 1030.5 mg/kg bw/day) via oral gavage, once daily for 90 days. Examinations during the study included mortality, clinical signs, body weight, food and water consumption, haematology, clinical chemistry, ophthalmoscope, organ weights, gross and histopathological examination, and biodistribution of Ag. No mortality or clinical signs were observed during the study. However, several treatment-related systemic effects of cAgNPs were observed in females and males treated with the highest dose. An increased white blood cell level (1.3-fold compared to control group) was found in females and decrease platelet level was

found in males treated with cAgNPs 1030 mg/kg bw/d compared with their control groups. Serum alkaline phosphatase was significantly increased both males and females treated with 1030 mg/kg bw/d. Also, rat treated with 1030 mg/kg bw/d had increased incidence of lymphocytic infiltration in the liver (8/12, male; 6/12, female) vs. controls (5/12, male; 4/12, female) and to a lesser extent in the kidney. Moreover, male and females treated with 1030 mg cAgNP/kg bw/d had decreased weight of the pituitary gland and the ovary, respectively.In addition, biodistribution analysis revealed significant concentrations of silver in the blood of all treatment group compared to the control group. Further, increased concentrations of sliver were found in spleen, lung and brain after cAgNP administration which showed a clear dose-response relationship in both male and female rats. In conclusion, a NOAEL (female) of 515.3 mg/kg bw/day was suggested, and the systemic toxicity of cAgNPS, including liver and kidney toxicity, might be explained by extensive systemic distribution of silver originating from the silver nanoparticles (REACH registration on silver, CAS: 7440-22-4).

Two OECD TG 407 28 day repeated dose oral toxicity studies were available from the OECD/WPMN (2015 part 7 and 2017 summary) dossier. In both studies female and male rats were orally administrated once daily with cAgNPs (0, 25, 100, 400 mg/kg bw/day - study 1) or (0, 30, 300, 1000 mg/kg bw/day – study 2). Study 1 showed no mortality or significant toxicity, while some changes in blood chemistry markers were observed in female rats. In study 2, no mortality or clinical signs were observed. However, microscopic examinations revealed a dose-dependent accumulation of AgNPs in the lamina propria of both the small and large intestine. Moreover, analysis suggests that cAgNPs are a powerful intestinal secretagogue and cablable of inducing an abnormal mucin composition in the intestinal mucosa. No NOAEL or LOAEL were suggested in both studies.

Additionally, two 28-day repeated oral toxicity studies (key studies) were obtained from the REACH registration on silver (CAS: 7440-22-4). In one study performed according to OECD TG 407 in rats, daily oral gavage to 0, 30, 300 and 1000 mg AgNPs/kg bw/day for 28 days resulted in effects on red blood cell parameters in females at a dose of 300 mg/kg bw/day, and indication of liver damage (increased alkaline phosphatase, cholesterol and total protein) in females at 300 or 1000 mg/kg bw/day. Also, incidences of bile duct hyperplasia around the central vein were observed in both female an male animals in a dose-dependent way. No NOAEL or LOAEL were suggested by the authors, even though dose dependent effects were observed from the dose level of 300 mg/kg bw/day (suggesting 300 mg/kg bw/day as LOAEL for both sexes).

In the other key study from the REACH registration on silver, repeated oral toxicity was assessed in rats by daily oral gavage with polyvinylpyrrolidone (PVP) stabilised AgNPs for a 28, no test guideline was followed. The doses were 2.25, 4.5 or 9.0 mg. No mortality or clinical signs were obsered in the study period. Sporadic differences in haematological parameters and organ weight were recorded for the AgNP- treated groups compared with the vehicle controls. However, the authors concluded that these results were not of concern and suggested a NOAEL ≥ 9 mg Ag/kg bw/day, the highest dose tested.

One OECD TG 422 study (Combined Repeated Dose Toxicity Study with Reproduction/ Developmental Toxicity Screening Test) was reported in the OECD/WPMN (2015, AgNP part 7 and 2017 summary) dossiers. Rats (50/sex/dose) were daily orally administered cAgNPs at 62.5, 125, and 250 mg/kg bw/day for 42 days (male) and 52 days (female). No changes in body weight gain, water and food consumption, mortality, clinical signs or significant toxicity period (haematology, serum biochemistry, histopathology, urinalysis and necropsy) or in the post-mortem analysis. An almost identical OECD TG 422 study was available from the REACH registration on silver (key study 4). Same dose-regime was applied in this study, but with fewer animal numbers (10/sex/dose), and an additional Ag biodistribution study was added. cAgNPs did not cause any clinical signs or significant toxicity. However, the biodistribution study of cAgNP in pregnant rats revealed a 213-fold increase in cAgNP accumulation in lung tissue, 34-fold increase in liver and a 13-fold increase in the kidneys compared to control rats.

Applicability of test methods

The OECD TGs on repeated oral exposure are considered applicable as no methodological difficulties have been reported in the conducted tests.

Applicability of GHS criteria and classification

The GHS criteria for STOT RE classification in relation to oral toxicity are considered applicable.

In the report by Lee et al. (2017), it was suggested to clasify AgNP as STOT RE category 2 based on the reporting a dose-dependent accumulation of AgNPs in the lamina propria of the intestine from a 28-day study and changes in mucin composition in goblet cells and abnormal mucin secretion in the intestine. Moreover, Lee et al.(2017) based their suggestion of a STOT RE 2 classification on NOAEL values of 30 mg/kg bw/day obtained from a 28 day and 90 day study – no references are given.

It is considered as unlikely that abnormal mucin production without any further description of the toxicological impact of this would warrant a STOT RE classification. Further as indicated below, other repeated dose toxicity studies have not found any adverse effects associated with this finding.

When considering other types of effects, the lowest LOAEL from the 28-day studies was 300 mg/kg bw/day based on effects on red blood cell parameters, increased alkaline phosphatase, cholesterol and total protein and bile duct hyperplasia. However, these effects regarding severity is not considered sufficient to warrant a STOT RE 2 classification (the GHS criteria stipulate an upper dose level of 300 mg/kg bw/day at which severe effects should be observed).

With respect to the three 90-day studies a lowest LOAEL of 125 mg/kg bw/day was found based on bile duct hyperplasia and focal inflammation in the liver, although dose-response relationship was not detected. However, from two other 90-day studies using dose levels up to 1030 mg/kg bw/day NOAELs of 400 and 515 mg/kg bw/day were found. Thus, the findings from the 90-day studies do not point towards severe effects at dose levels that would lead to a STOT RE 2 classification (upper GHS value for a STOT RE 2 classification is 100 mg/kg bw/day at which severe effcts should occur).

Overall, the data from the repeated oral dose testing do not warrant any STOT RE classification for repeated oral exposure.

Data gaps and uncertainties

AgNPs have been tested for repeated dose toxicity by oral exposure in five OECD TG 407 studies, three OECD TG 408 studies, and two OECD TG 422 studies. Thus, there is no data gap on testing.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nanospecific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.3.4.2 Inhalation exposure

Short summary of most relevant data

The OECD/WPMN (2015, AgNP part 7) describes one OECD TG 412, repeated inhalation 28 day study, and one OECD TG 413, repeated inhalation 90 day study:

In a study by Li *et al.* (2017), rats were exposed 6 h/day, 5 days/week for 4 weeks by inhalation to AgNP (purity of 99.98% and particle size below 20 nm) at the target concentration levels of

1.2×10^4 particles/cm^3 (1.2×10^6 nm^2/cm^2), 1.2×10^5 particles/cm^3, 8.5×10^7 nm^2/cm^2) and 1.2×10^6 particles/cm^3 (1.8×10^9 nm^2/cm^2) or fresh air as control (no mass metric dose levels indicated). According to OECD/WPMN (2015, AgNP summary 7), the following was concluded from the study:

"No significant gross pathological or organ weight changes were observed. Histopathological examination of the male rat livers revealed one case of cytoplasmic vacuolisation in the control, four cases in the low-dose group, and one case each in the middle- and high-dose groups, respectively. For the female rats, two cases of cytoplasmic vacuolisation were detected in the control and low-dose group, respectively, six cases in the middle dose group, and seven cases in the high-dose group. Two cases of hepatic focal necrosis were detected in the male rats of the high-dose group and one case in the female rats of the high-dose group. The other organs, including the kidneys, spleen, lungs, adrenals, heart, reproductive organs, brain, and nasal cavity were also examined histopathologically, with no distinct findings…. The LOAEL of silver nanoparticles was considered to be (1.2×10^6 nm^2/cm^2), 1.2×10^5 particles/cm^3 in Sprague-Dawley rats"

In a study by Sung et al. (2009), rats were exposed 6 h/day, 5 days/week, for 90 days by inhalation to AgNP (purity of 99.99% and particle sizes in the range of 6-55 nm) at dose levels of 0,49 µg/m^3 (0.6×10^6 particles/cm^3), 133 µg/m^3 (1.4×10^6 particles/cm^3) and 515 µg/m^3 (3.0×10^6 particles/cm^3) or to filtered air as control. According to OECD/WPMN (2015, AgNP summary 7), the following was concluded from the study:

"Liver and lung were appeared to be the target organs of inhaled silver nanoparticles. BAL analysis revealed concentration dependent increase in albumin, lactate dehydrogenase and total protein were found in female rats. Decreases in tidal volume were observed in the male rats, which was concentration-dependent. In case of female rats, such decrease was detected in the group exposed to 1.4×10^6 particles/cm^3. No change was detected in prothrombin time and activated partial thromboplastin time. Significant increase in erythrocyte clotting was found in female rats exposed to 3.0×10^6 particles/cm^3. Distribution in lung, kidney, liver, blood, brain and olfactory nerve was prominent and concentration dependent. The NOAEL of silver nanoparticles was $1.0 \times 10^{6*}$ particles/cm^3 or 100 µg/m^{3*} in Sprague-Dawley rats."
*corresponding to the analytical concentration of 133 µg/m^3 (1.4×10^6 particles/cm^3).

This study is also the key study in the REACH registration of silver that also concluded a NOAEC of 133 µg/m^3.

The original publication was consulted for further data:
In the publication by Sung et al. (2009), it is indicated that the histopathological findings showed a high incidence of chronic alveolar inflammation, mixed cell perivascular infiltrate, and alveolar macrophage accumulation in the high-dose male and female animals when compared with the controls. In males chronic inflammation was found in 3/10, 2/10 and 8/9 in low, medium and high dose versus 2/10 in controls, while in females the incidences were 2/10, 0/10, 8/10 versus 3/10 in controls indicating exposure associated effects at the highest exposure level in male and female rats. Further bile duct hyperplasia was observed in 5/9 males and 8/10 females at high dose versus 0/10 in male controls and 3/10 in female controls.

In an earlier pubication by Sung *et al.* (2008) the effects on lung function from the exposed animals were described in detail:

From the figures in this publication it can be seen that high exposure male rats at the end of the exposure period compared to controls showed significant decreases in tidal volume, in minute volume and in peak inspiratory flow at about 19, 20 and 26%, respectively (readings form figures in the publication). Female rats at highest exposure level showed significantly reduced minute volume (about 15%) and peak inspiratory flow (about 10%) whereas the tidal volume was not significantly altered compared to control females. In this publication the histopathological lesions were described as dose-dependent increases in lesions related to silver nanoparticle exposure,

including infiltrate mixed cell and chronic alveolar inflammation, thickened alveolar walls and small granulomatous lesions.

Applicability of test methods

The recent updated 2018-versions of OECD TG 412 28-day (subacute) inhalation toxicity study and OECD TG 413 90-day (subchronic) inhalation study specifically address the applicability and design of the tests methods for testing of nanomaterials.

The OECD TG 412 and OECD TG 413 are considered applicable for testing AgNP at low exposure levels. However, the very low exposure levels (highest dose level used was 515 µg/m^3 of AgNP) should be noted. No experience has been gained for higher exposure levels, so the applicability of the test methods for testing AgNP at higher exposure levels cannot be evaluated.

Applicability of GHS criteria and classification

There is no indications that the GHS criteria should not be applicable; however, AgNP has only been tested at very low exposure levels considerably below the GHS classification limit of 20 mg/m^3 for STOT RE 1 classification. At a dose level of 515 µg/m^3 of AgNP (i.e. 1/40 of the upper concentration limit for STOT RE 1) signs of chronic lung inflammation were seen in both male and female rats. Also, the lung function of the animals was significantly decreased at this dose level.

These findings could most relevantly be compared to the following relevant criteria points given in section 3.9.2.7.3 of the GHS (2015):

(b) 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);

(d) significant organ damage noted at necropsy and/or subsequently seen or confirmed at microscopic examination;

(e) multi-focal or diffuse necrosis, fibrosis or granuloma formation in vital organs with regenerative capacity;

(f) morphological changes that are potentially reversible but provide clear evidence of marked organ dysfunction (e.g. severe fatty change in the liver);

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

Based on the current data, the severity of the observed effects is considered to be mild/ moderate and cannot at the low exposure level used be considered life-threatening or severely affecting organ function. However, the effects are still to be considered adverse and if the effects further progress, more severe consequences can be anticipated.

As the effects have been observed at an exposure level 1/40 of the guidance level for a STOT RE classification, a considerably higher degree of severity is to be expected at a 40 times higher dose level.

Thus, signs of chronic lung inflammation and imparied lung function at very low dose levels indicate that STOT RE (with respect to inhalation and with the lungs as the target organ) of AgNP may be appropriate.

Data gaps and uncertainties

Further, data are needed for assessing the applicability of the test methods and for assessing the most appropriate STOT RE classification of AgNP.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria is warranted based on available information.

For evaluation of data from tests using very low exposure levels, more guidance is needed on how to use such data for classification purposes, i.e. how to extrapolate from mild adverse effects at low levels to severe adverse effects at higher dose levels.

4.3.5 Overview of findings for nano silver

An overview of the findings for nano silver is presented in Table 8.

Table 8 Overview of the findings in the evaluation of the test data for nano silver of selected hazard classes.

Nano silver				
Hazard class	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
Acute oral toxicity	++	++ (No classification)	No	No
Acute dermal toxicity	++	++ (No classification)	No	No
Acute inhalation toxicity	0	0 ?	Yes (No test data)	No
Skin Sensitisation	++ *in vivo* 0 *in vitro*	++ (No classification)	No	No
STOT RE oral	++	++ (No classification)	No	No
STOT RE inhalation	++ However only low exposure levels used	++ (STOT RE ?) (inhalation, lung)	Yes (Testing at higher dose levels)	No

** **0:** no assement due to lack of data **- :** not applicable **+:** applicable with limitations*
*++**:** fully applicable **(?)** classification cannot be concluded due to lack of data*

*** The indicated classification is not necessarily applicable for all types/qualities of AgNP as test data is related to one or several representatives of AgNP. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities/types of AgNP as well and that testing/ information for this hazard class is warranted in particular*

4.4 Nano zinc oxide

In the OECD/WPMN dossiers, physicochemical properties are described for four different types of zinc oxide particles, two uncoated nanoforms (MN110, NM112), one coated nanoform with triethoxycaprylylsilane (CAS # 2943-75-1, content 2%) (NM111, Z-Cote HP1), and one uncoated micro-sized form (NM113). The three nanoforms had particle sizes were in the range of 33.8 – 77.5 nm, specific surface areas in the range of 6.6 – 25.9 m^2/g, water soubilities measured after 2 days in the range of 764 – 2536 ng/g, and bulk densities in the range of 0.293 – 0.693 g/cm^3.

4.4.1 Overview of data availability for relevant health hazard classification

The toxicity data on nano zinc oxide on the prioritised health hazard classes, acute toxicity and STOT RE, were retrieved from the OECD/WPMN (2015, ZnO part 3) report, the publicly available data in the REACH registration dossier of zinc oxide, and from recent scientific publications found in web-based search regarding inhalational/ intratracheal exposure to nano zinc oxide.

Table *9* Number of studies on nano zinc oxide for the prioritised hazard classes.

Hazard classes	Number of studies (OECD-dossier)	Further studies retrieved for this project
Acute toxicity	Oral 1	1 (Pasupuleti et al. 2012; from REACH reg)
	Dermal 1	0
	(3 studies using alternative administration methods)	Inhalation 1 (REACH reg.)
Specific target organ toxicity repeated exposure	Oral 0	0
	Dermal 0	1 (Surekha et al. 2012; from REACH reg) 1 (Ryu et al. 2014)
	Inhalation 4	3 Adamcakova-Dodd et al. (2014) Morimoto et al. (2016) Monsé et al. (2018)

The assessment below is a summary of the findings in Appendix D, where more detailed descriptions of the data are given.

4.4.2 Acute toxicity

4.4.2.1 Oral exposure

Short summary of most relevant data

Acute oral toxicity of nano zinc oxide (particle size 20 nm) and sub-microscale zinc dioxide (particle size 120 nm) was studied in mice according to OECD TG 401. For nanoform zinc oxide no deaths occurred up to a dose level of 5000 mg/kg bw, whereas the LD50 value for for sub-microscale zinc oxide was in the range of 2000 - 5000 mg /kg bw (OECD/WPMN (2015, ZnO part 3).

In the REACH registration dossier a study by Pasupuleti et al. (2012) is reported. In an OECD TG 423 (Acute Oral toxicity - Acute Toxic Class Method with modifications regarding dose

levels) rats were orally gavaged with 5, 50, 300, 1,000 and 2,000 mg/kg bw. The average size of the nano ZnO was 63 nm determined in SEM analysis; the average size of nano ZnO (in solution) was 224.7 nm determined using dynamic light scattering (DLS). No mortality occurred and LD50 was concluded to be above 2000 mg/kg bw.

Applicability of test methods

The OECD test methods for acute oral toxicity are considered applicable to nano zinc oxide.

Applicability of GHS criteria and classification

The GHS criteria for acute oral toxicity are applicable to the test data on nano zinc oxide. As the oral LD50 value was found to be above 5000 mg/kg bw for nano zinc oxide no classification is warranted.

Data gaps and uncertainties

Two studies in relation to acute oral toxicity testing have been found for nano zinc oxide. Due to the lack of toxic potential, acute oral tocixity is not considered an end-point of concern for nano zinc oxide.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.4.2.2 Dermal exposure

Short summary of most relevant data

Acute dermal toxicity of a coated nano zinc oxide (coating: triethoxycaprylylsilane 2%) in rats was assessed in an OECD TG 402 study. Dermal exposure to 2000 mg/kg bw for 24 hours caused no indication of toxicity or death up to 14 days post exposure. Thus, an LD50 > 2000 mg/kg was concluded (OECD/WPMN 2015, ZnO part 3).

Applicability of test methods

The OECD TG 402 test method for acute dermal toxicity is considered applicable to nano zinc oxide.

Applicability of GHS criteria and classification

The GHS criteria for acute dermal toxicity are applicable to the test data on nano zinc oxide. No toxicity at a dose level of 2000 mg/kg bw for nano zinc oxide was found and thus no classification is warranted.

Data gaps and uncertainties

Only one study in relation to acute dermal toxicity testing has been found for nano zinc oxide. Due to the lack of toxic potential acute dermal toxicity is not considered an end-point of concern for nano zinc oxide.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance warranted based on available information.

4.4.2.3 Inhalation exposure

Short summary of most relevant data

No acute inhalation toxicity studies were available from the OECD/WPMN (2015, ZnO part 3) dossier; however, an acute inhalation toxicity study on the nanoform is included in the REACH registration of zinc oxide.

In an acute inhalation toxicity test conducted according to EPA OPP 81-3 five female and five male rats were exposed to 1.79 mg/L nano zinc oxide during 4 hours. The nano zinc oxide had a specific surface area of 30 m^2/g and an average particle size of 36 nm. The maximum attainable exposure concentration was 1.79 mg/L with aerosols having an average MMAD of 4.122 µm. No mortality occurred. Clinical signs included activity decrease, crust around eyes and nose, piloerection, ptosis and respiratory gurgle, no longer evident by day 4. No observable abnormalities were found at gross necropsy. The LC50 was concluded to be above 1.79 mg/L.

Intratracheal instillation
Studies using intratracheal administration were included in the OECD/WMPN (2015, ZnO part 3) dossier.

In two studies rats were intratracheally instilled with a single-dose of 0.2 mg to 5 mg nanoform zinc oxide/kg bw (Sayes,2007 and Morimoto et al., 2016). This resulted in signs of lung inflammation and lung damage at 24 hours and 72 hours post exposure. Further, one study also showed increased markers of oxidative stress in lung tissue 72 hours post exposure. However, after one and three months post exposure, these parameters had returned to baseline. No deaths were observed in either of the studies.

In a study by Jacobsen et al. (2016), intratracheal instillation or pharyngeal aspiration of non-coated nanoform zinc oxide in mice were assessed in three non-OECD TG studies using doses between 2- and 100 µg zinc oxide/mouse. Pharyngeal aspiration of 25, 50 and 100 µg nano zinc oxide (purity 99% and particle size 12 nm) caused death in 2, 3 and 5 animals in the respective dose groups (n=5) within 13 days post exposure. Also, instillation of 18 µg nano zinc oxide caused severe clinical signs 48 hours post exposure, and the instilled mice were humanely euthanised. Further, instillation of 2 µg to 15 µg caused acute lung inflammation and histopathological changes in the lung tissue.

Applicability of test methods

Acute inhalation toxicity test methods seems only to be technically applicable for nano zinc oxide at concentration levels up to about 1.8 mg/L.

