Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T

EC Number: -
CAS Number: -

CLH-O-0000007108-75-01/F

Adopted

18 March 2022

February 2023: latest version, replacing all previous versions

18 March 2022

CLH-O-0000007108-75-01/F

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

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

Chemical name: **Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T**

EC Number: **-**

CAS Number: **-**

The proposal was submitted by **Germany** and received by RAC on **5 March 2021.**

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

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **5 July 021**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **3 September 2021**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Tiina Santonen**

Co-Rapporteur, appointed by RAC: **Betty Hakkert**

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

The RAC opinion on the proposed harmonised classification and labelling was adopted on **18 March 2022** by **consensus**.

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

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio > 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T	-	-	Carc. 1B STOT RE 1	H350i H372 (lung)	GHS08 Dgr	H350i H372 (lung)			
RAC opinion	TBD	Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio > 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T	-	-	Carc. 1B STOT RE 1	H350i H372 (lung) (inhalation)	GHS08 Dgr	H350i H372 (lung) (inhalation)		STOT RE 1; H372 C $\geq$ 1 %; STOT RE 2; H373: 0,1 % $\leq$ C < 1 %	
Resulting Annex VI entry if agreed by COM	TBD	Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio > 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T	-	-	Carc. 1B STOT RE 1	H350i H372 (lung) (inhalation)	GHS08 Dgr	H350i H372 (lung) (inhalation)		STOT RE 1; H372: C $\geq$ 1 %; STOT RE 2; H373: 0,1 % $\leq$ C < 1 %	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

General considerations related to definition

Fibre paradigm

The Dossier submitter's (DS) proposal covers Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T. Thus, the scope is limited to synthetic graphite in tubular shape, multi-walled within the defined dimensional range and excludes other similar carbonaceous materials, such as carbon (nano)fibres, graphene, single walled carbon nanotubes (SWCNT) etc.

The DS proposal is largely based on the so called "fibre paradigm" that has evolved during several decades of studies on the pathogenicity of asbestos and other fibres (Stanton *et al* 1981, Donaldson *et al* 2010, 2011, 2013). The features of this paradigm and their relevance to the proposal are described briefly below.

The fibre paradigm can be seen as a time-tested structure: toxicity model that identifies the features related to fibre pathogenicity. The features identified in the fibre paradigm include:

- Sufficient length of the fibre.
- Thinness, allowing for a small enough aerodynamic diameter enabling deposition beyond ciliated airways.
- Biopersistence of the fibre (including retaining the fibre shape) allowing for prolonged effects of the deposited fibres and the accumulation of the fibres.

The WHO (1985) criteria for the fibre dimensions, for which the paradigm is applicable to, were originally applied in the 1960s as counting rules of asbestos fibres for occupational hygiene measurements. These criteria are also used by the DS for MWC(N)Ts. The dimensions are: length > 5 µm, diameter < 3µm, aspect ratio 3:1 as noted in the proposal.

The proposal deviates from the fibre paradigm by adding a requirement for rigidity. Fibre diameter is considered as a proxy for this feature; the thicker the fibre, the more likely it is to be rigid. The lower limit of 30 nm for the fibre diameter is based on studies on carbon nanotubes that seem to indicate that thinner fibres, presumably because of their flexibility, do not cause mesotheliomas after intraperitoneal (IP) injection, suggesting that pathogenetic mechanisms related to the fibre shape do not play a role in the pathogenicity of these fibres. The fibre paradigm in original form does not include this feature, probably because asbestos and other fibres where it has been applied are inherently rigid.

The fibre paradigm is not affected by the chemical composition of the fibre unless this affects biopersistence and thus the chemical composition is in general not as important. In addition, MWC(N)Ts all have basically the same chemical composition, being synthetic graphite in tubular form. The dimensions of MWC(N)Ts vary between each producer and they can also vary between production batches, meaning that the fibres produced are not uniform but represent a range of lengths and diameters. Therefore, RAC agrees with the DS and considers it unnecessary to evaluate fibre types in other respects than their dimensions. However, a decrease in biopersistence affects the pathogenicity of fibres. Decreasing biopersistence in fibres has been the main approach for achieving "safety by design" in fibre materials, notably man-made vitreous fibres (MMVF). It is not clear if this approach can be applied to MWC(N)Ts. The DS had considered

introduction of exemption criteria similar to Note Q for the classification of mineral wool. This note requires either *in vivo* biopersistence testing or intraperitoneal injection, or long-term inhalation testing. Since any pristine MWCNT is known to be quite bioresistant (see section "Toxicokinetics", below), this is an ambiguous criterion when proving fibre pathogenicity. Instead, the IP injection test is deemed highly informative but would likely need long-term follow-up. Therefore, the DS decided not to propose including Note Q but notes that in the event that industry provides scientific evidence, a negative IP test may be reconsidered as a valid exemption criterion. RAC acknowledges that IP and biopersistence testing are generally considered as possible tests to evaluate fibres in relation to their ability to cause pathogenicity by a fibre related mechanism. Negative results can be considered to show that the fibre paradigm (and therefore classification based on this mechanism) may not be applicable. However, it does not rule out effects, including carcinogenicity caused by other mechanisms.

The fibre paradigm is applicable to all pathologies resulting from the exposure to the appropriate fibres. For asbestos the main pathologies are lung cancer, mesothelioma, and lung fibrosis, all of these have been caused by MWC(N)Ts in animal experiments. Lung cancer and lung fibrosis can be caused by other mechanisms than fibre related ones while mesotheliomas are quite specific for asbestos exposure in humans and can be caused by other fibres in animal models. As mentioned above, an IP test is commonly used to evaluate fibres in relation to the to their ability to cause pathogenicity, specifically by a fibre related mechanism. The DS pays special attention to studies with mesotheliomas, most of these studies consists of intraperitoneal injection of MWC(N)Ts; studies where pulmonary exposure of MWC(N)Ts cause mesothelioma in animal experiments are rare.

<u>Definition of the "substance" covered in the proposal</u>

The definition of the substance and the dimensions were one of the main points commented on during the consultation of the CLH report: according to industry, the CLH proposal should be targeted only to MWCNT-7 type tubes since the majority of data comes from this type and they consider that extending to other types of MWC(N)Ts is not appropriate mainly due to lack of data. Two commenting Member States, on the other hand considered the possible extension of the scope to MWCNTs with a diameter of <30 nm. When considering the dimension issue, it is important to note that:

1) It is not possible to give a scientifically based lower limit for the "rigid" MWCNTs or MWCNTs causing asbestos like effects (incl. cancer) and there are data suggesting that also MWCNTs with a smaller diameter may have carcinogenic properties. The cut-off limit of 30 nm chosen by the DS is based on the available data on mesothelioma induction: the lowest diameter of MWCNTs that has been observed to cause mesothelioma is currently 37 nm (Rittinghausen *et al*., 2014). This issue will be discussed further later in the RAC opinion.
2) The range does not fulfil the EU recommendation for a definition of nanomaterial and which states that one external dimension (in this case the diameter) should be in the size range of 1-100 nm. The DS has extended the applicability domain of the CLH proposal to MWC(N)Ts with a diameter up to 3 µm, as it is expected, according to the fibre pathogenicity paradigm, that MWC(N)T with diameters fitting within the respirable range will possess similar fibre-like properties. In addition, there is evidence that MWC(N)T ~ 150 nm may induce mesothelioma. It is, however, not known if MWC(N)T beyond a diameter range of > 200 nm (in the constituent particles) are manufactured.
3) The diameter was used in the CLH proposal as a pragmatic surrogate for fibre rigidity since precise and reproducible method to express rigidity is not available and rigidity has

been suggested to be one critical point in lung and pleural pathogenicity of these types of fibres.

