Comments on behalf of the Vanadium Consortium[1] and Vanitec[2]

on the

CLH Report for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name: Divanadium pentaoxide
(Synonyms: vanadium pentoxide, V_2O_5)

Prepared by EBRC Consulting GmbH, Hannover (Germany)

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[1] The Vanadium Consortium is a voluntary industry association that aims to support its members to fulfil all obligations under Regulation (EC) No 1907/2006 (i.e. REACH), especially regarding the key requirement to provide data for a scientifically sound hazard and risk assessment that will enable registration.

[2] Vanitec is the technical and scientific committee representing companies involved in the mining, processing and manufacture of vanadium and vanadium-containing products for use mainly in the metals and materials manufacturing and chemical industries, world-wide.

1 Executive summary

The Vanadium industry, represented by Vanitec and the Vanadium Consortium, takes this opportunity to submit scientific comments on the Proposal for Harmonised Classification and Labelling of divanadium pentaoxide. Our comments are put forward in detail endpoint-by-endpoint further below.

We propose an alternative science-based, justifiable classification since we disagree with the unbalanced analysis, methodology, and findings in the CLH proposal. We have tabulated the existing classification together with the proposed classification by the Dossier Submitter (DS), and also the classification that the vanadium industry (the Registrant) deems appropriate as follows:

Table 1: Existing and harmonised classification and labelling proposed for V_2O_5 by dossier submitter and by the Registrant

Existing classification	Proposed by the dossier submitter	Revision proposed by the Registrant[#]
Acute Tox. 4, H302	Acute Tox. 3, H301	Acute Tox. 4, H302
Acute Tox. 4, H332	Acute Tox. 1, H330	Acute Tox. 4, H332
Muta. 2, H341	Muta. 1B, H340	Muta. 2, H341
Repr. 2, H361d	Repr. 1B, H360Fd	Repr. 2, H361fd
	Lact., H362	~~Lact., H362~~
	Carc. 1B, H350	Carc. 2, H351i
STOT SE 3, H335	STOT SE 3, H335	STOT SE 3, H335
STOT RE 1, H372	STOT RE 1, H372	STOT RE 1, H372
Aquatic Chronic 2, H411	Aquatic Chronic 2, H411	Aquatic Chronic 2, H411

[#]Please note that based on a reliable *in vivo* eye irritation study in rabbits (Leuschner, 2010), the Registrant has self-classified divanadium pentaoxide with “Eye Damage 1 (H318: Causes serious eye damage)”.

1.1 Acute toxicity via the oral route

The DS has selected an LD50 using the findings of an acute oral study in female rats with a pulverised product of technical grade (Leuschner, 1991a), thereby disregarding data available both for the pure substance, i.e. pulverised, analytical grade (Leuschner, 1991c) or the technical fused product as marketed (Leuschner, 1991b). The Registrant contends that for the assessment of the true intrinsic toxicity of a substance, the results of the oral toxicity of the pulverised “pure” analytical grade in female rats are more relevant and appropriate. Thus, based on the study by Leuschner (1991c), divanadium pentaoxide is already conservatively classified as **Acute Oral Toxicity Category 4 – H302**. This existing classification should be retained.

1.2 Acute toxicity via inhalation

In his classification proposal, the DS has chosen an unusual approach in deriving an LC50 by using only one gender-specific finding in only one acute inhalation study in mice, thereby disregarding reliable data for male mice in the same study (Sullivan, 2011a). This sex-specific derivation is not considered reasonable and is not in accordance with OECD guideline 436, since both male and female animals were tested and therefore results of both sexes should be pooled together.

In contrast, in the opinion of the Registrant the acute toxicity findings obtained in rats supported by further findings in mice (Sullivan 2011a,b) document that very fine divanadium pentaoxide powder should instead be classified as: Acute Inhalation Toxicity Category 2 “Fatal if inhaled” – H330”. However, the above mentioned very fine divanadium pentaoxide powder was artificially generated in a laboratory by milling, whereas commercially available grades are far coarser (<3% of particles (w/w) < 10 µm).

In the studies by Sullivan (2011a,b), granular divanadium pentaoxide was milled to produce a very fine powder (>96% of particles (w/w) < 10 µm) to conduct the inhalation exposures in rodents for comparative research purposes. These tests were not carried out with the substance in the form in which it is placed on the market and in which it can reasonably be expected to be used. According to the CLP Regulation Article 9(5), "when evaluating the available information for the purposes of classification, the manufacturers, importers and downstream users shall consider the forms or physical states in which the substance or mixture is placed on the market and in which it can reasonably be expected to be used."

Thus, based on the study by Leuschner (1991d,e,f), divanadium pentaoxide in the forms in which it is actually placed on the market and is used, is already adequately and conservatively classified as **Acute Inhalation Toxicity Category 4 – H332**. The existing classification should be retained.

1.3 Lack of evidence of a primary genotoxic mechanism of action for divanadium pentaoxide

The analysis of the available data as employed in the CLH report does not appear to transparently weigh the relevance, reliability and adequacy of the data sources. The selection of the studies to be compared against the CLP criteria remains unclear and unexplained, so that the decision on the classification lacks transparency.

The Registrant has undertaken a thorough evaluation of all available data and has compared the data against the classification criteria as laid down in the Guidance on the Application of the CLP criteria (ECHA, 2017) in a weight-of-evidence analysis. A detailed analysis is presented in chapter 2.5 below. The outcome of this weight-of-evidence analysis can be summarised as follows:

- No evidence for *in vitro* mutagenicity in bacteria
- Equivocal evidence for *in vitro* clastogenicity/aneugenicity
- No evidence for *in vitro* mutagenicity in mammalian cells
- No evidence for *in vivo* mutagenicity in transgenic rodents
- No evidence for site of *in vivo* contact genotoxicity after inhalation
- No evidence for *in vivo* clastogenicity, positive findings stem largely from unreliable studies with unphysiological route of exposure
- Positive findings were largely obtained from studies published by one and the same working group of E. Rojas and M. A. Altamirano-Lozano (University of Mexico City), whose study design and reporting shows recurring deficiencies

In contrast to the CLH report, the Registrant finds that the genetic toxicity studies actually support the conclusion that divanadium pentaoxide does not elicit any mutagenic activity.

The weight-of-evidence analysis of the entire genotoxicity database does not show any clear evidence of germ cell mutagenicity. Consequently, divanadium pentaoxide should not be classified in Category 1B. On the basis of the information obtained from relevant and reliable studies but also considering the remaining studies with all their limitations, the current classification with "**Mutagenicity Category 2 – H341**" already appears overtly conservative.

1.4 Carcinogenicity

In the view of the Registrant, the NTP (2002) inhalation study in rats and mice does not provide sufficient data to draw definitive conclusions regarding thresholds and/or mechanisms of action or mode of action. The relevance of the lung tumours in mice is severely confounded by the chronic and persistent inflammation, presumed to have occurred over the whole duration of the two-year exposure, and the lack of a dose-response relationship.

Divanadium pentaoxide reacts strongly acidic, so that this is likely to contribute to the chronic inflammation. Further, V_2O_5 is not expressing its carcinogenic activity via genotoxicity since tumours did not develop at other organ sites following 2-years of inhalation even though divanadium pentaoxide circulated systemically. Based on the absence of genotoxicity, divanadium pentaoxide is thought to exert genotoxicity by indirect mechanisms (secondary genotoxicity, i.e. chronic inflammation and oxidative stress).

The tumour promoting activity in mice, although strain-dependent (Rondini et al., 2010) may be considered part of that mode of action of V_2O_5. It is further noted that there is a high degree of discordance between rats and mice in the development of chemically induced lung tumours, as shown in a statistical analysis of 58 compounds tested in NTP two-year inhalation studies (Smith & Anderson, 2017). From this data, it has also been concluded that bronchioalveolar lung tumours induced only in mice by non-genotoxic chemicals are of low relevance for human lung cancer risk (Smith et al., 2018). Further, the genomic responses of mice to inflammation are of questionable comparability to those of humans (Seok et al., 2013).

Finally, the available carcinogenicity data relate only to inhalation exposure of rats and mice to divanadium pentaoxide, which affected only the respiratory tract, and with tumour formation only in mice lungs which is considered a substance-specific local effect. The NTP (2002) study does not report any treatment related lesions in other tissues whatsoever, despite measured vanadium levels in blood indicating systemic exposure.

In conclusion, the evidence of carcinogenicity in mice of both sexes (NTP, 2002) is not considered sufficient for classification of divanadium pentaoxide as a Category 1B substance. Instead, a **classification in Category 2 – H351i** appears to be most appropriate and adequately conservative. Additionally, any classification should be route-specific, i.e. restricted to inhalation.

1.5 Effects on fertility and effects via lactation

The Registrant contends that taking the information from several publications together, there is only limited and weak evidence from these studies that divanadium pentaoxide exposure by the inhalation and oral route may affect male and female fertility. This evidence is not considered strong enough to support classification in Cat 1B. It is therefore proposed to retain the already existing **Category 2 – H361f.**

For <u>adverse effects on development,</u> the Registrant is of the opinion that the current harmonised classification with **Repro. Category 2 H361d** for development is still justified and should be retained. This is supported by the available information on developmental toxicity.

For adverse effects on or via lactation, the publications referred to by the CLH report are all of low reliability (RL=3)[3], but the CLH report nevertheless comes to the conclusion that classification as H362: May cause harm to breast-fed children is warranted. This conclusion is derived from a limited data set without taking relevant uptake routes into consideration; however, an appropriate assessment is not possible due to a lack of any reported (no) effect level. The data summarised by the DS are not considered to represent clear evidence of adverse effects in the offspring due to transfer in the milk or adverse effect on the quality of the milk. There are also no reliable data that indicate the likelihood that the substance is present in potentially toxic levels in breast milk. Overall, the available data **do not justify classification for lactation effects**.

In a very recent publication by US NTP, three-month toxicity studies of tetravalent and pentavalent vanadium compounds in Hsd:Sprague Dawley SD rats and B6C3F1/N mice via drinking water exposure are described (Roberts et al. 2019). Data on the number of offspring from treatments, post implantation *in utero* and live births, birth weights, and weight gains were generated. The results for vanadium in its pentavalent form indicate that fetal effects are only seen at levels that are toxic to mothers, so that vanadate ions do not appear to be selectively affecting live births or to be selectively toxic to neonates. The full findings of these sub-chronic studies are not available yet, but are expected to be published in 2020. They can reasonably be expected to provide valuable information regarding conclusions on the effect of vanadium in its pentavalent form on fertility and effects via lactation, thereby closing a data gap.

Since the Registrant has been aware of these studies, a testing proposal was previously not submitted in the REACH registration of divanadium pentaoxide to avoid duplicate animal testing. The Registrant suggests that any decision on the classification for effects of divanadium pentaoxide on reproduction and via lactation should be deferred until the full study reports will be available for rats and mice.

[3] Reliability rating was performed according to the scoring scheme of Klimisch et al. (1997).

2 Detailed comments on the proposed classifications

The following represents a commentary section-by-section, paragraph-by-paragraph according to the sequence of the CLH report as they were considered relevant for each of the proposed classifications.

2.1 Considerations on the chemistry and stability of V_2O_5

The inorganic chemistry of vanadium is complex and should be considered in evaluating the relevance and reliability of studies.

- The aqueous chemistry of vanadium is highly dependent on pH, concentration and redox potential. Even in limited biological and environmental ranges of these parameters, multiple chemical species can form with different oxidation states and molecular charges, including anions and cations. It is important to give very careful consideration to the form of vanadium being studied. This error is common in studies purporting to be on V_2O_5 while the species measured is typically total, unspeciated vanadium. It should further be noted that V_2O_5 is not stable in water and instead reacts to form acidic solutions.

- In the test of V_2O_5 according to OECD 105 (Klawonn, 2010d), a water solubility of 0.92 g/L was determined at 20°C and steady state after 3 days with a resulting solution pH of 2.7. Thus, a pH decrease upon dissolution in water below the cut-off criteria for irritation, i.e. pH of 3, was observed. Severe irritation was also observed in the eye irritation test for V_2O_5 (Leuschner, 2010), based on which the substance meets CLP criteria of Eye Damage 1.

- Studies related to the direct exposure of animals to V_2O_5(s) are wholly appropriate for consideration in the hazard assessment. Studies involving “dissolved V_2O_5“ are much more problematic since the resulting acidic solutions of vanadic acid (in water) or vanadium complexes (if other ligands are present in a biological fluid or biological fluid surrogate) are not V_2O_5.

- Concentrated solutions of vanadium (above 1mM) contain complex vanadate oligomers of which decavanadate is the most common. This is a heavy hexa-anion (6- charge), which cannot be assumed to have properties similarly to neutrally charged V_2O_5. Studies on dissolved V_2O_5 need to consider that the materials has (i) reacted and (ii) may be present in very different chemical forms with varying concentration.

2.2 Toxicokinetic information

(CLH report Section 9.1, Page 14-20):
The dossier submitter (DS) has apparently chosen to refer primarily to secondary literature to derive conclusions on toxicokinetics (TK). As a result, some of the statements in the CLH dossier are somewhat inaccurate or perhaps misleading.

For example, on page 16 (9.1 a, Absorption), bioaccessibility studies are cited from an abbreviated review (VPRA, 2010), with the “disqualifying” statement “an incomplete reporting of these data does not allow to assess them more thoroughly”. However, the REACH dosser on V_2O_5 cites five very detailed bioaccessibility studies conducted under GLP at the German Fraunhofer Institute (Klawonn, 2010a,b,c, 2011, 2012); these are described in great detail in robust study summaries in the REACH dossier, and are informatively summarised in the respective endpoint summary.

Thus, direct reference to the REACH dossier and the primary literature cited therein allows appropriate up-to-date conclusions on the bioaccessibility and speciation of vanadium released from divanadium pentaoxide (and other vanadium substances) upon dissolution in physiological media to be drawn.

The most important conclusions on the very extensive bioaccessibility studies with vanadium substances of relevance for V_2O_5 can briefly be summarised as follows: all vanadium substances upon dissolution transform predominantly into pentavalent vanadate ions in all physiological media, except for artificial lysosomal fluid (ALF) in which tetravalent V appeared to be the predominant species present after 2 and 24h in bioaccessibility experiments; the ALF medium likely in this case underestimates the transition to the pentavalent vanadate anion due to the absence of oxidative mechanisms usually in place in lysosomes.

Some further incorrect statements in the CLH dossier are briefly highlighted:

(i) On p.16 (absorption, respiration route), the CLH dossier cites secondary literature and states “After inhalation exposure to divanadium pentaoxide in particle form, most of the substance is deposited and retained in the lungs”. This is incorrect. The REACH dossier on V_2O_5 cites in detail airborne particle size distribution measurements of all commercially available forms of V_2O_5 (ranging in particle size from d50 1.3-262.8um), combined with Multiple-Path Particle Dosimetry (MPPD) model predictions of respiratory tract deposition. In view of the particle size distribution data, only 0-0.3% of inhaled V_2O_5 would be deposited in the alveolar fraction of the lungs (and only 0-0.1% in the tracheobronchial region), whereas the majority (ca. >40-60%) would immediately be impacted in the extrathoracic (head) region, subsequently swallowed and therefore would be overwhelmingly subject to systemic uptake through the gastro-intestinal tract (EBRC 2010).

(ii) On page 16, the final sentence of the same chapter cites Greim (2006), who is also cited several times elsewhere in the text, with the remark: “report not available”. However, Greim (2006) summarises the derivation of the German MAK (an occupational exposure limit) value, and therefore this reference constitutes secondary literature and not primary data.

(iii) In several other text passages throughout the toxicokinetics chapter, statements are made that can appear as confusing: “After divanadium pentaoxide **inhalation** exposure of CD-1 mice, the tested compound was distributed to testis”, “After **oral** gavage application, divanadium pentaoxide was mainly distributed to bone, liver, muscle, kidney, spleen, and blood” and “Divanadium pentaoxide is shown to cross the placenta barrier”; in contrast, other parts of this CLH subchapter in fact correctly acknowledges the dissolution to pentavalent vanadate ions. Overall, this leads to several inconsistencies in the report.