Applicability of GHS criteria and classification

The GHS criteria can in principle be applied, however, because of technical limitations for obtaining higher exposure levels than 1-2 mg/L of nano zinc oxide, testing up to concentrations covering the range of numerical values in the classification criteria for Acute tox 4 (>1 and ≤ 5 mg/l) cannot be done. Current testing on a coated nanoform of zinc oxide indicated no mortality up to a concentration level of 1.79 mg/L and thus no classification is warranted for this quality of nanoform.

Data gaps and uncertainties

All available data on acute inhalation toxicity from animal experiments are obtained using only one nanoform zinc oxide “Z-COTE HP1” which is a nanosized zinc oxide coated with 2% triethoxycaprylyl-silane. Presently it is not known whether this specific form is representative for other nano zinc oxides. Data on non-coated nano zinc oxide indicate high acute toxicity in mice after intratracheal instillation or pharyngeal aspiration. Thus data from acute inhalation studies of non-coated nano zinc oxide is lacking and is needed for comparison to better understand the acute inhalation toxicity of nano zinc oxide.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.4.3 STOT RE

4.4.3.1 Oral exposure

No oral repeated dose toxicity studies are available neither from OECD/WPMN (2015, ZnO part 3) nor the REACH registration on zinc oxide.

4.4.3.2 Dermal exposure

Short summary of most relevant data

No data are available on repeated dermal exposure in the OECD/WPMN (2015, ZnO part 3) dossier, but a 28-day dermal toxicity study (Surekha et al. 2012) on the nanoform is included in REACH registration of zinc oxide and a further 90-day study (Ryu et al. 2014) was found in the literature.

In an OECD TG 410, Repeated Dose Dermal Toxicity study, rats were dermally exposed to 75, 180, and 360 mg/kg bw /day of nano zinc oxide (particle size 20 nm) 6h/day, 5 days/week for 28 days. Further, a group of rats were exposed to micro-size zinc oxide at a limit dose of 2,000 mg/kg bw/day. No gross pathology or histopathological lesions were observed. However, based on increase in clotting time and decrease in the collagen content in the skin in all the nano zinc oxide treated groups, a LOAEL of 75 mg/kg bw/day was established. The decreases in collagen content were inversely correlated to the dose levels. The effects were reversible in a period of 14 d (Surekha et al. 2012).

In an OECD TG 411 study, nano zinc oxide (citrate coated, particle size of 29 nm and zeta potential of -44.4 mV) was applied in a vehicle of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-citrate buffer on soaked gauze to the shaved skin of rats at dose levels of 0, 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw for 6 hour/day during 90 days. Clinical observations as well as weight and food consumption were measured and recorded daily. Hematology and biochemistry parameters were determined. Gross pathologic and histopathologic examinations were performed on selected tissues from all animals. No increased mortality in the experimental groups were observed. There was dose-dependent increase in irritation at the site of application resulting in skin crusts and areas with hyperkeratosis and papillomatosis at the highest dose level. No abnormal findings were recorded in other organs and a NOAEL for systemic adverse effects of 1000 mg/ kg bw/ day was concluded from the study (Ryu et al. 2014).

Applicability of test methods

The OECD TGs for repeated dermal exposure considered applicable to nano zinc oxide.

Applicability of GHS criteria and classification

The GHS criteria for STOT RE in relation to dermal toxicity are considered applicable to the test data on nano zinc oxide. The findings in relation to increased blood clotting time and decrease in collagen content of the skin are considered of concern, however, the interpretation in the context of the GHS criteria is unclear especially in terms of the severity of the effects. Further, the inverse dose-response relationship for effects on collagen content complicates a clear interpretation. Overall, it is concluded that the data do not warrant a STOT RE classification.

Data gaps and uncertainties

The implications of the findings regarding increase in blood clotting time and decrease in the dermal collagen content after dermal exposure to nano zinc oxide should be examined further in the context of the GHS criteria for STOT RE.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria or guidance is warranted based on available information.

4.4.3.3 Inhalation exposure

Short summary of most relevant data

From the OECD/WPMN (2015, ZnO part 3) dossier, the following studies on coated nano zinc oxide (Z-Cote HP1) are considered most relevant.

One OECD TG 413 (90 days inhalation study in rats) and one OECD TG 412 (14 days inhalation study in rats) have been conducted with the same quality of nano zinc oxide (coated with triethoxycaprylylsilane 2%) using exposure levels in the range of 0.3 mg/m3 to 8 mg/m3. Very slight to slight lung inflammation (neutrophil influx), lung damage (LDH), increased levels of reactive oxygen species in BAL fluid, and histopathological changes in the nasal and paranasal cavities were observed. However, all the observed effects were reversible within 14 -29 days post exposure. A NOAEC of 1.5 mg/m^3 for the 90 day study and a NOAEC of 2.0 mg/m^3 for the 14 day study was concluded OECD/WPMN (2015, ZnO part 3).

In a five-days inhalation study, rats were exposed to nano zinc oxide coated with triethoxycaprylylsilane 2% (Z-Cote HP1) at dose levels in the range of 0.5 - 12.5 mg/m^3 during 6h/day for 5 days/week followed by a three-week recovery period. Local inflammation and histopathological changes were observed in the lungs, and multifocal necrosis of the olfactory epithelium was also observed at all dose-levels. However, all effects were reversible within the recovery period. Thus, a LOAEC of 0.5 mg/m^3 was concluded (OECD/WPMN (2015, ZnO part 3).

Data on other qualities of non-coated nano zinc oxide were found from a literature search:

In a non-OECD guideline study, mice were exposed to nano zinc oxide at 3.5 mg/m^3 4h/day for 14- or 90 days and necropsied immediately or 21 days post exposure. In the 14- and 90 days studies the primary particle size was measured to be 15 and 26 nm, respectively. No neutrophilic inflammation or histopathological changes were observed in the lungs in any of the studies. However, there was a significant increase in macrophages, IL-12 and MIP-1-alpha in BAL fluid, indicating some state of inflammation (Adamcakova-Dodd et al. 2014).

In a non-OECD guideline inhalation study, rats were exposed to nano zinc oxide (2-10 mg/m^3 /6h/day for 5 days/week) for four weeks and euthanized at day 1, 3 or 1 ,3 and 6 months post exposure. The zinc oxide nanoparticles with a purity of 99.94 wt. % had a size diameter of 35 nm and a specific surface area of 31 m^2/g.Transient acute lung inflammation (neutrophil influx) and a transient increase in markers of oxidative stress (HO-1) in BAL fluid were observed at day three after exposure but returned to baseline one and three months post exposure **(**Morimoto et al. 2016).

In a study with healthy (non-smoking) human volunteers, nano zinc oxide particles (freshly generated by pyrolysis) were inhaled at exposure levels in the range 0.5 - 2.0 mg/m^3 for 4 h on 4 different days (included 2 hours of cycling with a low workload). The exposure caused flu-like symptoms and a dose-dependent increase in inflammatory markers in blood (Monsé et al.

2018). The findings from this study is difficult to interpret in relation to industrially manufactured nano zinc oxide as the findings were in relation to freshly generated nano zinc oxide fume.

Applicability of test methods

The recent updated 2018-versions of OECD TG 412 28-day (subacute) inhalation toxicity study and OECD TG 413 90-day (subchronic) inhalation study specifically address the applicability and design of the tests methods for testing of nanomaterials.

Repeated exposure inhalation testing with nano zinc oxide has only been conducted up to a highest dose level of 12 mg/m^3. However, acute inhalation toxicity testing has been performed up to a dose level of 1790 mg/m^3, indicating that higher dose levels can be used in repeated dose toxicity testing as well.

Applicability of GHS criteria and classification

In an OECD TG 413 (90-day inhalation) study, the highest dose of nano zinc oxide used was 4.5 mg/ m^3 6h/ day, i.e. 4-fold lower than the upper criteria value of 20 mg/m^3 for classification as STOT RE 1, and 44-fold lower than the upper criteria value of of 200 mg/m^3 for STOT RE 2 classification. Also, the highest dose levels used in the OECD TG 412 (14-day) inhalation study was 8 mg/m^3 6h/day, i.e. a very low concentration compared to the classification criteria.

The observed inflammatory responses in the lung in the OECD TG 412 and 413 were considered to be very slight and cannot be considered severe adverse effects that warrant a STOT RE classification. Effects in the nasal epithelium (multifocal very slight to slight degeneration of the olfactory epithelium) noted in the OECD 412 study at 8 mg/m^3 and also found in a five-day inhalation study using dose levels in the range of 0.5 - 12.5 mg/m^3, are considered of more concern, although these effects were still considered slight and reversible in the follow-up period. Thus, if tested at higher dose-levels these effects may very well develop to more serious effects.

Currently, the present data in an overall weight of evidence assessment indicate that there is not sufficient evidence to support classification as STOT RE by inhalation.

Data gaps and uncertainties

Most data for repeated inhalation exposure have been generated using a specific coated quality of nano zinc oxide (Z-Cote HP1). Thus it is difficult to evaluate to which extent other qualities of nano zinc oxide (coated and non-coated) would have a comparable toxicological profile. To fill this knowledge-gap, further testing using other qualities of nano zinc oxide is needed.

Most importantly, animal inhalation studies (90 days or 28 days) using higher dose-levels in the range 20-200 mg/m^3 are needed to clarify whether a STOT RE classification for nano zinc oxide would be needed as the current testing was more focused on NOAEC derivation in relation to inflammatory responses than examining the need for STOT RE classification.

Need for revison of GHS criteria or guidance in relation to CLP

No proposal for further work on nano-specific adaptions of GHS/CLP criteria is warranted based on available information.

In order to get more optimal use of the current data from experimental animal studies, guidance is needed on how to include other toxic parameters (e.g., markers for inflammatory responses and mild histopahtological changes) induced at low exposure levels to be used as markers for severe effects. Also, guidance is needed on how data from studies using intratracheal instillation or pharyngeal aspiration may be used as support for classification purposes.

4.4.4 Overview of findings for nano zinc oxide

An overview of the findings for nano zinc oxide is presented in Table 10.

Table 10 Overview of the findings in the evaluation of the test data for nano zinc oxide of selected hazard classes.

Nano zinc oxide				
Hazard class	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments** **Criteria/ guidance**
Acute Tox oral	++	++ (No classification)	No	No
Acute Tox dermal	++	++ (No classification)	No	No
Acute Tox inhalation	+ (high exposure levels not achievable in testing)	++ Testing limitations (No classification)	Yes (Data on non-coated qualities lacking)	No
STOT RE oral	0	0	Yes (No test data)	No
STOT RE dermal	++	++ (No classification)	Yes (Increase in blood clotting time and decrease in dermal collagen content to be further examined)	No
STOT RE inhalation	++	++ (No classification/ ?)	Yes (Further testing needed for different qualities, testing at higher dose levels)	For classification purpose: how to interpret mild toxic repsonse at low exposure levels. How to interpret data from instillation/ aspiration tests.

* ***0:*** *no assement due to lack of data* ***- :*** *not applicable* ***+:*** *applicable with limitations*
++: *fully applicable* ***(?)*** *classification cannot be concluded due to lack of data*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

******** *The indicated classification is not necessarily applicable for all types/qualities of nano zinc oxide as test data is related to one or several representatives of nano zinc oxide. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities/types of nano zinc oxide as well and that testing/ information on this end-point is especially warranted.*

5. Findings for the selected hazard classes

In this chapter the findings from the four nanomaterials will be presented under the headings of the hazard classes examined.

5.1 Acute toxicity

5.1.1 Acute oral toxicity

An overview of the findings is given in Table 11.

Table 11 Overview of the findings from the evaluation of the test data on acute oral toxicity of the four nanomaterials.

Acute oral toxicity				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
SWCNT	+ (only low dose level achievable for testing)	++ Testing limitations (?)	Yes (only test on one type of SWNCT available)	Guidance may be needed for testing voluminous NMs
Nano silicon dioxide	++	++ (No classification)	No	No
Nano silver	++	++ (No classification)	No	No
Nano zinc oxide	++	++ (No classification)	No	No

* ***0:*** *no assement due to lack of data* ***- :*** *not applicable* ***+:*** *applicable with limitations* ***++:*** *fully applicable* ***(?)*** *classification cannot be concluded due to lack of data.*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

From the Table 11 it can be seen that the current OECD test methods for acute oral toxicity testing in general are applicable to testing of nanomaterials. However, for voluminous nanomaterials (in this case SWCNT) since only low mass based dose levels can be technically achieved. It may not be possible to achieve high enough exposure levels that correspond to the Acute Toxicity estimates for the less severe hazard categories i.e Acute tox. 3/4/5. Thus, for such nanomaterials it may no based on the current mass-based GHS criteria be possible to differentiate the acute toxic potential. However, the studied data do not indicate whether other dose metrics such as specific surface area or particle(or fiber) number concentrations may be better metrics for obtaining acute oral toxicity classification in various categories.

5.1.2 Acute dermal toxicity

An overview of the findings is given in Table 12.

Table 12 Overview of the findings from the evaluation of the test data on acute dermal toxicity of the four nanomaterials.

Acute dermal toxicity				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
SWCNT	+ (only low dose level achievable for testing)	++ Testing limitations (?)	Yes (no gudeline testing avaialble)	Guidance may be needed for testing voluminous NMs
Nano silicon dioxide	++	++ (No classification)	No	No
Nano silver	++	++ No classification	No	No
Nano zinc oxide	++	++ (No classification)	No	No

* ***0:*** *no assement due to lack of data* ***- :*** *not applicable* ***+:*** *applicable with limitations* ***++:*** *fully applicable* ***(?)*** *classification cannot be concluded due to lack of data.*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

From the Table 12, it can be seen that the current OECD test methods for acute dermal toxicity testing in general are applicable to testing of nanomaterials. However, for voluminous nanomaterials (in this case SWCNT) only low mass based dose levels can be achieved. It may not be possible to achieve high enough exposure levels that correspond to the Acute Toxicity estimates for the less severe hazard categories i.e Acute tox. 3/4/5.

5.1.3 Acute inhalation toxicity

An overview of the findings is given in Table 13.

Table 13 Overview of the findings from the evaluation of the test data on acute inhalationtoxicity of the four nanomaterials.

Acute inhalation toxicity				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/guidance**
SWCNT	0	0 (?)	Yes (No test data)	Guidance may be needed for testing voluminous NMs
Nano silicon dioxide	+ (high exposure levels not achievable in testing)	++ Testing limitations (No classification)	No	No
Nano silver	0	0 (?)	Yes (No test data)	No
Nano zinc oxide	+ (high exposure levels not achievable in testing)	++ Testing limitations (No classification)	Yes (Data on non-coated qualities lacking)	No

* ***0:*** *no assement due to lack of data* ***- :*** *not applicable* ***+:*** *applicable with limitations* ***++****: fully applicable* ***(?)*** *classification cannot be concluded due to lack of data.*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard end-point indicates that this may be a relevant classification for other qulaitites of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

From the Table 13 it can be seen that the current OECD test methods for acute inhalation toxicity testing in general are applicable to testing of nanomaterials. However, for some nanomaterials (in this case nano silicon dioxide and nano zinc oxide) only relatively low mass based dose levels can be achieved. This aspect is assumed also to apply for SWCNT although no data on acute inhalation toxicity testing is available. Thus, it may not be possible to achieve high enough exposure levels that correspond to the Acute Toxicity estimates for the less severe hazardcategories i.e Acute tox. 3/4/5.

Thus, for such nanomaterials it may no based on the current mass-based GHS criteria be possible to differentiate the acute toxic potential.However, the data do not indicate whether other dose metrics such as specific surface area or particle (or fiber) number concentrations may be better metrics for obtaining acute toxicity classification in various categories.

5.2 Serious eye damage or eye irritation

Only data from SWCNT were evaluated. An overview of the findings is given in Table 14.

Table 14 Overview of the findings from the evaluation of the test data on eye irritation / eye damage of SWCNT.

Eye damage/ irritation				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/guidance**
SWCNT	*In vivo* - (only low dose level achievable for testing) *In vitro* + / ++	+ (At least Eye Irrit 2)	Yes (only two non-adequate in vivo tests available and only one in vitro test available)	Guidance may be needed for testing voluminous NMs both *in vivo* and *in vitro.*

** **0**: no assement due to lack of data **- :** not applicable **+:** applicable with limitations **++:** fully applicable*
***(?)** classification cannot be concluded due to lack of data.*
****** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

No overall observations of the findings can be made as only data on SWCNT have been evaluated.

5.3 Skin sensitisation

Only data on nano silver were evaluated. An overview of the findings is given in Table 15.

Table 15 Overview of the findings from the evaluation of the test data on skin sensitisation of nano silver.

Skin sensitisation				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
Nano silver	**Skin sensitisation** *In vivo*			
	++	++ (No classification)	No	No
	Skin sensitisation *In vitro*			
	0	0	0	0

** **0:** no assement due to lack of data **- :** not applicable **+:** applicable with limitations **++:** fully applicable*
***(?)** classification cannot be concluded due to lack of data.*
****** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard end-point indicates that this may be a relevant classification for other quaities of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

No overall observations from the findings can be made as only data on nano silver have been evaluated.

5.4 Specific target organ toxicity, repeated exposure

5.4.1 Oral exposure

An overview of the findings is given in Table 16.

Table 16 Overview of the findings from the evaluation of the test data on repeated oral exposure of the four nanomaterials.

STOT RE oral exposure				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
SWCNT	+ (only low dose level achievable for testing)	++ Testing limitations (?)	Yes (only test on one type of SWNCT available)	Guidance may be needed for voluminous NMs
Nano silicon dioxide	++	++ (No classification/ ?)	Yes (new study suggests effects at low exposure)	No
Nano silver	++	++ (No classification)	No	No
Nano zinc oxide	0	0	Yes (no test data)	No

* ***0:*** *no assement due to lack of data* ***- :*** *not applicable* ***+:*** *applicable with limitations* ***++:*** *fully applicable* ***(?)*** *classification cannot be concluded due to lack of data.*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qulaitites of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

From the Table 16 it can be seen that the current OECD test methods for repeated oral exposure in general are applicable to testing of nanomaterials. However, for voluminous nanomaterials (in this case SWCNT) only low mass based dose levels can be achieved. It may not be possible to technically achieve high enough exposure levels that correspond to the dose `guidance values` for the less severe hazard category 2 for STOT RE.

Thus, for voluminous nanomaterials it may no based on the current mass-based GHS criteria be possible to differentiate the potential for STOT RE. However, the data do not indicate whether other dose metrics such as specific surface area or particle (or fiber) number concentrations may be better metrics for obtaining differentiation in the STOT RE classification.

5.4.2 Dermal exposure

Only data on nano zinc oxide was available. The findings are shown in Table 17.

Table 17 Overview of findings from the evaluation of the test data on repeated dermal exposure on nano zinc oxide.

STOT RE dermal exposure				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
Nano zinc oxide	++	++ (No classification)	Yes (Increase in blood clotting time and decrease in dermal collagen content to be further examined)	No

** **0**: no assement due to lack of data **- :** not applicable **+:** applicable with limitations **++:** fully applicable **(?)** classification cannot be concluded due to lack of data.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard end-point indicates that this may be a relevant classification for other qulaitites of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

No overall observations of the findings can be made as only data on SWCNT have been evaluated.

5.4.3 Inhalation exposure

An overview of the findings is given in Table 18.

Table 18 Overview of the findings from the evaluation of the test data on repeated inhalation exposure on the four nanomaterials.

STOT RE inhalation exposure				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments** **Criteria/ guidance**
SWCNT	+ (only low dose level achievable for testing)	++ Testing limitations (STOT RE1)	Yes (only two 28-day studies available)	Guidance is needed for testing voluminous NMs. For classification pupose: how to interpret mild toxic repsonse at low exposure levels. How to interpret data from instillation/ aspiration tests)
Nano silicon dioxide	++	++ (STOT RE2, maybe RE1)	Yes (Data on precipitated qualities lacking, testing at higher dose levels)	No
Nano silver	++ (However only low exposure levels used)	++ STOT RE ? (inhalation, lung)	Yes (Testing at higher dose levels)	No
Nano zinc oxide	++	++ (No classification)	Yes (further testing needed for different qualities, testing at higher dose levels)	For classification purpose: how to interpret mild toxic repsonse at low exposure levels. How to interpret data from instillation/aspiration tests)

* ***0:*** *no assement due to lack of data* ***- :*** *not applicable* ***+:*** *applicable with limitations* ***++:*** *fully applicable*
(?) *classification cannot be concluded due to lack of data.*

Testing limitations*: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard end-point indicates that this may be a relevant classification for other qulaitites of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

From the Table 18 it can be seen that the current OECD test methods for repeated inhalation exposure are applicable to testing of nanomaterials, not at least as the most recent updates of OECD TG 412 and 413 specifically include parameters in relation to testing of nanomaterials. Furthermore, aspect regarding repeated inhalation testing is also addressed in the nanomaterial appendix to ECHA´s “endpoint specific guidance” (ECHA 2017).

However, for voluminous nanomaterials (in this case SWCNT) only low mass based dose levels can be achieved and it may not be possible to technically achieve high enough exposure concentrations that correspond to the dose `guidance values` for the less severe hazard category 2 for STOT RE. Thus, for voluminous nanomaterials it may no based on the current mass-based GHS criteria be possible to differentiate the potential for STOT RE. However, the

studied data do not indicate whether other dose metrics such as specific surface area or particle (or fiber) number concentrations may be better metrics for obtaining differentiation in the STOT RE classification.

5.5 Germ cell mutagenicity

Only data on SWCNT was evaluated. The findings are shown in Table 19.