Another specific issue raised in comments received during consultation of the CLH report was related to the classification threshold for the concentration of fibres fulfilling critical dimensions in a mixture. However, these concentrations are defined in Annex VI of the CLP regulation (EG) 1272/2008, which gives general concentration limits for different hazard classes. Thus, if the substance is present on its own, as a constituent/impurity or in a mixture at above the generic concentration limit defined in the CLP regulation for the specified hazard the substance or the mixture needs to be classified. In this case, it means that if these MWC(N)Ts are classified for carcinogenicity in category 1B, substances with 0.1% or more (w/w) of these MWC(N)Ts need to be classified for carcinogenicity in category 1B.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of the toxicokinetic data

Summary of the Dossier Submitter's proposal

The DS provided summaries of a number of toxicokinetic studies with MWCNT, a short overall summary and a statement on the overall relevance of this information for the proposed classification. According to the DS, the available studies show that pristine fibre-like MWCNT (mainly MWCNT-7) are retained in the deep lung and slowly relocate into e.g. the alveolar interstitium, lung-associated lymph nodes, parietal pleura and distant organs such as liver and kidney. Systemic exposure occurs mainly via the lymphatic system. MWCNT disposition and retention was concentration and time dependent and was related to the toxic response. Pleural migration and penetration have been shown for rigid MWCNT in several studies whereas thin tangled low-diameter MWCNT forming coiled agglomerates were not reported as migrating to the pleura. However, effects on visceral pleura were observed after inhalation of tangled Baytubes.

The DS stated that measuring the rigidity of MWCNT is a matter needing further research but proposed to use the outer diameter as a proxy because in general, the greater the number of walls which make up the MWNCT tube, the larger its diameter is and the less flexible it becomes. The diameter also depends on the size of the particle used as catalyst for the synthesis. The relationship between diameter and relocation to the pleura was confirmed, according to the DS, by the available toxicokinetic studies.

Furthermore, the tube length was considered critical as studies with other types of fibre have shown that fibres longer than 5 µm can reach and remain in the pleura but fibres of 4 µm can reach the pleura but are removed via the stomata in the parietal pleura. This aligns their toxicity with the WHO fibre definition.

Overall, the DS concluded that there is sufficient evidence proving that rigid poorly soluble MWCNT with fibre like morphology are retained in the lung and may reach the pleura and more distant organs after inhalation in a dose-dependent manner. This triggers inflammatory injury in the tissue and neoplastic processes in both the lung and the pleura comparable to other carcinogenic fibres, such as asbestos.

For other carbon nanotubes, the DS provided a summary of the available data on the non-functionalised form with emphasis on the biopersistence, intracellular uptake and distribution.

Comments received during consultation

No specific comments regarding the toxicokinetic properties of MWCNT were provided during the public consultation. However, a discussion emerged regarding the influence of the form (straight versus tangled and rigid versus flexible) on the proposed mode of action which in part depends upon the toxicokinetic behaviour.

One additional study containing toxicokinetic information was provided in the consultation, namely Saleh *et al*. (2020) which was taken into account.

Assessment

The proposed classification for carcinogenicity is based on the fibre paradigm as described above. It is assumed that a sufficient dose of biopersistent fibres with a certain diameter and length can induce lung cancer and the formation of mesothelioma. However, fibres may induce carcinogenicity in the lung via other mechanisms as shown for short tangled MWCNT (Saleh *et al*., 2020). As toxicokinetic data can be used to assess whether specific forms of MWCNT fulfil some of these properties, the focus of the RAC assessment was primarily on whether:

- the fibres can reach the alveoli
- the fibres are biopersistent
- the fibres can translocate to the pleura including whether the fibres were observed visceral, parietal or in the pleural cavity.

Therefore, the table below was made describing the tested MWCNT and whether one or more of the toxicokinetic properties, with focus on distribution, was shown. However, it should be noted that not all potential properties were determined in all studies.

***Table**: The types of fibres that were tested in the available toxicokinetic studies*

Name fibre	**Length**	**Diameter**	**MMAD**	**Form: straight or tangled**	**Distribution**	**Study**
MWCNT-7	Mean: 5.7 µm (48.7% > 5 µm)	Mean: 90.7 nm	1.4-1.6 µm	straight	alveolar visceral subpleura parietal pleura nasal cavity	Kasai *et al*., 2015
MWCNT-L	Mean: 7.34 µm	150 nm		needle shaped	alveolar visceral pleura pleural cavity parietal pleura extrapulmonary	Xu *et al*., 2014
MWCNT-S	3 µm	15 nm		cotton candy like aggregates	alveolar	Xu *et al*., 2014
MWCNT-M (=MWCNT-7)	median: 4.47 µm mean: 5.11 µm				alveolar pleural cavity lymph nodes	Xu *et al*., 2012
MWCNT-N	median: 3.02 µm				alveolar	Xu *et al*., 2012

Name fibre	Length	Diameter	MMAD	Form: straight or tangled	Distribution	Study
	mean: 3.64 µm				pleural cavity lymph nodes	
MWCNT-7		35.5 or 53 nm			alveolar	Aiso *et al*., 2011
^{14}C-MWCNT	mean: 3.9 µm (0.5 – 12 µm)	mean: 40 nm (10-150 nm)			Biopersistent Alveolar spleen and liver	Czarny *et al*., 2014
MWCNT-7	mean: 4.3 µm		1.5 µm		Biopersistent alveolar visceral pleura pleural cavity extrapulmonary (liver, lymph nodes)	Mercer *et al*., 2013a Mercer *et al*., 2013
MWCNT-7			1.5 µm		alveolar pleural	Porter *et al*., 2013
short straight MWCNT	0.5 – 2 µm	20-30 nm		straight	Cleared via lymphatics after interpleural administration	Murphy *et al*., 2011
long straight 2	max: 56 µm	165±5 nm		straight	Retained in parietal pleura after interpleural administration	
MWCNT-7	median: 3.86 µm	49±13 nm			alveolar pleural	Porter *et al*., 2010
MWCNT-7	median: 3.86 µm	49±13 nm			Biopersistent alveolar visceral pleura	Mercer *et al*., 2010
MWCNT	0.3-50 µm	30-50 nm (average)			Biopersistance alveoli pleural	Ryman-Rasmussen *et al*., 2009
MWCNT-A MWCNT-B	6.39±3.07 µm 1.04±0.71 µm	150±43 nm (213 walls) 7.4±2.7 nm (6-7 walls)		straight tangled	Biopersistance alveoli Biopersistance alveoli	Saleh *et al*., 2020

MWCNT-7 fibres were shown to reach the alveoli of rats after repeated inhalation exposure and were mainly present within the macrophages. In the highest dose group, occasionally MWCNT

could be observed in the visceral subpleural areas and in the parietal pleura at the diaphragm (Kasai *et al*., 2015).

The longer, needle-like MWCNT type (MWCNT-L) but not the shorter, "cotton candy"-type (MWCNT-S) was found in the pleural cavity and deposited primarily at the parietal pleura after repeated transtracheal exposure of rats during 24 weeks. MWCNT-S were found phagocytosed in alveolar macrophages close to the visceral pleura (Xu *et al*., 2014).

Both needle like MWCNTs and crocidolite asbestos translocated into the pleural cavity when administered into the rat lung after repeated transtracheal intrapulmonary spraying during 9 days. However, no fibres were observed in the parietal pleura. Only few MWCNT were observed penetrating through the visceral pleura (Xu *et al*., 2012).

MWCNT migrated to the right and left posterior mediastinal lymph nodes and – to a lesser extent – to the parathymic lymph node in rats after a single intratracheal installation. The deposition of MWCNT in these lymph nodes increased gradually and dose-dependently during the postexposure period (Aiso, 2011).

Twelve months after a single exposure of mice via pharyngeal aspiration to radioactive MWCNT, 10% of the estimated dose was retained in the lung. Also in other organs such as the spleen and the liver radioactivity and fibres were observed. The results indicate high biopersistance (Czerny *et al*., 2014).

Repeated whole body inhalation exposure of mice with a post-exposure period of almost one year showed high retention of MWCNT fibres in organs outside the lung with the highest concentration in the tracheobronchial lymph nodes (Mercer *et al*., 2013a). Fibres were also observed in the pleural lavage. In the same study, the total lung burden decreased by 36% over almost one year. During this period there was a shift of distribution of the MWCNT fibres from the alveolar macrophages towards the alveolar tissue (Mercer *et al*., 2013). MWCNT were also observed in the sub-pleural tissue region.