<u>Conclusions on toxicokinetics of divanadium pentaoxide:</u>

The CLH dossier does not seem to make use of the very detailed chapter on toxicokinetics of the V_2O_5 REACH Registration dossier; instead, it refers on numerous occasions to secondary literature sources with subsequent inaccurate or incomplete conclusions.

Based on reliable data presented in the REACH dossier, it is important to note:

- upon inhalation, only a very small fraction of inhaled material will actually be deposited in the lung alveolar fraction – the majority of inhaled material will be translocated to the gastro-intestinal tract.
- whether inhaled or ingested, any V_2O_5 will rapidly dissolve forming almost exclusively pentavalent vanadate anions, which are highly water soluble and readily distributed throughout the body.
- it is critical to distinguish between the <u>local</u> effects of V_2O_5 at the site of contact, and <u>systemic</u> effects mediated through vanadate ions; locally, the low amounts of V_2O_5 in contact with (moist) surfaces will readily dissolve – however, especially in the case of V_2O_5 this dissolution is associated with a strong acidic reaction which may explain its strong irritative properties.

2.3 Acute toxicity via oral route

(CLH report Section 10.1, Page 20-23):
The dossier submitter derived an LD50 using the finding of an acute oral study in female rats with a pulverised product of technical grade (Leuschner, 1991a), thereby disregarding data available for the purer product, i.e. pulverised, analytical grade (Leuschner, 1991c) or the technical fused product as marketed (Leuschner, 1991b).

Leuschner (1991a,b,c) tested three different grades of divanadium pentaoxide, i.e. technical grade pulverised, technical grade fused and analytical grade pulverised, according to OECD 401 and derived LD50 values for male rats of 313.8, 715.7 and 474.2 mg/kg bw and for female rats of 221.1, 658.4 and 466.9 mg/kg bw, respectively (see also Leuschner et al. 1994). To determine the true intrinsic toxicity of a substance, the results for the oral toxicity of the pure, analytical grade in female rats are more relevant, appropriate and support that divanadium pentaoxide is already conservatively classified as: Acute Oral Toxicity Category 4 “Harmful if swallowed – H302”.

Conclusion on classification and labelling of divanadium pentaoxide for acute oral toxicity:
Based on the study by Leuschner (1991c) and Leuschner et al. (1994), divanadium pentaoxide is already conservatively classified as **Acute Oral Toxicity Category 4 “Harmful if swallowed – H302”**, and the existing classification should be retained.

2.4 Acute toxicity – inhalation route

(CLH report Section 10.3, Page 25-28):
Decisive reference is made in the CLH dossier with respect to the proposed classification for Acute Tox. 1 ”fatal if inhaled” (H330) to an Acute Inhalation Toxicity Study according to OECD 436 in B6C3F1 mice (Sullivan, 2011a). The dossier submitter reported two LC50 values, i.e. a LC50 (4 hours, male mice) > 0.5 mg/l and a LC50 (4 hours, female mice) < 0.05 mg/l. However, no information on the procedure used for calculation of the LC50s is given, with respect to the assumed gender difference in mice. The approach for deriving LC50 values separately for male and female mice is actually not in compliance with the provisions specified in OECD 436. It is therefore considered unjustified to use solely the LC50 < 0.05 mg/l based only on the results from three female mice for an overall conclusion on acute toxicity classification.

The OECD guideline 436 foresees a stepwise procedure that tests three males and three females simultaneously at each step. At each step, decisions are based on the number of observed deaths from the <u>combined</u> group of six animals and either a classification is derived, or testing continues at the next higher or lower concentration, depending on the starting concentration. Based on the combined mortality results of that study in male and female mice and according to the scheme laid down in Annex 3b of the OECD Test Guideline 436, an LC50 (4 hours) of 0.25 mg/l was estimated for mice.

In a second acute inhalation toxicity study on aerosolised divanadium pentaoxide powder exposure conducted in rats in accordance with OECD 436, also an LC50 (4 hours) of 0.25 mg/l is derived.

According to Annex I, 3.1.2.2.1. of the CLP Regulation, “*the preferred test species for evaluation of acute toxicity by the oral and inhalation routes is the rat”.* In general, classification is based on the lowest acute toxicity estimate (ATE) derived using the LC50 where available.

The LC50 of 0.25 mg/l derived from the study in male and female rats is within the range of >0.05 – 0.5 mg/l (dusts and mists) established for classification as Acute Inhalation Toxicity Category 2 under the criteria of regulation (EC) 1272/2008.

However, in the studies by Sullivan (2011a,b), commercially available coarse divanadium pentaoxide was milled to produce a very fine powder to conduct the inhalation exposures in rodents for research purposes in the US. The most widely used commercial form is a fused flake, made by melting divanadium pentaoxide and then cooling it in sheets to form a dark brown glassy form, which has typical particle sizes of several millimeters thick and tens of millimeters length and width. Thus, the tests were not carried out on the substance in the form in which it is placed on the market and in which it can reasonably be expected to be used. According to CLP Regulation Article 9(5), “when evaluating the available information for the purposes of classification, the manufacturers, importers and downstream users shall consider the forms or physical states in which the substance or mixture is placed on the market and in which it can reasonably be expected to be used.”

In the following figure, particle size distributions (PSDs of aerodynamic diameters) of the milled test material as tested by Sullivan (2011a,b) (pink lines) are compared with PSDs of powders (3) and granules (19 as placed on the European market. For the milled material tested by Sullivan (2011a,b), 2 measurements were reported for each of the three dose groups, whereas PSDs were determined for 4 representative samples of marketed powders. Clearly, airborne dust generated from marketed powders and granules consists of coarser particles when compared with the milled material tested by Sullivan (2011a,b). Ninety percent (w/w) of the particles of the average PSD of the milled material tested by Sullivan (2011a,b) do represent less than 2 % of the average particle mass of marketed powders and granules (see green dotted lines).

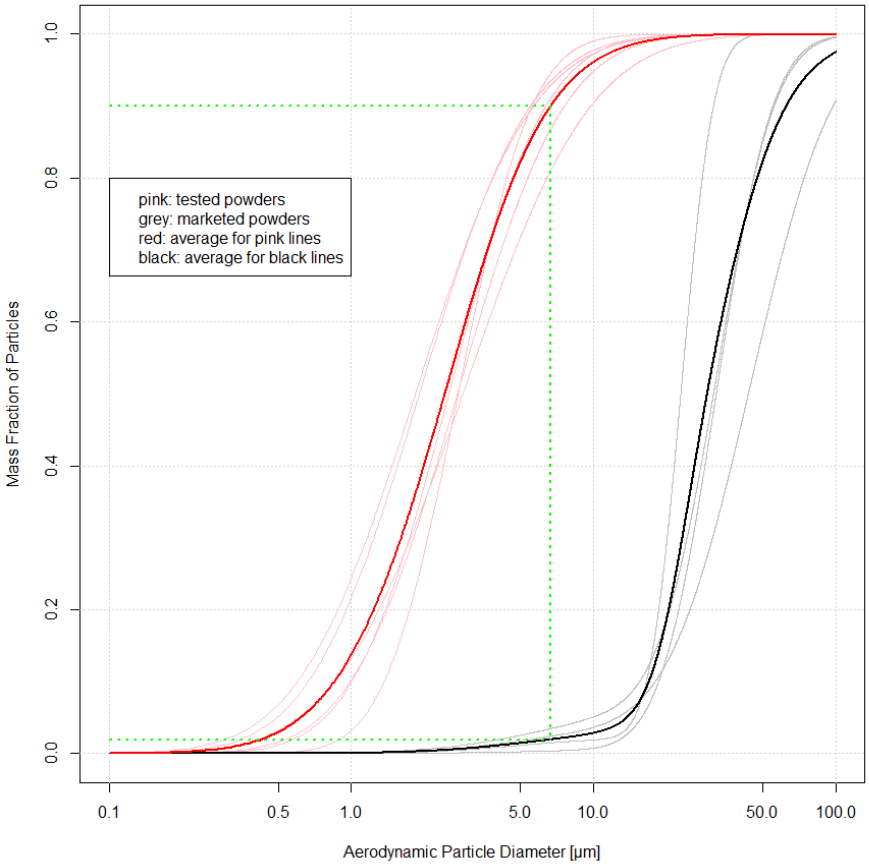

Figure: PSDs of powders tested by Sullivan (2011a,b) (red lines) vs. PSDs of marketed powders and granules (black lines)

Leuschner (1991d, e, f) tested three different grades of divanadium pentaoxide, i.e. analytical grade pulverised, technical grade fused, and technical grade pulverised, according to OECD 403 and derived 4-h LC50 values for male rats of 11.09, 16.19 and 4.4 mg/L air and 4-h LC50 for female rats of 4.29, 4.04 and 2.21 mg/L air, respectively (see also Leuschner et al. 1994).

Based on the studies by Leuschner (1991d,e,f) and Leuschner et al. (1994), divanadium pentaoxide (in the forms or physical states in which it is placed on the market and in which it can reasonably be expected to be used) is already conservatively classified as Acute Inhalation Toxicity Category 4 “Harmful if inhaled – H332”.

Conclusion on classification and labelling of divanadium pentaoxide for acute inhalation toxicity:
Based on the study by Leuschner (1991d,e,f) and Leuschner et al. (1994), divanadium pentaoxide is already conservatively classified as **Acute Inhalation Toxicity Category 4 “Harmful if inhaled – H332”**, and the existing classification should be retained.

2.5 Germ cell mutagenicity

(CLH report Section 10.8, Page 34-38):
The CLH report summarises the available genotoxicity data in a tabular format, separately for *in vitro, in vivo* and human data. However, only very brief conclusions on the observations are provided, thereby lacking essential information relevant for decision making, such as correlating any findings with the evaluation criteria, dose-dependency of a positive response, biological relevance and plausibility of findings. These summary tables also do not provide a justification for the reliability rating of each literature item, merely the Klimisch scores are given in the summary tables. When comparing the rating of the references, it does not appear that the quality criteria as given in the ECHA guidance R.4 were applied in a consistent manner as illustrated below.

The Registrant has undertaken to reassess all studies in a weight-of-evidence analysis and transparently weigh the relevance, reliability and adequacy of the references as summarised in the table below:

Table 2: Weight-of-evidence analysis for *in vitro* and *in vivo* genetic toxicity data

	RL=1	**RL=2**	**RL=3**	**RL=4**
Bacterial reverse mutation assay (OECD 471)	NEGATIVE; NTP 2002, V_2O_5			POSITIVE/ NEGATIVE; Sun 1987, V_2O_5
***In vitro* clastogenicity (OECD 473, 487, 479*)**	POSITIVE: Lloyd 2010a, V_2O_5	NEGATIVE; Gibson et al. 1997, V_2O_5 NEGATIVE; Rodríguez-Mercado et al. 2010, V_2O_5	NEGATIVE; Zhong et al. 1994, V_2O_5 (SCE) POSITIVE: Zhong et al. 1994, V_2O_5 (MN) POSITIVE: Roldán & Altamirano 1990, V_2O_5 POSITIVE: Ramírez et al. 1997, V_2O_5	
***In vitro* mutagenicity in mammalian cells (OECD 476, 490)**	NEGATIVE; Lloyd 2010b, V_2O_5		NEGATIVE; Zhong et al. 1994, V_2O_5 (-)	
In vitro* DNA damage (Comet)*			POSITIVE: Rodríguez-Mercado et al. 2011, V_2O_5 POSITIVE: Kleinsasser et al. 2003, V_2O_5 POSITIVE: Rojas et al. 1996, V_2O_5	
***In vivo* clastogenicity (OECD 474, 475, 478)**	NEGATIVE; Beevers 2011, V_2O_5 NEGATIVE; NTP 2002, V_2O_5		POSITIVE: Rojas-Lemus et al. 2014, V_2O_5 POSITIVE: García-Rodríguez et al. 2016, V_2O_5 POSITIVE: Altamirano-Lozano et al. 1996, V_2O_5	POSITIVE: Sun 1987, V_2O_5 (MN) POSITIVE: Sun 1987, V_2O_5 (DLA)
***In vivo* gene mutation (OECD 488)**	NEGATIVE; Manjanatha et al. 2015, V_2O_5			
***In vivo* DNA damage (OECD 489)**	NEGATIVE; Schuler et al. 2011, V_2O_5		POSITIVE: Altamirano-Lozano et al. 1999, V_2O_5 POSITIVE: Altamirano-Lozano et al. 1996, V_2O_5	
other data	NTP 2002; Devereux et al. 2002, V_2O_5 (oncogene alteration) Paramanik and Rajalakshmi 2013, V_2O_5 (DNA migration) Altamirano-Lozano 1993, V_2O_5 (SCE)			

* OECD 479 was deleted on 2nd April 2014
** no accepted guideline available

References, for which the Registrant does not concur with the reliability rating given in the CLH report, were reassessed for relevance and reliability in the following. A detailed justification for the proposed reliability rating is provided with the recommendation to re-consider the relevance in the overall weight-of-evidence evaluation for the endpoint germ cell mutagenicity.

In vitro genetic toxicity

In the CLH report, the publication by Roldan and Altamirano (1990) was rated as reliable with restriction (RL=2). However, the publication shows reporting deficiencies and deficiencies in the study design as follows:

- SCE test: the test item characterisation is insufficient (purity and supplier not stated), no information on the evaluation and scoring criteria is given, historical control data (if any) are not shown or discussed. No positive control is used to demonstrate the sensitivity of the test system; exposure duration is not unambiguously stated. The negative control is only specified as "control", without any further identification of the substance. No information on confounding factors (such as precipitation, pH, osmolality). The determination of the average generation time (AGT) and the mitotic index (MI) is not sufficiently described. The number of chromosomes and number of SCE per chromosome are not stated.
- CA assay: the item characterisation is insufficient (purity and supplier not stated); only a single treatment and harvesting time point (OECD TG 473, 1983 recommends treatment at various time points or alternatively single treatment with multiple harvesting time points). There is a complete lack of historical control data, which are not shown or discussed, likewise the evaluation and scoring criteria are not given. No positive control is used to demonstrate the sensitivity of the test system. The exposure duration is not unambiguously stated. The presence of the negative control is only stated as "control", without any further identification of the substance. No information on confounding factors (such as precipitation, pH, osmolality) is provided. The determination of average generation time (AGT) and mitotic index (MI) is not sufficiently described; only the total number of structural chromosomal aberrations is stated, without further specification on types and their occurrence. No information on statistical testing of differences in structural chromosomal aberration frequencies is available. Cytotoxicity is not discussed in correlation with the chromosomal aberrations observed.

Based on the above described deficiencies, the Registrant does not agree with the rating of the study by Roldan and Altamirano as given in the CLH report with “reliable with restriction” (RL=2) but proposes to reduce the reliability to “not reliable” (RL=3).

The publication by Zhong et al. (1994) was rated in the CLH report as reliable with restriction (RL=2). However, the publication shows severe reporting deficiencies and deficiencies in the study design:

- for the SCE test, the evaluation criteria for SCE scoring are partially missing and deviate from the OECD test guideline (i.e., only cells with 46 ± 2 centromeres of the modal chromosome number vs cells with 20-23 distinguishable and well-spread chromosomes, see OECD TG 479), historical control data are not shown or discussed, and any confounding effects are not discussed (such as precipitation, cytotoxicity, pH, osmolality); information on the cell line is insufficient (no data on mycoplasma contamination, karyotype stability, cell doubling times). The number of chromosomes and number of SCE per chromosome is not stated, the number of cells treated is unclear since this information is lacking; the concentration is specified in µg/mL, which is pointless for adherent cell cultures; the statistically significant increase of the SCE frequency at the intermediate concentration is not discussed or was not tested in an independent experiment.
- for the MN assay, historical control data are neither shown nor discussed, the evaluation and scoring criteria are not stated. Cytotoxicity, as indicated by the replication index (RI), was not determined; cytotoxicity/cytostasis, as indicated by the cytokinesis-block proliferation index (CBPI), was only determined in an isolated experiment, using an equation different from the OECD 487 test guideline, in which the intermediate concentration (2 µg/mL) was not tested. Confounding factors such as precipitation, cytotoxicity, pH and osmolality are not determined or discussed, the cytochalasin B concentration is not specified. Information on cell line is insufficient (no data on mycoplasma contamination, karyotype stability, cell doubling times).