Table 19 Overview of the findings from the evaluation of the test data on mutagenicity of SWCNT .

Germ cell mutagenicity				
Substance	**Applicability of test methods***	**Applicability of GHS criteria* (Classification)****	**Data gaps**	**Comments Criteria/ guidance**
SWCNT	***In vitro***			
	++ *In vitro* (Mammalian cells)			Guidance on NM testing for both *in vitro* and *in vivo* available
	In vivo			
	++ *In vivo*	++ (Muta. 2)	Yes (further testing on site-of-contact tissue)	

** **0:** no assement due to lack of data **- :** not applicable **+:** applicable with limitations **++:** fully applicable (?) classification cannot be concluded due to lack of data.*

*** The indicated classification is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard end-point indicates that this may be a relevant classification for other qulaitites of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*

No overall observations from the findings can be made as only data on SWCNT have been evaluated.

6. Overall findings from the project

Applicability of GHS criteria on the test data for the selected nanomaterials

An overview of the applicability of GHS criteria on the test data for the selected nanomaterials is given below in Table 20.

Table 20 Applicablity of GHS criteria for the selected nanomaterials and the examined hazard classes.

Applicability of the GHS classification criteria				
Hazard class	**SWCNT**	**Nano Silicon dioxide**	**Nano Silver**	**Nano zinc oxide**
Acute oral toxicity	++ Testing limitations	++	++	++
Acute dermal toxicity	++ Testing limitations	++	++	++
Acute inhalation toxicity	0	++ Testing limitations	0	++ Testing limitations
Eye damage/ irritation	- for *in vivo* testing +/++ for *in vitro* testing	NA	NA	NA
Skin sensitisation	NA	NA	++ *in vivo* 0 *in vitro*	NA
STOT RE oral exposure	++ Testing limitations	++	++	0
STOT RE dermal exposure	NA	NA	NA	++
STOT RE inhalation exposure	++ Testing limitations	++	++	++
Germ cell mutagenicity	++	NA	NA	NA

***- :** not applicable **+:** applicable with limitations **++:** fully applicable **NA**: not assessed **0**: no assessment due to lack of data*
***Testing limitations**: not technically feasible to test up to concentrations/ doses relevant for classification in the least severe category(-ies) of the hazard class.*

This overview indicates that in general the GHS is considered applicable for the data on the selected nanomaterials. However, it is noted that for voluminous nanomaterials (i.e. with relatively high specific surface areas and low pour densities) it may not be technically feasible to test up to dose levels that correspond to the less severe hazard categories for acute toxicity and STOT RE.

Application of the GHS criteria and classification of the selected nanomaterials

Based on the assessment of the data on the selected hazard classes the data allow for the following classification on the nanomaterials:

Table 21 Relevant classifications for the selected nanomaterials.

Classification* of the selected nanomaterials					
Hazard class	**SWCNT**	**Nano Silicon dioxide**	**Nano Silver**	**Nano zinc oxide**	**Comment**
Acute oral toxicity	Not sufficient data	No classification	No classification	No classification	-
Acute dermal toxicity	Not sufficient data	No classification	No classification	No classification	-
Acute inhalation toxicity	Not sufficient data	Not sufficient data	Not sufficient data	No classification	Testing has/can not be conducted at sufficient high exposure levels relevant for classification
Eye damage/irritation	At least Eye Irrit. 2 (based on *in vitro* testing)	NA	NA	NA	*In vivo* testing may be difficult to perform
Skin sensitisation	NA	NA	No classification**	NA	-
STOT RE oral exposure	Not sufficient data	No classification	No classification**	Not sufficient data	-
STOT RE inhalation exposure	STOT RE 1	STOT RE 1 or RE 2	STOT RE ?	Not sufficient data**	The classification proposed may be confirmed using testing at higher exposure levels than used.
Germ cell mutagenicity	Muta. 2	NA	NA	NA	-

**The indicated classifications is not necessarily applicable for all types/qualities of the nanomaterial but is related to one or several representatives of the specific nanomaterial. However, a classification for a specific hazard class indicates that this may be a relevant classification for other qualities of this nanomaterial as well and that testing/ information on this end-point is especially warranted.*
***indicate different conclusion compared to the initial proposed classification from the screening phase as indicated in table 1 and 2.*
NA: not assessed

It should be noted that data gaps for the examined nanomaterials have been identified in relation to acute toxicity testing by inhalation in particular, and that repeated dose toxicity testing by inhalation typically have not been conducted at dose levels relevant for classification purposes but at rather low expsoure levels more relevant for NOAEC/LOAEC identification.

When comparing to the starting point concerning proposed classifications from the screening phase of the project as indicated in Table 2 by Lee et al. (2017) it can be seen that identical classifications are suggested for SWCNT and silicon dioxide in Table 20 as in Table 2.

For nano silver the proposed classifications in Table 2 by Lee et al. (2017) as Skin Sens 1 and STOT RE 2 (oral, liver) are not considered to be supported by the available data , but there are

indications that classification in STOT RE may be appropriate for nano silver as indicated in Table 20.

For nano zinc oxide a classification STOT RE 1 was proposed in Table 2 by Lee et al. (2017), however, such a classification is not supported by the available data, although data points towards toxicity at low levels in the lung. Current data, however, is not considered sufficient for making a conclusion regarding STOT RE classification by inhalation as indicated in Table 20.

Overall observations and reflections

When having an overall look on the experience gained by this project some general findings/aspects can be highlighted:

a. In general, the current GHS classification criteria for the five evaluated hazard classes were found to be applicable to the generated data on SWCNT, nano silicon dioxide, nano silver and nano zinc oxide.

b. Differences in toxicity exist between the various types/qualities (e.g. related to production methods (e.g. silicon dioxide) or impurity profile (e.g. SWCNT)) of the same nanomaterials which may result in different classifications of the various types/qualities.

c. STOT RE is considered a highly relevant hazard class to examine for all the nanomaterials especially considering the lung as the target organ.

d. For the voluminous nanomaterials (i.e. nanomaterials with a relatively high specifc surface area and low density) testing at high dose levels may not be technically achievable. Hence, testing in accordance with OECD TG method covering all relevant dose levels for acute toxicity classification and STOT RE classification according to the GHS criteria values may not be possible. This is especially relevant for testing via inhalation route.

e. For acute toxicity and STOT RE the GHS criteria based on a mass-based dose metric can be applied for voluminous nanomaterials, however, the dose levels corresponding to the less severe hazard categories cannot be technically achieved. It may be examined whether another dose metric (e.g. specifc surface area or particle number concentrations) would be a better metric for enabling differentiation in toxicity and the classification of nanomaterials.

f. It is noted that most testing regarding repeated inhalation exposure has focused on identification of NOAEC/LOAEC levels and the examination of early signs of toxicity (e.g. various inflammatory markers) rather than establishing data for STOT RE classification. So mostly very low exposure levels compared to the STOT RE criteria have been used. Thus, there are data gaps for assessing the proper STOT RE classification of nanomaterials.

g. As support for a STOT RE classification it should be considered how to use an AOP or MOA approach using inflammatory signs/markers or mild/moderate histopathological effects induced in target organs at very low exposure levels for classification purpose.

h. Also, it may be examined how and under which circumstances data from e.g. intratracheal instillation or pharyngeal aspiration may be used as support for STOT RE classification if data from inhalation testing are limited or do not cover the relevant dose ranges for classification.

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APPENDICES

Appendix A. SWCNT

1. Overview of relevant data and hazard classes

For the assessment of SWCNT the focus was on data relevant for the following hazard classes:

- *Acute toxicity*
- *Eye irritation*
- *STOT RE*
- *Germ cell mutagenicity*

Relevant data for this assessment has been found from the OECD/WPMN (2016, SWCNT summary) and the OECD/WPMN (2015, SWCNT part 2) reports as well as from the REACH registration of the substance. No relevant data was obtained from the NanoReg project (EU 7th framework), the NanoSafety Cluster projects or from a focused web-based literature search (using substance name, toxicological endpoint and exposure route as relevant search terms).

Table A1. Number of studies/ test data on the prioritised hazard classes

Hazard class	Number of studies (OECD-dossier)	Further studies retrieved for this project
Acute toxicity (oral)	3	0
Acute toxicity (inhalation)	1	0
Acute toxicity (dermal) *However, data on Skin corrosion/irritation in vivo*	0 4	0 0
Serious eye damage/eye irritation	2	1 (REACH-reg. data)
Genetic toxicity (in vitro)	17	1 (REACH-reg. data)
Genetic toxicity (in vivo)	7	0
Specific target organ toxicity repeated exposure (oral)	1	0
Specific target organ toxicity repeated exposure (inhalation)	2	**0**

2. Substance identity and characterisation

Chemical name: SWCNTs, Single-Walled Carbon Nanotubes

CAS No.: 308068-56-6

In the OECD dossiers the substance identity and physicochemical properties are described for 15 different types of SWCNT from 14 different manufacturers (Lee et al. 2017).

Below physicochemical properties are given for two different SWCNTs based on data provided by OECD/ WPMN (2016, SWCNT summary):

Table A2. Physicochemical properties for two different SWCNTs

Endpoint	Nikkiso SWCNT	Super Growth SWCNT
Agglomeration/aggregation	---	particles were in bundle consisting of a few or dozens of SWCNTs. The average bundle length was 0.28 µm (SD=0.17) and the average bundle diameter was 9.3 nm (SD=4.5)
Water solubility	Insoluble in water	Insoluble in water
Dustiness	n/a	Respirable mass conc. 0.025 mg/m^3
Particle size distribution	Dia = 2.7 µm SD = 1.4 (measured by DLS)	Dia = 8.2 nm (SD = 1.7) Length = 0.23µm (SD=1.8) (measured by TEM)
Specific surface area	877.7 m^2/g	1064 m^2/g
Zeta potential	19.9 +/- 5.5	- 14.7 +/- 0.9
Surface chemistry	O/C = 0.022	O/C = 0.003
Porosity	Pore volume: 30.5 ml/g Davg = 190 nm	Pore volume: 18.67 ml/g Davg = 54 nm

3. Hazard classes

3.1 Acute toxicity

3.1.1 Oral exposure

Nikkiso SWCNT Acute oral toxicity study in rat according to OECD TG 423

The following summary on acute oral toxicity was given by OECD/ WPMN (2016, SWCNT summary):

"An acute oral toxicity study was conducted based on OECD TG 423. The maximum dose of 2000 mg/kg required by the guideline was impracticable because of very high specific volume of SWCNT. Three female Crl:CD(SD) rats were gavage dosed with Nikkiso SWCNT (suspended in 5% arabic-gum aqueous solution) at a total dose of 50 mg/kg bw (four equally divided doses at one-hour intervals). No deaths occurred, and no abnormalities were observed in the clinical condition during the observation period in any animals. The LD50 of Nikkiso SWCNT was considered to be greater than 50 mg SWCNT/kg bw."

Mammalian in vivo micronucleus tests according to OECD TG 474 of Nikkiso SWCNT and Super Growth SWCNT in mice (included as supporting information)

The following description was given by OECD/WPMN (2016, SWCNT summary):

"Male Crlj:CD1(ICR) mice (6 animals/group) were gavage dosed with Nikkiso SWCNT (suspended in the water and was diluted with 0.3% CMC-Na solution) at 5, 10 or 20 mg/kg bw/day two times in the interval of 24 hours (OECD TG 474) (Naya et al, 2011). Male Crlj:CD1(ICR) mice (5 animals / group) were gavage dosed with Super Growth SWCNT (suspended in PBS with 1% Tween 80) at 60 or 200 mg/kg bw/day two times in the interval of 24 hours (NEDO [#NN-30] unpublished study). No death or indicative of abnormality is observed in both studies."

3.1.2 Inhalation

No acute toxicity testing from single inhalation exposure available (OECD/ WPMN 2016, SWCNT summary)

3.1.3 Intratracheal exposure

The following summary on single exposure by Intratracheal administration was given with respect to inflammatory responses and histopathological findings in the lung by OECD/ WPMN (2016, SWCNT summary); (type of SWCNT is below high-lighted in **bold**):

*"A single dose of **Nikkiso SWCNT** by intratracheal instillation was conducted in male Wistar rats (20 rats/group) at 0.2 mg/rat (ca. 0.67 mg/kg bw) or 0.4 mg/rat (ca. 1.33 mg/kg bw) (NEDO [#NN-14] unpublished study). Inflammation and fibrosis were examined from the third day to six months after instillation. Both dose groups showed inflammatory cellular infiltration, and particularly it lasted for six months for high dose. Also, heme oxygenase-1 (HO-1) gene expression increased continuously in both dose groups. Observation is ongoing up to two years.*

*Kobayashi et al.42 (2012) reported two intratracheal studies conducted by NEDO project [#NN-05-1 and #NN-05-2]. Male SD rats were given **Super Growth SWCNT** by intratracheal instillation at 0.2 or 2 mg/kg, and observation was carried out from 24 h to 3 months after administration (1st study). Male SD rats were given **Super Growth SWCNT** by intratracheal instillation at 0.04, 0.2 or 1 mg/kg and observation was carried out from 3 days to 6 months after administration. There were dose-dependent inflammatory responses in the lungs in both studies. Significant increases in pulmonary tissue inflammation and inflammatory biomarkers persisted at 1 and 2 mg/kg for 6 months.*

*Male SD rats were exposed to **SWCNT (CNI, HiPco)** by intratracheal instillation at 0.2 or 1 mg/kg and observed for 3 months (NEDO [#NN-10] unpublished study). There was a dose-dependent inflammatory response in the lungs. The inflammation was recovered by one month after instillation at 0.2 mg/kg, but not at 1 mg/kg.*

*Lam et al.(2004) investigated the acute lung toxicity through intratracheal instillation test to male B6C3F mice using **three different SWCNTs [1: Rice Univ, HiPco; 2: Rice Univ, refined HiPco; 3: CaboLex]** made by different processes and containing different types and amount of residual catalytic metal at doses of 0, 0.1 and 0.5 mg/animal. All nanotube products induced dose-dependent epithelioid granulomas. In some cases, interstitial inflammation, peri-bronchial inflammation and necrosis that had extended into the alveolar septa were observed.*

*ICR mice were exposed to **SWCNT (unknown identification)** by intratracheal instillation at 0.5 mg/kg (Chou et al.45, 2008). Foamy macrophages phagocytosing CNT were observed at 3 days post-instillation and multifocal granuloma and foamy macrophages at 14 days.*

*Male Wistar rats were exposed to **SWCNT (CNI, HiPco)** by intratracheal instillation at 2.25 mg/rat (17.3 mg/kg) (Miyawaki et al, 2008). Formation of foreign body granuloma in the lungs was observed. Severity of pathological findings was similar at 7 days and 90 days post-instillation (not recovered)."*

3.1.4 Pharyngeal aspiration

The following summary on single exposure by Intratracheal administration was given with respect to inflammatory responses and histopathological findings in the lung by OECD/ WPMN (2016, SWCNT summary); (type of SWCNT is below high-lighted in **bold**):

*"NIOSH (Shvedova et al.47, 2005) demonstrated that pharyngeal aspiration of **SWCNT (CNI, HiPco)** elicited unusual pulmonary effects in female C57BL/6 mice that combined an acute inflammation with progressive fibrosis and granulomas. An early neutrophils accumulation*

followed by lymphocyte and macrophage influx, was accompanied by early elevation of proinflammatory cytokines followed by fibrogenic transforming growth factor. A rapid progressive fibrosis found in mice exhibited two distinct morphologies: 1) SWCNT-induced granulomas mainly associated with hypertrophied epithelial cells surrounding SWCNTs aggregates; and 2) diffuse interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWCNTs.

*Male and female Fischer 344 rats were exposed to **SWCNT (Helix Material Solution, CVD**) at 2 mg/kg by pharyngeal aspiration (Mangum et al48, 2006). Twenty-one days after aspiration, histopathological examination showed an indication of inflammation in the lung cells.*

***SWCNT (CNI, HiPco)** was given by pharyngeal aspiration in C57BL/6mice at a dose of 0 or 40 µg/mouse (Shvedova et al,50 2007; NIOSH). Inflammation, damage, and fibrosis were observed during 1 day to 3 months post-exposure. Followings are observed: rapid but transient inflammation and damage, rapid and persistent granulomas, and rapid and progressive fibrosis. Fibrosis is greater in vitamin E deficient mice and less in NADPH ox Kos.*

*Pharyngeal aspiration of **SWCNT (CNI, HiPco)** in C57BL/6 mice at 0-20 µg/mouse caused inflammatory response, oxidative stress, collagen deposition, and fibrosis as well as mutations of K-ras gene locus in the lung of mice (Shvedova et al. 2008; NIOSH).*

*NIOSH (Mercer et al.52, 2008) investigated an effect of dispersion of **SWCNT (CNI, HiPco).** Male C57BL/6 mice were exposed to SWCNT or dispersed SWCNT by pharyngeal aspiration at 10 µg/mouse. Lung sections and lavage cells demonstrated an early, transient neutrophilic and inflammatory phase that rapidly resolved and was similar to that observed with large agglomerates. No granulomatous lesions or epithelioid macrophages were detected. Dispersed SWCNT was rapidly incorporated into the alveolar interstitium and produced an increase in collagen deposition."*

Further a study where reported where C57BL/6 mice were exposed to **SWCNT (CNI, HiPco)** at 10 and 40 µg/mouse by intrapharyngeal instillation (Li et al.49 2007). In this study aortic mtDNA damage was developed at 7, 28, and 60 days after exposure. Further, a single intrapharyngeal instillation induced activation of heme oxygenase-1 (HO-1) in the lung, aorta, and heart tissue in O-1 reporter transgenic mice.

3.2 Eye Irritation

3.2.1 Eye Irritation *in vivo*

Nikkiso SWCNT eye irritation in vivo (OECD/WPMN 2015, SWCNT part 2)

This study report from 2011 was considered as a key study with a reliability score of 1 by OECD/WPMN (2015, SWCNT part 2).

In an OECD 405 *in vivo* study 0.1ml of 0.1 wt% *Nikkiso SWCNT (regarding characterisation see table 2)* solution was dropped into the conjunctival sac of left eye of three Kbl:NZW rabbits. The test sample was prepared as the maximum concentration allowing the instillation into the eyes in a media of non-irritating olive oil. The test specie was rinsed off with warm saline from cornea and conjunctival sac in an hour and the animals observed and scored at 1, 24, 48 and 72 hours after the instillation.

At none of the observations, an animal was scored above the lowest score of "0" with respect to each of the parameters: cornea score, iris score, conjunctivae score and chemosis score. Thus, no potential for eye irritation was found in this study.

Super Growth SWCNT Eye irritation in vivo (OECD/WPMN 2015, SWCNT part 2)

This study report from 2011 was considered as another key study with a reliability score of 1 by OECD/WPMN (2015, part 2).

In this OECD TG 405 *in vivo* study 0.1ml of 0.1 wt. % *Super Growth SWCNT (regarding characterisation see table 2)* solution was dropped into the conjunctival sac of left eye of three Kbl:NZW rabbits. The test sample was prepared as the maximum concentration allowing the instillation into the eyes in a media of non-irritating olive oil. The test specie was rinsed off with warm saline from cornea and conjunctival sac in an hour and the animals observed and scored at 1, 24, 48 and 72 hours after the instillation.

At none of the observation, an animal was scored above the lowest score of "0" with respect to each of the parameters: cornea score, iris score, conjunctivae score and chemosis score. Thus, no potential for eye irritation was found in this study.

3.2.2 Eye Irritation *in vitro*

TUBALL$^{(R)}$ SWCNT Eye irritation in vitro (REACH registration dossier 2018)

This study report from 2016 was considered as a key study with a reliability score of 1 in the REACH registration dossier of SWCNT. No further data for this end-point was reported (REACH registration dossier 2018).

An OECD TG 492 (Reconstructed Human Cornea-like Epithelium (RhCE) study was conducted using EpiOcular™ tissues. Exposure was approximately 50 mg (83.3 mg/cm^2 according to guideline) for 6 hours to the SWCNT (TUBALL$^{(R)}$: MMAD 1.6 nm; Tube length: 1 - 10 µm.

Relevant irritating effects were observed following 6 hours incubation with TUBALL®. The mean relative absorption value of the tissues corresponding to the cornea viability decreased to 26.5% compared with the value of the negative control (threshold for irritancy: ≤ 60%).

Under the experimental conditions reported, TUBALL® possesses an eye irritating potential and classification as Eye Irrit. 2 was concluded by the dossier submitter based on the GHS criteria.

3.3 STOT RE

3.3.1 STOT RE, inhalation

Nikkiso SWCNT. OECD TG 412 study from 2011 and indicated as a key study with a reliability score of 1 by OECD/WPMN (2015, SWCNT part 2)

Wistar male rats (15 rats / dose / observation period) were in a whole-body exposure chamber exposed to Nikkiso SWCNT (long-CNT) for 4 weeks (6 hours/day, 5 days/week) at a level of 0 mg/m^3; 0.08 +/- 0.014 mg/m^3 or 0.40 +/- 0.11 mg/m^3 of the particle weight concentration in the exposure chamber. SWCNT was suspended in the air using the pressurized nebuliser, and its aqueous component (distilled water including Triton) was removed by mist dryer. Observation and examinations were performed 3 days, 1 month and 3 months after exposure.

Body weight was recorded in observation periods, and wet organ weights of the lung, brain, nasal cavity, testis, liver, kidney and spleen were recorded on the day of autopsy. Histopathological examination was carried out on these organs. White blood cells and neutrophil cells were counted as biomarkers representing inflammation in blood, number of total cells of BALF, number of neutrophil cells of BALF, and concentration of HO-1 were recorded.