Repeated whole body inhalation exposure of mice with a post-exposure period of 4 days showed that these fibres could reach the pleural wall which they occasionally penetrated (Porter *et al*., 2013).

After a single intrapleural injection of MWCNT in mice, there was indirect evidence that passage through parietal stomata to mediastinal lymph nodes is dependent on MWCNT length. Thus short, tangled fibres were able to be cleared via the lymphatics, whereas long fibres are retained at the parietal pleura. The length-dependent pleural passage block was confirmed by nickel wire administration of defined lengths (Murphy *et al*., 2011).

Single pharyngeal aspiration of MWCNT in mice with post-exposure durations of up to 56 days showed that some fibres could reach the pleura (Porter *et al*., 2010).

Single pharyngeal aspiration of MWCNT in mice with post-exposure durations of up to 56 days showed that MWCNT can reach the pleura, as fibres were observed in the intrapleural space (visceral pleural surface) and the subpleural lymphatics at all observation times up to day 56 (Mercer *et al*., 2010).

Single inhalation exposure of mice (6 hours, 30 mg/m^3) resulted in the observation of MWCNT engulfed by macrophages and in mesenchymal cells in the subpleural region and MWCNT containing macrophages in mononuclear cell aggregates on the pleural surface on day 1 after exposure. These declined afterwards but some remained up to 14 weeks after exposure (last measurement). No such effects were observed after exposure to the low dose of 1 mg/m^3 (Ryman-Rasmussen *et al*., 2009).

Exposure of rats to straight and tangled MWCNT via intra-tracheal intra-pulmonary spraying once a week for 7 weeks at two different dose levels with a 2 year post-exposure follow up resulted in

clear biopersistence of both type of materials with a higher biopersistence for the tangled material in lung and mediastinal lymph node (Saleh *et al*., 2020).

Overall, there is evidence from the available toxicokinetic information that MWCNT which reach the alveoli, are taken up by macrophages and mesenchymal cells, are highly biopersistent and can translocate to the sub-pleural areas and into the pleural space and are retained there. However, it is unclear whether these toxicokinetic properties are applicable to all forms of MWCNT. In some of the studies, not all physical properties of the tested MWCNT were known. No information on the flexibility of the tested MWCNTs was available.

All tested MWCNT reached the alveoli. The diameter of rigid straight fibres is considered determinative for the ability of fibres to reach the alveoli as these fibres align with the airstream. Therefore, the diameter of the rigid straight MWCNT is also the diameter of the fibre particle. However, for tangled MWCNT, the diameter of the tube is much less than the diameter of the particles. Therefore, to measure the respirability of tangled rigid fibres, the Mass Median Aerodynamic Diameter (MMAD) is likely to be a better parameter than the diameter, which may overestimate the respirability. However, this is likely to become a relevant issue in classification only with thicker fibres (just below 3 µm in diameter) and is considered hypothethical, as fibres with a diameter just below 3 µm are not put on the market. For these pragmatic reasons, the proposed upper diameter of 3 µm is retained. The lower diameter of 30 nm cannot be based on the ability to reach the alveoli as there is evidence that MWCNT with smaller diameter can reach the alveoli (tangled 7.4 nm).

Information on the ability to reach the pleural cavity was available for straight MWCNT with a diameter of 30 - 150 nm (Ryman-Rasmussen *et al*., 2009 and Xu *et al*., 2014) but was shown not to apply to cotton-candy like MWCNT with a diameter of 15 nm (Xu *et al*., 2014).

Information on biopersistence is available for a range of diameters (7.4 – 150 nm) and for both straight and tangled MWCNTs (Saleh *et al*., 2020). No reduction in biopersistence is expected for fibres with a larger diameter.

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

Summary of the Dossier Submitter's proposal

The STOT RE 1 classification proposal is based on the available data for the cut off levels as chosen by the DS and a weight of evidence approach for rigid WHO fibre-like MWC(N)T.

A key study was a 90d-inhalation study which gives a LOAEC of 0.0002 mg/L for MWCNT-7. Concentration-dependent increases in inflammatory broncho-alveolar lavage (BAL) parameters (polymorphonuclear (PMN) cells and lymphocyte numbers, lactate dehydrogenase (LDH), alkaline phosphatase and total protein) were seen in both sexes starting from this dose level (the lowest dose level tested). Chronic retention of inhaled fibre-like MWCNT was associated with granulomatous lesions in the lungs starting from the lowest dose in males. Multifocal fibrosis of the alveolar wall was observed at 0.001 mg/L and above in both sexes.

This study was supported by shorter duration inhalation studies (Umeda *et al*., 2013; Porter *et al*., 2013; Mercer *et al*., 2013; Rydman *et al*., 2014) and studies applying intratracheal administration or pharyngeal aspiration. These have been listed as tables 14 (inhalation studies) and 15 (other studies) in the CLH report. The majority of the studies were performed using MWCNT-7 (Mitsui) with a diameter typically between 30-90 nm and length > 5 µm. This was considered to represent a prototype for fibre-like MWCNT of high diameter. Studies by Poulsen

et al. (2015); Rydman *et al*. (2013), Murphy *et al*. (2013); Porter *et al*. (2010) compared different types of MWCNTs, including MWCNT-7 and tangled MWCNTs with a diameter ≤15 nm. The study by Murphy *et al*. (2013) was considered particularly relevant as it provides evidence of an asbestos-like pathology (including alveolar as well as pleural fibrosis) for a long and straight MWCNT, other than MWCNT-7 with similar fibre-like characteristics, as well as its absence in the case of tangled or short MWCNT types. This observation was considered to justify the same classification for MWCNT-7 and other MWCNT with similar dimensions based on a weight of evidence assessment.

Comments received during consultation

There were no comments related specifically to the STOT RE classification proposal. However, general comments regarding the fibre dimensions defined in the CLH proposal are relevant also for STOT RE classification. Industry criticised the extension of the scope to all MWC(N)Ts with geometric tube diameter range $\geq$ 30 nm to < 3 µm and a length $\geq$ 5 µm and aspect ratio $\geq$ 3:1 and proposed to follow the IARC approach and classify only MWCNT-7 type tubes, since the majority of the data comes from this type of MWCNTs. It was also pointed out that not only do fibre dimensions define the morphology but also the production method and high diameter fibres may also be tangled. Two commenting Member States pointed out that a diameter of 30 nm should not be seen as a limit for hazardous vs non-hazardous materials. One MSCA emphasised the lack of data on fibres with a diameter of 15-30 nm and suggested to extend the lower limit to 15 nm. Another MSCA referred to the recent study by Saleh *et al*. (2020), which showed lung inflammation and carcinogenicity after Intra-Tracheal Intra-Pulmonary Spraying (TIPS) of MWCNTs with a diameter of 7.4 nm and asked to consider also these effects in CLH proposal.

Assessment and comparison with the classification criteria

The key study is a 90 day inhalation study (6 h/day, 5 days per week) in rats using MWCNT-7 (Mitsui) type tubes with dose levels of 0.0002, 0.001 and 0.005 mg/L (Kasai *et al*., 2015). The effects observed included increases in inflammatory BAL parameters and granulomatous changes starting from the lowest dose of 0.0002 mg/L. Multifocal fibrosis was observed at a dose of 0.001 mg/L in both sexes.

The same group also performed a 14 d inhalation study (with an additional 4 week recovery period) using the same test material and the same dose levels (Umeda *et al*., 2013). Granulomatous changes and slight alveolar fibrosis occurred in the lung at the highest dose.

The chronic (carcinogenicity) study by the same research group (Kasai *et al*., 2016) showed a similar dose-dependent increase in non-cancer lung effects, including epithelial hyperplasia, granulomatous change, localized fibrosis, and alterations in BAL parameters starting from the lowest dose-level of 0.02 mg/m3 (i.e. 0.00002 mg/L) in rats (see the carcinogenicity section).

