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INHALATION TOXICITY TESTING: EXPERT MEETING ON POTENTIAL REVISIONS TO OECD TEST GUIDELINES AND GUIDANCE DOCUMENT

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IOMC
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Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
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The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents;** and **Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety (June 2005). This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the Joint Meeting decided to hold a Workshop on the Safety of Manufactured Nanomaterials in December 2005, in Washington, D.C. The main objective was to determine the "state of the art" for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Based on the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19] it was recognised as essential to ensure the efficient assessment of manufactured nanomaterials so as to avoid adverse effects from the use of these materials in the short, medium and longer term. With this in mind, the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee in September 2006. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. This programme promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

This document is published under the responsibility of the Chemicals Committee of the OECD. It is intended to provide information on the outcomes and developments of the OECD programme on the safety of manufactured nanomaterials.

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OECD'S PROGRAMME ON THE SAFETY OF MANUFACTURED NANOMATERIALS

The OECD's Programme on the Safety of Manufactured Nanomaterials[1] was established in 2006 to assist member countries to efficiently and effectively address the safety challenges of nanomaterials. OECD has a wealth of experience in developing methods for the safety testing and assessment of chemical products.

The Programme brings together more than 100 experts from governments and other stakeholders from: a) OECD Countries; b) non-member economies such as China, the Russian Federation, Singapore, South Africa, and Thailand; and c) observers and invited experts from UNITAR, FAO, WHO, ISO, BIAC[2], TUAC[3], and environmental NGOs.

Although OECD member countries appreciate the many potential benefits from the use of nanomaterials, they wished to engage, at an early stage, in addressing the possible safety implications at the same time as research on new applications are being undertaken.

The Programme of Work is being implemented through specific projects to further develop appropriate methods and strategies to help ensure human health and environmental safety:

- OECD Database on Manufactured Nanomaterials to Inform and Analyse EHS Research Activities;
- Safety Testing of a Representative Set of Manufactured Nanomaterials;
- Manufactured Nanomaterials and Test Guidelines;
- Co-operation on Voluntary Schemes and Regulatory Programmes;
- Co-operation on Risk Assessment;
- The role of Alternative Methods in Nanotoxicology;
- Exposure Measurement and Exposure Mitigation; and
- Environmentally Sustainable Use of Manufactured Nanomaterials

Each project is being managed by a steering group, which comprises members of the WPMN, with support from the Secretariat. Each steering group implements its respective "operational plans", each with their specific objectives and timelines. The results of each project are then evaluated and endorsed by the WPMN, and subsequently by the OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

This document is the meeting report of the WPMN Expert Meeting of Inhalation Toxicity Testing for Nanomaterials, held on 19-20 October 2011 in The Hague, hosted by the Netherlands. Fifty experts from the WPMN as well as the WNT participated in the meeting.

More information about the work of the OECD's Programme on the Safety of Manufactured Nanomaterials, as well as OECD's publications regarding safety issues of nanomaterials, is available at www.oecd.org/env/nanosafety.

1 Updated information on the OECD's Programme on the Safety of Manufactured Nanomaterials is available at: www.oecd.org/env/nanosafety

2 The Business and Industry Advisory Committee to the OECD

3 Trade Union Advisory Committee to OECD

EXECUTIVE SUMMARY

The expert meeting on Inhalation Toxicity Testing for Nanomaterials was held on 19-20 October 2011 in The Hague, hosted by the Netherlands, with the aim of discussing the results of the OECD Sponsorship Programme (under the responsibility of SG3) on this specific topic and addressing issues relevant to inhalation toxicity. Fifty experts from the WPMN as well as the OECD Working Group of the National Coordinators for the Test Guidelines programme (WNT) participated in the meeting.

A preliminary report of the meeting was initially prepared by the host, the Netherlands, and presented at the 9th meeting of the WPMN (December 2011) and it was agreed that this report be circulated to the meeting participants for their comments with a view to its improvement reflecting the discussions and conclusions of the meeting. Accordingly, the meeting report has been reviewed by not only meeting participants but also the WPMN and the WNT members.

This document, the meeting report, includes summaries and recommendations from each of the presentations (Annex II) as well as suggested revisions to the OECD Test Guidelines (TGs 403, TG412, TG413 and TG436), the Guidance Document on Acute Inhalation Toxicity Testing [ENV/JM/MONO(2009)28] and the Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [ENV/JM/MONO(2010)25] (Annex IV). It also includes the meeting agenda (Annex I), and the Participants list (Annex III).