Pulmonary inflammation and collagen deposits (fibrosis) were analyzed by the point counting method. Urinary examination was carried out for 8-OHdG levels.

Inflammation and fibrotic response were examined at three days, one month or three months after the last exposure. Only observed effects were increased neutrophil cells in blood at 3 months after the administration in the high concentration group. Neither the low concentration exposure group nor the high concentration exposure group showed increase of the pulmonary wet weight, the infiltration of the inflammatory cell and increase of the HO-1 gene expression.

In OECD/WPMN (2015, SWCNT part 2) it is indicated that the results from this study are still under evaluation.

Super Growth SWCNT. OECD TG 412 Repeated dose toxicity by inhalation. From 2011 and assessed as a key study, however with no reliability score indicated (OECD/WPMN 2015, SWCNT part 2)

In a whole-body exposure chamber, Wistar male rats (10 rats/ dose) were exposed to Super Growth SWCNT for 4 weeks (6 hours/day, 5 days/week) at a level of 0 mg/m^3; 0.03 ± 0.003 and 0.13 ± 0.03 mg/m^3, respectively. The particle number concentrations in the two groups were 5.0 ± 0.7 x 10^4 and 6.6 ±2.1 x 10^4 particles/cm^3, respectively. SWCNT was suspended in the air using the pressurised nebuliser, and its aqueous component (distilled water including Tween 80) was removed by a mist dryer. The geometric mean value and geometric standard deviation of the particle length, the length measured along the particle shape, were 0.7 and 1.7 µm, respectively. Those for the width of the particles, the maximum size perpendicular to the length, were 0.2 and 1.7 µm, respectively.

After the exposure period and recovery, rats were sacrificed 3 days, 1 month, and 3 months, and dissected. Each group of 10 animals was divided into 2 subgroups of 5 animals for lung tissue analysis as one subgroup provided bronchoalveolar lavage from the right lung.

In this study no signs of inflammation in relation to neutrophils or the concentration of CINCs (cytokine-induced neutrophil chemoattractant-1 and -2), or HO-1 in the lung were induced. Also, no neutrophil infiltrations into the alveolar space, granulomatous lesion, interstitial collagen deposition or emphysematous changes were found during the observation period in either of the SWCNT-exposed groups. Alveolar macrophages that ingested SWCNTs were seen to a slight extent. Overall, it was concluded that well-dispersed SWCNTs did not induce neutrophil inflammation in the lung.

SWCNT (CNI, HiPco). Non-guideline repeated dose toxicity by inhalation. Reliability score of 2 (OECD/ WPMN 2015, SWCNT part 2 with reference to Shedova et al. 2008)

Summary from OECD/WPMN (2016, SWCNT summary):

"An inhalation exposure to SWCNT (CNI, HiPco) in C57BL/6 mice (4 days, 5h/day at 5.52 ±1.37 mg/m^3) resulted in qualitatively similar pulmonary reaction as pharyngeal aspiration (0-20 µg/mouse). However, SWCNT inhalation was more effective than aspiration in causing inflammatory response, oxidative stress, collagen deposition, and fibrosis as well as mutations of K-ras gene locus in the lung of mice (Shvedova et al.41 2008; NIOSH)."

However, it should be noted that these SWCNT was used "as produced" in the testing. The following description was given by OECD/WPMN (2015, SWCNT part 2):

"*The nanotubes were manufactured using the high-pressure CO disproportionation process (HiPco) and were used in the inhalation and pharyngeal aspiration studies as produced; i.e., without being purified or otherwise treated after the initial production process. Analysis indicates that these SWCNT contained elemental carbon (82% wt), Fe (17.7%). Trace elements present included Cu (0.16%), Cr (0.049%), and Ni (0.046%). The diameter of SWCNT measured by transmission electron microscopy (TEM) was 0.8 –1.2 nm. The length of SWCNT was 100–*

1,000 nm measured by Carbon Nanotechnology using atomic force microscopy. The surface area of SWCNT measured by the nitrogen absorption-desorption technique (Brunauer-Emmett-Teller method, BET) was 508 m^2/g.

In the original publication of this study, Shedova et al. (2008) evaluated the degree of pulmonary cytotoxicity caused by SWCNT inhalation by the LDH (lactate dehydrogenase) activity in the BAL fluid recovered from mice. Time course of LDH accumulation in BAL fluid of mice that inhaled SWCNT revealed a statistically significant 118%, 80%, and 71% increase over control groups throughout recovery time (1, 7, and 28 days post exposure). Regarding the histopathological observations four mice at 28 days post-exposure had bronchiolar epithelial cell hypertrophy with one mouse having both hypertrophy and hyperplasia, one mouse having peribronchiolar bronchiolization accompanying bronchiolar epithelial cell hypertrophy, and two mice having bronchiolar epithelial cell hypertrophy without other bronchiolar alterations. Further foci of granulomatous inflammation were noted with fibrosis seen in sections stained with Masson's trichrome.

Pharyngeal aspiration

CNI SWCNT repeated dose toxicity, pharyngeal aspiration. Reliability score of 2 (OECD/WPMN 2015, SWCNT part 2)

Summary from OECD/WPMN (2016, SWCNT summary):

"Repeated exposure to SWCNT (CNI, HiPco) was conducted at 20 µg/mouse once every other week for 8 weeks by pharyngeal aspiration in ApoE–/– transgenic mice (Li et al., 2007). Although SWCNT exposure did not modify the lipid profiles of these mice, it resulted in accelerated plaque formation in ApoE–/– mice fed an atherogenic diet. Plaque areas in the aortas, measured by the en face method, and in the brachiocephalic arteries, measured histopathologically, were significantly increased in the SWCNT treated mice."

3.3.2 STOT RE, oral exposure

Nikkiso SWCNT. OECD TG 407 28-day subacute repeated oral toxicity study. Reliability score of 1

Summary from OECD/WPMN (2016, SWCNT summary):

"The maximum dose of 1000 mg/kg required by the guideline was impracticable because of very high specific volume of SWCNT (Matsumoto et al.53, 2012). Male and Female Crl:CD rats (5 or 10 animals/sex/dose) were administered Nikkiso SWCNT (suspended in 5% guam acacia) by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). No treatment related changes of body weight, behavioral and blood biochemical parameters were observed. A few minor changes with statistical significance in white blood cells composition, organ weights and urine volume were detected, although no relevant pathological changes were observed. Based on the above findings, the NOAEL of repeated oral dose toxicity of the SWCNT was considered to be 12.5 mg/kg bw/day (the highest dose tested) in rats."

3.4 Germ cell mutagenicity

For the assessment of mutagenicity, a more focused approach will be used as description and discussion of each of the many individual *in vitro* and *in vivo* studies conducted with various types of SWCNT. The data reported in OECD/WPMN (2016, SWCNT summary) will be used to provide an overall view of the data):

In vitro

Table A3. Bacterial mutation tests

Substance /Manufacture	Type of study	Strains	Concentration (exposure period)	Result	Reference
Nikkiso SWCNT	Reverse gene mutation assay OECD TG471	*S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA	1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate +/- metab. act.	Non-mutagenic	AIST 2011a
Super Growth SWCNT	Reverse gene mutation assay Japanese Guideline	*S. typhimurium* TA 97, TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA/pkM101	12.5, 25, 50, 100, 200 and 500 µg/plate +/- metab. act.	Non-mutagenic	Naya et al. 2011
SWCNT (CNI, HiPco)	Reverse gene mutation assay	*S. typhimurium* YG1024 and YG1029	0-240 μ g/ plate +/- metab. act.	Non-mutagenic	Kisin et al. , 200)

Table A4. Chromosomal aberration tests

Substance /Manufacture	Type of study	Cell type	Concentration (exposure period)	Result	Reference
Nikkiso SWCNT	chromosomal aberration test OECD TG 473	Chinese hamster lung (CHL/IU) cells	6.25, 12.5, 25 and 50 µg/plate 6h: +/- metab. act. 24h: + metab. act.	negative	AIST 2011b
Super Growth SWCNT	chromosomal aberration test OECD TG 473 And Japanese guideline	Chinese hamster lung (CHL/IU) cells	300, 500 or 1000 µg/plate 6h: +/- metab. act. 24h: + metab. act	negative	Naya et al. 2011

Further studies were reported by OECD/WPMN (2016, SWCNT summary) report in the following table:

Table A5. Micronucleus tests

Substance /Manufacture	Type of study	Cell type	Concentration (exposure period)	Result	Reference
Nonfunctionalized SWCNT	Cytokinesisblock micronucleus (CBMN)assay	HDMEC (PromoCell)	25-150 µl/ml (68 h)	↑ Micronuclei at 25 and 50 µl/m. ↓ Proliferation potential (CBPI) of cells	Cveticanin et al. (2010)
Amide functionalized purified SWCNT	Cytokinesisblock micronucleus (CBMN)assay	HDMEC (PromoCell)	25-150 µl/ml (68 h)	↑ Micronuclei at 25-150 µl/m.	
SWCNT/Sigma-Aldrich	Cytokinesisblock micronucleus (CBMN)assay	Murine macrophage cell line RAW 264.7	0.01-100 µg /ml (24 h)	↑ Micronuclei at doses above 0.1 µg/ml	Migliore et al (2010)
>50% SWCNT , ~40% other CNT/Sigma-Aldrich	Cytokinesisblock micronucleus (CBMN)assay	Human bronchial epithelial BEAS 2B cell	36-360 µg /ml	Dose independent increases of micro-nucleated cells at three concentra-tions in the 48 h treatment. (The authors' conclusion is positive)	Lindberg et Al. (2009)
SWCNT (CNI, HiPco)	in vitro mammalian cell micronucleus	CHL (V79) cells	0, 12, 24, 48 or 96 μ g/cm2 for 24 h	negative	Kisin et al., 2007

Table A6a. DNA damage and/or repair

Substance /Manufacture	Type of study	Cell type	Concentration (exposure period)	Result	Reference
SWCNT (EliCarb®, Tomas Swan)	Comet assay	FE1-Muta™Mouse lung epithelial cell line	24 hr: 0-200 µg/ml 576 hr: 100 µg/ml	Increased FPG sensitive sites/oxidized purines No increased strand breaks Increase in No mutation in cII gene	Jacobsen et al. 2008
SWCNT (CNI, HiPco)	Comet assay	CHL (V79) cells	0, 24, 48 or 96 µg/cm2 3h and 24 h	At 24H: Dose-response DNA damage. Increased migrated DNA, tail length and tail moment	Kisin et al., 2007

Further studies were reported by the OECD/WPMN (2016, SWCNT summary) report in the following table:

Table A6b. DNA damage and/or repair

Substance /Manufacture	Type of study	Cell type	Concentration (exposure period)	Result	Reference
SWCNT/NIST	Double strand Breaks (DSB) assay	Normal human NM and malignant MM mesothelial cell	25 or 50 μg/ml (24 h)	↑ H2AX phosphorylation	Pacurai et al (2008)
	Comet assay	Normal human NM and malignant MM mesothelial cell	25 or 50 μg/ml (24 h)	↑ DNA migration	
Nonfunctionalized SWCNT	Double strand breaks (DSB) assay	HDMEC (PromoCell)	0.5-30 ul/ml (24 h)	↑ γ-H2AX foci	Cveticanin et al (2010)
Amide functionalised SWCNT	Double strand breaks (DSB) assay	HDMEC (PromoCell)	0.5-30 ul/ml (24 h)	↑ γ-H2AX foci	
SWCNT/COCO, Chinese Academy of Science	Comet assay	Primary mouse embryo fibroblast (BALB/c mouse)	5 or 10 μg/ml (24 h)	↑ %Tail DNA ↑ Tail length ↑ Tail moment	Yang et al (2009)
SWCNT/Heji	Comet assay	Human leukocytes	1, 5 or 10 μg/ml (6 h)	No effects	Zeni et al (2008)
>50% SWCNT , ~40% other CNT/Sigma-Aldrich	Comet assay	Human bronchial epithelial BEAS 2B cells	3.8-380 μg/ml (24, 48 or 72 h)	↑ % Tail DNA at 3.8 μg/ml and more	Lindberg et al (2009)

In vivo

Table A7. Micronucleus assays

Substance /Manufacture	Type of study	Species	Concentration (exposure period)	Result	Reference
Nikkiso SWCNT	micronucleus assay, OECD TG 474	Crlj:CD1(ICR) mice	5, 10 and 20 mg/kg/day oral gavage	Negative (abnormalities and micronucleus formation)	AIST 2011c
Super Growth SWCNT	micronucleus assay, OECD TG 474	Crlj:CD1(ICR) mice	60 or 200 mg/kg/day oral gavage	Negative (abnormalities and micronucleus formation)	Naya et al., 2011

Table A8. DNA damage and/or repair

Substance /Manufacture	Type of Study	Species	Concentration (exposure period)	Result	Reference
Nikkiso SWCNT	Comet assay Lung tissue	male Crl:CD (SD) rats	0.2 or 1.0 mg/kg once or 0.04 or 0.2 mg/kg for 5 times (once/week) by intratracheal administration	Negative (% tail DNA)	(NEDO [#NN-33] unpublished study
SWCNT (EliCarb®, Tomas Swan)	Comet assay, Lung tissue	ApoE-/- mice	54 μ g/ mouse instillation	No effects on strand breaks but significant increases in *Il-6, Mip-2* and *Mcp-1* mRNA at 3 h and 24 h following instillation	Jacobsen et al. 2009
SWCNT (CNI, HiPco)	K-ras mutation, lung tissue	C57BL/6 mice	4 days, 5h/day at 5.52 ±1.37 mg/m3 inhalation and 5,10 and 20 µg/mouse, pharyngeal aspiration	Increased K-ras mutations after inhalation but not after aspiration	Shvedova et al. 2008
SWCNT (CNI, HiPco)	Mouse spot test	C57BL/6 mice	10 and 40 µg/mouse by intrapharyngeal instillation	Aortic mtDNA damage developed at 7, 28, and 60 days after exposure	Li et al. 2007
SWCNT (Thomas Swan and Co)	DNA damage and repair mRNA expression	Fisher rats	0.064 or 0.64 mg/kg bw gavage	increased levels of oxidatively damaged DNA in liver and lung.	Folkman et al., 2009)

Overall summary, germ cell mutagenicity

Based on the above data, a summary was given by the OECD/WPMN (2016, SWCNT summary) report:

"Nikkiso and Super Growth SWCNTs did not induce gene mutation in bacterial in vitro tests (OECD TG 471 and Japanese guideline). Nikkiso and Super Growth SWCNTs did not induce chromosome aberrations in cultured Chinese hamster lung (CHL/IU) cells (OECD TG 473 and Japanese guideline). In vivo micronucleus assays (OECD TG 474) for Nikkiso and Super Growth SWCNTs showed negative results. Although a comet assay in lung tissues taken from rats given Nikkiso SWCNT intratracheally showed no effects on %tail DNA, many other studies (the comet assays, CBMN assays, DSB assays, Kras mutation test, mtDNA assay and oxidatively damaged DNA assay) indicated possible DNA damage caused by SWCNTs."

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AIST 2011b. Endpoint study record: SWCNT Chromosomal Aberration Test; Genetic toxicity in vitro.002, p 88

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Appendix B. Nano silicon dioxide

1. Overview of relevant data and hazard classes

For the assessment of nano silicon dioxide focus was on data relevant for the following hazard classes:

- *Acute toxicity*
- *STOT RE*

Relevant data for this assessment has been found from the OECD/WPMN (2016, SiO2 summary) and the OECD/WPMN (2016, SiO part1-6) as well as from data obtained from the NanoReg project (EU 7th framework) and the NanoSafety Cluster projects or from a focused web-based literature search (using substance name, toxicological endpoint and exposure route as relevant search terms).

Table B1. Number of studies/ test data on the prioritised hazard classes

Hazard class	Number of studies (OECD-summary dossier)	Further studies retrieved for this project
Acute toxicity (oral)	10	
Acute toxicity (inhalation)	6	
Acute toxicity (dermal)	1	
Specific target organ toxicity repeated exposure (oral, 90D)	3	1 (from NanoCluster) 1 (from NanoReg)
Specific target organ toxicity repeated exposure (inhalation, 28D, 90D, + 90D)	0,6, 1	

2. Substance identity and characterisation

Table B2. Identification/ characterisation of different types of silicon dioxide; synthetic amorphous silica, CAS No 7631-86-9 (OECD/WPMN 2016, SiO2 summary)

	NM-200 (precipitated) CAS: 112926-00-8	NM-201 (precipitated) CAS: 112926-00-8	NM-202 (pyrogenic) CAS: 112945-52-5	NM-203 (pyrogenic) CAS: 112945-52-5	NM-204 (precipitated) CAS: 112926-00-8
Composition	SiO_2 ≥ 96% 2.7% Na_2SO_4 0.87% Al	SiO_2 ≥ 97% 1.4% Na_2SO_4 0.74% Al	SiO_2 ≥ 99% 0.45% Al	SiO_2 ≥ 99% 0.43% Al	SiO_2 ≥ 98% 0.6% Na_2SO_4 0.48% Al
Crystallinic phase	Synthetic amorphous silica	Synthetic amorphous silica	Synthetic amorphous silica	Synthetic amorphous silica	Synthetic amorphous silica
Primary particle size	14 – 23 nm	17 – 19 nm	15 – 20 nm	13 – 45 nm	10 – 21 nm

	NM-200 (precipitated) CAS: 112926-00-8	NM-201 (precipitated) CAS: 112926-00-8	NM-202 (pyrogenic) CAS: 112945-52-5	NM-203 (pyrogenic) CAS: 112945-52-5	NM-204 (precipitated) CAS: 112926-00-8
Aggregate size, Medium diameter	31 nm	43 nm	53 nm	48 nm	No data
Specific surface areas, BET	189.16 m^2/g	140.46 m^2/g	204.11 m^2/g	203.92 m^2/g	136.6 m^2/g
Pour-density	0.12 g/cm^3	0.28 g/cm^3	0.13 g/cm^3	0.03 g/cm^3	0.16 g/cm^3
Water solu-bility mg/L	Precipitated nano silicon dioxide: 76 - 138 mg/L at 37ºC (REACH-registration). Pyrogenic nano silicon dioxide: 141-166 mg/L at 37ºC (REACH-registration).				

3. Hazard classes

3.1 Acute toxicity

3.1.1 Oral exposure

The OECD/WPMN (2016, SiO2 summary) reported ten OECD TG 401 oral acute toxicity tests in rats and mice covering five different qualities of nano silicon dioxide (NM-200, NM-201, NM-202, NM-203; NM-204) using maximum dose levels in the range of 3160 – 20 000 mg/kg bw in the tests, see table B3 below.

Table B3. Data on acute oral toxicity (table modified from OECD/WPMN (2016, SiO2 summary)

Study/ test substance	Dose levels	Results	Data source
Acute Oral Toxicity (OECD TG 401)*; Rat NM-200	Oral (gavage) 1000, 2500, and 5000 mg/kg bw (pre-study); 5000 mg/kg bw (main study)	LD50 > 5000 mg/kg bw No sign of toxicity	Data provided by BIAC. Tests performed by IFT (F) in 1986
Acute Oral Toxicity (OECD TG 401)*; Rat NM-200	Oral (gavage) 2000 and 5000 mg/kg bw	LD50 > 5000 mg/kg bw No sign of toxicity	Data provided by BIAC. Tests performed by Laboratorium für Pharmakologie und Toxikologie (LPT) (GER) in 1977
Acute Oral Toxicity (OECD TG 401)*; Rat NM-201	Oral (gavage) 1000, 2500, and 5000 mg/kg bw (pre-study); 5000 mg/kg bw (main study)	LD50 > 5000 mg/kg bw No sign of toxicity	Data provided by BIAC. Tests performed by IFT (F) in 1986

Study/ test substance	Dose levels	Results	Data source
Acute Oral Toxicity (OECD TG 401)*; Rat NM-201	Oral (gavage) 2000 and 5000 mg/kg bw	LD50 > 5000 mg/kg bw No sign of toxicity	Data provided by BIAC. Tests performed by Laboratorium für Pharmakologie und Toxikologie (LPT) (GER) in 1977
Acute Oral Toxicity (OECD TG 401)*; Rat NM-202	Oral (gavage) 2000 and 3300 mg/kg bw	LD50 > 3300 mg/kg bw. No signs of toxicity. Body weight: slight reduction of 4 - 8 %, measured at days 1, 2, and 14. Feed consumption reduced in the 2000 mg group (10 , 4, 6 % at day 1, 2 and 14)	Data provided by BIAC. Tests performed by Laboratorium für Pharmakologie und Toxikologie (LPT) (GER) in 1977
Acute Oral Toxicity (OECD TG 401)*; Mouse NM-202	Oral (gavage) 178, 316, 562, 1000, 1780 and 3160 mg/kg bw	LD50 > 3160 mg/kg bw No adverse signs of toxicity in any animal during the study, no macroscopic lesions upon necropsy after 14-d observation.	Data provided by BIAC. Tests performed by Hazelton Laboratories, (USA) in 1964
Acute Oral Toxicity (OECD TG 401)*; Rat NM-203	Oral (gavage) 2000 and 3300 mg/kg bw	LD50 > 3300 mg/kg bw Slight reduction of body weight of 4 - 8 %, measured at days 1, 2, and 14. Feed consumption reduced in the 2000 mg group (10, 4, 6 % at day 1,2 and 14)	Data provided by BIAC. Tests performed by Laboratorium für Pharmakologie und Toxikologie (LPT) (GER) in 1977
Acute Oral Toxicity (OECD TG 401)*; Mouse NM-203	Oral (gavage) 178, 316, 562, 1000, 1780 and 3160 mg/kg	LD50 > 3160 mg/kg bw No adverse signs of toxicity in any animal during the study, no macroscopic lesions upon necropsy after 14-d observation	Data provided by BIAC. Tests performed by Hazelton Laboratories, (USA) in 1964
Acute Oral Toxicity (OECD TG 401)*; Rat Substance equivalent to NM-200/201/204 (precipated quality)	Oral (gavage) Single administration 5110 mg/kg bw 237 mg/mL	LD50 > 5000 mg/kg bw No signs of toxicity	Data provided by BIAC. Tests performed by ASTA Pharma AG in 1990
Acute Oral Toxicity (OECD TG 401)*; Rat	Oral (gavage) 10000, 12600, 15800, and 20000 mg/kg	LD50 > 20 000 mg/kg bw no clinical symptoms; after 1 day the stools were white coloured (reversible after 2 days)	Data provided by BIAC. Tests performed by Huntingdon Research Center (HRC) in 1978

Study/ test substance	Dose levels	Results	Data source
Substance equivalent to NM-200/201/204 (precipated quality)			

*According to or comparable to OECD TG 401

No mortality was found in any of the tests, and no acute toxic potential was observed by oral exposure.