These three highly reliable studies were supported by three additional 4-12 day inhalation studies in mice.

Porter *et al* (2013) exposed mice to 0 or 0.010 mg/L of MWCNT-7 for 2, 4, 8, or 12 days and observed a dose-dependent increase in PMN leucocyte, LDH and albumin levels in whole lung lavage markers when compared to the controls. In lung histopathology bronchiolocentric inflammation, bronchiolar epithelial hyperplasia and hypertrophy, minimum to mild bronchiolocentric fibrosis, vascular changes and rare pleural penetration were seen. The effects were related to increasing cumulative dose when different exposure times were compared.

Mercer *et al* (2013) exposed mice to 0 or 0.005 mg/L of MWCNT-7 for 12 days with post- exposure observations at 1, 14, 84, 168, and 336 days. Inflammatory BAL parameters (PMN, LDH, and

albumin), were increased at day 1 post-exposure and declined slowly over the post-exposure period, being still significantly increased on day 168. Progressive alveolar fibrosis was also observed with an increase in connective tissue thickness in the alveolar region by 70 % 336 days after exposure.

Rydman *et al* (2014) exposed mice for 4 days (4h/d) to 0.0062-0.0082 mg/m^3 of rigid rod-like CNTs (rCNTs – MWCNT-7) and 0.0175-0.0185 mg/m^3 of flexible, tangled (tCNTs - MWCNTs 8-15 nm thick). rCNTs, but not tCNTs induced the recruitment of inflammatory cells, especially eosinophils accompanied by mucus hypersecretion, hyperresponsiveness and the expression of Th2-type cytokines, and up-regulation of genes involved in innate immunity and cytokine/chemokine pathways. Macrophages were found to undergo "frustrated phagocytosis" and form foreign-body giant cells. This was interpreted by the DS to suggest that MWCNTs may cause respiratory sensitization, which is, however, not the correct interpretation. This eosinophilic response, with eosinophilic crystals (similar to Charcot-Leyden crystals associated with chronic allergic asthma) and other features compatible with a Th2-type of inflammation, has so far been described for asbestos and for two high aspect ratio nanomaterials (HARNs), the MWCNT-7 and NM401 (Kobler *et al* 2015; Sabo-Attwood *et al* 2005; Rydman *et al* 2014; Rydman *et al* 2015). The response indicates a more persistent Th2 inflammation, which is a feature these types of CNTs share with the material originating the fibre paradigm, asbestos. It is, however, unclear if (and how) this is related to the long-term pathogenic processes caused by the fibres.

Although these studies mostly used the MWCNT-7 (Mitsui) type of nanotubes, there are additional studies using pharyngeal aspiration providing comparative data on other types of rigid nanotubes. These have been summarised in table 15 of the CLH report. The study by Murphy *et al*. (2013) provides comparative data on lung effects of various MWCNTs after bolus administration of a dose of 25 µg MWCNT/animal via pharyngeal aspiration with examination 1 and 6 weeks after exposure. MWCNTs included a tangled form with a diameter of 15 nm, one short straight form (length 0.5-2 µm, diameter 20-30 nm) and a MWCNT with a diameter of 165 nm and length 56 µm (76 % > 20 µm; 84 % > 15 µm) [*Note: in the CLH dossier it is stated that Murphy et al tested 5 different MWCNT but the original paper lists only three different types*]. Long straight MWCNT but not tangled or short MWCNT caused acute neutrophilic inflammation in BAL at 1 week and progressive thickening of the alveolar septa. An inflammatory response in the pleural lavage and lesions along the chest wall and diaphragm were seen after 6 weeks. The effects were comparable to those reported earlier after pharyngeal aspiration of 10, 20, 40 or 80 µg of MWCNT-7 in mice (Porter *et al*., 2010). Poulsen *et al* (2015) reported that also NM-401 (physicochemically similar to MWCNT-7 but more flexible) elicited an earlier onset of inflammation and induced more fibrosis and a unique fibrotic gene expression signature at day 28, compared to the tangled Nanocyl NC7000 (D: 11 ± 4.5 nm L: 0.85 ± 0.457 µm) in mice after intra-tracheal administration. However, both MWCNT elicited strong acute phase and inflammatory responses that persisted up to 28 days.

In addition, toxicokinetic data suggesting translocation of MWCNTs (both Mitsui type and other long rigid fibres, Kasai *et al*. 2015; Xu *et al*., 2012 and 2014) to the parietal pleura and induction of inflammation and mesothelial proliferation supports asbestos-like pleural pathogenicity of MWC(N)Ts. Parietal pleural penetration and related inflammatory and proliferative effects are generally considered to be a hallmark of fibre pathogenicity. No pleural penetration was observed in studies with MWCNTs with a diameter <15 nm but it needs to be noted that limited information is available on fibres between 15- 30 nm in diameter.

Comparison with the criteria

According to the CLH criteria, substances are classified in Category 1 for specific target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in

which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.

The guidance value for classification to STOT RE cat 1 by dust inhalation (in rats) is $\leq$ 0.02 mg/L/6h/day for a 90d study. If shorter duration studies (e.g. 4 wk study) are used for classification, Haber's rule can be applied (if considered applicable) and the cut off limit is elevated accordingly (e.g. the cat 1 limit is elevated to 0.06 mg/L in the case of a 4 week study). Comparison of the available inhalation data showing granuloma formation in 12-90 day studies with LOAECs between 0.0002- 0.01 mg/L, shows that STOT RE cat 1 criteria are clearly fulfilled for MWCNT-7 tested in these studies. Available evidence from the studies using intra-tracheal or pharyngeal aspiration shows inflammatory and fibrotic effects of similar magnitude also after exposure to other types of long, rigid carbon nanotubes. Toxicokinetic data showing translocation of both Mitsui type and other long rigid fibres to the parietal pleura also gives some support to the hypothesis that the fibre paradigm applies also to other fibres within the scope of the classification proposal and not only the Mitsui type MWCNTs.

Therefore, it is reasonable to assume that repeated inhalation exposure to these MWCNT will result in similar lung effects as shown for MWCNT-7 in the study by Kasai *et al*. (2015). Therefore, RAC supports the DS proposal to classify MWC(N)Ts specified in this proposal as STOT RE 1. These lung effects are likely to occur only after inhalation exposure, resulting in high retention of MWCNTs in the lungs. Therefore, it can be specified that these effects occur only when inhaled. Therefore RAC concludes that classification is warranted as **STOT RE 1;H372, Causes damage to lungs through prolonged or repeated exposure via inhalation**.

The DS did not propose a specific concentration limit for the STOT RE classification. According to CLP guidance, specific concentration limits (SCLs) for STOT RE may be set for substances inducing target organ toxicity at a dose level or concentration clearly (more than one magnitude) below the guidance values. This is the case with MWCNTs. Taking a LOEAC of 0.0002 mg/m^3 from the 90 day study by Kasai *et al*. (2015) as a starting point and applying the formula given in Section 3.9.2.6 of the CLP guidance (2017) for the determination of the SCL, an SCL of 1% for the classification to STOT RE cat 1 is derived. **RAC proposes an SCL of 1 % for STOT RE 1 and an SCL of 0.1% for the STOT RE 2 to apply to the classification of MWC(N)Ts.**

As discussed also elsewhere in this opinion, it should be noted that the lower diameter limit of 30 nm given by DS in the classification proposal is chosen based on the availability of carcinogenicity data (on mesothelioma induction). There are subchronic studies available for commercial tangled MWCNT types with lower diameter focusing on the lung effects caused by these nanotubes (Ma-Hock *et al*., 2009; Pauluhn *et al*., 2010; Pothmann *et al*., 2015). These studies have not been evaluated in the classification proposal of the DS and classification of these MWCNTs falling outside of this classification proposal needs to be considered separately. RAC notes, however, that effects have been observed outside the size range, as also noted by Member State comments in the consultation, although the mechanisms of pulmonary effects of thin, tangled fibres may differ from those of long rigid MWC(N)Ts (See also the related discussion under "carcinogenicity").