- for the gene mutation assay: historical control data are neither shown nor discussed, only three analysable concentrations are evaluated (at least four are recommended in OECD TG 476, 1984), evaluation criteria are not stated, confounding factors (such as precipitation, pH, osmolality) are not determined. The results are not discussed in correlation with the cytotoxicity observed. Information on cell line is insufficient (no data on mycoplasma contamination, karyotype stability, cell doubling times).

Based on the above described deficiencies, the Registrant does not agree with the rating of the study by Zhong et al. as given in the CLH report with “reliable with restriction” (RL=2) but proposes to reduce the reliability to “not reliable” (RL=3).

In the CLH report, the publication by Rodrigues-Mercado et al. (2011) was rated as reliable with restriction (RL=2). However, the publication shows reporting deficiencies and deficiencies in the study design as follows:

- First of all, there is currently no guideline available for the conduct of the *in vitro* comet assay. A review by Lovell and Omori (2008) on the robustness of the *in vitro* comet assay has revealed a number of issues that need to be considered in test planning, and the review by Frötschel R (2015) highlighted that there is no advantage of using the *in vitro* comet over the established *in vitro* mutagenicity assays and that in the cases investigated, the *in vitro* comet had no relevance for the hazard assessment.
- In addition to the generic drawbacks of the *in vitro* comet assay as detailed above, the study by Rodrigues-Mercado et al. (2011) shows further shortcomings: the evaluation criteria are not specified, resulting in unclear evaluation results, since information on scoreable vs. un-scoreable cells is not provided and the occurrence of hedgehogs is not given. Crucial experimental details on e.g. alkali unwinding time and electrophoresis conditions are not provided. Characterisation of the cells is insufficient and, as numbers of donors are not specified, it is unclear whether samples are pooled. Confounding factors (precipitation, pH, osmolality) are not discussed, positive control results are not shown or discussed, type of negative control is not clearly specified. According to the authors, the frequency of cells with DNA damage was only 4% in the cultures treated with the test material. These data are, however, not shown or discussed in the context of the findings.; laboratory’s proficiency in preparing and analysing comets is not demonstrated, since e.g. historical control data are not shown or discussed.

Based on the above described extensive deficiencies, the Registrant (i) considers the above described non-standard assay as poorly conducted and (ii) of no relevance for classification purposes. Overall, the Registrant does not agree with the rating of the study by Rodrigues-Mercado et al. as given in the CLH report as “reliable with restriction” (RL=2) but proposes to reduce the reliability to “not reliable” (RL=3).

The publication by Kleinsasser et al. (2003) on an *in vitro* comet assay in human nasal epithelial cells and lymphocytes was rated in the CLH report as reliable with restriction (RL=2). However, the publication shows major reporting and methodological deficiencies as well as deficiencies in the study design.

- Test item characterization is insufficient (purity not stated; physical appearance, test material origin and CAS No. not specified). The age of donors was too high (21-61) compared with the recommendations given for other *in vitro* genotoxicity assays, i.e. recommending a range of 18-35 years of age for the donors. There is unclear information on donor samples. It is only stated that 17 patients were included in this study (14 males, 3 females, 21-61 years of age) and that six patients donated mucosal cells, six patients donated lymphocytes, and five patients donated both. Thus, eleven nasal mucosal cell samples and eleven lymphocytes sample were available and used for the assay. However, it remains unclear which donors contributed to which samples. Thus, the donors’ sex and age cannot be correlated with the samples used.
- Confounding factors such as smoking behaviour are not documented. Further, the authors stated that “mucosa was harvested during surgery for restoring the nasal air passage”, and further “patients were otherwise healthy”. Based on the information given, the health status from each patient is not fully clear, especially for those “patients”, which donated only blood samples. Moreover, the reason for the surgery was not provided and it is therefore unclear whether the tissue was void of pre-existing damage.
- Apparently, no positive control was applied, which does not allow a conclusion on the sensitivity of the test system. The results are only represented as median with standard deviation. Means are, however, not shown. Statistical testing results are not specified for each concentration and a trend test was not performed. Information on scoring and evaluation criteria is missing. Occurrence of hedgehogs is not specified. Historical control data is not shown or discussed. Confounding factors (precipitation, pH, osmolality) are not discussed.

Based on the above described deficiencies, the Registrant does not agree with the rating of the study by Kleinsasser et al. as given in the CLH report as “reliable with restriction” (RL=2) but proposes to reduce the reliability to “not reliable” (RL=3).

The publication by Ramirez et al. (1997) was rated in the CLH report as reliable with restriction (RL=2). However, the publication shows major reporting and methodological deficiencies as well as deficiencies in the study design.

- First, the study solely investigates the presence of aneuploidy in cultured peripheral blood lymphocytes of two donors via FISH assay. However, this investigation is commonly used in *in vitro* micronucleus assays in cases when positive findings are received, to further differentiate between a clastogenic and aneugenic effect. Consequently, the study does not comply with a standard *in vitro* micronucleus test (OECD 487) and can therefore not be rated for reliability due to a complete lack of compliance with accepted guidelines for comparison.
- In addition to the (formal) non-guideline compliance, the study shows additional shortcomings: The age of the donors is not specified and it is unclear if and how the samples were pooled, since results are reported for 2 donors, although the methods section reports 3 (2 females, 1 male). It is further known that female donors show a higher clastogenic background level than males, which was not controlled for. The authors used colchicine as positive control substance for aneuploidy, which is very difficult to dose as positive control due to its very narrow therapeutic index, which is why vincristine is usually used as positive control for aneuploidy. The percentage of numerical aberration in chromosome 1 and 7 appears excessively high (approx. 10-15% in negative control and 14-20% in positive control for both hyper- and hypoploidy). Test material is insufficiently characterised (purity, physical appearance not specified), procedure on preparation of the test material is not described (unknown vehicle/data). Evaluation and scoring criteria are not given, vehicle control data and cytotoxicity were not measured or are not reported and authors do not provide historical control data to validate the findings. No information on hybridisation overlaps, potentially resulting in false negatives/positives. Confounding factors (such as precipitation, pH, osmolality) are not discussed and measurements were performed on single cultures as no replicates were used. Results are given without standard deviation or confidence interval.

Based on the above described shortcomings, the results of this publication cannot be rated for their quality, due to a missing guideline and serious experimental and reporting deficiencies. Consequently, this reported study should be rated as not reliable (RL=3) and disregarded for the weight-of-evidence assessment.

<u>*In vivo* genetic toxicity</u>

The publication by Devereux et al. (2002) merely constitutes an investigation on the association of lung carcinoma with MAPK activity, K-ras mutation and loss of heterozygosity in the K-ras region. In the CLH report this is misinterpreted as oncogene alterations indicative of divanadium pentaoxide-induced mutagenicity. This is an incorrect interpretation of the data, whereby negative results were obtained in the micronucleus assay in mice exposed via inhalation for 13 weeks (NTP, 2002). Since the study did not follow any guideline, a rating according to Klimisch does not apply.

The gene mutation study in transgenic Big Blue mice by Manjanatha et al. (2015) was rated in the CLH report as not reliable (RL=3), without further explanation how this rating was derived. The publication reports an *in vivo* study according to OECD 488 with a test procedure in accordance with generally accepted scientific standards and the materials, methods and results described in sufficient detail. The Registrant does not agree with the rating of this study as not reliable. Given the quality of the study and its documentation, the Registrant proposes to change it to reliable without restriction (RL=1).

The study by Altamirano-Lozano et al. (1999) concluded that vanadium pentaoxide (V_2O_5) induced DNA damage in several organs and tissues. The sensitivity to vanadium pentaoxide-induced DNA damage varied between the organs. The publication by Altamirano-Lozano et al. was rated in the CLH report as reliable with restriction (RL=2). However, the publication shows some major reporting and methodological deficiencies as well as deficiencies in the study design.

The administration of the test item via intraperitoneal injection is a non-physiological route of exposure (see also comment on administration routes further below) and should not be considered relevant for hazard assessment of industrial chemicals. The authors make contradictory statements on dosing, stating identical numbers but with different units (g/g bw vs. µg/g bw). The assessment of cell viability was done with a dual stain technique, which provides information on membrane integrity and also on metabolic activity (derived from esterase activity). Although information on viability and vitality is available, only cell viability is reported. The viability for all six tissue control samples is 100%, which appears implausible knowing that cells obtained from spleen or heart require intensive mechanical manipulation and/or enzymatic digestions whereas cells obtained from bone marrow are obtainable without further manipulation.
The authors draw questionable conclusions from the study results:

- "In heart cells a significant dose-response increase [tailed cells; according to statistics section and start of the section] was found." However, the values were 69.0, 43.9, 33.8 and 26.3% at 0, 5.75, 11.5 and 23 µg/g, respectively. The Pearson correlation (not included in the publication) showed a factor of -0.9, indicating in fact a <u>negative</u> dose-response.
- Most tissues showed a markedly increased proportion of untailed cells (compared to negative control), especially at high doses (liver: 1.4-fold, lung: 3-fold, heart: 2.4-fold, kidney: 2.1-fold). These findings are contradictory to the other results, indicating higher damage and higher proportion of cells with high damage. In the lung, for example, there were three times more cells untailed, whereas the proportion of cells with low damage was about 5.4-fold (ca. 12.5% vs. 67.5%) lower, when compared to concurrent control. This finding is somewhat implausible. It would be anticipated that a DNA-damaging substance would increase DNA damage in a dose-response relationship and not to markedly increase cells with high damage at a single treatment while simultaneously cells did not have any damage at that very treatment.
- The authors do not provide information on scoring criteria, scoreable cells or "hedgehog" occurrence. The methods section lacks details, since information on temperature and voltage [V/cm] of electrophoresis, time elapsed between cell harvest and preparation is missing. The scoring was not performed automatically or semi-automatically, and criteria for data evaluation are not stated. Information on animal husbandry, application volumes, clinical signs is not provided.

Based on the above described deficiencies, the Registrant does not agree with the rating of the study by Altamirano-Lozano et al. as given in the CLH report as "reliable with restriction" (RL=2) but proposes to reduce the reliability to "not reliable" (RL=3).

The publication by Altamirano-Lozano et al. (1996) reports on results obtained in an *in vivo* comet assay as well as a dominant lethal assay – both are reported separately in the CLH report. Since both studies show similar shortcomings in study design, reporting and interpretation, they are discussed together here. The CLH report rates both studies as reliable with restriction (RL=2), which in the light of the shortcomings appears unjustified.

- In both studies, the (poorly characterised) test substance divanadium pentaoxide was administered via intraperitoneal injection. Intraperitoneal injection is a non-physiological route of exposure (see also comment on administration routes further below) and should not be considered relevant for hazard assessment of industrial chemicals.
- The health status of the male CD-1 mice may be questioned, since authors state a weight of 26-29 g for animals aged 45 days – information from a commercial breeder suggests a mean body weight of 34-36 g for a 6-7 weeks CD-1 mouse. No further information on the health status, housing conditions and clinical observations of the animals was provided.

Comments specifically on the comet assay:

- The cell isolation and processing as well as the electrophoresis and scoring is insufficiently described, since information on: time elapsed between cell harvest and preparation, neutralization step after alkalic treatment, scoring system, scoring criteria and "hedgehog" occurrence is missing. The degree of DNA damage was assessed by migration length, which is not the recommended parameter for DNA damage (OECD 489 recommends %DNA in tail). Cytotoxicity as a potential confounder of the read-out was not determined and is not discussed. However, in the repeated exposure experiment of the same study, sperm counts decreased by 75% compared to controls.

- As generic comment on the suitability of the comet assay for germ cells, the OECD guideline 489 (2016) states: "*Whilst there may be interest in genotoxic effects in germ cells, it should be noted that the standard alkaline comet assay as described in this guideline is not considered appropriate to measure DNA strand breaks in mature germ cells. Since high and variable background levels in DNA damage were reported in a literature review on the use of the comet assay for germ cell genotoxicity (Speit et al. 2009), protocol modifications together with improved standardization and validation studies are deemed necessary before the comet assay on mature germ cells (e.g. sperm) can be included in the test guideline.*"

Comments specifically on the dominant lethal assay:

- For the fertility assessment, data on general toxicity are missing. Mortality, clinical signs, and body weights are not specified. However, when interpolating the data from the sperm assessment, it can be seen that in this study, 60 days of treatment lead to reduced body weights in the male mice. The body weights were reduced by 10.2% compared to the initial body weight and were 21% lower, when compared to the vehicle control group. Similarly, in the sperm assessment, clinical signs are not specified, and the body weight data is only partially discussed. The body weights are only compared within the test groups (initial vs terminal), however, testis weight and sperm count/motility/morphological abnormalities are compared between groups. Comparison of the terminal body weights revealed that all treatment groups show distinctly lower terminal body weights (66.8-87% of the control group). Moreover, the absolute testis weights are found to be reduced. However, the relative testis weight is similar compared to the control animals. Only limited information was presented on the female animals.

Based on the above described deficiencies, the Registrant does not agree with the rating of the study by Altamirano-Lozano et al. as given in the CLH report as "reliable with restriction" (RL=2) but proposes to reduce the reliability to "not reliable" (RL=3) for both experiments reported.

The publication by Altamirano-Lozano et al. (1993) reports results obtained in a study similar to a sister chromatid exchange assay. In the CLH report, the study is rated as reliable with restriction (RL=2), which in the light of the shortcomings discussed below appears unjustified.

- The publication appears to be a short communication, since relevant information in the material and method and results section is missing. A full quality assessment of the study is therefore not possible due to the lack of data. The administration of the test item via intraperitoneal injection is a non-physiological route of exposure (see also comment on administration routes further below) and should not be considered relevant for hazard assessment of industrial chemicals.

Based on the lack of information to allow a complete quality assessment, the reference should be rated as not rateable (RL=4).

The publications by Paramanik and Rajalakshmi (2013), Rodríguez-Lara et al. (2013 and 2016a), Sun (1987, DNA synthesis inhibition assay) and Cano-Gutiérrez et al. (2012) merely investigate mechanistic endpoints for which the authors claim relevance for the mutagenic/carcinogenic potential of divanadium pentaoxide. Whereas these publications may be of possible relevance for the mode of action analysis and risk assessment of divanadium pentaoxide, they do not represent state of the art mutagenicity studies. Since none of these studies were conducted in accordance with an accepted guideline, a rating according to Klimisch is not possible and should therefore not be presented here.

Conclusions on *in vivo* genotoxicity

It appears that the CLH report considers *in vivo* studies using the intraperitoneal route of administration equally relevant and adequate for classification purposes as studies using a physiological route of exposure. In contrast, the Registrant proposes an evaluation in-line with (i) the provisions laid down in regulation (EC) 1272/2008 (ii) the test guidelines specified in Article 13(3) of the REACH regulation and (iii) ECHA guidance (Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.4):

i. the CLP Regulation states in Annex I: 3.5.2.3.9 "*The relevance of the route of exposure used in the study of the substance compared to the most likely route of human exposure shall also be taken into account.*". For industrial chemicals, the inhalation, dermal and oral route are considered relevant for human exposure. The intraperitoneal injection may only be relevant for substances used in pharmaceutical applications, which is clearly not the case for inorganic vanadium substances. Consequently, only studies via physiological routes should be considered relevant as foreseen in the CLP Regulation.

ii. all recent OECD Test Guidelines for *in vivo* genetic toxicology testing (OECD 474, 475, 483, 488 and 489) state in section "Administration of doses" that "*Intraperitoneal injection is generally not recommended since it is not an intended route of human exposure, and should only be used with specific scientific justification.*". As stated above, the intraperitoneal route may be relevant for pharmaceutical applications, but not for industrial chemicals. None of the studies using the intraperitoneal (IP) route described in the CLH report provides a justification for this route of exposure. Consequently, these studies should be considered non-compliant with the validated and accepted test guidelines and not relevant for the purposes of classification.

iii. the ECHA Guidance on IR & CSA, Chapter R.4: Evaluation of available information (2011) provides guidance on the reliability rating of studies (Chapter 4.2, page 3). Studies are considered not reliable in case organisms/test systems were used which are not relevant in relation to the exposure (e.g. non-physiological pathways of application).