INHALATION TOXICITY TESTING: EXPERT MEETING ON POTENTIAL REVISIONS TO OECD TEST GUIDELINES AND GUIDANCE

Summary

1. The OECD WPMN Expert Meeting on Inhalation Toxicity Testing for Nanomaterials took place on 19th – 20th October 2011 in The Hague, the Netherlands. This event was hosted by the delegation of the Netherlands.

2. The meeting agenda was prepared by the host, the Netherlands, and chaired by Tom van Teunenbroek and Flemming Cassee. The rapporteurs were Yvonne Staal (TNO, the Netherlands), Peter Kearns (OECD) and Ilse Gosens (RIVM, the Netherlands). There were 50 participants from OECD member countries, and other stakeholders from industry, academia and environmental NGOs. See ANNEX III for the list of participants.

3. At the 8th meeting of the OECD WPMN held in March 2011, it was agreed to hold a horizontal meeting on the inhalation toxicity testing for manufactured nanomaterials that included participation from OECD member countries, the Business and Industry Advisory Committee (BIAC) and NGOs. The aim of the OECD Working Party on Manufactured Nanomaterials (WPMN) is to promote international co-operation on human health and environmental safety aspects of manufactured nanomaterials, in order to assist in their safe development. The workshop was intended to share expertise knowledge from experiences of OECD Sponsorship Programme (SG3) and provide information useful for development of OECD Test Guidelines specific for manufactured nanomaterials.

4. With respect to updating the "Preliminary Guidance Notes on Sample Preparation and Dosimetry [ENV/JM/MONO(2010)25]" and OECD test guidelines, suggestions for revision were prepared on beforehand of the meeting by the Netherlands (see Annex IV). These suggestions considered how to generate test atmospheres for nanomaterials depending on the use of the toxicity tests. While inhalation toxicity tests with aggregates or agglomerates of nano-sized particles seem adequate for risk assessment for handling of powders, it may be relevant for risk assessment of nanoparticles in the production phase to include inhalation toxicity of aerosols consisting of single nanoparticles as much as possible; the same applies for hazard assessment. Care should be taken that at high mass concentrations size distributions will shift towards larger particles due to agglomeration as a function of time and particle number concentrations.

5. Recommendations were made for adapting current guidelines and the guidance document for inhalation toxicity testing of nanomaterials that include deletion of a lower cut-off of the size range and minimal mass concentrations. Specifically, the expert group mentioned the need for careful aerosol generation and characterization. Most experts agreed more explicitly to incorporate the application of biokinetics into the guideline and detailed pathology of the brain and other parts of the central nerve system, while bronchoalveolar lavage fluid analysis should be included as a mandatory requirement.

Objectives of the Workshop

6. There were three main objectives: i) discuss whether the "Preliminary Guidance Notes on Sample Preparation and Dosimetry" should be updated on the basis of the experiences obtained in the sponsorship (SG3) or other new developments in the area of inhalation toxicity testing and assessment; ii) identify whether there is a need for specific guidance or a guidance document (GD) for inhalation toxicity testing of nanomaterials or adaptation of existing guidance documents; and iii) to discuss whether there is a need to update current (OECD) inhalation Test Guidelines or development of new ones. If updates are necessary, discuss what kind of changes or developments would be needed and which further steps need to be taken.

Recommendations raised by the speakers for the discussion

7. Various recommendations were raised by the speakers that served as points for discussion. These recommendations do not necessarily reflect a general agreement. The full summary of the presentations by the speakers is given in annex II.

- "Provide explicit guidance for the generation of aerosols (sample preparation) based on the exposure scenario". Hans Muijser

- "Generation of a test atmosphere should have workplace characteristics, but should be adapted to adjust for rodent respirability". Günter Oberdörster

- "A choice for a dry aerosol or a liquid aerosol should depend on the given test substance and planned test approach (hazard- or risk driven)". Otto Creutzenberg

- "Aerosol characterization should include size distribution, mass, number and morphology of the material". Günter Oberdörster

- "Mass concentration is not sufficient for comparison of nanomaterials of the same chemical composition". Flemming Cassee

- "Dry powders will appear as agglomerate upon aerosolization, which needs to be addressed in the sample preparation guidelines". Flemming Cassee

- "Dissolution behaviour of the test substance should be assessed in physiological fluids mimicking various lung-specific pH ambiences (neutral, acid)". Otto Creutzenberg