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS provided a non-exhaustive database of *in vitro* and *in vivo* genotoxicity and mutagenicity studies to support the proposed classification for carcinogenicity. However, assessment of the need for classification as a germ cell mutagen was not intended and was therefore outside the scope of the assessment by RAC.

The DS concluded that the genotoxicity data show that exposure to fibre-like MWCNT may favour clastogenic as well as aneugenic effects in the lung when inhaled. DNA damage *in vivo* may be directly induced and/or may be secondary due to oxidative stress and an inflammation response, whereas it is plausible to assume direct interference of nanotubes with the mitotic spindle formation, resulting in aneuploidy. Based on the available data, a genotoxic mode of action cannot be excluded, supporting the carcinogenic potential of MWCNT.

Comments received during consultation

As germ cell mutagenicity was outside the scope of the CLH proposal, no specific comments on genotoxicity and/or mutagenicity were provided. It was commented that genotoxicity has been observed in rats and mice for MWCNT with a length less than 5 µm (the proposed lower limit). Furthermore, some additional references to genotoxicity and mutagenicity studies were provided.

Assessment

As this hazard class was outside the scope for this proposal, the RAC assessment was focused on the mutagenicity data as support for the classification for carcinogenicity. Therefore, the focus of the RAC assessment was on *in vivo* studies and on the lung tissue. In addition, the effect of the dimensions of the MWCNT to induce mutagenicity *in vivo* was assessed to support the definition of the MWCNT to which such a classification would be applicable.

The available *in vitro* studies indicate that MWCNT induce numerical chromosomal changes and polyploidy in chromosome aberration tests. Micronucleus tests and comet assays gave inconsistent results, depending on the tested cell line and the dimensional properties of the MWCNT.

In vivo results in lung tissue after inhalation exposure were mainly positive in several types of tests including a transgenic rodent assay, a micronucleus assay and several comet assays. The results indicated the formation of reactive oxygen species which could either be related to inflammation but also to the presence of metal impurities in the MWCNT. Based on the positive outcome of some *in vivo* micronucleus tests in lung cells and the in numerical chromosomal changes *in vitro*, clastogenic and/or aneugenic results were observed. The aneugenic effect could be explained by interference of the fibres with the mitotic spindle apparatus. The available *in vivo* studies with multiple MWCNTs indicated some differences in mutagenicity between straight and tangled MWCNT and the related fibre dimensions with stronger effects in straight MWCNTs. In a direct comparison, Catalan *et al* (2016) showed that straight MWCNT (mean 7 µm * 71 nm) induced DNA strand breaks in lung cells, as detected in the comet assay after a single pharyngeal administration in mice (high dose only), whereas a decrease was observed for a tangled type of MWCNT (mean 0.37 µm * 21 nm) in lung cells and bronchoalveolar lavage cells. After inhalation exposure, straight MWCNTs induced an increase in DNA strand breaks in lung and bronchoalveolar lavage cells and an increase in micronucleated alveolar type II cells. No such increases in strand breaks was observed for the tangled type of MWCNT. The formation of micronuclei was not determined for tangled MWCNTs. Poulsen *et al*. (2015) compared the effects of small curled MWCNT (mean length 0.8 µm) with large thick MWCNT (mean length 4 µm) at several dose levels and several timepoints (days 1, 3 and 28) after a single intratracheal exposure. Large MWCNTs induced an increase in strand breaks in lung cells only at day 1 (all dose levels) whereas small MWCNTs induced an increase on day 3 (middle and top doses). As indicated by transcriptome analysis and histopathology, both MWCNT types induced an acute phase and a persistent inflammatory response. There were some indications that the mutagenic effect was also dependent upon the presence of metal impurities.

Overall, the available *in vivo* mutagenicity tests support lung carcinogenicity of rigid type MWCNTs via a mutagenic mechanism. It is to be noted that on basis of the information available, no strict borders for tube dimensions can be determined, as positive results were also observed for thin tangled MWCNT (Poulsen *et al*., 2015).

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS proposal on the classification as Carc. 1B is based on the evidence from animal studies on several different types of MWC(N)T with fibre dimensions ($\geq$ 5 µm in length and a diameter range $\geq$ 30 nm to < 3 µm) including:

- Significantly increased incidence in lung tumours in rats following long-term inhalation of MWCNT-7 (Kasai *et al*., 2016).
- Experimental mesothelioma formation in rats and (mutant) mice following intraperitoneal injection (Nagai *et al*.,2011; Huaux *et al*, 2016; Rittinghausen *et al*., 2014; Tagaki *et al*., 2008 and 2011).
- Preneoplastic changes (fibrosis) of lung and pleura tissue and tumour types strikingly similar to those after asbestos exposure.
- Pleural drift and retention following inhalation exposure.
- Promotion of lung tumour development after short term inhalation exposure of initiated mice (Sargent *et al*., 2014).
- Mesothelioma formation after transtracheal intrapulmonary spraying (Suzui *et al*., 2016)
- Pathogenicity progress after inhalation and intratracheal instillation similar to asbestos.

The critical studies used by the DS are listed in the table below (modified from table 13a in the CLH report, but with the data on thin, tangled MWCNTs excluded).

Test material	Length	Diameter	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWCNT-7 **(Mitsui)**	5.2 or 5.7 µm (45.1 or 48.7 % of tubes > 5µm	83.8 or 90.7 nm	rat	Inhalation (whole body) 104 weeks	Yes Lung carcinoma and adenoma (mainly bronchiolo-alveolar) No pleural mesothelioma	Kasai *et al*. (2016)
MWCNT-7 **(Mitsui)**	5.11 µm (mean)		rat	Transtracheal intrapulmonary spraying 9 days	Yes Hyperplastic visceral mesothelial proliferation	Xu *et al*. (2012)
MWCNT-N **(Nikkiso)**	3.64 µm		rat	Transtracheal intrapulmonary spraying 9 days	Yes Hyperplastic visceral mesothelial proliferation	Xu *et al*. (2012)
MWCNT-L **(synthesized in lab)**	8 µm	150 nm	rat	Transtracheal intrapulmonary spraying 24 weeks	Yes Patchy parietal mesothelial proliferation	Xu *et al*. (2014)
MWCNT-N **(Nikkiso)**	4.2 µm 2.6 µm	30-80 nm	rat	Transtracheal intrapulmonary spraying	Yes	Suzui *et al*., 2016

Test material	Length	Diameter	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
				2 weeks + 109 weeks p.e.	Bronchiolo-alveolar lung adenomas and carcinomas Pleural malignant mesotheliomas	
NT_{long2} **(Univ. Manchester)**	> 15 µm (85 % of fibres)	20-100 nm (165 nm, measured)	mouse	Intrapleural injection	Yes Mesothelial proliferation in parietal pleura of chest wall and diaphragm 24 weeks p.e.	Murphy *et al*. (2011)
MWCNT-7 **(Hodogaya)**	Several µm	Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm.	mouse	inhalation	Yes (Promoting activity) Pulmonary adenomas and adenocarcinomas as well as systemic sarcomatous mesotheliomas 17 months p.e.	Sargent *et al*. (2014) [Porter *et al*., 2013]
NT50a **NT-50a(-agg)** **= MWCNT-7, Mitsui)**	5.29 µm	49.95 nm	rat	Intraperitoneal injection	Yes Malignant mesothelioma after 1 year	Nagai *et al*. (2011)
NT145 **(Showa Denko)**	4.34 µm	143.5 nm	rat	Intraperitoneal injection	(Yes) However mesothelioma incidence after 1 year of 1mg dose much lower and latency of tumour-induced mortality higher compared to 1 mg NT50a and equivalent fibre dose of NT50a(-agg*). Furthermore, in contrast to NT50a, fibrosis index of NT 145 was not significantly increased against controls, and did not pierce mesothelial cells.	Nagai *et al*. (2011)
NT50b **(Showa Denko)**	4.6 µm	52.4 nm	rat	Intraperitoneal injection	Yes Malignant mesothelioma after 1 year (1 mg not tested)	Nagai *et al*. (2011)
MWCNT A **(synthesized)**	8.57 µm (WHO fibres) 2.72 µm (all fibres)	85 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 98/90 % of rats (low/high dose) Mean survival time: 213/194 days First detection of morbidity: 5/5 months p.e.	Rittinghausen *et al*. (2014)