In a weight-of-evidence approach, studies using a non-physiological route of administration should therefore be considered to contribute to the overall assessment to a much lesser extent than information obtained respecting the relevant test guidelines and guidance documents.

In accordance with regulation (EC) 1272/2008, Annex I: 3.5.2.3.3 test systems assessing heritable alterations of DNA (such as *in vitro* gene mutation in the hprt locus) and systems investigating the induction of unspecific and transient DNA damage (such as comet assay) should be assessed differently: "*Classification for heritable effects in human germ cells is made on the basis of well conducted, sufficiently validated tests, preferably as described in Regulation (EC) No 440/2008 adopted in accordance with Article 13(3) of Regulation (EC) No 1907/2006 ('Test Method Regulation') such as those listed in the following paragraphs.". In vitro* DNA damage tests are neither validated nor were these tests conducted in accordance with an accepted guideline. The information gained from such systems should therefore be evaluated with great care and regarded to contribute to the overall evidence to a much lesser extent than information gained from tests using standardised, validated and accepted guidelines.

The summary presented in the CLH report does not take into account the rating of the studies according to their (i) intrinsic reliability and (ii) adequacy for classification purposes, but merely claims performing a weight-of-evidence analysis taking into account all evidence in the database: "*that these studies were considered in a weight of evidence analysis of the all genotoxic database*". Such an approach should adequately evaluate the quality and reliability of the evidence from all sources and come to a balanced conclusion (see also comment on lack of quality assessment given in the "General comments").

The Registrant notes that a publication on the assessment of the weight-of-evidence by the dossier submitter exists (ANSES, 2015), which describes how a weight-of-evidence assessment approach can be conducted. The procedure described therein foresees four sequential steps, which include a thorough quality assessment. This procedure has apparently not been followed during the compilation of the section on genetic toxicity.

The listing of the (allegedly) substance-specific effects of divanadium pentaoxide is a repetition of the summary tables presented on pages 34-45 in free text form. The ECHA Guidance on the preparation of CLH dossiers (Version 2.0, August 2014) and the CLH report template provided on the ECHA website[4] highlights that this section is meant to "*conclude on the relevance and uncertainty or controversy of the provided data. If ambiguous results are presented please discuss why different results are observed in different tests and the basis of the final conclusion on whether the substance is genotoxic or not. If applicable, please consider the significance of any deviations from the guideline*", which was not provided by the authors of the CLH report.
The section "Comparison with the CLP criteria" is intended to compare the evidence obtained in the weight-of-evidence analysis of all available data with the classification criteria given in the regulation (EC) 1272/2008. As discussed above, the weight-of-evidence analysis as employed in the CLH report does not weigh the relevance, reliability and adequacy of the reported studies and thereby does not conclude on the hazard for genetic toxicity in a balanced manner.

Finally, this section merely cites four studies out of the 35 summarised in the previous tables for the comparison with the CLP criteria. It is unexplained why this selective choice of studies was made. It appears somewhat biased that, although all available evidence was presented in the previous pages, only four studies are considered adequate for the comparison with the CLP criteria.

The Registrant would therefore appreciate a balanced weight-of-evidence assessment for classification purposes.

<u>Human data relevant for germ cell mutagenicity</u>

The CLH report refers to a study by Ehrlich et al. (2008). The Registrant is of the opinion that this study has several substantial shortcomings, which limits its usefulness for this classification severely.

The following is only a short summary but a comprehensive review and evaluation of the findings by Ehrlich et al. are attached in Appendix I. The study relates to a plant that manufactured multiple metal containing products. It is important to note that there are no data documenting divanadium pentaoxide exposure; only measurements of blood vanadium are reported with no speciation or data on evidence that could correlate it to V_2O_5 exposure. Workers were in fact exposed to various metal-containing raw materials, intermediate products and finished materials, but also to other chemicals used in the production process and to other chemical elements and compounds contained in the raw materials. Thus, correlating any observed effects exclusively to divanadium pentaoxide exposure without a detailed and careful analysis of other potential confounders cannot be considered appropriate. This study cannot be included in the assessment of divanadium pentaoxide without documentation of exposure and of the absence of co-exposure to other substances.

As a result, the findings and conclusions should be considered inconclusive. There is no unequivocal evidence presented to support attribution of the differences in the control jail warden group compared to the workers as being caused by divanadium pentaoxide. Differences in age, smoking and plasma vitamin B12 and B6 levels raise questions about the suitability of the comparison of the two groups. Whereas the frequency of micronuclei and other endpoints were different in workers compared to jail warden controls, the authors failed to find any correlation of these effects with the measured vanadium level of the blood. The comet assay, reported to be a sensitive indicator of DNA damage in human lymphocytes *in vivo* was not different in workers compared to ail warden controls even though other affected endpoints reported, such as micronuclei and nuclear bridges, are indicative of such effects.

[4] CLH report template (with explanations) (updated 13/02/2017):
https://echa.europa.eu/documents/10162/13563/clh_report_template_en.doc/b89cfbb2-aa3c-4a5c-9c15-0b1d7c166028

Conclusions of the weight-of-evidence approach:

(i) The Registrant concludes that the weight of evidence for the *in vitro* genetic toxicity database can be summarised as follows:

- the study by Sun (1987) is only available as a very short communication. Despite numerous efforts by both the DS and the Registrant, no copy of the original study could be obtained. Consequently, an assessment of the quality of the work is not possible, and thus the reliability must be rated as “not assignable” (RL=4) and this information should consequently not be considered in the hazard assessment.

- none of the test systems investigating gene mutations such as the Ames test and gene mutation assays in mammalian cells returned a positive finding, irrespective of the studies’ quality (Lloyd, 2010b; NTP, 2002; Zhong et al. 1994).

- a positive finding was obtained in a reliable *in vitro* micronucleus test in human peripheral blood lymphocytes as well as in a human lymphoblastoid cell line (Lloyd, 2010b). This is considered as a concern with regard to the clastogenic effects of divanadium pentaoxide, triggering the need to consider corresponding available *in vivo* data.

- however, the positive micronucleus findings in human lymphocytes could not be repeated in two further studies rated as reliable with restriction when reporting the results of an *in vitro* micronucleus and *in vitro* chromosomal aberration test in mammalian cells (Gibson et al. 1997; Rodríguez-Mercado et al. 2010).

- mixed results were received in three studies on *in vitro* clastogenicity/aneugenicity rated as not reliable (Zhong et al. 1994; Roldán & Altamirano 1990; Ramírez et al. 1997)

- positive results were received in three *in vitro* DNA damage tests, rated as not reliable (Rodríguez-Mercado et al. 2011, Kleinsasser et al. 2003, Rojas et al. 1996)

Based on the overall evidence for *in vitro* test systems, it is concluded that divanadium pentaoxide is non-mutagenic in bacterial and mammalian cell systems. However, there is some indication for concern seen in a reliable micronucleus study, showing a positive *in vitro* response in human lymphocytes.

(ii) The weight of evidence for the *in vivo* genetic toxicity database can be summarised as follows:

- no increase in mutant frequencies was seen in lungs investigated in a Big Blue mouse assay (reliable without restriction), when exposed to otherwise tumorigenic concentrations of divanadium pentaoxide for up to 8 weeks (Manjanatha et al. 2015).

- negative results were obtained in an *in vivo* micronucleus test (reliable without restriction) in bone marrow after oral exposure of divanadium pentaoxide to male rats by gavage up to the maximum tolerated dose of 20 mg/kg/day (Beevers 2011).

- negative results were likewise observed in an *in vivo* micronucleus test (reliable with restriction) in peripheral blood after sub-chronic inhalation exposure of divanadium pentaoxide to mice at five concentrations between 1 and 16 mg/m³ (NTP, 2002).

- negative results were received in the comet assay of lavage cells of female mice exposed via nose only inhalation of concentrations of 0.25 to 4 mg/m³ divanadium pentaoxide (Schuler et al. 2011).

- positive results were noted in a number of *in vivo* clastogenicity and DNA damage tests, which however exhibit severe shortcomings in study design and reporting, and which were largely achieved by applying the substance via a non-physiological route of exposure. Further, the majority of these publications stems from the same working group and can therefore not be viewed as independent experiments (Altamirano-Lozano et al. 1996, Altamirano-Lozano et al. 1999, García-Rodríguez et al. 2016, Rojas-Lemus et al. 2014).

- a number of studies show mixed results for mechanistic investigations not directly linked to heritable mutations (such as changes in the cytoskeleton or cellular morphological changes. Since these studies are of no relevance for classification purposes, they should be disregarded (e.g. Devereux et al. 2002, Paramanik & Rajalakshmi 2013, Altamirano-Lozano 1993).

The negative outcome in the mutation study in Big Blue mice (Manjanatha et al. 2015) corroborates the negative findings in the *in vitro* assays and thereby confirms that divanadium pentaoxide is not a primary acting mutagen (Non-DNA reactive). Two reliable *in vivo* micronucleus studies via oral and inhalation route of exposure did not show an increase in micronucleus frequency in peripheral blood (NTP, 2002; Beevers, 2011). Thus, the clastogenic potential of divanadium pentaoxide *in vitro* has been addressed by negative *in vivo* micronucleus results.

Based on the overall database of genetic toxicity studies, it is concluded that divanadium pentaoxide does not elicit any mutagenic activity either in bacterial or mammalian test systems. Equivocal evidence indicates that genotoxic (non-DNA reactive) effects are induced *in vitro*, manifest as DNA strand or chromosome breaks. These are considered secondary to inflammatory changes and do not represent direct genotoxicity. Based on strong negative evidence from reliable *in vivo* studies and by applying a weight-of-evidence approach, it can be concluded that effective protective processes exist *in vivo* to prevent genetic toxicity with relevance for humans from divanadium pentaoxide.

Conclusion on classification and labelling for germ cell mutagenicity:
The weight-of-evidence analysis of the entire genotoxicity database does not show a clear evidence of germ cell mutagenicity with relevance for humans. Consequently, divanadium pentaoxide should not be classified in Category 1B. On the basis of the information obtained from highly relevant and reliable studies and the limitations of the remaining studies, the current classification as **Germ Cell Mutagenicity Category 2** appears as overtly conservative.

2.6 Carcinogenicity

(CLH report Section 10.9, Page 50-56):
In the CLH proposal, the decisive study with respect to the proposed classification for **Carc. 1B, May cause cancer (H350)** is the 2-year carcinogenicity study in F344/N rats and B6C3F1 mice (NTP, 2002) (cf. Table 18). The details of this study are described in Annex I to the CLH report “Proposal for Harmonised Classification and Labelling, Divanadium pentaoxide (Version 2.0, August 2019).”

These NTP studies in rats and mice with aerosolised divanadium pentaoxide are used as key studies in sections 10.9.1: Short summary and overall relevance of the provided information on carcinogenicity, 10.9.2 Comparison with the CLP criteria, and 10.9.3 Conclusions on classification and labelling for carcinogenicity. In brief, male and female F344/N rats and B6C3F1 mice were exposed to atmospheres containing aerosols of 0, 0.5, 1 or 2 mg/m^3 (rats) or 0, 1, 2, or 4 mg/m^3 (mice) of respirable particulates of crystalline divanadium pentaoxide by chamber inhalation for 6 hours/day, 5 days/week for two years.

By applying a weight-of-evidence approach, the evidence of carcinogenicity in mice of both sexes (NTP, 2002) is not considered to be sufficient for classification of divanadium pentaoxide as a Category 1B substance. Instead, taking into account a number of facts and limitations discussed in this comment, a **classification in Category 2** seems to be appropriate and adequately conservative. Additionally, in the opinion of the Registrant, a route-specific classification for carcinogenicity (i.e. inhalation) is considered justified by the existing animal and toxicokinetic data and the likely mode of action for the induced mouse lung tumours.

Comments on tumour formation in the lungs of rats and mice

Rats:

The incidences of alveolar/bronchiolar adenoma (including multiple) in males were 4/50, 8/49, 5/48, and 6/50 for the exposure concentrations of 0, 0.5, 1, and 2 mg/m3, respectively. Incidences of alveolar/bronchiolar carcinoma (including multiple) were 0/50, 3/49, 1/48, and 3/50, and of alveolar/bronchiolar adenoma and carcinoma (combined) 4/50, 10/49, 6/48, and 9/50 for the respective concentrations. In females, the incidences of alveolar/bronchiolar adenoma were 0/49, 3/49, 1/50, and 0/50, and those of alveolar/bronchiolar adenoma and carcinoma were 0/49, 3/49, 1/50, and 1/50 at the respective concentrations.

Initially, NTP (2002) stated that there were no statistically significant pairwise differences in the poly-3 test of lung tumour rates between treated and concurrent control group rats of either sex. The conclusion drawn by NTP that "some evidence of carcinogenic activity in male F344/N rats" and "equivocal evidence of carcinogenic activity in female F344/N rats" was based on comparisons of divanadium pentaoxide-treated group incidence rates with limited incidence lung cancer rate ranges for historical control groups from 9 (or 10, for female rats) previous carcinogenicity studies conducted with the NTP-2000 diet (as used in the V_2O_5 bioassay) and from the earlier NTP historical database using the NIH-07 diet (16 studies).

Later, NTP (2011) published a revised summary report of tumour incidence rates in control groups of F344/N rats fed the NTP-2000 diet in 25 carcinogenicity studies performed subsequent to the V_2O_5 study (NTP, 2002). Using these additional and more relevant NTP historical control data (NTP 2011) for supplementation of the NTP-2000 historical control data summarized in the V_2O_5 bioassay report (NTP 2002), Starr et al. (2012) re-evaluated the evidence regarding the carcinogenicity of divanadium pentaoxide in rats in relation to this larger and more relevant historical lung tumour database:

The re-analysis with the pooled historical control tumour incidence data from 34 NTP studies (all using the NTP-2000 diet) resulted in a widening of the background tumour incidence ranges reported in the V_2O_5 Technical Report 507 (NTP 2002). In particular, the historical control range for male rat alveolar/bronchiolar carcinoma increased from 0-2% (NTP TR 507) to 0-6%. Thus, the alveolar/bronchiolar carcinoma incidence rates in the low and high V_2O_5 concentration groups of male rats (3/49 and 3/50, respectively) were at the upper end but within the updated historical control range for this tumour, not three times above it, as concluded on the limited NTP TR 507 database. With respect to the incidence of alveolar/bronchiolar adenoma in the 0.5 mg/m^3 V_2O_5 group of female rats (3/49), this incidence is within the updated historical control range for this tumour of 0-8%, not at the upper end of the range. The analyses also characterized the concurrent male rat control group from the V_2O_5 bioassay as possibly being an "outlier" relative to the expanded historical control database, at least in relation to lung tumour incidence. That would account for the fact that the unadjusted male alveolar/bronchiolar adenoma incidence rate (8/49) at 0.5 mg/m^3 and the unadjusted male alveolar/bronchiolar adenoma or carcinoma incidence rates at 0.5 and 2 mg/m^3 (10/49 and 9/50, respectively) exceed the upper bound of the appropriate historical control incidence rate ranges (0-12% and 0-14%, respectively) for these tumours.