3.1.2 Dermal exposure

In a standard acute dermal toxicity test (from 1978) no systemic toxicity was noted in rabbits after dermal application of nano silicon dioxide (comparable to the qualities NM-200, NM-201, NM-203) under occlusive conditions for 24 hours at dose levels of 2000, 3000, 4000 and 5000 mg/kg bw. Local effect: very slight erythema (score 1 of 4), reversible after 2 days or 5 d in one or a few animals. No systemic signs of toxicity or organ toxicity (OECD/WPMN 2016, SiO2 summary).

3.1.3 Inhalation

The OECD/WPMN (2016, SiO2 summary) reported six OECD TG 403 inhalation toxicity tests in rats performed in the period 1981-1983 covering four different qualities of nano silicon dioxide (NM-200, NM-201, NM-202, NM-203), see table B4 below.

Table B4. Data on acute inhalation toxicity (table modified from OECD/WPMN (2016, SiO2 summary)

Study/ test substance	Dose levels	Results	Data source
Acute Inhalation Toxicity (OECD TG 403) Rat NM-200	4 h maximum attainable concentration: 691 mg/m^3 (range: 650 - 725 mg/m^3) Nominal concentration: 36.7 g/m^3 (highly reduced air exchange rate in inhalation chamber)	LD50 ≥ 0.69 mg/L air (analytical) No clinical symptoms except some restlessness and eye closing. Body weight gain was not affected in males, but females hardly gained weight during two days after exposure, however, subsequently, showed normal development. No findings at autopsy after 14 d post-treatment.	Data provided by BIAC. Tests performed by TNO Division of Nutrition and Food Research, Zeist (NL) in 1983
Acute Inhalation Toxicity (OECD TG 403) Rat NM-201	4 h maximum attainable concentration: 691 mg/m^3 (range: 650 - 725 mg/m^3) Nominal concentration: 36.7 g/m^3 (highly reduced air exchange rate in inhalation chamber)	LD50 ≥ 0.69 mg/L air (analytical). No clinical symptoms except some restlessness and eye closing. Body weight gain was not affected in males, but females hardly gained weight during two days after	Data provided by BIAC. Tests performed by TNO Division of Nutrition and Food Research, Zeist (NL) in 1983

Study/ test substance	Dose levels	Results	Data source
		exposure, however, subsequently, showed normal development. No findings at autopsy after 14 d post-treatment.	
Acute Inhalation Toxicity (OECD TG 403) Rat NM-202	4 h maximum technically attainable analytical concentration: av. 139 mg/m^3 (range 110 – 190 mg/m^3) Nominal concentration: 16.7 g/m^3 (highly reduced air exchange rate in inhalation chamber)	LC0 ≥ 0.14 mg/L air LD50 ≥ 0.14 mg/L air (analytical) Restlessness, half-closed eyes Slight decrease or stagnation on day 2, but not related to previous exposure (note: by mistake animals were deprived of water for 16 h directly after exposure.). No clinical symptoms and no findings at autopsy after 14 d post-treatment.	Data provided by BIAC. Tests performed by TNO Division of Nutrition and Food Research, Zeist (NL) in 1983
Acute Inhalation Toxicity (OECD TG 403) Rat NM-202	4 h Analytical concentration: 2.08 mg/L (average of 10 samples with a range from 1.63 to 2.70 mg/L, one outlier with 0.45 mg/L) Nominal concentration: 58.8 mg/L (exposure concentration was limited for technical reasons)	LC0 ≥ 2.08 mg/L air (analytical) LD50 > 2.08 mg/L air (analytical) LC0 > 58.8 mg/L air (nominal) LD50 > 58.8 mg/L air (nominal) No animals died. Nasal discharge during exposure, crusty eyes, crusty nose and alopecia at days post-exposure. No macroscopic organ lesions, but in one animal discoloration of the lung.	Data provided by BIAC. Tests performed by Toxigenics Inc., (USA) in 1981
Acute Inhalation Toxicity (OECD TG 403) Rat NM-203	Maximum technically attainable analytical concentration: av. 139 mg/m^3 (range 110 - 190 mg/m^3) Nominal concentration: 16.7 g/m^3	LC0 ≥ 0.14 mg/L air LD50 ≥ 0.14 mg/L air (analytical) Restlessness, half-closed eyes. Slight decrease or stagnation on day 2, but not related to previous exposure (note: by mistake animals were deprived of water for 16 h directly after exposure.) No clinical symptoms and no findings at autopsy after 14 d post-treatment.	Data provided by BIAC. Tests performed by TNO Division of Nutrition and Food Research, Zeist (NL) in 1983

Study/ test substance	Dose levels	Results	Data source
Acute Inhalation Toxicity (OECD TG 403) Rat NM-203 (pyrogenic)	4 h Analytical concentration: 2.08 mg/L Nominal concentration: 58.8 mg/L (exposure concentration was limited for technical reasons)	LC0 ≥ 2.08 mg/L air (analytical) LD50 > 2.08 mg/L air (analytical) LC0 > 58.8 mg/L air (nominal) LD50 > 58.8 mg/L air (nominal). No animals died. Nasal discharge during exposure, crusty eyes, crusty nose and alopecia at days post-exposure. No macroscopic organ lesions, but in one animal discoloration of the lung.	Data provided by BIAC. Tests performed by Toxigenics Inc., (USA) in 1981

*According to or comparable to OECD TG 403

No mortality was found in the tests at the maximum attainable concentrations (limited by technical reasons) of 0.14 - 2.08 mg/L (OECD/WPMN 2016, SiO2 summary).

In two tests (with NM-202 and NM-203) the highest attainable concentration was 0.14 mg/L and in two tests (with NM-200 and NM-201) the highest attainable concentration was 0.69 mg/L. In tests with N-202 and N-203 the highest attainable concentration was 2.08 mg/L. Higher doses were not technically achievable.

3.2 STOT RE

3.2.1 Oral exposure

In the OECD/WPMN (2016, SiO2 summary) reports three identical OECD TG 408 90-day oral toxicity tests with NM-200, NM-201 and NM-202 conducted in 1981. The test results from these studies are shown in table B5. Further a non-guideline 84 days study was retrieved from the NanoCluster publication web-site and an OECD TG 408 was retrieved from the NanoReg publication web-site.

Table B5. Overview of oral repeated exposure studies from the OECD/WPMN (2016, SiO2 summary) report

Test substance, Test guideline Species (Reference)	Dose regimen	NOAEL / LOAEL	Observations
NM-200 OECD TG 408 (Repeated Dose 90-Day) Rat (Wistar, male and female) (OECD/WPMN 2016, SiO2 summary)	Oral (feed) Continuous 13 weeks exposure Approx. 0.5, 2 and 6.7 % Si in the diet	NOEL 6.7 % in feed NOEL highest dose ca. 4000 ≤ 4500 mg/kg bw/day (nominal)	No clinical symptoms or other findings including haematological, bloodchemical and urinary parameters. Mean food intake was slightly increased in the female top-dose group (some +5 % after 4 wks) with no corresponding body-weight gain, but barely seen in males. The reduced food efficiency may be due to the rather high amount of inert test substance. Water consumption was normal throughout. Gross and microscopical examinations did not reveal any (histo-) pathological changes that could be attributed to the feeding of the test substance.
NM-201 OECD TG 408 (Repeated Dose 90-Day) Rat (Wistar, male and female) (OECD/WPMN 2016, SiO2 summary)	Oral (feed) Continuous 13 weeks exposure Approx. 0.5, 2 and 6.7 % Si in the diet	NOEL 6.7 % in feed NOEL highest dose ca. 4000 ≤ 4500 mg/kg bw/day (nominal)	No clinical symptoms or other findings including haematological, bloodchemical and urinary parameters. Mean food intake was slightly increased in the female top-dose group (some +5 % after 4 wks) with no corresponding body-weight gain, but barely seen in males. The reduced food efficiency may be due to the rather high amount of inert test substance. Water consumption was normal throughout. Gross and microscopical examinations did not reveal any (histo-)pathological changes that could be attributed to the feeding of the test substance.
NM-202 OECD TG 408 (Repeated Dose 90-Day) Rat (Wistar, male and female) (OECD/WPMN 2016, SiO2 summary)	Oral (feed) Continuous 13 weeks exposure Approx. 0.5, 2 and 6.7 % Si in the diet	NOEL 6.7 % in feed NOEL highest dose ca. 4000 ≤ 4500 mg/kg bw/day (nominal)	No clinical symptoms or other findings including haematological, bloodchemical and urinary parameters. Mean food intake was slightly increased in the female top-dose group (some +5 % after 4 wks) with no corresponding body-weight gain, but barely seen in males. The reduced food efficiency may be due to the rather high amount of inert test substance. Water consumption was normal throughout. Gross and microscopical examinations did not reveal any (histo-)pathological changes that could be attributed to the feeding of the test substance.

Thus, in these studies no adverse effects were noted up to the highest dose level used of about 4000 mg/kg bw/day.

Oral repeated dose non-guideline of study of SAS and NM-202 in rat, van der Zande et al. (2014)

A recent repeated oral dose non-guideline study was conducted by van der Zande et al. (2014). In this study groups of 5 male Sprague Dawley rats were fed with Synthetic Amorphous Silica (SAS) at 0,100, 1000 and 2500 mg/kg bw/day or NM-202 at 100, 500, and 1000 mg/kg bw/day for 28 days. Further, two groups of five male rats were dosed with the highest dose levels of SAS and NM-202 for 84 days. The Synthetic Amorphous Silica was food-grade quality with a purity ≥ 99.8%, a primary particle size of 7 nm and specific a surface area of 380 m^2/g, whereas NM-202 had a purity ≥99.9%, a primary particle size of 10-25 nm, and a specific surface area of 200 m^2/g.

The exposure to SAS or NM-202 did not result in clearly elevated tissue silica levels after 28-days of exposure. However, after 84-days of exposure to SAS, but not to NM-202, silica accumulated in the spleen. Biochemical and immunological markers in blood and isolated cells did not indicate toxicity, but histopathological analysis, showed an increased incidence of liver fibrosis after 84-days of exposure, which only reached significance in the NM-202 treated animals. This observation was accompanied by a moderate, but significant increase in the expression of fibrosis-related genes in liver samples.

Thus, from the study by van der Zande et al. (2014) adverse effects relevant for STOT RE classification purpose were obtained at an exposure duration for 84 days at a dose level of 1000 mg/kg bw/day.

Oral 90-day repeated dose toxicity of NM-203 according to OECD TG 408 in rats, NanoReg (2016)

NanoReg (2016) reported an OECD TG 408 90-day oral toxicity in which rats were exposed by oral gavage to NM-203 at dose levels of 0, 2, 5, 10, 20 and 50 mg/kg bw/ day. No signs of general toxicity were noted. In males the body weight gain was significantly reduced at 20 mg/kg bw per day at sacrifice and absolute and relative liver weight was significantly decreased at 10 mg/kg bw/day. Also, lung, adrenal and kidney relative weights were significantly increased at 10 mg/kg bw day in males. In females, uterus weight was significantly increased at 10 mg/kg bw / day. From histological examinations of the liver from males increased hepatocyte vacuolization/steatosis at 50 mg/kg bw per day, Intralobular lymphoid infiltration at 2 and 10 mg/kg bw/day, enlarged sinusoids at 10, 20 and 50 mg/kg bw/ day, congestion of sinusoids at 2, 20 and 50 mg/kg bw/ day, and focal intralobular necrosis in the middle zone of liver lobule at 10 mg/kg bw/day were found. In female liver, intralobular lymphoid infiltration and enlarged sinusoids were increased at 2, 10, 20, 50 mg/kg bw/ day, congestion of sinusoids was increased at 5 and 10 mg/kg bw/ day, and focal intralobular necrosis at 20 mg/kg bw/ day. Overall, the authors concluded that no NOAEL was found but at the same time it was noted that no clear dose-response-relationship of the effects was found either. It is noted that the description of the findings is qualitative in nature and that quantitative data as to the incidences of the findings are lacking.

3.2.2 Inhalation

OECD/WPMN (2016, SiO2 summary) reported six OECD TG 413 90-day inhalation toxicity tests performed in the period with NM-200, NM-201, NM-202, NM-203 and NM-204. Further, in 1969 a non-guideline one-year inhalation study was conducted with NM-202. The test results from these studies are shown in table B6.

Table B6. Overview of inhalation repeated exposure studies from the OECD/WPMN (2016, SiO2 summary) report

Test substance, Test guideline Species Year (Reference)	**Dose regimen**	**NOAEC / LOAEC**	**Observations**
NM-200 **NM-201** **NM-204** OECD TG 413 (Subchronic inhalation toxicity: 90-Day Study) Rat (Wistar, male and female) 1987 (OECD/WPMN 2016, SiO2 summary, reference to Reuzel et al. 1991 and test data on Sipernat 22S)	Inhalation 90 days 6h/day; 5 days/week 35 mg/m^3 Post exposure recovery up to one year	No NOAEC	Identical obeservations were reported for each of the NMs according to OECD / WPMN (2016, SiO2 summary): *"A slight decrease of body weight (- 5 %) by 13 weeks exposure was observed (still at – 4 % after 52 weeks post exposure). No significant effects in haematology were detected but white blood cells count elevated in both male and female groups at the end of exposure period, but it was not clearly attributable to the increase in the number of neutrophilic leukocytes.* *After 13 weeks recovery, neutrophil count still tended to be higher in males and females, and normalised by 28 weeks of recovery. No changes in heart, thyroids, adrenals, testes, brain, spleen and kidneys weights were observed, but the relative mean of lungs weighs slightly increased (≈ x 1.3). Thymus weight increased as well. Swollen lungs and enlarged mediastinal lymph nodes were noted. The effects gradually subsided after the exposure period. Lung weights were normalised after 13 weeks recovery in males and females. In the lung, accumulation of alveolar macrophage, intra-alveolar polymorphonuclear leukocytes and increased septal cellurarity in males and females were noted. Treatment related microscopic changes in the nasal region were found at the end of the exposure, such as very slight local necrosis and slight atrophy of the olfactory epithelium, intracystoplasmic proteinaeceous droplets.* *Accumulation of macrophages was seen in the mediastinal lymph nodes (disappearing after 39 week post exposure).* *Collagen content in the lungs slightly increased at the end of exposure. During the recovery period, all changes disappeared mostly within 13 – 26 weeks post exposure. Silica could be detected in lungs only in relatively small amounts at the end of the exposure periods. On average 0.5 mg per lung in male and 0.35 mg per lung in female groups, decreasing over time and no longer measurable after 39 weeks post exposure were found"* (OECD/WPMN 2016, SiO2 summary)
NM-202 **NM-203** OECD TG 413 (Subchronic inhalation toxicity: 90-Day Study) Rat (Wistar, male and female) 1987	Inhalation 90 days 6h/day; 5 days/week 1.3, 5.9, 31 mg/m^3 analytical 1, 6 and 30 mg/m^3 target concentrations	NOAEC = 1.3 mg/m^3 Air (analytical) NOEC < 1.3 mg/m^3 Air (analytical) LOAEC = 5.9 mg/m^3 Air (analytical)	Identical obeservations were reported for each of the NMs according to OECD / WPMN (2016, SiO2 summary): *"The respiration rate was increased (concentration related). No effect in female weights in all dose levels was detected. Depressive effects on males weight were found (1 mg/m^3 slightly at day 14 (- 5 %), 6 mg/m^3 slightly from day 49 to 77 (- 6 to -5 %), 30 mg/m^3 significant throughout exposure (-10 to - 7 %)). No difference from control at day 45 observed.* *No haematology effects were found for 1 mg/m^3 group. For the 6 mg/m^3 group, white blood cell count elevated in males and females due to increase in the number of neutrophilic leukocytes but concentration response relation was poor. After 3 months recovery, these blood parameters were normalised. For 30 mg/m^3 group, red blood cells and haemoglobin were statistically higher in males, but not in females. White blood cells count elevat ed in males and females due to increase of the number of neutrophilic leukocytes at 3 months of recovery. In females, a slight increase above the control group still existed after 6 months of recovery.*

Test substance, Test guideline Species Year (Reference)	Dose regimen	NOAEC / LOAEC	Observations
(OECD/WPMN 2016, SiO2 summary, reference to Reuzel et al. 1991 and test data on Aerosil 200 and Aerosil R974)	Post exposure recovery up to one year		*Swollen lungs and enlarged mediastinal lymph nodes at the end of recovery was found (treatment related degrees of severity). No lung weight effect was found for 1 mg/m3 group, but an increase was observed for the 6 (1.7 x for males and 1.4 x for females) and 30 mg/m^3 (2.3 x for males and 2.0 x for females) groups. For the 6 and 30 mg/m^3 groups, collagen content in the lungs was clearly increased, most pronounced in males. The above mentioned effects gradually subsided after the exposure period. But in males exposed to 6 to 30 mg/m^3, the collagen content was still above control values at the end of the study. Granuloma like lesions were seen in animals at the end of exposure period and after the 13 weeks of recovery. They did not show fibroblastic activity and hyanilisation and regressed during recovery. Accumulation of macrophages was seen in the mediational lymph nodes (disappeared week 39). Treatment related microscopic changes in the nasal region were occasionally found at the end of exposure period such as focal necrosis and slight atrophy of the olfactory epithelium. Interstitial fibrosis was not noted directly after the exposure period but appeared with a delay. It was observed for the first time after 13 weeks post exposure, increasing incidence especially for 30 mg/m^3 and less for 6 mg/m3. It decreased in severity and frequency until the end of the study. All types of pulmonary lesions were more marked in males than in females. The level of 1.3 mg/m^3 induced only slight changes after 13 weeks post exposure which generally recovered quickly. Morphological changes after 13 weeks exposure are considered statistically significant at 1.3 mg/m^3. Silica could be detected in lungs only in relatively small amounts at the end of exposure period. Only one male exposed to 30 mg/m^3 showed a small amount of silica in the regional lymph nodes. 90 days after termination of exposure, no silica could be recovered from any animal."*
NM-200 OECD 413 Subchronic inhalation toxicity: 90-Day Study Rat (male) 2014 (OECD WPMN 2016, SiO2 summary)	Inhalation 90 days 6h/day; 5 days/week 1, 2.5 and 5 mg/m^3	NOAEC (extrapolated) = 0.6 mg/m3 LOAEC (analytical) = 1 mg/m^3	Only very fragmented information is given by OECD/WPMN (2016, SiO2 summary) "BAL analysis: Benchmark approach based on PMN increase on 21d post-exposure Histopathology of nasal cavities (day 1 and 91 post-exposure): Hyperplasia (epithelial + mucous cell); epithelial hyaline droplets; epithelial inflammatory cell infiltration" No further data on this study could be found in OECD/WPMN (2016, SiO2 part1) covering information on NM-200

Test substance, Test guideline Species Year (Reference)	Dose regimen	NOAEC / LOAEC	Observations
NM-202 No guideline test Repeated inhalation, Female Sprague-Dawley Rat 1969 (OECD/WPMN 2016, SiO2 summary)	Inhalation 6 weeks, 18 weeks and 12 months 5h/day; 5 days/week 50 - 55 mg/m3 (total dust) = approx. 30 mg/m3 (respirable) Weekly exposure frequency was reduced to 2-3 times/week Due to losses from severe bronchitis and inflammation.	No NOAEC.	OECD / WPMN (2016, SiO2 summary) reported: *"After 12-months exposure, about 1 % of administered total respirable dust was estimated to be still retained in the* *lung. The increase in lung deposition was low from 18 weeks to 12 months of exposure (18 wk: 1.2 mg SiO2, 12 months: 1.37 mg SiO2). Mediastinal lymph nodes contained about 0.13 mg SiO2 after 12 months. After 5 months post-exposure, mean levels of SiO2 were 0.16 mg/lung and 0.047 mg/lymph node, i.e. a reduction at some 88 % in the* *lung and more than 50 % in the lymph nodes.* *Microscopically visible small dust foci could be observed under the pulmonary pleura, mediastinal lymph nodes were moderately enlarged. In the interior of alveoles, numerous macrophages accumulated, partially normal, partially destroyed, associated with deposition of cell debris ("desquamation catarrh"). Perivascular and peribronchiolar small dust foci of macrophages were associated with mild and moderate formation of connective tissue (ranked as grade I to II, based on a ranking system according to Belt & King). In the alveolar septa the collagen formation was increased. In some cases, collagenic fibrosis was detected, partially showing decay. There were no signs of typical silicosis. In the mediastinal lymph nodes, foci and clusters of phagocytes, partially normal, partially showing decay, were observed"* No further data on this study could be found in OECD/WPMN (2016, SiO2 part3) covering information on NM-202

From the above descriptions, the *pyrogenic derived nano silicon dioxide* qualities (NM-202 and NM-203) seems to be of highest toxicological concern. At an exposure level of 1.3 mg/m^3 pulmonary morphological changes after 13 weeks exposure were statistically significant and granuloma-like lesions were seen in animals at the end of exposure period and after the 13 weeks of recovery at the dose level of 30 mg/m^3. Further, pulmonary interstitial fibrosis was observed after 13 weeks post exposure with significant increasing incidence at 30 mg/m^3 and less for 6 mg/m^3. Treatment related degrees of severity of swollen lungs and enlarged mediastinal lymph nodes were observed at the end of recovery period.