Test material	Length	Diameter	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWCNT B **(synthesized)**	9.3 µm (WHO fibres) 2.13 µm (all fibres)	62 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 92/90 % of rats Mean survival time: 294/207 days First detection of morbidity: 6/5 months p.e.	Rittinghausen *et al*. (2014)
MWCNT C **(synthesized)**	10.24 µm (WHO fibres) 4.18 µm (all fibres)	40 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 84/94 % of rats Mean survival time: 415/265 days First detection of morbidity: 10/6 months p.e.	Rittinghausen *et al*. (2014)
MWCNT D **(synthesized)**	7.91 µm (WHO fibres) 2.53 µm (all fibres)	37 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 40/70 % of rats Mean survival time: 666/585 days First detection of morbidity: 20/11 months p.e.	Rittinghausen *et al*. (2014)
MWCNT-7 (Mitsui)	7.1 µm	75 nm	rat	Intraperitoneal injection	Yes First incidences of mesotheliomas after 6 months, ~ 100 % of animals positive after 12 months (crocidolite asbestos negative)	Huaux *et al*. (2016)
Short MWCNT-7	2.8 µm	75 nm	rat	Intraperitoneal injection	Yes Lower incidences and longer latency compared to unground MWCNT-7 (crocidolite asbestos negative)	Huaux *et al*. (2016)
MWCNT-7 **(Mitsui)**	< 20 µm (27.5 % > 5 µm	100 nm	mouse (transgenic)	Intraperitoneal injection	Yes Invasive mesothelioma after 25 weeks (100 % mortality)	Takagi *et al*. (2008)
MWCNT-7 **(Mitsui)**	< 20 µm (27.5 % > 5 µm	100 nm	mouse (transgenic)	Intraperitoneal injection	Yes Dose-dependent mesothelioma induction 1 year p.e., with cumulative incidence of mesotheliomas of 19/20, 17/20 and 5/20, respectively.	Takagi *et al*. (2012)

Test material	Length	Diameter	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWCNT-7 **(Mitsui)**	1-4 µm (72.5 %)	82 % in range 70-110 nm	rat	Intrascrotal injection	Yes Mesothelioma in 6/7 animals after 52 weeks	Sakamoto *et al*. (2009)

The proposal for classification for carcinogenicity is restricted to the inhalation route as according to present knowledge the inhalation route is the only relevant one. It is highly improbable that exposure by the dermal or even oral route would lead to a carcinogenic response, taking into account that long-term deposition of MWC(N)T in the tissues, as can occur in lung, is a prerequisite for carcinogenicity. According to present knowledge, there is no evidence that other carcinogenic fibres meeting the WHO definition have carcinogenic properties after oral or dermal exposure.

Comments received during consultation

According to several industry comments, the CLH proposal should be targeted only to MWCNT-7 type tubes since majority of data comes from this type of MWCNTs and they consider that extension to other type of MWC(N)Ts is not appropriate mainly due to lack of data. One industry organisation also considered Cat 2 more appropriate in line with the IARC 2B classification. Two commenting Member states agreed with the proposed cat 1B classification but were considering the possible extension of the scope to MWCNTs with a diameter <30 nm. To support this, the recent study by Saleh *et al* (2020) was cited.

Assessment and comparison with the classification criteria

Key studies for the assessment of the carcinogenicity of MWCNTs are:

1. Kasai *et al*. (2016), which is a guideline based, full 2-year inhalation carcinogenicity study in rats. It showed a significantly increased incidence in lung tumours (mainly bronchiolo-alveolar carcinoma, and combined carcinomas and adenomas) after exposure to MWCNT-7 (Mitsui). In males, increased incidences of tumours were seen at 0.2 and 2 mg/m^3 and in females at 2 mg/m^3. A summary of the malignant tumour findings is provided in the table below. The absence of induction of pleural mesothelioma is likely to indicate the lack of sensitivity of the study design to properly detect this slowly developing tumour type by a fibre-related pathology. Low numbers of MWCNT were detected in the pleural region after 104 weeks of inhalation compared to the numbers after high bolus doses administered in positive intraperitoneal injection studies. However, a concentration-dependent increase in mesothelial hyperplasia of the parietal and ventral pleura, focal fibrosis of the parietal pleura and the diaphragm as well as inflammation of the mediastinum were observed in male rats. Females showed a statistically significantly increased incidence of focal fibrosis of the ventral pleura. The authors did not comment the peritoneal mesotheliomas which were observed in males, especially at the low-dose.

***Table**: Tumour findings in the study by Kasai et al. (2016).*

	Male				Peto test	Female				Peto test
Dose (mg/m^3)	**0**	**0.02**	**0.2**	**2**		**0**	**0.02**	**0.2**	**2**	
No. of animals examined	**50**	**50**	**50**	**50**		**50**	**50**	**50**	**50**	
Neoplastic lesions										
Lung										
Bronchiolo-alveolar carcinoma	1	1	8*	10**	↑ ↑	0	1	0	5**	↑ ↑
Adenosquamous carcinoma	0	0	0	1		0	0	0	1	
Poorly differentiated adenocarcinoma	0	0	0	0		0	0	0	1	
Squamous carcinoma	0	0	0	0		0	0	0	1	
Total carcinoma	1	1	8*	11**	↑ ↑	0	1	0	8**	↑ ↑
Bronchiolo-alveolar cell adenoma	1	1	7*	5		3	1	4	3	
Total adenoma and/or carcinoma	2	2	13**	16**	↑ ↑	3	2	4	11**	↑ ↑
Peritoneum										
Malignant mesothelioma	0	3	1	1		0	0	0	0	

2. Sargent *et al*. (2014) is a tumour promotion study in B6C3F1 hybrid mice. Mice were pre-treated with the tumour initiator methylcholanthrene (MCA, 10 µg/g bw by single intraperitoneal injection) and exposed one week later by inhalation to MWCNT-7 (5 mg/m^3, 5 hours/day, 5 days/week) for 15 days. After 17 months post-exposure, they were sacrificed and examined for tumour formation. As can be seen from table 2, 90.5 % of MCA+MWCNT-7 -exposed mice developed one or the other tumour type, compared to 51.9% of MCA-only treated mice or 26.5% of MWCNT-7-only mice. Bronchio-alveolar adenocarcinomas developed in 14 % of mice without MCA pre-treatment, which was close to the incidence in the air-control group (13 %), 22% without MWCNT-7 treatment and in 62% of mice treated both with MCA and MWCNT-7. Several pre-treated mice also developed malignant serosal tumours consistent with sarcomatous mesothelioma.