Taken altogether, the use of historical control data from carcinogenicity studies performed subsequent to a given bioassay in evaluating the evidence regarding the carcinogenicity of a substance is argued for as follows (Starr et al. 2012):

None of the poly-3 adjusted pairwise comparisons of alveolar/bronchiolar tumour incidences in treated and concurrent control groups of male or female rats from the V_2O_5 study were statistically significant (NTP 2002). Further, the carcinogenicity conclusions drawn by NTP for male and female F344/N rat alveolar/bronchiolar tumours in the V_2O_5 study (NTP 2002) as providing "some" and "equivocal" evidence of carcinogenic activity, respectively, are entirely based on the comparisons of the treated group tumour incidence rates in the V_2O_5 bioassay with tumour incidence rate ranges for earlier historical control groups, only a limited number of which were fed the same NTP-2000 diet. Importantly, NTP (2002) recognized that the additional inclusion and use of the earlier NTP-07 diet historical control database might be inappropriate because tumour incidence rates can be affected by diet.

The use of additional historical control data for tumour incidences from 25 NTP carcinogenicity studies with F344/N rats conducted subsequent to the V_2O_5 study, all of which employed the NTP-2000 diet (NTP 2011), resulted in an extended database for assessment of the historical control incidence rate ranges of alveolar/bronchiolar neoplasms.

Based on their comparative re-evaluation of the observed lung tumour incidences in rats with pooled historical control tumour incidence data from 34 NTP studies, Starr et al. (2012) came to the conclusion that there is "no" evidence of divanadium pentaoxide carcinogenicity in male or female F344/N rats, which is in clear contradiction to the statement in the CLH Report with respect to lung tumour formation based on the NTP (2002) conclusions.

Mice:

In male mice, the incidences of alveolar/bronchiolar adenoma (including multiple) were 13/50, 16/50, 26/50, and 15/50 for the exposure concentrations of 0, 1, 2 and 4 mg/m^3, respectively. Incidences of alveolar/bronchiolar carcinoma (including multiple) were 12/50, 29/50, 30/50, and 35/50. Alveolar/bronchiolar adenoma and carcinoma (combined) occurred with statistically significant increased incidences in all exposed males (22/50, 42/50, 43/50, 43/50). In female mice, the incidences of alveolar/bronchiolar adenoma (including multiple) were 1/50, 17/50, 23/50, and 19/50, and those of alveolar/bronchiolar carcinoma (including multiple) were 0/50, 23/50, 18/50, and 22/50. Alveolar/bronchiolar adenoma and carcinoma (combined) occurred with incidences of 1/50, 32/50, 35/50, and 32/50.

The incidences of alveolar/bronchiolar carcinoma and alveolar/bronchiolar adenoma or carcinoma (combined) were significantly increased at a maximum level across all three tested concentrations in exposed groups of male and female mice. The incidences of alveolar/bronchiolar adenoma were significantly increased in males exposed to 2 mg/m^3 and in all groups of exposed females (NTP, 2002).

The significantly increased rates of tumours (alveolar/bronchiolar carcinoma) seen in the lung of all mice (males and females) exposed to 1, 2, or 4 mg/m^3 as compared with those in the chamber controls amounted to 58%, 60%, 70% vs. 24% for males and 46%, 36%, 44% vs. 0% for females, respectively, thus exceeding the historical ranges for controls (all routes) given the NTP-2000 diet.

NTP concluded that there was clear evidence of carcinogenic activity of divanadium pentaoxide in male and female B6C3F1 mice (NTP, 2002). However, a flat plateau-like dose response for lung tumour formation in mice, especially in males, was seen with increasing V_2O_5 concentrations. These tumour rates do not appear to be dose-related over the narrow range of V_2O_5 concentrations that were tested. Poly-3-adjusted Cochran-Armitage trend test analyses without inclusion of the lung tumour incidence data from control group mice confirmed the absence of any significant dose-related effect on mouse lung tumour incidence in the study groups exposed to 1, 2 or 4 mg/m^3 V_2O_5 (Starr & MacGregor, 2014).

NTP chronic study designs are comprehensive and include evaluation of an extensive list of tissues from all animals at all levels tested. Following these extensive tissue microscopic evaluations, only the respiratory tract was found to be affected in this 2-year chronic divanadium pentaoxide inhalation study (NTP, 2002). There was evidence of carcinogenic activity of V_2O_5 in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms. Exposure to divanadium pentaoxide caused a spectrum of non-neoplastic lesions in the respiratory tract (nose, larynx, and lung) including alveolar and bronchiolar epithelial hyperplasia, inflammation, fibrosis, and alveolar histiocytosis of the lung in male and female mice. Hyperplasia of the bronchial lymph node occurred in female mice. No other tissues showed macroscopic or microscopic treatment-related lesions.

Importantly, no increase in tumours was seen at any other target site in mice as well as in rats. This is noteworthy because vanadium levels were measured in blood, indicating internal systemic exposure and that the form of vanadium circulating would appear not to be toxic at the levels measured (NTP, 2002).

Lung tumour formation in mice, especially in males, showed a flat, plateau-like dose response with increasing V_2O_5 concentrations (NTP, 2002). Further, the absence of any significant dose-related effect on mouse lung tumour incidence in the study groups exposed to concentrations of 1, 2 or 4 mg/m^3 V_2O_5 was confirmed by poly-3-adjusted Cochran-Armitage trend test analyses without inclusion of the lung tumour incidence data from control group mice (Starr & MacGregor, 2014). Thus, the tumour response in B6C3F1 mice exposed to 1-4 mg/m^3 V_2O_5 is considered to be due, at least in part, to other factors than V_2O_5 airborne concentration or vanadium lung burden. The incidences of alveolar epithelial hyperplasia and bronchiolar epithelial hyperplasia were both significantly increased in a dose-dependent manner. However, chronic inflammation and alveolar histiocytic infiltration were observed in lungs of nearly all exposed mice at the end of the NTP study (NTP, 2002), e.g., male mice showed nearly maximum incidences of 42/50, 45/50, 47/50, and 36/50, 45/50, 49/50, respectively, at exposure levels of 1, 2, and 4 mg/m^3. Based on these clear inflammatory responses, it was suggested that all of the tested V_2O_5 concentrations were excessively high, particularly for the low and intermediate tested dose groups, thus resulting in saturation of certain inflammatory mechanisms leading to toxicity.

Findings of the so-called 16-day special study in female mice (NTP 2002, cf. MacGregor et al., unpublished) indicated the occurrence of lung inflammation by Day 13 at exposure levels of 2 mg/m^3 and above. Because the 16-day inhalation study in female B6C3F1 mice by Schuler et al. (2011) showed lung inflammation as well as increased cell proliferation at the same exposure levels, the interpretation of tumour data is confounded by the presence of long-term chronic inflammation, presumably over the whole duration of the two-year exposure duration.

The formation of lung tumours is secondary to chronic inflammatory changes which developed shortly after initiating exposure and persisted throughout the study. Similar to mice, rats had comparable vanadium lung burdens and also showed similar increased lung inflammation and epithelial hyperplasia in response to V_2O_5 exposure for two years, but lung tumorigenicity was not substantiated, indicating a species difference in response to this persistent chronic inflammation.

In a tumour initiation/promotion study in several strains of male mice (Rondini et al., 2010), divanadium pentaoxide administered repeatedly via oropharyngeal aspiration did not initiate lung tumours, but increased tumour multiplicity initiated by methylcholanthrene in A/J and BALB/cJ mice, but not in C57BL/6J mice. Hence, V_2O_5 operated as a tumour promoter in A/J and BALB/cJ mice. These sensitive mice were also found to be more susceptible to V_2O_5-induced pulmonary inflammation.

The genotoxicity experiments (*S. typhimurium* gene mutations and *in vivo* mouse peripheral blood micronucleus assay) included in the NTP (2002) study showed negative results for mutagenic effects. There is also a substantial weight of evidence from other *in vivo* and *in vitro* investigations (Assem & Levy, 2009) that the mode of action underlying the lung tumours in mice in the NTP 2002 study is by non-DNA reactive mechanism(s). The study in female B6C3F1 mice by Schuler et al. (2011) and the initiation-promotion study by Rondini et al. (2010) provide further evidence of a non-genotoxic mechanism for tumour induction by divanadium pentaoxide. Whereas some vanadium compounds have been shown to produce a range of chromosome damages, guideline-conforming, state-of-the-art *in vitro* gene mutation studies performed with all three valency states of vanadium have unequivocally demonstrated an <u>absence</u> of such effects, thus ruling out direct DNA interactions (Lloyd, 2010).

The lack of significant induction of *cII* mutant frequencies in the lungs of male transgenic Big Blue (BB) mice exposed to 1 mg/m^3 V_2O_5 (tumorigenic concentration) by inhalation for up to 8 weeks suggests that divanadium pentaoxide is unlikely to act via a mutagenic mode of action (Manjanatha et al. 2015). Further, the lack of significant changes in levels of *Kras* codon 12 mutations (GGT→GAT or GGT→GTT) following exposure of male BB mice at V_2O_5 concentrations of 1 mg/m^3 for 8 weeks supports the idea that the accumulation of additional *Kras* mutants is not an early event in mouse lung carcinogenesis, and/or that the proliferative advantage of *Kras* mutant clones requires either longer expression times or larger cumulative V_2O_5 exposures (Banda et al. 2015). Furthermore, the data do not provide support either for any direct genotoxic effect of V_2O_5 on *Kras* in the context of the exposure conditions used, or for any early amplification of pre-existing mutation as being involved in the genesis of V_2O_5-induced mouse lung tumours.

Additional evidence that genotoxicity is not a driving force in lung tumour formation by divanadium pentaoxide comes from genomic analyses of lung tissue of female B6C3F1 mice following 90-day V_2O_5 exposure at tumorigenic concentration (2 mg/m^3) (Black et al., 2015). V_2O_5 exposure resulted in 1026 differentially expressed genes, 483 of which were unique to V_2O_5. Functional ontology enrichment indicated amongst others several possible effects on inflammatory pathways. These functional ontology results are entirely consistent with evidence of epithelial hyperplasia, degeneration and inflammation in mouse lung, as evidenced in the NTP (2002) study, but were not indicative of processes traditionally related to tumour initiation. There was no evidence for enrichment of pathways associated with changes in cell cycle arrest/proliferation, DNA-damage, or oxidative stress related pathways with the V_2O_5 differentially expressed genes.

In summary, the *in vivo* genotoxicity studies in mice following inhalation exposure do not support a genotoxic (DNA-reactive) mode of action for divanadium pentaoxide.

Conclusions on carcinogenicity of divanadium pentaoxide

In the view of the Registrant, the NTP (2002) study conducted over only a very narrow and high dose range of divanadium pentaoxide (1 to 4 mg/m^3 in mice, and 0.5 to 2 mg/m^3 in rats) did not provide sufficient data to draw definitive conclusions regarding thresholds and/or mechanisms of action or mode of action. An interpretation of the relevance of the lung tumours, seen in mice at all three concentrations tested, is confounded by the chronic and persistent inflammation, presumed to have occurred over the whole duration of the two-year exposure, and the lack of a dose-response relationship.

Taken together, chronic inhalation exposure of rats and mice to divanadium pentaoxide resulted in inflammation, histiocytosis, hyperplasia and formation of tumours in the lungs only of mice. Taking into consideration the available data on non-neoplastic and neoplastic effects it can be hypothesised that the cancer mode of action for divanadium pentaoxide-induced lung tumours in mice involves chronic inflammation, cell proliferation, hyperplasia of the alveolar and bronchiolar epithelia, alveolar/bronchiolar adenomas, and alveolar/bronchiolar carcinomas. Given that divanadium pentaoxide in contact with aqueous media yields an immediate and strongly acidic pH (Klawonn, 2010d), due to chronic inflammation promoted by this pH effect divanadium pentaoxide may act through an increase in inflammatory-related oxidative stress. However, the reasons for the discordance between rats and mice with respect to formation of lung tumours are currently not elucidated.

Based on the evaluation of available genotoxicity data divanadium pentaoxide is thought to exert genotoxicity by indirect mechanisms (secondary genotoxicity, i.e. chronic inflammation and oxidative stress). Divanadium pentaoxide is not expressing its carcinogenic activity via genotoxicity since tumours did not develop at other organ sites following 2-years of inhalation even though divanadium pentaoxide circulated systemically. The tumour promoting activity in mice, although strain-dependent (Rondini et al., 2010) may be considered part of that mode of action of V_2O_5.

The contribution of hypoxia as further mechanism of carcinogenesis might be suspected because abnormal breathing was observed in some mice, particularly in those exposed to 2 or 4 mg/m^3 (NTP, 2002).

A high degree of discordance between rats and mice in development of chemically induced lung tumours became evident from a statistical analysis of 58 compounds tested in NTP two-year inhalation studies (Smith & Anderson, 2017). Further evaluations of that database suggested that bronchioalveolar lung tumours induced only in mice by non-genotoxic chemicals are of low relevance for human lung cancer risk (Smith et al., 2018). Furthermore, the genomic responses of mice to inflammation are of questionable comparability to those of humans (Seok et al., 2013).

The high degree of discordance between rats and mice with respect to divanadium pentaoxide-induced formation of lung tumours presents a species discordance, and the human relevance of this species difference needs to be elucidated.

For the assessment of a need for classification on carcinogenicity, the criteria of the CLP Regulation were applied, i.e. “A substance is classified in Category 1A for carcinogenicity if the substance is known to have a carcinogenic potential for humans, largely based on human evidence” (Annex I, Table 3.6.1 and 3.6.2.2.3.(a)).

There is inadequate evidence from the available studies on exposure of humans to divanadium pentaoxide for classification into Category 1A, thus the **criterion for category 1A is not fulfilled**.

The evidence relevant to carcinogenicity in experimental animals should be classified into one of the following categories (CLP Regulation, Annex I, 3.6.2.2.3.(b)):

- **Sufficient evidence of carcinogenicity:**

“a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.”

There is clear evidence of induction of benign and malignant tumours in the lung of both sexes of mice in the NTP (2002) study, which is a GLP study. However, the dose selection, which caused persistent chronic inflammation at all three dose concentrations and is clearly against the advice of the OECD TG for such an effect, compromised this study being described as a “well-conducted study” in all other design aspects. A flat, plateau-like dose response for lung tumour formation was seen with increasing divanadium pentaoxide concentrations. However, these tumour rates did not appear to be dose-related over the narrow range of V_2O_5 concentrations that were tested. Poly-3-adjusted Cochran-Armitage trend test analyses without inclusion of the lung tumour incidence data from control group mice confirmed the absence of any significant dose-related effect on mouse lung tumour incidence in the study groups exposed to concentrations of 1, 2 or 4 mg/m^3 V_2O_5 (Starr & MacGregor, 2014).

With respects to rats, there is doubt on the interpretation of the statistically insignificant lung tumour incidences in male and female rats in the GLP-compliant NTP (2002) study because of the limited incidence rate ranges for historical control groups from 9 (or 10, for female rats) previous carcinogenicity studies conducted with the NTP-2000 diet as in the V_2O_5 study. Based on the comparative re-evaluation of the observed tumour incidences in the NTP (2002) study in rats with pooled historical control tumour incidence data from 34 NTP studies (Starr et al., 2012), the conclusion was drawn that there is “no” evidence of divanadium pentaoxide carcinogenicity in male or female F344/N rats.

In conclusion, in view of the above, only the clear evidence of carcinogenicity in both sexes of mice, i.e. in a single species, can be considered for the classification of divanadium pentaoxide. The presence of a dose-response relationship, as well as statistically significant increase over controls, is important to support a claim of causality by a known chemical exposure. However, such a significant dose-related effect on mouse lung tumour incidence was not observed with increasing V_2O_5 concentrations. Thus, the criteria for category 1B are not fulfilled.

- **Limited evidence of carcinogenicity:**

“the data suggest a carcinogenic effect but are limited for making definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increase the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.”