- "Data analysis should include interpretation of aerosol characteristics, NOAEL, risk assessment implications, mode of action and a strategy for dosimetric extrapolation to humans. The inclusion of biokinetic data is important". Günter Oberdörster

- "Include biokinetics in the guidance, since different distribution patterns in the whole organism are likely dependent on physicochemical characteristics of nanoparticle aerosols and the dose at the target site will therefore be different. This will allow the assessment of accumulation of nanomaterials in the body at low exposure levels and long-term exposure. A way to perform it is by radiolabelled materials, chemical elemental analysis to determine organ concentrations and transmission electron microscopy". Wolfgang Kreyling. Others who have suggested inclusion of biokinetics or recognized the importance were Otto Creutzenberg, Frieke Kuper, Günter Oberdörster and David Warheit.

- "Retention and clearance should also be considered for inclusion in repeated dose studies". Flemming Cassee

- "Measurements of lung lavage parameters as well as lung weight are sensitive and robust endpoints to assess adverse health effects. Bronchoalveolar lavage fluid (BALF) analysis should be mandatory. Inflammatory cells (differential cell count for total and percentage of cells) in BAL fluids should be a requirement in the test guidelines for all particles (and is relevant for other substances as well)". Otto Creutzenberg. Inclusion of mandatory BAL analysis was also raised by Frieke Kuper, David Warheit and Craig Poland.

- "Expand endpoints for genotoxicity and include assessment of oxidative damage of lung epithelial cells by immunohistochemistry (8-OH-dG)". Otto Creutzenberg

- "Analysis of the olfactory bulb is suggested to be added to the existing guidelines, but there is a research need to assess the best way to examine the olfactory bulb. The brain is already included in guidelines 412/413 and guidance document 125, but may be more specifically assessed. An approach should be developed on how translocation to the brain should be examined". Frieke Kuper

- "Consideration of cell proliferation studies (of various components of the respiratory tract) as recommended supplementary/complementary components to traditional subchronic inhalation toxicity studies (OECD 413 studies) with particles and nanoparticles". David Warheit

- "Development of better guidance criteria for establishing No Adverse Effect Levels in subchronic inhalation toxicity studies (OECD 413 studies) with particles and nanoparticles". David Warheit

- "The apparent density of the particle (agglomerate or assemblage) appeared to be the most decisive dosimetric endpoint. Particle-displacement volume determines the degree of lung overload and inflammogenic potency accordingly. The most robust exposure dose metrics appears to be the mass concentration (external, internal) which can readily converted to the cumulative retained particle volume by using the known relationship of particle volume = mass divided by agglomerate density". Juergen Pauluhn

- "Both the study design (dose selection) and the selection of the post exposure recovery period should take into account the kinetic behavior at non-overload and overload conditions. Animal numbers and study costs can be significantly reduced by executing hypothesis-based (PBPK-modeling) repeated exposure inhalation studies". Juergen Pauluhn

- "Bronchoalveolar lavage and specialized approaches differentiating extravasated collagen (inflammatory) and that produced locally by myofibroblasts (fibrogenic) are important adjuncts worthwhile to consider". Juergen Pauluhn

- "GD39 should differentiate between acute and repeated dose toxicity and focus on cumulative lung burden". Juergen Pauluhn

- "It is suggested to examine pleural effects (e.g., pleural lavage) and to develop guidance on how to do this should be included in the test guidelines as a useful addition, but not compulsory. This would only be relevant to high aspect particles (an aspect >~10µm) such as fibres, plates, tubes and low density bundles". Craig Poland.

Discussions among all participants

8. The discussion on day 1 was chaired by Juan Riego Sintes and Flemming Cassee. The discussion points included:

- A proposal of suggested revision for OECD Test Guidelines and Guidance Documents Drafted by Hans Muijser (TNO Triskelion bv) and Flemming R. Cassee (RIVM). After the discussion several minor adjustments have been made to the proposal and the final version can be found in Annex IV.

- Should single nanoparticles or aggregates/agglomerates be tested? Should this be focussed on hazard? And if so, are smaller particles more hazardous or not? Or should a realistic scenario be followed, either from a technical view (aerosol generation in the lab) or for human exposure? Single particles will agglomerate during aerosol generation. If single particles are desired, than also indications on concentration limits and dose metrics should be provided in the guidelines. Better guidance should be given in OECD documents for sample preparation, depending on the purpose of the test (e.g. hazard assessment at the site of production or during storage and handling).