***Table**: Tumour findings in the study by Sargent et al. (2014).*

	Air	**MCA**	**MWCNT**	**MCA + MWCNT**
No. of animals	56	54	49	42
No. of animals with focal adenomatous alveolar hyperplasia	7	8	14*	26*
No. of bronchiolo-alveolar adenoma	6	18*	9	32*
% of mice with one or more of bronchiolo-alveolar adenoma	11 %	33 %*	18 %	76 %*
No. of bronchiolo-alveolar adenocarcinomas	7	12*	7	26*
% of mice with one or more of bronchiolo-alveolar adenocarcinomas	13 %	22 %*	14 %	62 %*
No. of bronchiolo-alveolar adenoma and/or adenocarcinomas	13	28*	13	38*
% of mice with lung tumours	23.2 %	51.9 %*	26.5 %	90.5 %*

3. The third set of evidence comes from IP studies (and from one intra-scrotal study) showing mesothelioma induction with MWCNT-7 (Mitsui). As described in section 'RAC general comment' in this opinion, the IP test is commonly used test to evaluate fibres in relation to their ability to cause pathogenicity specifically by a fibre -related mechanism.
    a. Huaux *et al*. (2016) injected Wistar rats intraperitoneally with MWCNT-7 (NRCWE-006; median length: 7.1 µm) or a ground short fibre fraction thereof as a single dose of 6 mg (= 2 x 10^9 WHO fibres and 0.36 x 10^9 WHO fibres, respectively). Crocidolite served as a positive control. Both CNT-7 and short CNT-7 induced mesothelioma, the latter to a lesser extent. The majority of animals developed tumours after a latency of 12 months, the first tumours occurring after 6 months (no exact figures provided). Only one animal developed mesothelioma 12 months after crocidolite injection.
    b. Takagi *et al*. (2008) used a p53 heterozygous asbestos-sensitive mouse model to explore the mesotheliomagenic potential of MWCNT-7. IP injection of MWCNT-7 (3 mg/mouse) resulted in a rapid induction of mesotheliomas, which were invasive to the abdominal wall, diaphragm, liver parenchyma and pancreas, and in some case involving the thoracic cavity. Distant metastasis was not observed (day 172 after injection). The overall mesothelioma incidence at day 84 post-treatment was even higher for MWCNT (87.5 %) compared to Crocidolite (77.8 %).
    c. Takagi *et al*. (2012) used the same p53 heterozygous asbestos-sensitive mouse model as in their previous study. IP injection of MWCNT-7 at doses 3, 30 and 300 µg/mouse resulted in a dose dependent increase in mesotheliomas (0/20, 5/20, 17/20, 19/20 mesotheliomas for controls and three dose groups, respectively). Most mesothelioma were lethal. The 15 surviving mice at low dose treatment showed focal mesothelial atypical hyperplasia. No mesothelioma was observed in the vehicle control group.
    d. Nagai *et al*. (2011) treated rats intraperitoneally with three different types of MWCNT ("NT50a": D:50 nm, L: 5.3 µm, long-crystalline fibres = MWCNT-7, "NT145": D: 145 nm, L: ~4.3 µm, thick tubes or NTtngl: D: 15 nm, L: 3 µm tangled tubes). In addition, a sub-fraction of non-aggregated NT50a fibres was tested at a number concentration equivalent to 1 mg NT145 ("NT50a-agg*). Injections with NT50a(-agg*) or 1 mg of NT50a (= MWCNT-7) induced malignant mesothelioma with a higher frequency and earlier progression than injections with 1 mg of NT145. No mesotheliomas were observed with 10 mg of NTtngl.
4. Sakamoto *et al*. (2009) administered 1 mg/kg bw MWCNT-7 to Fischer rats by intrascrotal injection. MWCNT treatment induced mesotheliomas in 6 of 7 treated rats that died prior to the end of the 52 week observation period. Apart from these mesothelial proliferative lesions, granulomas with high cellularity, including macrophages and multinucleated giant cells were observed. Rats treated similarly with 2 mg/kg bw crocidolite also developed granulomas but no mesotheliomas. The authors explained the absence of mesotheliomas with the low particle number concentration of crocidolite in their study. Further evidence on the fibre-like pathogenicity of MWCNTs comes from short term studies providing information on the pleural penetration and inflammatory effects. Most relevant are the studies by Xu *et al*. (2012 and 2014). Increased cellularity in pleural fluid and mesothelial proliferation were demonstrated in rats after transtracheal intrapulmonary spraying with MWCNT-7 (Mitsui), MWCNT (Nikkiso), lab synthesized 150 nm (in diameter) NTs but not with tangled 15 nm NTs (Xu *et al*., 2012 and 2014). This means that CNTs, and the positive control, crocidolite, were able to enter the pleural fluid at high enough levels to cause inflammation, resulting in mesothelial proliferation. The mesothelial proliferation can be seen as an early precursor event to mesotheliomas.

It is noted that the study by Saleh (2020, submitted during the consultation) showed only non-significant increases in combined lung adenoma and carcinoma after once-a-week intra-tracheal intra-pulmonary spraying (TIPS) of a total dose of 0.5-1.0 mg of MWCNT-7. Also, the positive control crocidolite (total dose 1.0 mg) remained negative in this study. However, the study included only 20 animals per dose group.

Although the majority of studies have been performed with MWCNT-7 (Mitsui) type nanotubes, the mesotheliomagenic potential is not restricted to MWCNT-7. There are several studies showing that also other, rigid MWCNTs fulfilling WHO fibre criteria are also able to induce mesothelioma. These include the following studies:

1) Rittinghausen *et al*. (2014) tested several different synthesized MWCNTs with a diameter between 37 nm-85 nm and a length between 7.91-10.24 µm. They all resulted in high incidences of peritoneal mesotheliomas after IP injection in rats. This study also provides the lowest diameter (37 nm) shown so far to cause mesothelioma in an IP test.
2) Nagai *et al*. (2011, see more detailed description above) evaluated four different types of MWCNTs including MWCNT-7 (Mitsui), Shova Denko NT-145 (145 nm), NT-50 and tangled (D: 15 nm; L: 3 µm) nanotubes. All caused mesothelioma after IP administration except the tangled CNTs. The potency of 145 nm nanotubes was, however, lower compared to MWCNT-7 and NT-50.
3) Suzui *et al*. (2016) performed an intrapulmonary spraying study in rats using three different sieve fractions of MWCNT (Nikkiso), which has dimensions similar to MWCNT-7 but with 10-times lower iron content. The diameter range of these nanofibers was 30-80 nm, an "unfiltered" fraction had a mean tube length of 4.2 µm and a "flow-through" fraction had a mean tube length of 2.6 µm. The "retained" fraction contained agglomerates, which precluded length determination. Exposure to a total dose of 1 mg/animal of all these three fractions resulted in the induction of lung tumours and pleural malignant mesothelioma, with a combined incidence of 52.6% (control incidence 0%).
4) Murphy *et al*. (2011) showed induction of asbestos-like inflammation, pleural proliferation and fibrosis after intrapleural administration of MWCNT-7 (Mitsui), long straight MWCNTs (mean diameter 165 nm, Univ of Manchester) in mice. No similar effect was observed with short (length 0.5–2 µm) or two tangled (D: 15 nm, L:1-5 µm or 5-20 µm) MWCNTs.
5) Xu *et al*. (2012) and (2014) studies (see above) showed increased cellularity in pleural fluid and mesothelial proliferation in rats after transtracheal intrapulmonary spraying with MWCNT-7 (Mitsui), MWCNT (Nikkiso), lab synthesized 150 nm (in diameter) NTs but not with tangled 15 nm NTs.

All these studies together with evidence indicating high biopersistence and associated non-neoplastic findings provide strong evidence for a fibre-like pathogenicity similar to asbestos. The DS has also summarised several *in vitro* studies trying to further elucidate the mechanisms of the induction of cancers by the MWCNTs. These are, however, of less importance for this classification proposal.

As discussed in the 'RAC general comments'-section, the fibre paradigm is not affected by the chemical composition of the fibre unless this affects biopersistence. Therefore, RAC agrees with the DS that the chemical composition and other material specific factors are in general secondary to fibre dimensions and of lesser importance.

It is important to note that the available data on mesotheliomagenic potential comes from the fibres of which the mean diameter is 37 nm or above. Fibres with a diameter of 15 nm or below has not caused mesothelioma when tested using IP exposure (Muller *et al*., 2009, Nagai *et al*., 2011, 2013), intrapleural injection (Murphy *et al*. 2011) or transtracheal intrapulmonary spraying (Xu *et al*., 2014) for mesothelioma induction. There is a lack of data on fibres between 15-30 nm. Although thin, tangled fibres do not cause mesothelioma and other effects via the mechanism

related to fibre paradigm, they may cause cancer by other mechanisms. Evidence for this is provided by the recent study (Saleh *et al*., 2020), submitted during consultation of the CLH report. This study was a 2-year comparative carcinogenicity study with either straight-type MWCNT (approximately 150 nm in diameter) or tangled-type MWCNT (7.4 nm in diameter) administered via intra-Tracheal Intra-Pulmonary Spraying (TIPS) to rats (once a week over a 7 week period, followed by a 2-year observation period). Crocidolite asbestos was used as the reference material. The rats administered straight type MWCNT or asbestos did not have a significant increase in bronchiolo-alveolar hyperplasia or tumours in the lung. However, tangled MWCNT did have significantly elevated incidences of bronchioloalveolar hyperplasia and tumours in the lung (the incidence of adenoma and adenocarcinoma combined being 1/19, 5/20, and 7/20 in the control, low dose and high dose groups, respectively). Malignant pleural mesothelioma was not induced in any of the groups. Overall, the results of this initial study suggests that also tangled-type MWCNT are carcinogenic to the rat lung when administered via the airway but probably via a different mechanism.