When considering that

1) the carcinogenicity is restricted to a single experiment in mice without dose-response relationship,
2) the tumours induced in mice are a local, site-specific response (no tumours at other sites in mice (or rats) in spite of evidence of ample tissue exposure to the vanadate moiety),
3) tumour induction by secondary mechanisms (presumably non-genotoxic) is suggested,
4) the tumour formation in mice is accompanied by long-standing chronic inflammation,
5) there are questions whether the genomic responses of mice to inflammation are comparable to those of humans,
6) bronchioalveolar lung tumours induced only in mice by non-genotoxic chemicals are of low relevance for human lung cancer risk, and
7) absence of reliable human data,

the Registrant's judgement is that the **classification of divanadium pentaoxide in Carcinogenicity Category 2** would be most appropriate.

According to Annex I, 3.6.2.2.8 of the CLP Regulation, the route of exposure should be limited if it is conclusively proven that no other routes of exposure cause the hazard. Carcinogenicity data by the oral or dermal route are lacking for divanadium pentaoxide. The data available relate only to inhalation exposure of rats and mice to divanadium pentaoxide, which however actually affected only the respiratory tract, and with tumour formation only in mice lungs which is considered a substance-specific local effect.

The extensive tissue microscopic evaluations undertaken in the NTP (2002) study showed no treatment-related lesions in other tissues whatsoever. This is noteworthy because vanadium levels were measured in blood, indicating internal systemic exposure and that the form of vanadium circulating would appear to be not toxic at the levels measured.

In conclusion, any classification of divanadium pentaoxide is proposed to be route-specific, for the following reasons: oral administration of V48-labelled divanadium pentaoxide by gavage results in an absorption rate of about 3% of the administered dose (cf. section 9.1). Low absorption is equally expected by the dermal route. The hypothesized mode of action of V_2O_5 is related to inflammatory processes mediated by the local acidic reaction of V_2O_5 in lung tissues. However, as detailed deposition modelling for V_2O_5 has shown, the overwhelming majority of inhaled V_2O_5 will translocate to the gastro-intestinal tract – there, detailed histopathological evaluation in the NTP studies documented a complete absence effects in the gastro-intestinal tract. Finally, upon contact with aquatic media and moist epithelial surfaces, V_2O_5 will rapidly transform into pentavalent vanadate ions which will be readily distributed throughout the body. The absence of any systemic tumour formation in the NTP studies does not indicate the need to consider any effect other than the observed lung tumour formation in mice for cancer classification purposes.

Additional considerations for classification

Some further factors have to be considered for classification justification (CLP Regulation, Annex I, 3.6.2.2.6.)

- *Tumour type and background incidence:* only lung tumours in mice (adenoma and carcinoma), statistically significant in mice of both sexes, high background incidences of adenoma (26%), carcinoma (24%) and adenoma or carcinoma (44%) in male mice. No statistically significant increases in lung tumours in rats of both sexes.
- *Multi-site responses:* no, neoplasms only in the lung of mice
- *Progression of lesions to malignancy:* yes, carcinoma observed
- *Reduced tumour latency:* yes
- *Whether responses are in single or both sexes:* clear evidence in male and female B6C3F1 mice
- *Whether responses are in a single species or several species:* clear evidence in male and female B6C3F1 mice. No evidence in male or female F344/N rats (Starr et al., 2012). NTP (2002) statistics did not show a significant increase in tumours in either male or female rats. NTP conclusions relied on limited and mainly inappropriate historical data comparisons most of which came from bioassays with a different rodent diet.
- *Structural similarity to a substances(s) for which there is good evidence of carcinogenicity:* no information

- *Routes of exposure:* inhalation, data on oral and dermal route are lacking
- *Comparison of absorption, distribution, metabolism and excretion between test animals and humans:* metal compounds like divanadium pentaoxide are not metabolised. Divanadium pentaoxide will be dissolved in the mammalian body and reduced and/or (re-)oxidated by redox-reactions to different vanadium species.
- *Possibility of a confounding effect of excessive toxicity at test doses:* Very probable. Because the NTP study in mice showed a comparably high degree of chronic lung inflammation and histiocytosis at all exposure levels (e.g. in males 84-94% and 72-98%, respectively), the interpretation of tumour data is confounded by the presence of long-standing chronic inflammation, presumably over the whole two-year exposure duration.
- *Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity:* The discordance between rats and mice with respect to divanadium pentaoxide-induced formation of lung tumours presents a species discordance and the human relevance of this species difference is not known. Analysis of the NTP database of 2-year inhalation studies suggested that lung tumours induced only in mice and not in rats by non-genotoxic chemicals are of low relevance for human lung cancer risk (Smith et al. 2018).

Conclusion on classification and labelling for carcinogenicity:
By applying a weight-of-evidence approach, the evidence of carcinogenicity in mice of both sexes (NTP, 2002) is not considered to be sufficient for classification of divanadium pentaoxide as a Category 1B substance. Instead, when considering a number of facts and limitations discussed in this comment, a **classification in Category 2** seems to be appropriate and adequately conservative. Additionally, in the opinion of the Registrant, a route-specific classification for carcinogenicity (i.e. inhalation) is considered justified by the existing animal and toxicokinetic data and the likely mode of action for the induced mouse lung tumours.

2.7 Reproductive toxicity - Adverse effects on sexual function and fertility

The Registrant considers that the selection of studies for this endpoint in the CLH is not entirely fit for purpose, for the following reasons:

CLH report Section 10.10.1, Page 62-70):
In-line with (i) the provisions laid down in Regulation (EC) 1272/2008, (ii) the test guidelines specified in Article 13(3) of the REACH Regulation, and (iii) ECHA guidance (Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.4), *in vivo* studies using intraperitoneal administration are not equally relevant and adequate for classification purposes as studies using physiological routes of exposure:

The CLP regulation actually states in Annex I, 3.5.2.3.9 "*The relevance of the route of exposure used in the study of the substance compared to the most likely route of human exposure shall also be taken into account.*" For industrial chemicals, only the inhalation, dermal and oral route are considered relevant for human exposure.

In the OECD test guidelines, e.g. OECD 443 Extended One Generation Reproductive Toxicity Study, it is explicitly stated "*Selection of the route should take into consideration the route(s) most relevant for human exposure. Although the protocol is designed for administration of the test chemical through the diet, it can be modified for administration by other routes (drinking water, gavage, inhalation, dermal), depending on the characteristics of the compound and the information required.*" The intraperitoneal route is not listed as a relevant route in this guideline. The CHL report however provides no justification as to why a study using the intraperitoneal route is appropriate for exposure to V_2O_5.

In a weight-of-evidence approach, studies using a non-physiological route of administration should therefore be considered to contribute to the overall assessment to a much lesser extent than information obtained respecting the relevant test guidelines and guidance documents.

Comments on section 10.10.2, Page 70-74, Reproductive toxicity - Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

No guideline-conforming reproduction toxicity studies are available specifically for V_2O_5. Therefore, information is taken from 90-day toxicity studies investigating effects on male and female reproductive organs and various publications of animal studies to evaluate a potential effect of vanadium on fertility and reproduction. Publications of human studies were regarded as not relevant for classification because of contradictory results, thus only animal studies were considered for classification of reproduction toxicity in the CLH report.

Female fertility

With respect to **female fertility**, only a very limited data set is available. Two 90-day NTP studies (2002) in F344 rats and B6C3F1 mice with inhalation exposure (V_2O_5) and a reliability score of 1, and one publication (Altamirano et al. 1991) of a study in rats with IP administration and a reliability score of 3 were considered. The NTP study can be regarded as most relevant for classification and labelling, because of its high reliability, while the study by Altamirano et al. 1991 is of low reliability and was conducted with a non-relevant route of exposure and should therefore by disregarded.

In the NTP study, there was no effect on estrous cycle length in female mice; the statistically significant effect on estrous cycle length in female rats was without clear relation to dose and treatment. Thus, since the effects in female animals were observed only in a single species (rat) and not in the other species (mice), they should be regarded as of limited evidence for postulating a V_2O_5-induced effect on female fertility.

In addition, one study (Morgan & El-Tawil, 2003) with administration of ammonium metavanadate (NH_4VO_3) via drinking water in female Sprague-Dawley rats was considered, although this study shows limitations and is categorized with a reliability score of 3. The results of this study seemingly indicate an effect on female fertility because mating and fertility indices were reduced in treated females mated with untreated males, and the estrous cycle was disturbed in treated females and the total number corpora lutea was reduced.

In conclusion, the evidence of an effect of vanadium exposure on female fertility must be regarded as weak based on the data available for V_2O_5 (NTP, 2002; Altamirano et al. 1991). In one study with inhalation exposure, there was an effect on the estrous cycle length in rats but without any clear relation to dose and treatment. In another study with a non-relevant route of exposure (IP) there was an effect on the ovulation rate, but not on the oestrus cycle. In addition, no effect on estrous cycle length was observed in mice. Therefore, the reported findings clearly lack consistency and may in fact be incidental.

However, the evidence of an effect on female fertility is supported by a study with ammonium metavanadate in rats exposed via drinking water (Morgan & El-Tawil 2003). Although the study shows limitations with respect to the methodology used, the results can be regarded as an indication.

Male fertility

With respect to **male fertility**, two NTP studies (2002) with 90 days of exposure via inhalation in F344 rat and B6C3F1 mice with a reliability of 1 were considered. In summary, the finding in male rats and mice are of only limited evidence for assessing vanadium induced effects on male reproductive organs, and it is doubtful whether this would result in any functional deficit in fertility. In addition, the findings are not consistent between species. The effects in male animals were observed only in a single species (mice) and not in the other species (rat). There was no clear relation to dose and treatment, and thus the findings must be regarded as limited evidence for postulating a vanadium induced effect on male fertility.

A number of other publications with exposure of CD1 mice via inhalation for 12 weeks are considered (Fortoul et al., 2007; Mussali-Galante et al. 2005; Bizarro-Nevares et al. 2016; Rodriguez-Lara et al. 2016b), but the publications are all from the same research group and it is totally unclear, based on the available information published, to understand how many different studies the published data are referring to. All four publications were rated with a reliability score of 3 because of severe limitations. The study design described in these publications is essentially similar and data on the inhalation exposure system and results appears to indicate that only one study (perhaps two) served as the basis for all of the four publications showing different mechanistic endpoints. This assumption is supported by presentation of the exposure level in testes which show essentially the same profile in two of the publications.

Two other studies coming from the same group of researchers (Altamirano-Lozano et al. 1996 and Altamirano et al. 1991) with intraperitoneal injections in mice, rats and another study in (Uche et al. 2008) in male guinea pigs were considered, and the reliability score was judged in the CLH report to be 2 (Ushe et al. 2008, Altamirano-Lozano et al. 1996) or 3 (Altamirano et al. 1991). It is explicitly noted here that the studies show limitations and were conducted via the IP route and should therefore be disregarded, because a non-relevant route of exposure was used.

Further studies in animals exposed via the oral (Morgan & El-Tawil, 2003, Domingo et al. 1986) or IP route (Chandra et al. 2007a, b, 2010) with other inorganic vanadium substances were considered according to the described read-across concept.

In the study published by Morgan & El-Tawil (2003), mating and fertility indices were reduced in treated males paired with untreated females. Reduced weight of testes, epididymis, prostate gland, seminal vesicles, ($p<0.05$), with no reduction in body weight between control and treated males were observed. It should be noted that the mating period was only 5 days in this study. In regulatory toxicity studies, the mating period is 3 weeks and animals are placed only in the study when mating is confirmed. The study was conducted only with one dose which clearly represents an effect level and is not designed to establish a no effect level. In addition, the number of females used for investigations of effects on male mating and fertility indices as well as other developmental endpoints was very low compared to guideline studies. No information on general toxicity is presented, and therefore the study is considered to be RL=3 and of only of limited value for regulatory purposes such as CLH.

The second study conducted with an appropriate route of exposure and with 3 intragastric dose levels of sodium metavanadate published by Domingo et al. (1986) is presented in the CLH report, but not considered at all in the argumentation. In this study, fertility was <u>not</u> affected by treatment of male and female rats with sodium metavanadate. However, this non-guideline reproduction toxicity study with treatment of male and female Sprague-Dawley rats is only of RL=3, because it was conducted only in a small number of animals, and only a limited number of endpoints to evaluate fertility were investigated. Nevertheless, based on this study an effect on fertility cannot be postulated.

It should be noted here, that there is another publication available (Llobet et al. 1993) with treatment of male and female swiss albino mice by the oral route via drinking water at different dose levels of sodium metavanadate which is equally not considered at all in the CLH report. Animals were subjected to mating and evaluation of sperm parameters. In this study, although limited in the way the study was conducted and presented (RL=3), effects on male reproductive performance were observed only at 60 and 80 mg/kg bw/d by a reduction of the percentage of pregnant females, and no effects were observed at 40 mg/kg/bw/d.

Three publications coming from the same group of researchers (Chandra et.al.) and published in 2007 and 2010 refer to one and the same study conducted via IP injections in male Sprague-Dawley rats. Direct comparison of the individual data is not possible, because they are not available in the publications, but looking into the tables of the publication it becomes obvious that the data for the vanadium treated groups (0.4 mg V/kg bw for 26 days) are similar making it likely that the publications are based on the same study. Although the study is well conducted and documented and therefore sufficient information for regulatory purposes is given, the route of exposure is IP and thus not relevant for CLH purposes. Therefore, the studies should be disregarded.

In conclusion:

- there are only two reliable (RL=1) 3-month studies (NTP, 2002) with inhalation exposure of V_2O_5 in male and female rats and mice available which give only limited evidence of a direct effect of vanadium exposure on reproductive organs and fertility by exposure through a relevant route.

- there is some evidence that the four following additional references published by a group of researchers from the National University of Mexico (Fortoul et al. 2007, Mussali-Galante et al. 2005, Bizarro-Nevares et al. 2016, Rodriguez-Lara et al. 2016b) showing results obtained in CD1 mice after inhalation exposure with V_2O_5 and a reliability score of 3 are probably based only on one (perhaps two) study, and that different results from this study are presented in four different papers. Therefore, this should be judged as only one additional inhalation study with a low reliability score showing only supportive evidence.
- there is one other study (Morgan & El-Tawil, 2003) with ammonium metavanadate treatment via drinking water in rat in which male and female animals were paired and effects on fertility evaluated. This study is of RL=3 and thus only supportive, because limitations with respect to cohabitation, dose level and reporting are evident.

- another study (Domingo et al. 1986) conducted with an appropriate route of exposure (0, 5, 10 or 20 mg/kg bw/d sodium vanadate intragastric) in rats, but with RL=3 and thus of supportive evidence, which did not indicate an effect on male and female fertility is not considered in the argumentation for classification and labelling in the CLH report for unknown reason. A further study (Llobet et al. 1993), not considered in the CLH report, conducted by the oral route in mice indicated a no effect level of 40 mg/kg kg bw/d.

- the study published by the group of Chandra et al. (2007a, b, 2010) presented in three papers was conducted with a non-relevant route of exposure (IP) and is thus to be disregarded.

Taking this information from 3 studies (NTP, 2002 (rate, mice); Morgan & El-Tawil, 2003) together, there is only limited evidence from several studies conducted by an appropriate route of exposure but mostly with low reliability (except for the two studies by NTP (2002)).

<u>Comments on section 10.10.3, Page 74-76, Reproductive toxicity - Comparison with CLP criteria</u>

This section of the CLH report compares the evidence obtained in the weight-of-evidence analysis of all available data with the classification criteria given in the regulation (EC) 1272/2008. As discussed above, the view of the registrant is that the weight-of-evidence analysis as employed in the CLH report does not weigh the relevance, reliability and adequacy of the available studies and thereby does not conclude on the hazard for reproduction toxicity in a balanced manner.