- It was discussed whether acute testing is meaningful, which may be for hazard identification. There was no clear consensus, but it seems that the general view is that it is not a top priority for nanomaterials.

- It was agreed that, by default, exposure should be the maximum respirable exposure concentration, without a change in size distribution due to increasing agglomeration. In addition, the human deposition should be mimicked as much as possible (if human exposure data are available) for hazard assessment in realistic exposure scenarios. Particle size should be below 3µm, but a lower cut off should not be given. There were no major comments to the suggested revisions for Test Guidelines.

- With respect to the Guidance Document, there were three changes proposed for the guidance document on acute testing:

i. It was discussed whether dose metrics other than mass and mass based size distributions should be included. Other metrics should be chosen dependent on the endpoints to be assessed and therefore all could be applicable. Number concentration was generally considered mandatory, although it was argued that it might not tell as much as the system keeps changing.

  - The deposited dose and the surface area might be important.
  - The number size distribution is also considered important.
  - Morphology and geometry using TEM or SEM for aerosols.
  - Lung burden should be assessed.
  - Total volume of aerosols in relation to overload may also be important. It was questioned whether this can be generalized.

It was questioned where the limit should be for nano-specific dose metrics.

It was discussed whether nose-only or whole body exposure should be used. Nose-only may induce stress, but this may not always be the case especially in view of the fact that exposure occurs during the light period of the day when rodents are less active. Whole body exposure may also result in dosing through other routes (e.g. oral exposure by cleaning of the fur). It was indicated that methods used for respiratory exposure should be well substantiated. It was also recognized that this is not a nanomaterial specific issue.

ii. Some participants suggested that having only a single document is to be preferred and that it should be avoided to have to jump from one document to another while designing a new study, as opposed to having information in several documents.

- It was discussed whether kinetic data should be included. All participants acknowledged the importance of this information in the assessment of nanomaterials, although for some participants there was reluctance for inclusion due to the possibly increasing number of animals and costs. The question whether kinetic studies on nanomaterials should rather be a separate study and hence a separate guidance document was not answered.

9. The discussion on day 2 was chaired by Flemming Cassee. Proposed endpoints to add or revisions to the existing endpoints are:

- Histopathology of the olfactory bulb and brain from optional to mandatory based on the scientific evidence that nanomaterials can affect the physiology of these organs. It was mentioned that at present the precise meaning of effect on these parameters is not fully understood.

- Measurements of cell differentials (absolute numbers and fractions) in bronchoalveolar lavage fluid (BALF) are considered sensitive for inflammatory effects and should be considered to be mandatory. Other parameters such as cytotoxicity (e.g. lactate dehydrogenase (LDH)) and lung permeability (total protein) should be considered as mandatory measures by most experts, but consensus on this matter has not been reached. Standardization of the protocol for BALF should be applied and should be sorted out by a working group.

- Histopathology of the parietal pleura and the subpleural proliferation of lung tissue can be worthwhile to add for certain type of fibre-like structures, but only in repeated dose studies.

- Fibrosis or collagen accumulation may be added, but it should be clarified what a fibrotic change is and how this should be graded.

- Biokinetics is considered necessary for proper risk assessment, although it was recognized that this might imply additional costs and animals. There was some discussion on the exact conduct and the parameters to be included. It was suggested that it is not feasible to include biokinetics now, as more research has to be done first. Lung burden (measured, not calculated) may help as a start as it is considered advantageous to know the distribution.

10. Recommendations following this meeting for Phase 2 of OECD Sponsorship Programme were discussed and the issues were:

- The importance/preference to have results from phase 1 has been acknowledged.

- Although it was acknowledged that for the safety assessment of engineered nanomaterials (ENM), information of their effects after chronic exposure is of eminent importance. It is also acknowledged that the chronic testing is not necessarily a topic in the sponsor project, since one of the important tasks of the sponsor project is the assessment of the adequacy of test guidelines for nanomaterials, which does not necessarily mean the conduct of studies.

- Development of guidance on how to assess olfactory bulb and the brain is suggested.

- There is a need to gain more knowledge on the effects on the immune system.

- Feasibility of the concept of grouping of nanomaterials based on either common physicochemical properties or toxicological properties should be addressed.

- Assess whether there is a relation between particle size and toxicity.

- Insight in nanomaterial biokinetics in different organisms should be obtained.