For the precipitated nano silicon dioxide quality the most prominent findings were swollen lungs and enlarged mediastinal lymph nodes that gradually subsided after the exposure period. Lung weights were normalised after 13 weeks recovery in males and females. In the lung, accumulation of alveolar macrophage, intra-alveolar polymorphonuclear leukocytes and increased septal cellularity in males and females were noted.

For both pyrogenic and precipitated nano silicon dioxide treatment related focal necrosis and slight atrophy of the olfactory epithelium, were found at the end of the exposure.

Also, in a 12 months inhalation study the daily exposure to 50 - 55 mg/m^3 had to be reduced to an exposure frequency of 2-3 times per week due to mortality of the animals caused by severe bronchitis and inflammation.

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Appendix C. Nano silver (AgNP)

1. Overview of relevant data and hazard classes

In this evaluation regarding classification of nano silver (AgNP) focus will be placed on the evaluations of the data relevant for:

- *Acute toxicity*
- *Skin Sensitisation*
- *STOT RE*

Relevant data for this assessment has been found from the OECD/WPMN (2017, AgNP summary) and the OECD/WPMN (2016, AgNP part 7) as well as from data obtained REACH registration dossier or from a focused web-based literature search (using substance name, toxicological endpoint and exposure route as relevant search terms).

Table C1. Number of studies/ test data on the prioritised hazard classes

Hazard class	Number of studies (OECD-summary dossier)	Further studies retrieved for this project
Acute toxicity (oral)	2	0
Acute toxicity (inhalation)	0	0
Acute toxicity (dermal)	1	0
Sensitisation	2	0
Specific target organ toxicity Oral repeated exposure	5	5 key studies form REACH-reg. data
Specific target organ toxicity Inhalation repeated exposure	3	1 key study from REACH-reg. data Sung et al. (2009) Sung et al. (2008)

2. Substance identity and characterisation

Table C2. Identification/ characterisation of three different types of silver NPs; citrate capped silver NP, powder silver NP and NM-300, CAS No 7440-22-4 (OECD/WPMN 2017, AgNP summary)

	Citrate capped silver nanoparticles (cAgNPs) CAS: 7440-22-4	Silver powder (AgNPs) CAS: 7440-22-4	NM-300 CAS: 7440-22-4
Composition		Generated from silver wire (ISO 10801)	Nano-Silver colloidal dispersion with a nominal silver content of 10 w/w%
Primary particle size	10 nm, -39mV Average diameter <30nm (in water measured by DLS) GSD = 18.48 and 1.45 (in air measured by SMPS)	6-55 nm	< 20 nm D90: >3 - < 20 nm
Specific surface areas, BET	NA	NA	NA

	Citrate capped silver nanoparticles (cAgNPs) CAS: 7440-22-4	Silver powder (AgNPs) CAS: 7440-22-4	NM-300 CAS: 7440-22-4
Zeta potential	-60 mV (water) -20 mV (MEM) -50 to -30 mV (pH 6)	NA	NA
Water solubility mg/L	3.6 µg/L after 28 days at pH6 (particle size 1.9 µm (from REACH registration on silver)		

3. Hazard classes

3.1 Acute toxicity

3.1.1 Oral exposure

Acute oral toxicity study on cAgNP, (Key. NIER 2010) OECD/WPMN (2016, AgNP part 7)

"In an acute oral toxicity study performed according to OECD Guideline 423, groups (6 male rats/dose) of Sprague Dawley rats were given a single oral (gavage) dose of Citrate capped silver nanoparticles (cAgNPs) at 300 and 2000 mg/kg bw. Animals were then observed for mortality and clinical signs for 14 days.

No mortality or clinical signs were observed. In this study, the oral LD50 of test item was considered to be higher than 2000 mg/kg bw in male rats."

Acute oral toxicity study on cAgNP, (Key. MKE 2010) OECD/WPMN (2016, AgNP part 7)

"In an acute oral toxicity study performed according to OECD Guideline 423 and in compliance with GLP, groups (6 female rats/dose) of Sprague Dawley rats were given a single oral (gavage) dose of Citrate capped silver nanoparticles (cAgNPs) at 300 and 2000 mg/kg bw. Animals were then observed for mortality and clinical signs for 14 days.

No mortality or clinical signs were observed. In this study, the oral LD50 of test item was considered to be higher than 2000 mg/kg bw in female rats."

3.1.2 Dermal exposure

Acute dermal toxicity study on cAgNP, (Key. MKE 2010; Kim, 2013), OECD/WPMN (2016, AgNP part 7), OECD/WPMN (2017, AgNP summary)

This OECD TG 402 study report from 2010 was considered as a key study with a reliability score of 2 in the OECD/WPMN (2016, AGNP part 7) dossier. In the OECD/WPMN (2017, AgNP summary) report the following executive summary was given:

"Acute dermal toxicity study was conducted according to OECD TG 402 and in compliance with GLP (MKE, 2010; Kim et al., 2013). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) were applied to five male and female Sprague-Dawley rats of 8 weeks and 11 weeks of age, respectively. No deaths or abnormal findings were observed at the maximum concentration for 14 days. Although the dose tested was specified as 2,000 mg/kg bw, 2,400 mg/kg bw was administered. It is because the specific gravity of 1.2 was not

considered in preparation for the test material. LD50 of cAgNPs was considered to be higher than 2,000 mg/kg bw in rats"

3.1.3 Inhalation

No information available

3.2 Skin Sensitisation

Two studies were available from the OECD/WPMN (2016, AgNP part 7) dossier. Both studies were performed in accordance to OECD TG 406 and GLPBoth studies are key studies with a reliability score of 2.

The following executive summaries was given by OECD/WPMN (2017, AgNP Summary):

Skin sensitisation study on cAgNP, (Key. NIER)

"One skin sensitization study was conducted according to OECD TG 406 and in compliance with GLP (NIER, 2011). CrlOri:HA guinea pigs(20 males/dose) were induced dermally with 0.4 mL of Citrate capped silver nanoparticles(cAgNPs, ABC Nanotech Co., Ltd., Korea) in a 2.5 x 2.5 cm2 occlusive patch. This induction process was performed on the 27th day with a 0.1% (w/v) 1-chloro-2,4-dinitrobenzene(DNCB) and the test substance. For negative control, however, distilled water was applied while for the positive control, 1% (w/v) DNCB was applied instead of the test substance. 10 males for positive control group and 10 males for negative control group were used. Skin reaction was graded according to "Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions" in OECD TG No. 406. No skin reaction was observed in any of the treated groups at 24 and 48 hours after the challenge. 1%(w/v) DNCB induced skin sensitization. cAgNPs were a weak sensitizer in Guinea pig"

For both the dermal induction and the dermal challenge a concentration of 20.48% of cAgNP was used.

Skin sensitisation study on cAgNP, (Key. MKE 2010; Kim et al., 2013)

"The other skin sensitization study was conducted according to OECD TG 406 and in compliance with GLP (Kim et al., 2013). Hartley guinea pigs (20 males) were induced with 3 pairs of intradermal injection. Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) of 0.1 mL volume (20.48%) were given in the shoulder region at 1st induction. The volume of the test substance used for a 2 x 4 cm2 occlusive patch was 0.5 mL at 2nd induction and challenge phase. The patch was lasted for 48 hours at 2nd induction phase and lasted for 24 hours at challenge phase. For negative control (10 males), 1.0% citrate solution was applied instead of the test material. Interval between the first and second induction was 1 week and, after 2 weeks later, challenge was performed. Skin reaction was graded according to "Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions" in OECD TG 406. One out of 20 test animals (5%) exhibited grade 1 erythema at 24 or 48 hours after challenge, but no other skin reaction was observed in other animals. In this study, cAgNPs were a weak sensitizer in Guinea pig"

For both the dermal induction and the dermal challenge a concentration of 20.48% of cAgNP was used.

3.3 STOT RE

3.3.1 Oral exposure

In table C3 an overview is given of five oral repeated dose toxicity studies included in the OECD/WPMN (2015, AgNP part 7) dossier and of five oral repeated dose toxicity studies included in the REACH registration dossier of metallic silver.

Table C3. Summary data from repeated oral toxicity studies with AgNP

Test substance, Test guideline, Species, (Reference).	**Dose regimen**	**NOAEL / LOAEL**	**Observations**
cAgNPs (ABC Nanotech Co., Ltd., Korea), Particle size: <30nm, OECD TG 407 (Repeated Dose 28-Day), Rat (Sprague -Dawley, male and female (5/sex/dose)), (OECD/WPMN 2017, AgNP summary, NIER no. 2010-49-1224).	Oral administration via drinking water. Daily exposure to: 0, 25, 100, 400 mg/kg bw/day	NOAEL > 400 mg/kg bw/day (nominal), No significant toxicity observed	From OECD/WPMN (2017, AgNP summary): *"In a repeated dose oral toxicity study conducted according to OECD 407(NIER No. 2010-49-1224), citrate capped silver nanoparticle (cAgNPs, ABC Nanotech Ce., Ltd., Korea) were administered by oral via drinking water to Sprague-Dawley rats (5/sex/dose) at 0, 25, 100 and 400 mg/kg bw/day for 28 days. Examinations during the study included mortality, toxic effects, body weight, clinical biochemistry and macroscopic examination. No significant toxicity or mortality was observed. No significant difference in body weight was observed in any of the dose groups. Serum GPT was increased while blood urea nitrogen (BUN) was decreased in female rats, which showed dose-dependency. cAgNPs tested did not induce any toxicity in a repeated dose 28-day oral toxicity study in rodents."*
cAgNPs (ABC Nanotech Co., Ltd., Korea), Particle size: <30nm, OECD TG 408 (Repeated Dose 90-Day), Rat (Sprague -Dawley, male and female (10/sex/dose)), (OECD/WPMN 2017, AgNP summary, NIER no. 2010-49-1224).	Oral administration via drinking water. Daily exposure to: 0, 25, 100, 400 mg/kg bw/day	NOAEL > 400 mg/kg bw/day (nominal), No significant toxicity observed	From OECD/WPMN (2017, AgNP summary): *"In a repeated dose oral toxicity study conducted according to OECD 408 (NIER No. 2010-49-1224), citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Ce., Ltd., Korea) were administered by oral via drinking water to Sprague-Dawley rats(10/sex/dose) at 0, 25, 100 and 400 mg/kg bw/day for 90 days. Examination during the study included mortality, toxic effects, body weight, clinical biochemistry and macroscopic examination. No significant toxicity or mortality was observed. No significant difference in body weight was observed in any of the dose groups. Serum triglyceride (TG) was decreased while total bilirubin levels were increased in treated groups. However, there was no dose-dependent tendency and statistical significance was not tested. cAgNPs tested did not exhibit any toxicity in a repeated dose 90-day oral toxicity study in rodents."*
cAgNPs (ABC Nanotech Co., Ltd., Korea), Particle size: <30nm, OECD TG 422 (Repeated Dose 42-Day), Rat (Sprague -Dawley, male and female (50/sex/dose)), (OECD/WPMN 2017, AgNP summary, NIER and Hong 2014)	Oral administration. Daily exposure to: 62.5, 125, and 250 mg/kg bw/day	NOAEL > 250 mg/kg bw/day (nominal), No significant toxicity observed	From OECD/WPMN (2017, AgNP summary): *"Repeated dose oral toxicity studies were conducted according to OECD 422 and in compliance with GLP (Hong et al., 2014). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) were administered by oral route to Sprague-Dawley rats (50/sex/dose) at 62.5, 125 and 250 mg/kg bw/day, once daily for 42 days(Male: 14 days before mating, 14 days during the mating and 14 days of post-mating, Female: 14 days before mating, during the mating and gestation and 4 days of lactation). A daily application volume (10 ml/kg) was adjusted according to the most recent body*

			weight and the vehicle control rats were treated with an equivalent volume of distilled water. Examinations during the study included clinical observations, mortality, bodyweight, food and water consumption, haematology, blood chemistry, organ weight, gross and histopathological examinations. No signification toxicity or mortality was observed. Alopecia was observed in the vehicle control and treatment groups of both sexes. Salivation was observed in 1 female of the 250 mg/kg bw/day group on Day 1 of gestation. No significant differences in body weight, food and water consumption were observed in any of the dose groups. No statistically significant changes in haematological analysis were observed in any of the treatment groups, including, recovery group. In the serum biochemical analysis and urinalysis, no treatment related changes were observed. In both sexes, decreases in IP and AST and an increase in CI were also considered to be spontaneous because these were neither dose-related nor within the normal ranges. In recovery groups, a statistically significant increase in absolute and relative weights of liver was observed in males, and an increase in absolute weights of kidneys and adrenal glands were observed in females. No gross or histopathological findings were observed at necropsy. Under the test conditions, the NOAEL of AgNPs was considered to be higher than 250 mg/kg bw/day in Sprague-Dawley rats."
cAgNPs Silver nanoparticles (52.7–70.9 nm, average; 60 nm (Namatech Co., Ltd., Korea), OECD TG 407 (Repeated Dose 28-Day), Rat (Sprague -Dawley, male and female (50/sex/dose)), (OECD/WPMN 2017, AgNP summary, Jeong 2010)	Oral administration Daily exposure to: 0, 30, 300, 1000 mg/kg bw/day	NOAEL/LOAEL Not determined	From OECD/WPMN (2017, AgNP summary): *"Repeated dose oral toxicity studies were conducted according to the OECD Guideline 407 and in compliance with GLP (Jeong et al., 2010). Silver nanoparticles (cAgNPs, Namatech Co., Ltd., Korea) were administered by oral route to Sprague-Dawley rats (10/sex/dose) at 0, 30, 100 and 1,000 mg/kg bw/day (dosing volumes were 10 ml/kg), once daily for 28 days. Examination during the study included mortality, toxic effects, body weight, macroscopic and microscopic examination. No significant difference in body weight was observed in any of the dose groups. The treated samples showed luminal and surface particles and the tissue also contained silver nanoparticles. A dose-dependently increased accumulation of silver nanoparticles was observed in the lamina propria of both the small and large intestine, and also in the tip of the upper villi in the ileum and protruding surface of the fold in the colon. The cAgNPs treated rats exhibited a higher number of goblet cells that had released their mucus granules than the controls, resulting in more mucus materials in the crypt lumen and ileal lumen. Moreover, cell shedding at the tip of the villi was frequent. Lower amounts of neutral and acidic mucins were found in the goblet cells in the cAgNPs treated rats. Plus the amount of sialomucins was increased, while the amount of sulfomucins was decreased. In particular, in the colon of the sample treated rats,*

			sialyated mucins were detected in the lamina propria, the connective tissue under the epithelia. cAgNPs are a powerful intestinal secretagogue and induce an abnormal mucin composition in the intestinal mucosa."
Silver powders (Namatech Co., Ltd., Korea), Particle size: 60 nm, OECD TG 408 (Repeated Dose 90-Day), Rat (Fisher 344, male and female (10/sex/dose)), (OECD/WPMN 2017, AgNP summary, MKE 2008, Kim 2010) and REACH registration dossier on silver casn: 7440-22-4, key study 1 repeated dose toxicity: oral.	Oral administration (gavage) Daily exposure to: 0, 30, 125, 500 mg/kg bw/day	NOAEL < 30 mg/kg bw/day LOAEL = 125 mg/kg bw/day	From OECD/WPMN (2017, AgNP summary): *"Repeated dose oral toxicity studies were conducted according to the OECD Guideline 408 and in compliance with GLP (MKE, 2008; Kim et al., 2010) Silver powder (Namatech Co., Ltd., Korea) was administered by oral gavage to Fischer 344 rats(10/sex/dose) at 0, 30, 125 and 500 mg/kg bw/day for 90 days. Examinations during the study included mortality, clinical signs, body weight, food and water consumption, haematology, clinical chemistry, ophthalmoscope, organ weights, gross and histopathological examinations. No mortality or clinical signs were observed. Decrease in body weight gain was observed in male rats treated with 500 mg/kg bw/day after 4, 5 and 7 ~ 13 weeks. No significant differences in food and water consumption were found between treated and control groups. Increase of monocyte number and decrease of reticulocytes were detected in female rats treated with 500 mg/kg bw/day and in female rats treated with 30 mg/kg bw/day, respectively. No difference in prothrombin time and activated partial thromboplastin time was observed among groups. Total cholesterol and total bilirubin were elevated in male rats treated with 125 mg/kg bw/day of silver powder, and total cholesterol was increased in male rats treated with 500 mg/kg bw/day. The decreases in magnesium, total protein, and inorganic phosphorus were detected in female rats treated with 125 mg/kg bw/day. The increases in total cholesterol and alkaline phosphatase and the decreases in magnesium, total protein, and inorganic phosphate were observed in female rats treated with 500 mg/kg bw/day of silver powder. No significant organ-weight changes were observed in either the male and female rats except for an increase in the weight of the left testis in the 500 mg/kg bw/day male rats, and for decrease in the weight of right kidney in the 30 and 125 mg/kg bw/day female rats. No significant difference in gross pathology was observed between treated and control groups. Bile duct hyperplasia and focal inflammation in liver were prominent, although dose-response relationship was not detected. The LOAEL and NOAEL of silver powder were 125 mg/kg bw/day and < 30 mg/kg bw/day, respectively in Fischer 344 rats."*

Silver powders (Namatech Co., Ltd., Korea), Particle size: 60nm, OECD TG 407 (Repeated Dose 28-Day), Rat Sprague -Dawle, male and female (10/sex/dose)), REACH registration dossier on silver casn: 7440-22-4, key study 2 repeated dose toxicity: oral. (Kim, Y.S.; et al., 2008)	Oral administration (gavage) Daily exposure to: 0, 30, 300, 1000 mg/kg bw/day	LOAEL = 300 mg/kg bw/day	One study was performed according to OECD TG 407 where rats were daily orally gavage with 0, 30, 300 and 1000 mg AgNPs/kg bw/day for 28 days. Effects on red blood cell parameters were observed in females at a dose of 300 mg/kg bw/day, and indication of liver damage (increased alkaline phosphatase, cholesterol and total protein) was observed in females treated with 300 or 1000 mg/kg bw/day. Also, incidences of bile duct hyperplasia around the central vein were observed in both female and male animals in a dose-dependent way. No NOAEL or LOAEL were suggested by the authors.
AgNP stabilised with polyvinylpyrrolidone (PVP), Particle size: 14 nm, No guideline followed 28 days. Rat Wistar Hannover Galas, male and female (6 - 10 animals/group), REACH registration dossier on silver casn: 7440-22-4, key study 3 repeated dose toxicity: oral. (Hadrup et al., 2012)	Oral administration (gavage) Daily exposure to: Vehicle control group (11.5 mg/mL PVP) (females and males) PVP-stabilised AgNPs: - 2.25 mg Ag/kg bw/day: (females only) -4.5 mg Ag/kg bw/day: (females only) - 9.0 mg Ag/kg bw/day: (females and males)	NOAEL > 9.0 mg/kg bw/day	No effects were observed at the highest dose level.
cAgNPs (ABC Nanotech Co., Ltd., Korea), Particle size: 7.9 ± 0.95 nm (TEM), OECD TG 422 (Repeated Dose 42-Day), Rat (Sprague -Dawley, male and female (10/sex/dose)), REACH registration dossier on silver casn: 7440-22-4, key study 4 repeated dose toxicity: oral. (unknown publication 2013)	Oral administration, Daily exposure to: 62.5, 125 and 250 mg/kg bw/day Additional Ag analysis: Tissues including liver, kidney and lung were obtained from four female rats sacrificed after 4 days lactation. The concentration of Ag in these tissues was analysed using ICP-MS	NOAEL > 250 mg/kg bw/day	One OECD TG 422 study (Combined Repeated Dose Toxicity Study with Reproduction/ Developmental Toxicity Screening Test) Rats (10/sex/dose) were daily orally administrated cAgNPs at 62.5, 125, and 250 mg/kg bw/day for 42 days(male) and 52 days (female). No changes in body weight gain, water and food consumption, mortality, clinical signs or significant toxicity (haematology, serum biochemistry, histopathology, urinalysis and necropsy) were observed during the study. Additionally, an AgNP biodistribution study was included. However, the biodistribution study of cAgNP in pregnant rats revealed a 213-fold increase in cAgNP accumulation lung tissue (4461.22 ± 2726.42 ng/g vs. control: 20.91 ± 7.47 ng/g), 34-fold increase in liver (treatment: 1117.55 ± 381.68 ng/g vs. control: 449.78 ± 151.29 ng/g), and a 13-fold increase in the kidneys (treatment: 449.78 ± 151.29 vs. control: 34.81 ± 13.97 ng/g) compared to control rats.

cAgNPs (ABC Nanotech Co., Ltd., Korea), Particle size in medium: 19.0 ± 4.6 nm (DLS), OECD TG 408 (Repeated Dose 90-Day), Rat (Sprague -Dawley, male and female (12/sex/dose)), REACH registration dossier on silver casn: 7440-22-4, key study 5 repeated dose toxicity: oral.	Oral administration (gavage) Daily exposure to: 0, 257.6, 515.3, and 1030.5 mg/kg bw/day	NOAEL (female) = 515.3 mg/kg bw/day, (Based on White blood cell level in females treated with the highest dose of 1030 mg/kg bw/day)	The study was performed according to OECD TG 408 and female and male Sprague -Dawley rats (12/sex/dose) were exposed to cAgNPs (0, 257.6, 515.3, 1030.5 mg/kg bw/day) via oral gavage, once daily for 90 days. Examinations during the study included mortality, clinical signs, body weight, food and water consumption, haematology, clinical chemistry, ophthalmoscope, organ weights, gross and histopathological examination, and biodistribution of Ag. No mortality or clinical signs were observed during the study. However, several treatment-related systemic effects of cAgNPs were observed in females and males treated with the highest dose. An increased white blood cell level (1.3-fold compared to control group) was found in females and decrease platelet level was found in males treated with cAgNPs 1030 mg/kg bw/d compared with their control groups. Serum alkaline phosphatase was significantly increased both males and females treated with 1030 mg/kg bw/d. Also, rat treated with 1030 mg/kg bw/d had increased the incidence of lymphocytic infiltration in the liver (8/12, male; 6/12, female) vs. controls (5/12, male; 4/12, female) and to a lesser extent in the kidney. Moreover, male and females treated with 1030 mg cAgNP/kg bw/d had decreased weight of the pituitary gland and the ovary, respectively. In addition, biodistribution analysis revealed significant concentrations of silver in the blood of all treatment group compared to the control group. Further, increased silver concentrations of sliver were found in spleen, lung and brain after cAgNP administration which showed a clear dose-response relationship in both male and female rats. In conclusion, a NOAEL (female) of 515.3 mg/kg bw/day was suggested and the systemic toxicity of cAgNPS, including liver and kidney toxicity, might be explained by extensive systemic distribution of silver originating from the silver nanoparticles.