Comparison with the criteria

Since classification in Carc. 1A category requires human evidence, this category is not applicable in this case.

Carc. 1B applies, if the substance is presumed to have carcinogenic potential for humans and classification is largely based on animal evidence. A basic requirement is an increased incidence in malignant neoplasms in at least two species of animals, or at least two independent studies in one species. In this case, the key study is the study by Kasai *et al* 2016, showing a clear induction of lung tumours in rats after long term inhalation exposure both in males and females. Further evidence is provided by a number of supporting studies; the tumour promotion study by Sargent *et al*. (2014) in mice and studies demonstrating the mesotheliomagenic potential of MWCNTs in rats or mice after IP administration (Huaux *et al*. 2016, Takagi *et al*., 2008, 2012, Nagai *et al* 2011, Rittinghausen *et al*., 2016), after intra-scrotal administration (Sakamoto *et al*. (2009) or after transtracheal intrapulmonary spraying (Suzui *et al*., 2016). Also studies by Xu *et al*. (2012, 2014) and Murphy *et al*., (2011) showing pleural inflammation and mesothelial proliferation similar to asbestos support the fibre like carcinogenic potential of MWC(N)Ts.

RAC agrees with the DS that these data provide sufficient evidence for the **classification of MWCNTs specified in the proposal in category 1B for carcinogenicity.**

Although the key findings come from MWCNT-7 (Mitsui) type tubes, RAC agrees with the DS that there is sufficient evidence that the carcinogenicity is not limited only to MWCNT-7 (Mitsui) but also other, rigid MWC(N)Ts fulfilling WHO fibre dimensions can cause cancer by the same fibre pathogenicity paradigm related mechanism. This has been demonstrated by the studies by Rittinghausen *et al*. (2016), Nagai *et al*. (2011), Murphy *et al*. (2011) and Suzui *et al*. (2016), and are supported by the toxicokinetic and mechanistic evidence (e.g. studies by Xu *et al*., 2012 and 2014).

RAC agrees with the DS that the classification for carcinogenicity should be restricted to the inhalation route, resulting in hazard statement **H350i** (May cause cancer by inhalation). Considering the MoA related to the fibre pathogenicity paradigm, which requires high biopersistence resulting in a high long-term deposition of MWC(N)T in the lung, it is highly unlikely that exposure by the dermal or even oral route would lead to a carcinogenic response. This is also in accordance with our knowledge on the carcinogenicity of other carcinogenic fibres meeting the WHO fibre definition and in line with classification of other fibres encompassing WHO fibre dimensions.

RAC notes that the doses at which induction of lung tumours was observed in the study by Kasai *et al* (2016) are very low and in some IP studies, MWCNTs showed even higher mesotheliomagenic potency than crocidolite. This suggests a high carcinogenic potency of MWCNTs, which could justify the setting of an SCL (0.01%) instead of applying the generic concentration limit of 0.1%. However, RAC notes that the current guidance for the potency evaluation for the setting of SCL is designed primarily for systemic, non-threshold genotoxic carcinogens and may not be fully applicable to local carcinogens – and especially for fibres - with multiple modes of action playing a role in the carcinogenicity. The guidance for the setting SCLs for substances which are carcinogenic via inhalation is currently under development but may not be applicable to fibres in any case. Additionally, in the case of MWC(N)Ts, the verification that the concentration limit of fibres >30 nm in diameter is not exceeded is likely to require electron microscope (SEM/TEM) evaluation and it is not known how achievable percentages below 0.01% are in practice.

Finally, RAC notes that there is a gap in knowledge concerning fibres with a diameter between 15-30 nm. Thus, there is an apparent need to study the ability of fibres to cause cancer. On the other hand, RAC further notes that even though MWCNTs <30 nm in diameter have not been included in the current CLH proposal, MWCNTs with a mean diameter <30 nm are still subject for classification if they contain $\geq$0.1% (GCL) of individual MWCNTs with the dimensions specified in this proposal. Although fibres of 15 nm or below have not caused mesothelioma, they may cause lung cancer (via other mechanisms) as suggested by the study of Saleh *et al* (2020). Therefore, RAC identifies a need to evaluate the available data on MWCNTs with a diameter of <30 nm for their possible classification according to CLP.

Additional references

Donaldson K, Murphy FA, Duffin R, Poland CA. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part Fibre Toxicol. 2010 Mar 22;7:5. 1-17 doi: 10.1186/1743-8977-7-5.PMID: 20307263

Donaldson K, Poland CA, Murphy FA, MacFarlane M, Chernova T, Schinwald A. Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences.

Adv Drug Deliv Rev. 2013 Dec;65(15):2078-86. doi: 10.1016/j.addr.2013.07.014. Epub 2013 Jul 27.PMID: 23899865

Donaldson K, Murphy F, Schinwald A, Duffin R, Poland CA. Identifying the pulmonary hazard of high aspect ratio nanoparticles to enable their safety-by-design Nanomedicine (Lond). 2011 Jan;6(1):143-56. doi: 10.2217/nnm.10.139.PMID: 21182425 Review

Køble C, Poulsen SS, Saber AT, Jacobsen NR, Wallin H, Yauk CL, Halappanavar S, Vogel U, Qvortrup K, Mølhave K. Time-dependent subcellular distribution and effects of carbon nanotubes in lungs of mice. PLoS One. 2015 Jan 23;10(1): e0116481.

Rydman EM, Ilves M, Vanhala E, Vippola M, Lehto M, Kinaret PA, Pylkkänen L, Happo M, Hirvonen MR, Greco D, Savolainen K, Wolff H, Alenius H. A Single Aspiration of Rod-like Carbon Nanotubes Induces Asbestos-like Pulmonary Inflammation Mediated in Part by the IL-1 Receptor. Toxicol Sci. 2015 Sep;147(1):140-55. doi: 10.1093/toxsci/kfv112.

Sabo-Attwood T, Ramos-Nino M, Bond J, Butnor KJ, Heintz N, Gruber AD, Steele C, Taatjes DJ, Vacek P, Mossman BT. Gene expression profiles reveal increased mClca3 (Gob5)

expression and mucin production in a murine model of asbestos-induced fibrogenesis. Am J Pathol. 2005 Nov;167(5):1243-56.

Saleh DM, Alexander WT, Numano T, Ahmed OHM, Gunasekaran S, Alexander DB, Abdelgied M, El-Gazzar AM, Takase H, Xu J, Naiki-Ito A, Takahashi S, Hirose A, Ohnishi M, Kanno J, Tsuda H. Comparative carcinogenicity study of a thick, straight-type and a thin, tangled-type multi-walled carbon nanotube administered by intra-tracheal instillation in the rat. Part Fibre Toxicol. 2020 Oct 15;17(1):48. doi: 10.1186/s12989-020-00382-y

Stanton MF, Layard M, Tegeris A, Miller E, May M, Morgan E, Smith A. Relation of particle dimension to carcinogenicity in amphibole asbestoses and other fibrous minerals. J Natl Cancer Inst. 1981 Nov;67(5):965-75. PMID: 694625

WHO, World Health Organization (1985) Reference Methods for Measuring Airborne Man-made Mineral Fibres (MMMF) (Environmental Health Series 4), Copenhagen

ANNEXES:

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

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