- Flags for chronic diseases – how can long term effects be detected in shorter term studies by adding certain parameters.

- Cardiovascular and neurotoxicological effects should be addressed, and use of sensitive species for these endpoints, also in relation to detection of promotion of diseases or identification of susceptible groups in the population.

- Compare different toxicity tests, e.g. 5-day, 28-day and 90-day studies.

- Develop a new, high-throughput rapid screening methodology. In vitro studies may be valuable in this.

- Assessment of impact of repeated exposure to ENM (in vivo) and evaluate long-term effects.

- Extrapolation possibilities between similar ENMs with varying physicochemical properties.

- To develop principles for safety assessment for surface modified ENMs.

ANNEX I: MEETING AGENDA

Wednesday 19 October 2011	
8:45-9:15	**Registration at Hotel Mercure, The Hague**
9:15-9:30	Welcome, general introduction and agenda by chair Tom van Teunenbroek (Ministry of Infrastructure and the Environment)
Session 1	
9:30-10:00	Proposal of revisions for the OECD guideline on inhalation toxicity testing and the Guidance document on sample preparation and dosimetry – Hans Muijser (TNO)
10:00-10:30	Aerosol science in TiO2 inhalation toxicology studies: Wolfgang Kreyling (Helmholtz Centre Munich)
10:30-11:15	**Coffee break**
11:15-11:30	Sample preparation of CeO2: Hans Muijser (TNO)
11:30-12:15	General notes on sample preparation and dosimetry with specific notes on carbon nanotubes – Gunter Oberdorster (University of Rochester)
12:15-13:30	**Lunch**
Session 2 : Contributions by inhalation experts from the OECD sponsorship program	
13:30-14:00	Cerium oxide – Flemming Cassee (RIVM)
14:00-14:30	Zinc oxide – Otto Creutzenberg (Fraunhofer Institute)
14:30-15:00	Carbon nanotubes – Takuya Igarashi (AIST)
15:00-15:30	**Coffee break**
15:30-16:15	General discussion part 1 Proposal of revisions on OECD guidelines and guidance document on sample preparation: the technical view
16:15-17:00	General discussion part 2 Preparation of an inventory of contributions of Sponsorship programme and possible suggestions for guidance documents: the implementing view
19h00	Dinner to be offered and hosted by the Netherlands: Thai Restaurant "Spize"

Thursday 20 October 2011	
Session 3	
9:00-9:05	***General introduction and agenda by chair Flemming Cassee***
9:05-9:30	General comments on endpoints in inhalation studies – Frieke Kuper (TNO)
9:30-10:00	90-day inhalation study with carbon nanofibers – David Warheit (Dupont)
10:00-10:30	Common mechanism – based study design for inhalation toxicity testing of poorly soluble (nano)particles – Juergen Pauluhn (Bayer Healthcare)
10:30-11:00	*Coffee break*
11:00-11:30	Low aerodynamic diameter, high aspect ratio nanoparticles and their potential hazards – Craig Poland (Institute of Occupational Medicine)
11:30-12.00	Rat 2-week Toxicity of MWCNT by Whole-body Inhalation – Shoji Fukushima (Japan Industrial Safety and Health Association)
12:00-12:30	Study design for biokinetics – Wolfgang Kreyling (Helmholtz Centre Munich)
12:30-13:30	**Lunch**
Session 4: Round-up plenary session	
13:30-14:30	Unresolved issues from discussions
14:30-15:00	**Coffee break**
15:00-16:00	Recommendations for sponsorship programme phase 2
16:00-16:30	Any other business

ANNEX II: SUMMARY OF PRESENTATIONS

Session 1 - General Presentations: Setting the Scene

- Hans Muijser and Flemming Cassee from the Netherlands prepared suggestions for nanospecific revisions for OECD Technical Guidelines 403, 412, 413 and 436, and, guidance documents GD39 and GD24 (draft). The proposed revisions have been updated after discussion with the group in the afternoon of day 1 (see Annex IV). Additionally, Hans Muijser showed that even using a venturi and a subsequent jet mill (which is considered as maximum reasonable effort to aerosolize a dry powder) dry powders consisting of nanomaterials cannot be aerosolized such that the aerosol consists mainly of free primary particles. Freshly produced single nanoparticles will aggregate rapidly and only if the inhalation exposure is performed close to the production process, one might see large numbers of particles < 100 nm. Therefore the exposure scenarios should be taken into account when tests for safety assessment are requested. During the discussion, the suitability of acute inhalation toxicity tests for nanoparticles was questioned – especially in light of the restrictions on the achievable mass concentrations. **Recommendations**: provide explicit guidance for the generation of aerosols (sample preparation) based on the exposure scenario. e.g.: if a bag filled with a powder consisting of nanoparticles drops from a shelf and the powder is set free, mainly aggregates and agglomerates of particles will be found in the air. The worst case scenario, an atmosphere of single nanoparticles is therefore not realistic. For such a calamity risk assessment should be based on aerosolization of a powder using a venturi only, since that represents already more effort for deagglomeration than just dropping a powder.