Taking the information from several publications together, there is some evidence from these studies that vanadium exposure by the inhalation and oral route may affect male and female fertility (NTP, 2002; Morgan & El-Tawil, 2003), but in another study (Domingo et al., 1986), no effects were observed on fertility or a no effect level was defined (Llobet et al., 1993). Thus, the data available are not regarded as strong enough to support a classification into Cat 1B, because there are only two studies with a relevant route of exposure and a reliability score of 1 (NTP, 2002), and all other studies are only of supportive relevance, at best, because they show significant limitations with methodology and/or reporting (RL=3) and/or were conducted with an inappropriate route of exposure. Therefore, there is only limited and insufficient evidence that exposure of humans by inhalation or the oral route may result in effects on male or female fertility.

Therefore, it is proposed by the Registrant to retain **Category 2** based on criteria of the CLP Regulation: "*Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, … and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification*" (Annex I, Table 3.7.1(a)).

Whereas any evidence from mechanistic IP studies might be of academic interest, the exposure route is nevertheless not appropriate and therefore these studies should be disregarded and not be taken into consideration for classification of divanadium pentaoxide.

Comments on section 10.10.6, Page 86-89, Reproductive toxicity - Comparison with CLP criteria

"The classification of a substance in … Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on … development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate" (Annex I, Table 3.7.1(a)).
In the view of the Registrant, the low reliability of the available studies does not support a classification in Category 1B. Instead, retaining of the existing Category 2 is judged more appropriate.

"Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on … development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects" (Annex I, Table 3.7.1(a)).

The Registrant considers that the current harmonised classification as Repro. Cat 2 for development is still justified and should be retained. The justification for classification and maintenance of Repro. Cat 2 for development is supported.

2.8 Reproductive toxicity - Adverse effects on or via lactation

(CLH report Section 10.10.7, Page 89-93):
No studies are available for divanadium pentaoxide, which is why studies with sodium metavanadate were used as the basis for classification, which is generally acceptable according to the applied read-across concept.

All studies referred to for this endpoint in the CLH report (Soaza & Garcia, 2007; Cuesta et al. 2013; Olopade et al. 2011; Mustapha et al. 2014; Wang et al. 2015; Todorich et al. 2011; Morgan & El-Tawil, 2003; Edel & Sabbioni,1989) are of reliability score 3 and therefore can be regarded at best only of supportive evidence for classification and labelling. They all show deficiencies with respect to methods (e.g. only one dose level) and reporting of results. It needs to be noted that in these mechanistic studies, the exposure of lactating mothers was conducted by IP injections in order to attain sufficient secretion into the milk which is a non-relevant route of exposure, whereas only exposure of suckling pups via their mother's milk is relevant for this endpoint. In other words, the non-physiological administration circumvents the sequestration by physiological uptake mechanisms which is known to limit oral absorption to low levels (approx. 3%).

These non-regulatory mechanistic studies by Soazo & Garcia (2007) and Cuesta et al. (2013) were conducted with only one effective dose level of 3 mg $NaVO_3$/kg body weight/day (1.25 mg V/kg bw/day) in comparison to a control group and were not designed to evaluate a dose-response relationship or a no effect level. Therefore, a NO(A)EL or BMD cannot be obtained for this endpoint based on the available studies.

The publications by Soaza & Garcia (2007) and Cuesta et al. (2013) all come from the same group of researchers at the National University of Rosario, Argentina, and refer most probably to one and the same study with different endpoints published in two papers, although quite some time elapsed between the publications. There are two other publications coming from a group of researchers (Olopade et al. 2011 and Mustapha et al. 2014) from the University of Nigeria conducted in rats and mice.

Five (Soazo & Garcia, 2007; Mustapha et al. 2014; Olopade et al. 2011; Morgan & El-Tawil, 2003; Garcia et al. 2004) of 6 studies cited in the CLH report were conducted with suckling pups, directly exposed pups or adult rats, and effects were observed to a different degree on behaviour and motor coordination in open field tests. The effect on negative geotaxis investigated in three studies (Soazo & Garcia, 2007; Mustapha et al. 2014; Olopade et al. 2011) was not consistent and rotarod performance was affected in pups (exposed via milk or directly), but not in adult rats (Garcia et al. 2004).

In addition, histochemistry and immunohistochemistry investigations were conducted in these studies. There were occasions of a negative effect of vanadium treatment on myelin fibre density or demyelination in different brain areas in suckling pubs (Mustapha et al. 2014) as well as in adult rats (Garcia et al. 2004). Astrogliosis was observed in four studies in pups with varying degree, and oligodendrocytes were shown to be sensitive against vanadium treatment with oligodendrocyte progenitor cells (*in vivo* and *in vitro*) being more sensitive to vanadium exposure than astrocytes or mature oligodendrocytes (Cuesta et al. 2013; Mustapha et al. 2014; Olopade et al. 2011; Todorich et al. 2011). Lipid peroxidation was shown in adult rats but could not be detected in suckling pups (Soaza & Garcia, 2007). In addition, a few other single endpoints were evaluated.

The suggestion that exposure of suckling pups to vanadium via the milk of their mothers may result in neurophysiological effects, as evidenced by behavioural changes, myelin damage and astrogliosis under the conditions of these studies, is highly doubtful: all these studies were not conducted in a regulatory context and are of low reliability (1 dose only, purity of the applied compound not given, no documentation of general unspecific toxicity, irrelevant route of exposure etc.), and should therefore be judged as only of indicative value and should be evaluated critically with respect to classification and labelling.

It has been attempted to establish evidence that relevant amounts of vanadium are excreted into the milk of lactating animals and nursing mothers in amounts representing a toxicologically relevant exposure level by taking pharmacokinetic studies in animals (Edel & Sabbioni, 1989; Elfant & Keen, 1987) and humans (Anke, 2004) into consideration. However, there is only limited and indirect evidence that exposure of offspring rat via milk from their mothers (IP treatment) occur (Edel & Sabbioni, 1989), by the presence of significant amounts of vanadium in different tissues of suckling rats (including brain tissue), and the statement of a bioavailability of 70% made in one publication (Elfant & Keen, 1987) is not supported by any data whatsoever, thus must be regarded with caution.

Finally, it is stated in an article published by Anke (2004) that lactating women secreted 17% of their vanadium intake into the milk based on their lower excretion (79% in faeces and 4% in urine) in comparison to non-nursing woman (91% in faeces, 9% in urine). Based on the small number of individuals and the limited documentation, these results should also be considered with doubt.

Overall, taking the three publications together (Edel & Sabbioni, 1989; Elfant & Keen, 1987; Anke, 2004), there is only very limited information available on excretion of vanadium into milk of nursing animal or women and uptake by the suckling offspring based on studies with a very low reliability score. Based on the available information, it is not possible to define the bioavailability of vanadium in suckling rats or to make a comparison to adult rats. In addition, there are no valid data on a realistic exposure of breastfed children to vanadium.

Comments on section 10.10.9, Page 95-97, Reproductive toxicity - Comparison CLP criteria

With respect to classification and labelling there are no regulatory studies (one or two generation studies according to OECD and GLP) available to support an effect of vanadium on suckling offspring via lactation, but there are some publications of low reliability (RL=3) which indicate adverse effects in the offspring due to exposure via their nursing mothers. Nursing mothers were exposed IP only during lactation to pentavalent vanadium compounds, and effects were observed in pups exposed by the relevant route of exposure via lactation but with exposure to worst case amounts. Impairment in behavioural tests with evidence of myelin damage and astrogliosis could be demonstrated in these studies.

The following CLP criteria were taken into consideration as basis for classification. The CLP Regulation defines in Annex I, Table 3.7.1(b):

- *"absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk"*

as one classification criterion.

This statement is supplemented by two toxicokinetic studies (Edel & Sabbioni, 1989; Elfant & Keen, 1987) in rats and human data (Anke, 2004) which appear to indicate that vanadium exposure via the milk of the mother can occur and is a potential route of exposure. However, taking the three publications together, there is only very limited information available about the amount of excretion of vanadium into milk of nursing animals or women and uptake by the suckling offspring based on studies with a very low reliability score.

Therefore, the criterion in Annex I, Table 3.7.1(b):

- "*indicate the likelihood that the substance is* ***present in potentially toxic levels in breast milk"***

is not considered to be fulfilled based on this evidence, since the evidence of vanadium being present in mother's milk is limited and, in particular, there is no reliable and consistent data to conclude that the levels in human breast milk are of toxicological relevance.

The following ECHA Guidance on the Application of the CLP Criteria (2017) was also applied for conclusions on the classification:

- *"There may be toxicokinetic and toxicodynamic reasons why* ***neonates may potentially be more or less vulnerable to a particular adverse effect than adults****... Therefore, the relative sensitivity of neonates and adults to a substance must be judged on a case by case basis using expert judgement.*"

It is stated in the CLH report that suckling rats absorb higher fractions of vanadate compared to adult animals (Edel & Sabbioni, 1989) and therefore should be assumed to be at higher risk. This statement is not supported by the data presented in the publication. Whereas it could be demonstrated in the study that (i) vanadium is excreted via the milk of nursing mothers, and (ii) that absorption of vanadium by suckling rats is suggested by the presence in various organs, it is however not possible based on the data presented in the study publication to define the extent of bioavailability of vanadium in suckling rats or to make a comparison with adult rats.

The mechanisms of absorption in suckling rats are discussed in the publication: vanadium in maternal milk appears to have similarities with iron, apparently binding similar to transferrin-like proteins (lactoferrin), and that this form of vanadium is assumed to be available to the newborn during lactation. Vanadium in lactoferrin could also act as a metabolic promotor for the absorption itself, and the uptake may be owing to the relative immaturity of the intestinal mucosa at birth. In addition, vanadium in the cytosol of the intestines during the first period of uptake is mainly present in low molecular weight components, which may present an easily absorbable and mobile form, but the high molecular weight mobile form increases after 14 days.

In a study published by Edel et al. (1984), higher tissue levels of vanadium were observed in 21d old rats compared to rats of 115d of age, but significant decreases with age were found for kidney, liver and lung, while the variations in brain, heart, testes and spleen were generally regarded as small. It is stated in the publication that the mechanism responsible for higher levels of vanadium in younger rats is not clear, but some physiological factors were suggested e.g. higher gastro-intestinal absorption (lactoferrin), non-selective permeability of the gastro-intestinal tract, difference in absorption rates for several trace metals, higher retention capacity of undeveloped tissues, lower elimination rates.

The statement made by Elfant and Keen (1987) about increased vanadium absorption from the gastro-intestinal tract of the suckling, "bioavailability of vanadium added to milk is greater than 70% in suckling rat pups" must be regarded with caution, because it is not supported by any data whatsoever.

Whereas all this evidence supports some level of bioavailability of vanadium in suckling animals, and there is also some indication that tissue concentrations may be higher in younger animals than in adults, it however does not seem appropriate to draw the conclusion that these suckling pubs are assumed to be more vulnerable to vanadium than adults.

Based on the publication by Todorich et al., 2011 it is argued that there is a higher sensitivity of the newborn because of the maturation of the oligodendrocytes during the early life period. It is suggested by the authors that a high content of iron and ferritin in developing oligodendrocytes make them uniquely vulnerable to vanadium exposure, and that the destruction of oligodendrocyte progenitors (OPCs) may be the key contributor to vanadium-induced developmental hypomyelination.

This mechanistic study supports the elucidation of a potential mechanism of demyelination in the developing brain. It is also argued that vanadium exposure in the context of iron deficiency may become a clinically relevant health problem and that decreased iron and associated hypomyelination potentially exacerbated by vanadium toxicity could be an important health issue that “should receive additional investigation”. However, whereas this hypothesis may be of academic interest in the interpretation of demyelination in brain observed in other studies, it is not considered to be of relevance as an argument for an enhanced vulnerability of suckling newborns to vanadium exposure.

In this context, the CLP Regulation defines in Annex I, Table 3.7.1(b):

- *“Substances,… which may be present (including metabolites) in breast* ***milk in amounts sufficient to cause concern for the health of a breastfed child****, shall be classified and labelled to indicate this property hazardous to breastfed babies.*”

as classification criterion. The following ECHA Guidance on the Application of the CLP Criteria (2017) was also applied for conclusions on the classification:

“The mere presence of the substance in the milk alone, without a strong justification for a concern to offspring, would normally not support classification for effects on or via lactation.”

Animal studies as well as human data show that vanadium, unsurprisingly, is excreted via the milk of lactating mothers and taken up by suckling pubs. There are also studies available which appear to indicate an effect of vanadium in sucklings (neurophysiological effects evidenced by behavioural changes, myelin damage and astrogliosis), but the question is how far realistic exposure scenarios in humans result in a concern for the health of breastfed children remains totally open. The available studies are not designed and conducted to define no effect levels and there is also no information available on exposure of animals in these studies. The data on the amount of vanadium in breast feeding woman are also very weak and there is also no firm data about bioavailability in suckling pups.

Given that there are no valid data arising from a realistic route of exposure, it is questionable whether the observed effects on behaviour and oxidative brain damage reported in newborn rats and mice via exposure by suckling can be regarded as an indication of potential serious health impact in breastfed children.

It is argued in the CLH report that toxicokinetic studies (Edel and Sabbioni, 1989; Elfant and Keen, 1987) and human data (Anke, 2004) indicate that vanadium is transferred to human milk and that relevant absorption occurs. But it is also mentioned that absolute effective doses and a dose response relationship have not been established, and that potency comparisons to similar doses in the adult have not been performed.

In conclusion, there are no reliable data available which form the basis for this proposed classification. The studies presented in the CLH report are all of a reliability score of 3 and must therefore be considered as only supportive, because of their limitations and are thus not considered suitable for classification and labelling purposes.

An appropriate assessment is not possible due to the lack of a no effect level, and because reliable exposure levels and bioavailability data are not available. Unfortunately, the classification criteria for lactation do not define any cut-off criteria with respect to quantitative exposure and is solely based on the identification of a potential hazard and not on the risk of this hazard when put into the context of an exposure level.

Comments on section 10.10.10, Page 97-99, Conclusion on classification and labelling for reproductive toxicity

In a very recent publication by US NTP, three-month toxicity studies of tetravalent and pentavalent vanadium compounds in Hsd:Sprague Dawley SD rats and B6C3F1/N mice via drinking water exposure are described (Roberts et al. 2019). Data on the number of offspring from treatments, post implantation *in utero* and live births, birth weights, and weight gains were generated. The results for vanadium in its pentavalent form indicate that fetal effects are only seen at levels that are toxic to mothers, so that vanadate ions do not appear to be selectively affecting live births or to be selectively toxic to neonates. Effects were also not observed for vanadium in its tetravalent form, which however was tested at lower levels. The full findings of these sub-chronic studies are not available yet but are expected to be published in 2020. They can reasonably be expected to provide valuable information regarding conclusions on the effect of vanadium in its pentavalent form on fertility and effects via lactation, thereby closing a data gap. Since the Registrant has been aware of these studies, a testing proposal was previously not submitted in the REACH registration of divanadium pentaoxide to avoid duplicate animal testing. The Registrant suggests that any decision on the classification for effects of divanadium pentaoxide on reproduction and via lactation should be deferred until the full study reports will be available for rats and mice.

Conclusion on classification and labelling for adverse effects on sexual function and fertility:
Taking the information from several publications together, there is only some indication that vanadium exposure by the inhalation and oral route may affect male and female fertility, but the data available are not regarded as strong enough to support classification into Cat 1B, because there are only two studies with a relevant route of exposure and a reliability score of 1, and all other studies are only of supportive evidence because they show limitations with methodology and/or reporting (RL=3) and/or were conducted with an inappropriate route of exposure. Therefore, there is not enough evidence that exposure of humans by inhalation or the oral routes may result in effects on male or female fertility. Therefore, the Registrant proposes to retain **Category 2 – H361f**.

Conclusion on classification and labelling for adverse effects on development of the offspring:
The justification for classification and maintenance of Repro. Cat 2 for development is supported. However, the overall classification, because of the observed effects on sexual function and fertility and on developmental toxicity, of "**Repro. 1B, H360Fd"** is not supported based on the argumentation above. Therefore, for effects on sexual function and fertility, it is proposed by the Registrant to retain the classification of **Repro. Cat. 2 - H361fd**.