The five first studies in Table C3 are from the OECD /WPMN dossiers on AgNP. From these studies the most significant findings are from the Kim (2010) study using dose levels of 0, 30, 125, 500 mg/kg bw/day of AgNP. From OECD/WPMN (2017, AgNP summary) dossier:

"Total cholesterol and total bilirubin were elevated in male rats treated with 125 mg/kg bw/day of silver powder, and total cholesterol was increased in male rats treated with 500 mg/kg bw/day. The decreases in magnesium, total protein, and inorganic phosphorus were detected in female rats treated with 125 mg/kg bw/day. The increases in total cholesterol and alkaline phosphatase and the decreases in magnesium, total protein, and inorganic phosphate were observed in female rats treated with 500 mg/kg bw/day of silver powder. No significant organ-weight changes were observed in either the male and female rats except for an increase in the weight of the left testis in the 500 mg/kg bw/day male rats, and for decrease in the weight of right kidney in the 30 and 125 mg/kg bw/day female rats. No significant difference in gross pathology was observed between treated and control groups. Bile duct hyperplasia and focal inflammation in liver were prominent, although dose-response relationship was not detected. The LOAEL and NOAEL of silver powder were 125 mg/kg bw/day and < 30 mg/kg bw/day, respectively in Fischer 344 rats."

This 90 day study was also included in the REACH registration of silver as a key study 1 that concluded the target organ to be the liver as slight liver damage was indicated by significant dose-related changes in alkaline phosphatase and cholesterol levels in both males and females at 125 mg/kg bw/d and above. Further histopathology revealed slightly higher incidences of bile-duct hyperplasia with or without necrosis, fibrosis and/or pigmentation in treated animals together with a dose-dependent accumulation of silver in all tissues examined.

As indicated in the table four additional repeated dose toxicity studies were included in the REACH registrations of silver. An OECD TG 408 90 day study in rats was included as a key study in the REACH registration where dose levels of 0, 257.6, 515.3, and 1030.5 mg/kg bw/day were used. In this study signs of toxicity in liver and kidneys was seen as increased lymphocyte infiltration and increased serum alkaline phosphatase. A NOAEL of 515.3 mg/kg bw/day was concluded.

Table C4. Summary data from repeated inhalation toxicity studies with AgNP

Test substance, Test guideline, Species, (Reference).	**Dose regimen**	**NOAEL / LOAEL**	**Observations**
Silver powder AgNP (Daedeok Science, Korea- Analytical purity: 99.98 %, particle size <20 nm), OECD TG 412 (repeated dose 28/14 day study), Rat (Sprague - Dawley, male and female (10/sex/dose)), (OECD/WPMN 2016, AgNP part 7 and summary, Ji et al., 2007).	Inhalation Once a day, 6 h/day, 5 days/week, for 4 weeks -Fresh air control, -Low-dose group (target dose, 1.2 $\times 10^4$ particles/cm^3, 1.2×10^6 nm^2/cm^2), -Middle-dose group (target dose, 1.2×10^5 particles/cm^3, 8.5×10^7 nm^2/cm^2), -High-dose group (target dose, 1.2×10^6 particles/cm^3, 1.8×10^9 nm^2/cm^2).	LOAEL = 1.2×10^4 particles/cm^3, (1.2×10^6 nm^2/cm^2) i.e. low dose, (effect based on increased blood leukocytes)	From OECD/WPMN (2016, AgNP part 7): *"In a repeated dose toxicity study conducted according to the OECD 412 Guideline and in compliance with GLP,Silver nanoparticles was administered by inhalation to groups of Sprague-Dawley rats (10 animals/sex/dose) at the concentrations of low-dose group (target dose, 1.2×10^4 particles/cm^3, 1.2×10^6 nm^2/cm^2), middle-dose group (target dose, 1.2×10^5 particles/cm^3, 8.5×10^7 nm^2/cm^2), and high-dose group (target dose, 1.2×10^6 particles/ cm^3, 1.8×10^9 nm^2/cm^2) for 6 h/day, 5 days/week, for 90 days. Fresh-air used as control. Examinations during the study included: mortality, clinical observation of animals, body weight change, monitoring of food and water consumption, laboratory investigations: haematology, blood clinical chemistry, gross pathology, measurement of organ weights and histopathology. No mortality or clinical signs were observed. No significant difference in body weight and food/water consumption was observed in any of the dose groups. There were no significant dose-related changes in the hematology values for the male rats. The percentage of neutrophils and eosinophils increased significantly ($p < 0.05$) in female rats in the low-dose group when compared with the control. The MCH in the female rats in the middle-dose group increased significantly ($p < 0.05$) when compared with the female rats in the high-dose group. The high-dose group revealed significantly increased ($p < 0.05$) calcium in both the male and female rats when compared with the control,and increased total protein ($p < 0.05$) in the male rats when compared with the control. Meanwhile, the low-dose group of male rats showed increased gamma-GT ($p < 0.05$) when compared with the control group. The exact meaning of these differences was impossible to clarify. No significant gross pathological or organ weight changes were observed. Histopathological examination of the male rat livers revealed one case of cytoplasmic vacuolization in the control, four cases in the low-dose group, and one case each in the middle- and high-dose groups, respectively. For the female rats, two cases each of cytoplasmic vacuolization were detected in the control and low-dose group, respectively, six cases in the middle dose group, and seven cases in the high-dose group. Two cases of hepatic focal necrosis were detected among the male rats in the high-dose group and one case among the female rats in the high-*

			dose group. The other organs, including the kidneys, spleen, lungs, adrenals, heart, reproductive organs, brain, and nasal cavity, were also examined histopathologically, *with no distinct findings. The silver concentration in the lung tissue from the groups exposed to silver nanoparticles for 28 days revealed a statistically significant ($p < 0.01$) dose-dependent increase. Although no clear blood silver concentrations were detected for any of the dose groups, a clear increase ($p < 0.05$) was observed in the liver silver concentration for the high-dose group, along with a statistically significant ($p < 0.01$) increase in the brain silver concentration. The olfactory bulb, which showed higher silver-concentration levels than the brain, also revealed a dose-dependent increase ($p < 0.01$) in both the male and female rats.* *Under the test conditions, the Lowest Observed Adverse Effect Level (LOAEL) of Silver nanoparticles was considered to be 1.2×10^4 particles/cm^3 ($=1.2\times10^6$ nm^2/cm^2) in Sprague-Dawley rats."* In the publication by Ji et al. (2007) the upper dose level of 1.2×10^6 particles/cm^3 was indicated to by equal to a dose level of 61 µg/m^3.
Silver nanoparticles (52.7–70.9 nm, average; 60 nm Analytical purity: 99.98 %), (Namatech Co., Ltd., Korea) Rat (Fischer 344, male andfemale (10 rats/dose)), (OECD/WPMN 2016, AgNP part 7 ,Kim et al., 2009).	Vehicle 0.5% Carboxymethyl cellulose (CMC) -Low (30 mg CMC/kg/d), --Intermediate (125 mg CMC/kg/d), and -High (500 mg CMC/kg/d) Basis NB! Only gross pathology and histopathology were the solo endpoints of the study	NOAEL (NOEL): Not determined LOAEL (LOEL): Not determined	The study was performed as a repeated dose toxicity study according to the OECD Guideline 413 and in compliance with GLP. AgNPs were administered by inhalation to four groups of Fischer 344 rats (10 rats/group; males and females) at the concentrations of 0 (vehicle control - 0.5 % carboxymethyl cellulose), 30, 125 and 500 mg/kg bw/day, once daily for 90 days. No information about exposure time. Gross pathology and histopathological examination were performed. No mortality was observed, and the main results and conclusion were that female rats showed a higher accumulation of Silver nanoparticles in all kidney regions, including the cortex, outer medulla, and inner medulla. Especially, the glomerulus in the cortex contained a higher accumulation in females compared to males.
Silver nanoparticles (particle size 6-55 nm, CMD 18.48nm GSD 1.45 nm, analytical purity: 99.99 %), (particles generated from silver wire ISO/10801) OECD TG 413 (repeated dose 90 day study), Rat (Sprague -Dawley,, male and female (10 rats/dose)), (OECD/WPMN 2016, AgNP part 7 ,Sung et al., 2009).	Inhalation once daily, Continuous exposure, 6h/day 5days/week for 90 days, 0, $0.6x10^6$ particles/cm^3 (= 49 µg/m^3), $1.4 x10^6$ particles/ cm^3 (=133 µg/m^3) $3.0 x10^6$ particles/ cm3 (=515 µg/m3)	NOAEL: 100 µg/m^3 (1.0×10^6 particles/ cm3)	From OECD/WPMN (2016, AgNP part 7): *"In a repeated dose toxicity study conducted according to the OECD Guideline 413 and in compliance with GLP, Silver nanoparticles was administered by inhalation-particulate to groups of Sprague-Dawley rats (10 animals/sex/dose) at the concentrations of 0, 0.6×10^6 particles/cm^3($=49$ µg/m^3), 1.4×10^6 particles/ cm^3($=133$ µg/m^3) and 3.0×10^6 particles/ cm^3($=515$ µg/m^3)by continuous exposure, 6 h/day, 5 days/week, for 90 days. HEPA filtered clean air was supplied to negative control group. Examinations during the study included: mortality, clinical observation of animals, body weight change, monitoring of food and water consumption, laboratory investigations: haematology, blood clinical chemistry, ophthalmological examination, urinalysis, gross pathology, measurement of organ weights and histopathology.*

			No mortality or clinical signs were observed. Decrease in body weight in females exposed to 1.4 x 10^6 particles/cm^3 from 3 week exposure. No significant difference in food consumption was observed in any of the dose groups. No significant difference in haematology and urinalysis were observed between treated and control groups. Increases of creatinine and total protein and decreases of chloride were detected in male exposed to 0.6 x 10^6 particles/cm^3. However, all the values were within normal range and dose-response was not detected. No abnormalities were observed in ophthalmoscopic examination. In male, cervical lymph node congestive spot, spleen hypertrophy, bladder congestive spot, intestinal nodule, and brain retraction were detected. In female, cyst in ovary, spleen hypertrophy, black spot in liver and adrenal gland, and intestinal nodule were observed. No significant difference was observed in organ weight s in any of the dose groups. Liver and lung were appeared to be the target organs of inhaled silver nanoparticles. BAL analysis revealed concentration dependent increase in albumin, lactate dehydrogenase and total protein were found in female rat. Decreases in tidal volume were observed in the male rats, which was concentration-dependent. In case of female, such decrease was detected in the group exposed to 1.4 x 10^6 particles/cm^3. No change was detected in prothrombin time and activated partial thromboplastin time. Significant increase in erythrocyte clotting was found in female rats exposed to 3.0 x 10^6 particles/cm^3. *Distribution in lung, kidney, liver, blood, brain and olfactory nerve was prominent and concentration dependent.* *Under the test conditions, the NOAEL of Silver nanoparticles was 1.0 x 10^6 particles/cm^3 or 100 $\mu g/m^3$ in Sprague-Dawley rats."*

3.3.2 Inhalation exposure

OECD/WPMN (2016, AgNP part 7) provides information of three repeated dose inhalation studies. Summaries of the findings from these studies are given in the table C4 below.

From the studies above the most significant findings from OECD/WPMN (2017, AgNP summary) were in relation to the Sung et al. (2009) 90-day study using dose levels of 0, 49 µg/m^3, 133 µg/m^3 and 515 µg/m^3 or AgNP particle number concentrations of 0, 0.6×10^6 particles/cm^3, 1.4 x 106 and 3.0 x 10^6, respectively:

"Liver and lung were appeared to be the target organs of inhaled silver nanoparticles. BAL analysis revealed concentration dependent increase in albumin, lactate dehydrogenase and total protein were found in female rats. Decreases in tidal volume were observed in the male rats, which was concentration-dependent. In case of female rats, such decrease was detected in the group exposed to 1.4 x 10^6 particles/cm^3. No change was detected in prothrombin time and activated partial thromboplastin time. Significant increase in erythrocyte clotting was found in female rats exposed to 3.0 x 10^6 particles/cm^3. Distribution in lung, kidney, liver, blood, brain and olfactory nerve was prominent and concentration dependent. The NOAEL of silver nanoparticles was 1.0 x 10^6 particles/cm^3 or 100 µg/m^3 in Sprague-Dawley rats."

This study is also the basis for the key study in the REACH registration of silver, that concluded a NOAEC of 133 µg/m^3.

In the publication by Sung et al. (2009), it is indicated that the histopathological findings showed a high incidence of chronic alveolar inflammation, mixed cell perivascular infiltrate, and alveolar macrophage accumulation in the high-dose male and female animals when compared with the controls. In males chronic inflammation was found in 3/10, 2/10 and 8/9 in low, medium and high dose versus 2/10 in controls, while in females the incidences were 2/10, 0/10, 8/10 versus 3/10 in controls indicating exposure associated effects at the highest exposure level in male and female rats Further bile duct hyperplasia was observed in 5/9 males and 8/10 females at high dose versus 0/10 in male controls and 3/10 in female controls. Further, the histopathological findings were described as dose-dependent increases in lesions related to silver nanoparticle exposure, including infiltrate mixed cell and chronic alveolar inflammation, thickened alveolar walls and small granulomatous lesions.

In an earlier publication by Sung et al. (2008) the effects on lung function from the exposed animals were described in detail:

From the figures in this publication high exposure male rats at the end of the exposure period compared to controls showed significant decreases in tidal volume, in minute volume and in peak inspiratory flow at about 19, 20 and 26%, respectively (readings form figures in the publication). Female rats at highest exposure level showed significantly reduced minute volume (about 15%) and peak inspiratory flow (about 10%) whereas the tidal volume was not significantly altered compared to control females. In this publication the histopathological lesions were described as dose-dependent increases in lesions related to silver nanoparticle exposure, including infiltrate mixed cell and chronic alveolar inflammation, thickened alveolar walls and small granulomatous lesions.

The study by Kim et al. (2009) as reported by OECD/WPMN (2016, AgNP part 7) is not considered relevant for further discussion in relation to classification as severe limitations of the reporting was noted: no details were given about inhalation chamber, housing and feeding conditions, clinical observations, body weight, food/water consumption, clinical pathology, ophthalmoscopy and individual and summary tables of results. Also, the study was not taken further from the part 7 dossier and included in the repeated dose section of the OECD/WPMN (2017, AgNP summary) dossier.

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Appendix D. Nano zinc oxide

1 Overview of relevant data and hazard classes

For the assessment of nano zinc the focus was on data relevant for the following hazard classes:

- *Acute toxicity*
- *STOT RE*

Relevant data for this assessment has been found from the OECD/WPMN (2015, ZnO part 3) as well as from the REACH registration of the substance. No relevant data was obtained from the NanoReg project (EU 7th framework) or the NanoSafety Cluster projects. From focused web-based search (using substance name, toxicological endpoint and exposure route as relevant search terms) three relevant scientific publications were extracted from PubMed . One publication using intrathecal instillation, Wang et al., 2017 was disregarded due to different exposure methodology (intrathecal puncture). Only publications after 2014 published in English and containing lung histopathology were included.

Table D1. Nano zinc oxide studies and hazard classes

Hazard class	Number of studies (OECD-dossier)	Further studies retrieved for this project
Acute toxicity (oral)	1	1 (Pasupuleti et al. 2012; from REACH-reg. data)
Acute toxicity (inhalation)	Alternative adm. methods: 3	1 (REACH-reg data)
Specific target organ toxicity repeated exposure (oral)	0	0
Specific target organ toxicity repeated exposure (dermal)	0	1 (Surekha et al. 2012; from REACH-reg. data) 1 (Ryu et al. 2014)
Specific target organ toxicity repeated exposure (inhalation)	4	3 Adamcakova-Dodd et al. (2014) Morimoto et al. (2016) Monsé et al. (2018)

The REACH registration dossier indicates the CLP classification on 3 different qualities of ZnO. One ZnO quality ("ZnO lower grade") contained impurities of lead, whereas the two other qualities ("ZnO standard" and "ZnO nano") did not contain impurities. Data from "ZnO standard" and "ZnO nano" did not trigger any classification on human health. However, data on "ZnO lower grade" (not discussed further in this report) triggered classification on human health, as Acute tox. 4 (inhalation and oral), Repr. 1A, and STOT RE 2 in the REACH registration dossier. Impurities of lead in "ZnO lower grade" quality is most likely the reason for classification as Repr. 1A.

2. Substance identity and characterisation

Chemical name: Zinc Oxide Nano (ZnO); IUPAC name: oxozinc; CAS No.: 1314-13-2
In the OECD dossiers, the substance identity and physicochemical properties are described for four different types of zinc oxide particles, two uncoated nanoforms (MN110, NM112), one coated nanoform with triethoxycaprylylsilane (CAS # 2943-75-1, content 2%) (NM111), and one uncoated micro-sized form (NM113).
The physicochemical properties of ZnO are extensively characterized in dossier (OECD/WPMN 2015, ZnO part 1). As examples, some key physicochemical parameters are shown in Table 1 for the four different qualities.

Table D2. Selected physicochemical properties of ZnO. Adapted from OECD/WPMN (2015, ZnO part 1)

	NM-110, Z-COTE, zinc oxide nano uncoated,	**NM-111, Z-COTE HP1, zinc oxide nano coated triethoxycaprylylsilane (2%),**	**NM-112, zinc oxide nano,**	**NM-113, zinc oxide uncoated non-nanosized ZnO**
Purity	99%	96%	99.5%	-
Particle size, nm	77.5 ± 18	75.2 ± 7.6	33.75 ± 6.2	149.7 ± 25
Specific surface area, m^2/g	6.6 ± 0.3	11.8 ± 0.2	25.9 ± 0.3	4.0 ± 0.15
Water solubility, dest. water, 2 - 22 days, ng/g	2536 - 5030	-	764 - 1607	1864-6007
Bulk density, g/cm^3	0.293	0.693	0.415	0.646

3. Hazard classes

3.1 Acute toxicity

3.1.1 Oral exposure

Acute oral toxicity study on Z-COTE HP1 (NM-111), (Key. Wang et al. (2008), OECD/WPMN 2015, ZnO part 3)

This OECD TG 401 study from 2008 was considered as a key study with a reliability score of 2 in the OECD/WPMN (2015, ZnO part 3) dossier.
In the OECD/WPMN (2015, ZnO part 3) dossier the following summary was given:

"Wang et al. (2008) treated 5 mice per sex and dose orally by gavage with 1000, 2000, 3000, 4000 and 5000 mg/kg bw of nanoscale ZnO powder (particle size: 20 nm) and submicroscaled ZnO powder (particle size: 120 nm), respectively. The study was performed according to OECD

guideline 401 but under non-GLP conditions. After the 14-days observation period the oral LD50 of 20 nm ZnO was estimated to be > 5000 mg/kg bw while the LD50 of 120 nm ZnO was > 2000 and < 5000 mg/kg bw in mice."