- Wolfgang Kreyling from Germany reported that particle number concentration is a good indicator for exposure but given the fact that nanoparticle coagulation dynamics is a function of time and concentration, other measures such as number size distributions have to be provided. Mass concentrations are relatively low for nanoparticles. Therefore concentrations of 5 g/m^3, as was recommended by OECD TG 39, are impossible to achieve for nanoparticle aerosols because this would correspond to number concentration >1E10 part. per cm^3 coagulating in milliseconds. As nanoparticles deposit by diffusion, they have a much more uniform deposition in the lung than micro-sized particles. 20-40% of inhaled nanoparticles (<100nm) deposit in the alveoli. The extrapolation to human exposures is easier for nanomaterials compared to microsized particles since diffusion is the dominating process of deposition. Microsized particles are rapidly phagocytized by alveolar macrophages (within 4-6 hours), whereas nanoparticles at the same mass concentrations are far more abundant, probably less well recognized by macrophages and as a result more likely to interact with other cell types such as those of the epithelium. Cellular uptake and translocation to the circulation become therefore more important processes. Inhalation studies are expensive and laboursome. The alternative might be the use of intratracheal instillation of suspensions of nanomaterials. When using this technique, the dose rate is very much higher compared to that of inhalation exposure and it is more likely that host defence systems respond differently. Inhalation exposure results in much lower exposure to the individual cell at similar mass dose. Due to physical properties as well as biological mechanisms, the hazard of nanomaterials may very well differ from larger sized materials, even if the chemical composition is the same. **Recommendations**: Include biokinetics in the guidance, since intrinsic toxicity may not change that much for nanomaterials compared to the bulk, but they will have

different distribution patterns in the whole organism and the dose at the target site will therefore be different. This will allow the assessment of accumulation of nanomaterials in the body at low exposure levels and long term exposure.

- Hans Muijser from the Netherlands showed how nanoparticle test atmospheres with cerium dioxide particles were generated for the Dutch input in the OECD sponsorship programme. Three types of cerium dioxide were used, e.g. a microsized form from Sigma Aldrich (particle size <5µm), an aggregated form from Umicore (nanograin ceria, particle size 40nm), and another aggregated form from Antaria (Ceria dry, particle size 5-10 nm). For aerosolization a turntable dust feeder, with a venturi and a jet mill were used, which represented the maximum reasonable effort to generate small particles. SEM pictures of particles on glass fibre filters showed that the Sigma Aldrich material contained small and large aggregates and some individual particles, the Umicore material showed a few primary particles and mainly aggregates and the Antaria material showed aggregates and smaller particles. There was a large particle size distribution. Finally, a SEM picture of electric spark generated single cerium dioxide particles was shown (not used for the OECD sponsorship program).

- Günter Oberdörster from the University of Rochester (New York), United States, presented general notes on sample preparation of CNT. Inhalation and instillation exposure were compared. Inhalation has the disadvantage of a high demand in costs and expertise, but is based on realistic aerosol exposure (compared to bolus) and low dose rate whereas the same dose delivered as bolus is done at an unrealistic and extraordinary high dose rate. Nose-only exposure results in undesirable stress from being restrained as evidenced by a number of publications, whereas whole body exposure leads to oral exposure via cleaning of the fur which is in addition to gastro-intestinal tract exposure via normal mucociliary escalator clearance. An alternative, but less optimal, intratracheal inhalation exposure requires anesthetized animals and circumvents the upper respiratory tract.
  For instillation studies, nanomaterials have to be dispersed in an aqueous suspension which is often supplemented with surfactants to improve the dispersion and for which high energy dispersion techniques are used. Sonication duration affects particle size, resulting in increase or decrease which also depends on the solvent. All these preparatory steps can affect toxicity. An example was given for titanium dioxide. Instillation of TiO_2 (25 nm primary particle size) resulted in PMN responses which were lower with DPPC/albumin dispersion than with the saline dispersion and even lower when the same dose was deposited as inhaled aerosol. Respirable particle sizes by nasal or oral breathing are largely different in rats and humans, and lung deposition differs as well. A dosemetric should be chosen which is appropriate for the toxicological indicator.
  Recommendations: Whole-body inhalation is preferred, in particular for chronic and also subchronic exposures, using the rat and inclusion of a recovery or observation period. This view was not shared by the full audience. Generation of a test atmosphere should have workplace characteristics but be adapted to adjust for rodent respirability; changes in morphology should be avoided. Aerosol characterization should include size distribution, mass, number and morphology. Data analysis should include interpretation of aerosol characteristics, NOAEL, risk assessment implications, mode of action and a strategy for dosimetric extrapolation to humans. Important is the inclusion of biokinetic data. Measurements of lung lavage parameters (neutrophils in particular) as well as lung weight are sensitive and robust endpoints to assess adverse health effects.