Conclusion on classification and labelling for adverse effects on or via lactation:
It should be noted that for this endpoint, classification and labelling in the CLH report is based on a limited data set with low reliability. However, an appropriate assessment of the hazard is not possible due to the lack of a no effect level, and because relevant and reliable exposure and bioavailability data are not available. In the absence of valid data in the context of realistic routes of exposure, **the effects described in the CLH report should not be regarded as an indication of serious health impacts for breastfed children**.

3 General comments

The Registrant would like to make the following general comments on observations made during this CLH reporting process:

3.1 Non-compliance with the provisions for proposing a harmonised classification in accordance with Regulation (EC) 1272/2008 and the underlying guidance documents

The CLH report does not appear to comply with Regulation 1272/2008 (Annex I: 1.1.1.4) and ECHA Guidance on the preparation of dossiers for harmonised classification and labelling (Version 2.0, August 2014) and Guidance on the Application of the CLP Criteria (Version 5.0, July 2017): a relevance, reliability and adequacy screening apparently was either not performed or at least not documented transparently for the selected references/studies.

Due to the extent of the vanadium database for most endpoints under review, a weight-of-evidence approach is required to resolve the question of an appropriate classification according to the explicit instructions in the underlying guidance documents. Such evidence-based approach should involve an assessment of the relative values/weights of different pieces of the available information. To this end, the value of each piece of information needs to be assessed. The weight given to the available evidence will be influenced by factors such as the quality of the data, consistency of results/data, nature and severity of effects, and relevance of the information for the given regulatory endpoint. In all cases, the relevance, reliability and adequacy for the purpose must be considered (ECHA, 2011).

In addition, a mode of action analysis should be performed to assess the overall level of concern (Regulation (EC) 1272/2008, Annex I: 3.6.2.2.6). Such a mode of action for carcinogenesis is a biologically plausible sequence of key events leading to an observed effect, supported by robust experimental observations and mechanistic data (Sonich-Mullin et al. 2001, Boobis et al. 2006).

3.2 Considerations on the socio-economic impact of the CLH proposal if adopted

Additional human health classification of divanadium pentaoxide should be thoughtfully considered regarding the welfare of the European environment and society. Overly conservative classification of CMR properties will hamper an efficient handling and processing of substances at the workplace leading to increased costs and unwanted effects on incentives for European research and development. Divanadium pentaoxide is of critical importance for energy storage, exhaust air purification and as precursor for the chemical industry. The following sectors are expected to further grow and induce increased demand for the substance:

- Today's society is shifting towards renewable and environmentally sustainable energy sources such as wind and solar (photovoltaics). Renewable power generation depends on efficient energy storage. Divanadium pentaoxide is directly or as pre-cursor used for the production of large-scale industrial energy storage systems, i.e. vanadium redox flow batteries. Vanadium redox flow batteries are already widely used in the EU (e.g. AT, CZ, DK, DE, IT, NL, PT, SK, SI, ES, SE and UK).
- The growing "clean air" movement has led to stricter environmental emission permits in the EU. A competitive and sustainable European industry therefore depends on efficient air purification systems to prevent environmental emission related to industrial activities. Divanadium pentaoxide as DeNOx-catalyst plays an important role in exhaust air purification, e.g. in the selective catalytic reduction in power plants.
- Divanadium pentaoxide is the most effective catalyst for the oxidation of sulfur dioxide to sulfur trioxide and thereby sulfuric acid, one of the most important of all chemicals.
- Divanadium pentaoxide is a key raw material for the manufacture of some specific frits and complex inorganic pigments and cannot be substituted. These substances are exclusively manufactured at industrial sites by trained workers and their main end uses are in ceramic articles, glass, metals, plastics and paints or coatings.

Thus, divanadium pentaoxide is critical for the competitiveness and sustainable growth of the European economy and for meeting objectives of the European Climate Change Programme. Given the ongoing data generation, complexity of the hazard assessment and wealth of (non-GLP, not guideline-conforming, unreliable) data of V_2O_5, generated for non-regulatory purposes under other jurisdictions (including Argentina, Mexico, Nigeria) but applied in the decision-making, it would appear that this CLH proposal may in fact compromise European competitiveness.

3.3 Procedural aspects of the proposed classification

It is noted here for the sake of completeness that the Vanadium Consortium and Vanitec closely coordinate all research activities in order to avoid duplication of work, to realize synergies and to obtain the required research elements that address current data gaps and improve our overall knowledge on vanadium and vanadium compounds.

The Vanadium Consortium and Vanitec learned about the upcoming CLH process in February 2018 via ECHA Weekly on 21.03.2018. By publishing the intentions for CLH, ECHA aims to facilitate that "*anyone with information relevant to the proposed hazard classification for a substance may bring this to the attention of the party submitting the CLH proposal during the early stages of the process*" (ECHA webpage https://echa.europa.eu/regulations/clp/harmonised-classification-and-labelling).

Accordingly, the Vanadium Consortium and Vanitec contacted the Ministère du travail (20.06.2018 and 29.06.2018) and the Risk Assessment Department of ANSES (26.09.2019) to:

(i) offer access to all study reports and data available and responding to questions if needed, and
(ii) meet so that relevant issues for the assessment could be discussed.

However, we received no response from the Ministère du travail and ANSES to our inquiry. In response to the published CLH Report, the Vanadium Consortium, Vanitec and actively involved REACH registrants of divanadium pentaoxide developed this commentary and an alternative classification proposal (i.e., in the name of the "Registrant") as summarised in Table 1.

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Appendix 1 Review of study by Ehrlich et al. (2008)

The study by Ehrlich et al. (2008) reported the results of a study of workers at one facility involved in vanadium processing. It was conducted by scientists at the Medical University of Vienna and funded by the Austrian Workers Compensation Board.

In this study, a series of biomarkers of DNA or cellular damage were measured in blood lymphocytes from workers involved in vanadium production and compared to the results from jail wardens who served as controls. Endpoints measured in 52 workers and 52 jail warden controls were 1) DNA migration in the comet assay, 2) oxidized bases 3) sensitivity to DNA damage induced by in vitro exposure to bleomycin and 4) repair of the bleomycin-induced lesions. A micronucleus assay using cytokinesis-blocked lymphocytes was also performed on a subset of 24 of the workers and 23 of the jail wardens. In this subset, in addition to the above tests, the following were measured: the incidences of 5) micronuclei in binucleate cells, 6) nucleoplasmic bridges, 7) nuclear buds, and 8) apoptotic or necrotic cells (Ehrlich et al. 2008). Of the eight biomarkers of DNA or cellular damage measured in the lymphocytes from blood samples, seven were reported to be altered in workers relative to the control population. Most noteworthy, the micronucleus incidence and the incidence of nucleoplasmic bridges were increased in the worker subgroup, indicating the presence of chromosomal damage to the DNA. The comet assay, a test also indicative of DNA damage, was reported as negative when conducted under standard conditions (Ehrlich et al. 2008).

The findings reported in this publication are attributed by the authors to exposure to divanadium pentaoxide, as apparent from the title of the manuscript, ("Inhalative Exposure to Vanadium Pentoxide Causes DNA Damage in Workers: Results of a Multiple End Point Study"). However, the only reported exposure information is the level of vanadium in the plasma, which was significantly higher in the workers than the levels in the control population of jail wardens. There is no indication of whether exposures to other substances which might confound the study results, and hence its interpretation, were considered. The measure of exposure reported is plasma vanadium with the median (25th-75th percentile) reported to be 0.3 µg/L (range = 0.24- 0.39) in the jail wardens and 2.2 µg/L (1.54-3.89) in the workers. Whereas this difference in elemental vanadium is statistically significant and supports exposure to either elemental vanadium or one or more vanadium-containing substances, no evidence is offered that it is exposure to divanadium pentaoxide per se that is the cause of the difference in vanadium plasma levels. Furthermore, there is no description of the facility or its operations and it is quite likely that in a vanadium processing facility there exists a range of exposures to a number of vanadium compounds and other non-vanadium substances (Slobodin et al. 2004). The processing of vanadium raw materials would likely involve exposures to other metal compounds, potentially including salts of potassium, aluminum, silicon and magnesium and impurities including nickel, molybdenum, chromium and iron (Slobodin et al. 2004). Uranium and arsenic are also known impurities in some vanadium-rich raw materials. Each of these metals could be present in a number of oxidation states. Workers would also be exposed to associated process materials including refractories and additives. Since solvent extraction and aqueous chemical processing are typically part of the manufacturing process, employees could also be exposed to a range of organic solvents, organic chemicals and inorganic chemicals including acids and bases. As measurements in the workroom air of divanadium pentaoxide or other substances were not presented, it is not possible to determine that the route of exposure was solely inhalation or whether other routes were involved. Although it is noted by the authors that "Workers are exposed to vanadium dust during the entire shift (8hr) and are required to wear protective masks and gloves.", it is unclear whether there is a lack of compliance with this requirement or what the route or combination of routes of exposure might be.

As exposures to substances in the facility were either not measured or commented upon, attributing causation of any differences in any of the parameters reported is not possible at this time. In fact, statistical analyses showed no correlations between formation of micronuclei with oxidized bases (a likely mode of action if vanadium were the responsible agent) and the authors also failed to find a correlation between plasma levels of vanadium and the reported effects (see Discussion and Conclusions p. 1692). Thus, even if the results of the biomarker component of the study were real, the effects observed cannot be attributed to inhalation exposure of divanadium pentaoxide or even to total vanadium compounds, with any certainty. Given these uncertainties, characterization of the range of exposures in the vanadium processing facility are needed.

The rationale for selection of jail wardens as a suitable control population was not provided in the publication and is unclear. However, they are stated to be "matched" to the workers. Table 1. (p1690) presents "The Distribution of selected characteristics in exposed subjects and controls" for the study groups as a whole. There are some noteworthy differences in the characteristics in the exposed subjects compared to the jail wardens. For example, the age of the control group was 5 years younger on average, smoked less on average, and had markedly lower levels of vitamin B12. The fact that the levels of vitamin B12 were so different in the

two groups (reported to be a median of 665 ng/L in the jail wardens and 968.0 ng/L in the workers with the difference statistically significant at a p value = 0.013; Table 1) is perplexing. Mean values for vitamin B6 were also higher in the workers and statistically significantly different from the control group. No explanation for these population differences was offered. Such differences raise questions about inherent differences in the two groups, and about other factors that may potentially have played a role. As an example, statistically significant difference in vitamin levels in the blood of the worker group compared to the group of jail wardens supports the conclusion that some substantive differences exist between these populations with respect to nutritional and/or lifestyle factors. Vanadium is frequently added to vitamin and mineral supplements and therefore ingestion of supplements may have contributed to the difference in vanadium plasma levels noted. Normal levels of vanadium in serum and plasma are reported to vary widely (Heydorn, 1990) with levels of 2.2- 3.9 µg/L in the serum fraction reported (Stroop et al. 1982). By comparison, the values in the control jail warden group of 0.3 µg/L for the median of 25th to 75th percentile and the range of 0.24- 0.39 µg/L for plasma would appear low.

It should be noted that the measurements derived from the micronucleus test were evaluated on only approximately half of the total available study populations and the rationale and method for the selection of this subset was not provided. The distribution of the characteristics in the exposed and controls in Table 1 are not shown for the subset tested in this part of the study, so it is not possible to compare the characteristics of the controls and workers studied for these endpoints. When the technique to measure micronuclei in lymphocytes in human populations was developed, it was demonstrated that there is a significant increase in the frequency of micronuclei with age (Fenech, 1993) and the influence of age has been noted as an important confounder in lymphocyte micronuclei studies in human populations (Battershill et al., 2008). As the 52 workers were reported to have a median age of 5 years older (25th to 75th percentile), the age difference of the subpopulation of the 24 workers that had micronucleus evaluations is not known. Age differences are important and the populations need to be carefully age matched in order for any comparisons to be meaningful. A review of the factors that can impact the frequency of micronuclei in human peripheral blood lymphocytes, pointed out the influence of many factors (Battershill et al. 2008). Careful matching of characteristics in controls to the worker population would be essential in order to eliminate differences that could influence the results.

It is noteworthy that the frequency of micronuclei in lymphocytes reported in the study by Ehrlich et al. was unusually low. Ehrlich et al. report the frequency of micronuclei in 2000 binucleated cells from the workers as 5.0 (median of 25th to 75th percentile) and claimed this to be elevated over the frequency of 2.0 measured in the jail warden population (2008). However, both these values are extremely low compared to those reported in the literature (Fenech, 1993; Bonassi et al. 2001). In a large international study of 25 laboratories representing 16 countries and 4899 nonexposed individuals, the overall median micronuclei frequency in binucleated cells from normal subjects was 6.5 per 1000 cells and the interquartile range was between 3 and 12 per thousand (Bonassi et al. 2001). This is equivalent to a median of 13 per 2000 cells compared to only 5 and 2 (median of 25th to 75th percentile) and for workers and jail wardens respectively reported in Ehrlich (2008). This supports substantial underscoring in the Ehrlich evaluation which could render the small difference reported between the worker and jail warden groups as not meaningful.

In addition, the description of the methodology employed does not make it clear that the biological samples were appropriately handled, blinded, and microscopically scored. Because micronuclei and cytoplasmic bridges are formed during the period of in vitro culture (after the blood samples are obtained) it is critical in this assay that samples from workers and control subjects be obtained, stored, transported, and ultimately cultured under identical conditions and for the same periods of time. Significant differences in any of these parameters between the control and worker's samples, such as prolonged storage, temperature variations during transportation to the laboratory, or exposure to exogenous factors such as sunlight, could modify the parameters measured. The publication does not specify that these factors were appropriately controlled, and does not even specify the physical locations from which samples were obtained and the methods by which they were stored and transported. The higher incidence of necrotic cells in the worker samples (20.9% vs. 13.5% in controls) could be consistent with such sample stress.

Also, because the microscopic scoring of micronuclei has been demonstrated to be quite variable among different laboratories and scorers even on identical samples (Fenech et al. 2003; Dertinger et al., 2006; MacGregor et al., 2006) it is essential that control and worker's samples be blinded and randomized prior to scoring. This is a requirement for the conduct of a mammalian in vivo micronucleus test in regulatory settings (OECD guideline 474). The Ehrlich et al. 2008 publication does not specify that control and worker samples were scored concurrently in a randomized and blinded fashion, and does not specify the time periods during which each set of samples were obtained (i.e., whether they are concurrent, which is necessary to assure control of these types of experimental and scorer biases).

It is also noted that the spontaneous frequency of micronucleated cells in the control population is significantly lower than that reported for normal subjects in the literature, whereas the values for the workers while low is within the previously reported normal range. Thus, it is critical to establish that the samples were in fact randomized and blinded appropriately and that the reported difference is not due to a difference in scoring. Additionally, it is critical that the samples were obtained, processed, and scored during the same time period and under conditions of blinding and randomization that would have been controlled for scoring variability and also sample handling. Such experimental differences might also affect the other endpoints measured.

Considering the factors discussed above, the findings and the conclusions reported in the publication by Ehrlich et al 2008, should be considered inconclusive. There is no unequivocal evidence presented to support attribution of the differences in the control jail warden group compared to the workers as being caused by divanadium pentaoxide. Differences in the age, smoking and plasma vitamin B12 and B6 levels raise questions about the suitability of the comparison of the two groups. While the frequency of micronuclei and other endpoints were different in the workers compared to the jail warden controls, the authors reported that they had failed to find any correlation of these effects with the level of vanadium measured in the blood. The comet assay, reported to be a sensitive indicator of DNA damage in human lymphocytes in vivo (Milković et al. 2009), was not different in the workers compared to the jail warden controls even though the other affected endpoints reported, such as micronuclei and nuclear bridges, are indicative of such effects.