Acute oral toxicity of zinc oxide nanoparticles (Pasupuleti et al. (2012), REACH registration dossier on zinc oxide)

In an OECD TG 423 (Acute Oral toxicity - Acute Toxic Class Method with modifications regarding dose levels) rats were orally gavaged with 5, 50, 300, 1,000 and 2,000 mg/kg bw. The average size of the nano ZnO was 63 nm determined in SEM analysis; the average size of nano ZnO (in solution) was 224.7 nm determined using dynamic light scattering (DLS). No mortality occurred and LD50 was concluded to be above 2000 mg/kg bw.

3.1.2 Dermal exposure

Acute dermal toxicity study on, Z-COTE HP1 (NM-111), (Bellmann (2010), OECD/WPMN (2015, ZnO part 3) and REACH registration dossier on zinc oxide)

This OECD TG 402 study from 2010 was considered as a key study with a reliability score of 1 in the OECD/WPMN (2015, ZnO part 3) dossier.

In the OECD/WPMN (2015, ZnO part 3) dossier the following executive summary was given for a coated form of nano zinc oxide (coating: triethoxycaprylylsilane 2%);

"2000 mg/kg bw of the test item was semiocclusively administered for 24 hours as pasty formulation in corn oil to the shaved and defatted back of 5 females and 5 male rats in a GLP/guideline conform study. After the end of the 24 -hour exposure period the test item paste was recovered as effectively as possible using water and the animals were observed for 14 days. During the present study no mortality occurred and there were no indications of systemically toxicity, no effects regarding the body weight and neither clinical signs nor pathological findings observed. The LD50 is therefore estimated to be > 2000 mg/kg bw."

3.1.3 Inhalation

No acute inhalation toxicity studies were available from the OECD/WPMN (2015, ZnO part 3) dossier on ZnO, however, an acute inhalation toxicity study is included in the REACH registration of ZnO.

Acute inhalation toxicity, (EPA OPP 81-3), (Unnamed report 1997, REACH registration dossier on zinc oxide)

In an acute inhalation toxicity test conducted according to EPA OPP 81-3 five female and five male rats were exposed to 1.79 mg/L nano zinc oxide for 4 hours. The nano zinc oxide had a specific surface area of 30 m^2/g and an average particle size of 36 nm. The maximum attainable exposure concentration was 1.79 mg/L with aerosols having an average MMAD of 4.122 µm. No mortality occurred. Clinical signs included activity decrease, crust around eyes and nose, piloerection, ptosis and respiratory gurgle, no longer evident by Day 4. No observable abnormalities were found at gross necropsy. The LC50 was concluded to be above 1.79 mg/L.

Intratracheal instillation study, WoE. Nano. (Sayes 2007, OECD/WPMN 2015, ZnO part 3)

The study consists of an *in vitro* and an *in vivo* part where only the latter is discussed in the report. The study was assigned a reliability score of 4 because of not following an OECD-test guideline, as a non-GLP study and due to the likelihood of nanoparticle interference in the *in vitro* tests. The test material was Nano-ZnO (Sigma-Aldrich), purity > 99% with a reported primary particle size 50-70 nm. Measured in vehicle (physiological saline) the particle size was

90-283 nm. In addition, a fine size ZnO quality was used with a reported particle size <1000 nm and a calculated particle size of 111 nm.

Male SD rats (5 rats/group) were intratracheally instilled with a single-dose of 0, 1, or 5 mg nano ZnO/ kg bw. BAL cells (PMNs) and LDH were measured at one day, one week, 1- and 3 months post exposure. After 24 hours post-exposure a significant increase in the number of neutrophils (PMNs) and total cells were observed in BAL fluid from high-dose groups, indicating pulmonary inflammation. After one-week post exposure the recruitment of neutrophils was diminished, and no measurements were performed at one and three months, indicating the resolution of the pulmonary inflammatory response. Further, a significant increase in LDH release was observed at 24 hours and one-week post exposure.

Intratracheal instillation study, (Jacobsen et al., (2015), derived from web-based literature search)

The study was not performed in accordance to test guidelines and was performed under non-GLP conditions, and with limited or different investigated endpoints.

The publication consists of data from three studies investigating toxicity in mice after pulmonary exposure to nanoscale ZnO.

Study I: female C57Bl/6 mice (n=6/ ZnO exposure group, n=12/control group and n=8/ unexposed group) were intratracheally instilled with a single dose of 0, 2, 6, and 18 µg nanoscale ZnO or 162 µg Carbon Black. Nanoscale ZnO with an average size diameter of 12 ± 3 nm, and with a purity of 99% (according to manufacturer) was used. The mice were euthanized, and endpoints assessed at day one and three after exposure. Toxicity was assessed as cellular and acellular biomarkers in BAL fluid, liver and lung histopathology.

Study II: female BALB/c mice (n=8-12/ ZnO exposure group) were intratracheally instilled with a single dose of 0, 5 and 15 µg nanoscale ZnO. The ZnO particles had an average size diameter of 24-71nm, (according to manufacture). The mice were euthanized, and endpoints assessed at day one after exposure. Toxicity was assessed as cellular and acellular biomarkers in BAL fluid, and markers of oxidative stress in the lungs.

Study III: female C57Bl/6 mice (n=5/ ZnO exposure group, n=8/ positive control group and n=8/ unexposed group) were exposed by means of pharyngeal aspiration with a single dose of 0, 12.5, 25, 50 and 100 µg nanoscale ZnO or 2.5mg crystalline silica (positive control), average size diameter of 12 ± 3 nm, with a purity of 99% (according to manufacture) were used. The mice were euthanized, and endpoints assessed two months after exposure. Toxicity was assessed as cellular and acellular biomarkers in BAL fluid and total lung collagen (as Oh-proline).

Following results were obtained:

Study I: The group instilled with 18 µg was euthanized prematurely two days after exposure due to severe clinical signs. On day 3 mice instilled with 2- and 6 µg nanoscale ZnO had significantly increased cell numbers of neutrophils and macrophages in BAL-fluid this was accompanied by increased levels of BAL-fluid proteins. With respect to histopathologic examinations signs of pulmonary and liver damage was observed in all exposure groups, however, no severity score was given.

Study II: The group instilled with 15 µg showed signs of systemic inflammation, increased cell numbers of neutrophils and platelets in the blood. Strong pulmonary inflammation assessed as neutrophils and LDH was also observed in the group instilled with 15 µg ZnO. In addition, markers of oxidative stress were observed in the group exposed to 15 µg.

Study III: All mice exposed to 100 µg and 3 of 5 mice exposed to 50 µg died within 5 days post exposure to nanoscale ZnO, and 2 of 5 mice in the 25 µg group died within 13 days post

exposure. None of the mice in the negative and positive control group died before schedule. Two months post exposure there were no signs of pulmonary inflammation (in BAL-fluid) among animals of the remaining exposure groups. However, there were positive indications of pulmonary fibrosis, although this was not statistically significant due to the low statistical power.

3.2 STOT RE

3.2.1 Oral exposure

No oral repeated dose studies are available neither form OECD/WPMN (2015, ZnO part 3) nor the REACH registration dossier on zinc oxide.

3.2.2 Dermal exposure

No studies included in OECD/WPMN (2015, ZnO part 3).

Repeated dose dermal toxicity study of nano zinc oxide with Sprague-Dawley rats. (Surekha et al. (2012), REACH registration dossier on zinc oxide)

In this OECD TG 410 (Repeated Dose Dermal Toxicity: 21/28-Day Study) study rats were dermally exposed to 75, 180, and 360 mg/kg bw /day of nano zinc oxide (particle size 20 nm) 6h/day, 5 days/week for 28 days. Further, a group of rats were exposed to micro-size zinc oxide at a limit dose of 2,000 mg/kg bw/day. No gross pathology or histopathological lesions were observed. Based on increase in clotting time and decrease in the collagen content in the skin in all the nano zinc oxide treated groups, a LOAEL of 75 mg/kg bw/day for systemic toxicity was established. The decreases in collagen content of the skin were inversely correlated to the dose levels. All effects were reversible in a period of 14 days.

90-day repeated-dose dermal toxicity study in rats (Ryu et al. (2014), derived from web-based literature search)

In this OECD TG 411 study (with modifications) nano zinc oxide (citrate coated, particle size of 29 nm and zeta potential of -44.4 mV) was applied using a vehicle of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-citrate buffer on soaked gauze to the shaved skin of rats at dose levels of 0, 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw 6 hour/day for 90 days. Clinical observations as well as weight and food consumption were measured and recorded daily. Hematology and biochemistry parameters were determined. Gross pathologic and histopathologic examinations were performed on selected tissues from all animals. Analyses of tissue were undertaken to determine target organ tissue distribution. No increased mortality in the experimental groups were observed. There was a dose-dependent irritation at the site of application resulting in skin crusts and areas with hyperkeratosis and papillomatosis at the highest dose level. No abnormal findings were recorded in other organs. Increased concentrations of ZnO in the liver, small intestine, large intestine, and feces were thought to result from oral ingestion of ZnO NPs via licking. A NOAEL for systemic adverse effects of 1000 mg/ kg bw/ day was concluded from the study.

3.2.3 Inhalation

14-days repeated dose toxicity study; Z-Cote HP1 (NM 111) and Z-Cote (NM 110). CEFIC (2013) in OECD/WPMN (2015, ZnO part 3)

This study report from 2013 was considered as a key study with a reliability score of 2 in the OECD/WPMN (2015, ZnO part 3) dossier.

In the OECD/WPMN (2015, ZnO part 3) dossier the following executive summary was given:

"A GLP-conform study according to OECD 412 (Repeated Dose Inhalation Toxicity: 28/14-Day) was conducted. 45 male Wistar rats per dose group were treated with 0.5, 2, and 8 mg/m³ Z-COTE HP1 and with 8 mg/m³ of the reference items Z-COTE and microscale ZnO for 14 days on 5 consecutive days per weeks for 6 hours per day. No indication of systemic toxicity was observed. Test substance-related histopathological effects were detected especially in the high dose group and were restricted to the respiratory tract indicating reactions in the lung. These effects were comparable for all of the three test items and fully reversible within 14 days. A bronchoalveolar lavage fluid (BALF) analysis was conducted, which revealed statistically significant increases of polymorphonuclear neutrophils and lactic dehydrogenase, ß-glucuronidase and total protein levels and also an increase of absolute numbers of macrophages in the high dose groups of Z-COTE HP1, Z-COTE and microscale ZnO 1 day after end of exposure. However, all these effects were reversible and had returned to control levels at the 14-day post exposure sacrifice date. With regard to oxidative stress, the secretion of ROI was enhanced in the 0.5 and 2 mg/m³ NM-111 treated animals as compared to clean air controls. An increased concentration of the stimulatory cytokines CINC-1, tumour necrosis factor-α, interleukin-6 and the more deregulating mediator transforming growth factor-β (TGF-β) was measured in the Z-COTE HP1 and microscale ZnO treated animals. Under the conditions of this test an NOAEL of 2 mg/m3 for Z-COTE HP1 was derived (decisive endpoints: BAL: Cellular and enzymatic response; histopathology: bronchiolo-alveolar hyperplasia and mononuclear cell infiltration)."

Further in the section regarding histopathology it was reported:

"One day post-exposure 2/5 males of the high-dose (8 mg/m³) Z-COTE HP1 and 1/5 male each of the Z-COTE and microscaled ZnO group showed (multi)focal very slight to slight degeneration of the olfactory epithelium (level 2 to 5 of the nasal cavity sections). These effects were fully reversible within 14 days"

A NOAEC of 2 mg/m³ was established for Z-COTE HP1 based on the reversible effect on cellular and acellular BALF parameters but also on histopathology. The degree of severity of the reported histopathological effects was very slight to slight.

90-day repeated dose toxicity study. Z-COTE HP1 (NM-111). CEFIC, Creutzenberg (2011) in OECD/WPMN (2015, ZnO part 3´) and REACH registration on zinc oxide

This OECD TG 413 study (Subchronic Inhalation Toxicity: 90-Day) from 2011 was considered as a key study with a reliability score of 2 in the OECD/WPMN (2015, ZnO part 3) dossier.

In the OECD/WPMN (2015, ZnO part 3) dossier the following executive summary was given:

"A 90-day inhalation study was conducted in rats using nose-only exposure according to OECD TG 413 under GLP conditions. Additional endpoints (bronchoalveolar lavage, cell proliferation, electron microscopy analysis, toxicokinetics) were included to investigate potentially nano-specific aspects of toxicity. The animals (n=65/group) were treated with 0.3, 1.5 and 4.5 mg/m³ Z-COTE HP1 (coated nanoscale ZnO), respectively, as well as 4.5 mg/m³ non-coated microscale ZnO for 3 months, 5 consecutive days per week (6 h/d). Fresh air treated animals served as concurrent control. In conclusion, coated nanoscale (Z-COTE HP1) and non-coated microscale ZnO caused comparable and reversible histopathological findings restricted to the respiratory tract. The retained material was completely solved and eliminated rapidly since no increased Zn contents were detected in any body-compartment after the post-exposure period. The status of the respiratory burst of alveolar macrophages was temporarily increased by treatment with nano- and microscale ZnO and was fully reversible for Z-COTE HP1. Markers for cellular damage and inflammation were reversibly increased to a higher extent by microscale ZnO than by Z-COTE HP1. Based on the results of the present study the NOAEC for Z-COTE HP1 was assessed to 1.5 mg/m³."

As stated in the executive summary the study was performed according to the OECD TG 413 and a NOAEC of 1.5 mg/m^3 was concluded. The most notable effects, although described as very slight to slight effects, found in the present study were found in the BALF and the nasal and paranasal cavities. At day one after exposure increased levels of endogenous reactive oxygen species intermediates (ROI), infiltration of PMNs were found in the high dose nano- and microscale ZnO groups, elevated levels of hyaline droplets in nasal and paranasal cavities were also observed. All effects were observed in the respiratory tract at the highest dose level one day post last exposure they were reversible 29 days after exposure. One exception was the hyaline droplets in the nasal cavities, they were equally increased in both the high-dose group (7/10 rats) and control group (7/10 rats) 29 days after exposure. Further, it is commonly recognized that the number of hyaline droplets increases with age in control mice and rats.

5-day short-term repeated dose toxicity study. Z-Cote HP1 (NM 111) DRF study. CEFIC (2009) in OECD/WPMN (2015, ZnO part 3)

This study report from 2009 was considered as a key study with a reliability score of 2 in the OECD/WPMN (2015, ZnO part 3).

In the OECD/WPMN (2015, ZnO part 3) dossier the following executive summary was given:

"A non-GLP conform dose range finding study was conducted, where 5 male Wistar rats per dose group were exposed for 5 consecutive days and 6 hours per day with 0, 0.5, 2 and 8 mg/m^3 of Z-COTE HP1 by nose-only-inhalation. The study was conducted according to OECD 412 in due consideration of animal treatment for only 5 consecutive days and a reduced spectrum of investigated endpoints. The lung weight/body weight ratio was increased in the low and high dose group. Histopathological findings concerning nasal and paranasal activities, lungs and lung-associated lymph nodes were also observed. No NOAEC was identified in this dose range finding study."

5-days short-term repeated dose study. Z-Cote HP1 (NM 111). Ma-Hock et al. (2008) in OECD/WPMN (2015, ZnO part 3)

This study report from 2008-2010 was considered as a key study with a reliability score of 2 in the OECD/WPMN (2015, ZnO part 3) dossier.

In the OECD/WPMN (2015, ZnO part 3) the following executive summary was given concluded:

"Z-COTE HP1 (target concentration: 0.5, 2.5, and 12.5 mg/m^3) and ZnO powder (12.5 mg/m^3) were tested in a 5-days nose-only lung toxicity study like OECD 412 under non-GLP conditions. 17 male rats per group were treated 6 h per day for 5 consecutive days followed by a 3 weeks observation time. Z-COTE HP1 caused local inflammations in the lungs of the rats, indicated by changes in several parameters in the bronchoalveolar lavage fluid (BALF) and histological examinations. Secondary to the effect in the lung, activation of the draining lymph nodes and minimal to moderate necrosis of the olfactory epithelium was noted. These effects were in a concentration-related manner and reversible within the recovery period. Only a multifocal increase in alveolar macrophages was still present at the end of the recovery period. Similar effects were also observed in the animals exposed to ZnO powder. At the low concentration of 0.5 mg/m^3 Z-COTE HP1, increased levels of a few mediators in the BALF and serum were determined. Moreover, minimal multifocal necrosis of the olfactory epithelium was noted in the nasal cavity in one animal. Therefore, the lowest target concentration of 0.5 mg/m^3 was considered to be the Low Observed Adverse Effect Concentration (LOAEC)"

Overall, OECD/WPMN (2015, ZnO part 3) summarised thsese findings:

"Z-COTE HP1 and microscaled ZnO were tested in repeated dose inhalation studies with different durations: 90 days guideline/GLP conform study (CEFIC, 2011c), 5 days guideline conform study (Ma-Hock et al. 2008, BASF SE 2010) and 14 days guideline/GLP conform study (CEFIC, 2013). Both test items caused comparable and reversible histopathological findings

restricted to the respiratory tract, clearly related to the inhalation of particles rather than to the chemical entity. The effects were fully reversible within the recovery period. In addition, Z-COTE was also investigated in the 14-day inhalation study (CEFIC, 2013). The effects were comparable to Z-COTE HP1 and microscaled ZnO. The NOAEC for Z-COTE HP1 was assessed to be 1.5 mg/m^3 and 2.0 mg/m^3 based on the results of the 90-d study and 14-d study, respectively. The LOAEC of Z-COTE HP1 is 0.5 mg/m^3 determined from the 5-d study based on minimal multifocal necorsis of the olfactory epithelium in the nasal cavity in one animal and increased levels of a few mediators in the brochoalveolar lavage fluid (BALF) and serum. The 5-day dose range finding study (CEFIC, 2009) supports the results from the 90-d, 5-d and 14-d studies and revealed histopathological findings restricted to the respiratory tract."

Further data on other qualities of *non-coated nano zinc oxide* has been found from our literature search:

Toxicity assessment of zinc oxide nanoparticles using sub-acute and sub-chronic murine inhalation models, Adamcakova-Dodd et al. (2014) derived from web-based literature search

The study was performed under duration similar to OECD TG 412 and 413 under non-GLP conditions, and with limited or different investigated endpoints.

C57/Bl6 mice (n=6-8/group) were exposed to nanoscale ZnO 3.5 mg/m^3, 4 hours/day for 2 or 13 weeks and necropsied either immediately or 3 weeks after exposure. Toxicity was assessed as cellular and acellular biomarkers in BAL fluid, lung histopathology, pulmonary mechanics and generation of ROS in the lungs. ZnO particle size was 15 - 26 nm, and the aerosol size distribution was GM=46 nm (GSD=1.8) in the 2-weeks study and GM=36nm (1.8nm) in the13 week study. Both 2-weeks and 13-weeks exposure induced a significant increase in recruitment of total white blood cells (leukocytes) to the lung, the main increase represented macrophages, IL-12 and MIP-1-alpha. There was no difference in other markers of pulmonary inflammation, and no histopathological changes were observed between ZnO exposed animals (2- and 13-weeks) and control animals.

Evaluation of Pulmonary Toxicity of Zinc Oxide Nanoparticles Following Inhalation (repeated dose) and Intratracheal Instillation (Single dose). Morimoto et al. (2016) derived from web-based literature search

The study was not performed in accordance to test guidelines and was performed under non-GLP conditions, and with limited or different investigated endpoints. The ZnO nanoparticles with a purity of 99,94 wt. % had a size diameter of 35 nm and a specific surface area of 31 m^2/g.

Male fisher 344 rats (n=5/group) were exposed to ZnO nanoparticles by inhalation for 4 weeks (0, 2 or 10 mg/m^3 6 hours/day for 5 days/week or by intratracheal instillation with a single dose of 0 mg/kg, 0.2 mg/kg (0.8 mg/rat) or 1 mg/kg (4 mg/rat). The rats were euthanized, and endpoints assessed at day three, one month, three months and six months after last exposure. Toxicity was assessed as cellular and acellular biomarkers in BAL fluid, lung histopathology, and markers of oxidative stress in the lungs. Inhalation and intratracheal instillation of the highest dose of ZnO nanoparticles caused a transient increase in neutrophil influx in the lung and a transient increase in the concentration of HO-1 in BALF at day three after exposure. At one and three months after exposure, these parameters had returned to baseline.

Repeated dose systemic toxicity by inhalation of nano-sized ZnO in human volunteers, Monsé et al. (2018) derived from web-based literature search

A recent publication in Particle and Fibre Toxicology, Monsé et al. (2018), provide evidence that inhalation of ZnO nanoparticles in healthy human volunteers induces dose-dependent acute phase response in humans at mass-based doses well below- the occupational exposure limits of many European countries.

Sixteen young healthy non-smoking males (n=8) and females (n=8) were exposed to filtered air and ZnO particles freshly generated by pyrolysis at exposure levels of 0.5, 1.0 and 2.0 mg/m^3 for 4 h on 4 different days, including 2 h of cycling with a low workload. The effects were assessed at three-time points: before, immediately after, and about 24 h after each exposure. Measured effect parameters were FLU-like symptoms, body temperature, inflammatory markers and clotting factors in the blood, and lung function. Results included concentration-dependent increases in FLU-like symptoms, body temperature, and acute phase proteins and neutrophils in blood were detected after ZnO inhalation. Significant effects were detected with ZnO concentrations of 1.0 mg/m^3 or higher, with the most sensitive parameters being inflammatory markers in blood. It was concluded that low dose exposure to nanosized ZnO in young healthy causes systemic inflammation at doses below the OEL in many European countries.

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