Session 2 - Contributions by inhalation experts from the OECD Sponsorship Programme

- Flemming Cassee from the Netherlands presented the results of a 28-day nose-only inhalation study, performed according to OECD TG412, with samples of cerium oxide (OECD NM211, 212 and 213), using one concentration and three exposure durations to obtain 3 dose levels and including a 14-day recovery period. Despite the different size distributions of the primary particles, the mass median aerodynamic diameters were comparable. The material with the largest specific surface area showed the most pronounced response also after a recovery period. Macrophages and neutrophils in BAL were increased and inflammatory effects and signs of cytotoxicity were observed, also after recovery. To assess whether persistence of the inflammation, was caused by accumulation and retention in the body, data on biodistributions were collected. Cerium oxide levels in lung reflected the exposure concentration and were slightly different for each material. Cerium oxide was also found in other organs (liver, kidney, spleen, testis) at very low levels and very slow clearance after termination of the exposure. The toxic response is not determined by the total deposited amount, there may be differences in clearance. Steady state levels differ between materials. Discussion points were the validity of using C times T, how the inhaled dose was determined and the fate. Larger particles may stay in the lungs. It was suggested that differences in surface area result in differences in absorption of gaseous materials. **Recommendations**: Mass concentration is not sufficient for comparison of nanomaterials of the same chemical composition. Retention and clearance should also be considered to be included in repeated dose studies. Dry powders will appear as aggregates upon aerosolization, which needs to be addressed in the sample preparation guidelines.

- Otto Creutzenberg from Germany presented the results of Zinc Oxide. Three materials were used: Z-COTE HP1 (coated), Z-COTE (uncoated) and ZnO 205532 (micro-sized). A 5 day range finding study (0.5 – 2 – 8 mg/m^3) with the coated material showed histopathological effects in olfactory epithelium and nasal cavities and alveolar accumulation of macrophages. A 14-day study was done at the same concentrations and also including the other 2 materials at the high concentration level. Lavage parameters showed an increase on the day after exposure, but returned to control levels after a 14-day recovery period. PMN and protein were only increased at high concentration levels for all compounds and no effects were found on macrophages and lymphocytes. Accordingly, CINC-1 showed a dose-dependent increase 1 day and return to normal on day 14 post-exposure. IL6, TGFbeta and TNFalpha in BAL showed some increases on day 1 and 14, but had low absolute values. Histopathological effects were found in the nose and nasal cavities of animals exposed to the coated material. Macrophage accumulation was found in the lungs. All materials showed a fast dissolution in artificial lung fluid, so a distinct detection of ZnO particles in lung cells was impossible. Genotoxicity tests (MN within 14-day test; CA and MLA in vitro) were overall negative. A 90-day study was done with the coated material (0.3 – 1.5 – 4.5 mg/m^3) and microsized material (4.5 mg/m^3). The high dose groups showed increase of LDH in BAL and hyperplasia and mononuclear cell infiltration. These effects were stronger for microsized particles. Translocation was not detectable. An extended design for the OECD413 for nanoparticles gives a better mechanistic understanding. **Recommendations**:

  i. Dissolution behaviour of test item (also if considered as “noble metal”) should be assessed in physiological fluids mimicking various lung-specific pH ambiences (neutral, acid)
  ii. BAL to be included mandatory.
  iii. Toxicokinetics (chemical analysis + TEM).
  iv. Expand endpoint pattern for genotoxicity (immunohistochemistry on oxidative damage of lung epithelial cells; MN in vivo for soluble NP.
  v. Dry aerosol or a liquid aerosol to be used? Depending on given test item and planned test approach (hazard- or risk driven).

It was discussed whether the lymph nodes were also assessed (they were but did not show effects) and what would be expected from the effects on ROS, which is often inconclusive for particle exposures. The physical chemical characterization was discussed and the importance of dissolution and it was mentioned that validation of immunohistochemistry (validation for e.g. quartz dusts existing, however, parallel reference within study helpful).

- Takuya Igarashi from Japan presented Carbon Nanotubes (MWCNTs and SWCNTs) in a wet-dispersion using whole-body exposure for a 4-week exposure period (6 hours/day, 5days/week) followed by a 3-month observation period. The average length of the MWCNT in suspension was 0.94μm with an average diameter of 48nm. The average length of the MWCNT in the exposure aerosol was 1.1μm and average diameter of 63nm. Mass concentration of the aerosol exposed was 0.37 mg/m^3. Results showed a transient and mild increase of lung weight and inflammation/fibrosis related gene (HO-1) expression. The NOAEL was determined as 0.37 mg/m^3. A suspension with longer MWCNT was also prepared and showed a similar inflammatory response in intratracheal instillation tests. For SWCNT inhalation exposure tests, mass concentrations of the aerosol were 0.03 and 0.13 mg/m^3 for SWCNT of primary diameter 3nm and 0.08 and 0.4 for SWCNT of 1.8nm. MWCNTs, DWCNTs and SWCNTs of different manufacturers caused an increase in neutrophils in the BAL after intratracheal instillation followed by a 2-year observation period. This is a typical endpoint for inflammation. A quantitative analysis method of MWCNT in lung tissue was developed to examine the clearance of MWCNT from the lung, which is essential for kinetic modelling. **Recommendations**: The combination of inhalation and instillation is effective in dealing with the diversity of nanomaterials with limited budget and time.

Session 3 - Studies on Inhalation Toxicity

- Frieke Kuper from the Netherlands gave suggestions to update the inhalation test guidelines to make them more appropriate for testing of nanomaterials. Nanoparticles may have properties, which lead to unique deposition and distribution, and interaction with tissues. Particles may enter the brain through the olfactory bulb – which implies that intratracheal instillation is an inadequate method for detecting these effects. Therefore inclusion of the olfactory bulb in OECD 412 was proposed, possibly by histopathological examination or by PET/MRI imaging (which may be expensive and difficult). BAL should be included to assess neutrophils, which is a sensitive method and easy to conduct. It was also suggested to 'synchronize' OECD412 with OECD407, especially with respect to immune parameters. Information about the fate in the body is important, so some kind of kinetics should also be included. As a research need it was suggested to add flags for chronic or delayed effects in sub-acute studies (e.g. immune diseases, fibrosis, emphysema, cancer, cardiovascular effects and brain diseases). The current studies may not be sufficient for sensitive groups, and it should therefore be considered whether the guideline has to be augmented with compromised animals (sensitive groups for asthma, COPD, CVA). If there is more information on the mode of action in in vivo studies, with identification of biomarkers, translation to humans may be improved. Frieke suggested that progressive fine tuning and flexibility in the guideline is needed. **Recommendations**: The following parameters were suggested to be added to the existing guidelines: the olfactory bulb, BAL and kinetics. Research needs include: the best way to examine the olfactory bulb and brain, which parameters should be measured in BAL, the relation between kinetics and toxicity testing, flags or biomarkers for chronic diseases and the need to test compromised animals.
  It was discussed whether the olfactory bulb is sensitive enough as the brain may be more sensitive. The brain is already included, but may be more specifically assessed. An approach should be developed on how translocation to the brain should be examined.

- David Warheit from BIAC presented the results of an OECD413 study with carbon nano fibres (CNF) and additionally assessed cardiovascular and pulmonary endpoints. Male and female rats were exposed to 0, 0.54, 2.5 or 25 mg/m^3 CNF for 13 weeks and evaluated for toxicological endpoints; additional groups of high- level exposed, as well as air-exposed controls were assessed at 3 months postexposure. Bronchoalveolar lavage fluid PMN, LDH, MTP protein and alkaline phosphatase levels were increased only at the high concentration (25 mg/m^3), and still after a 90-day recovery period. However no differences vs. control values were measured at the 0.54 or 2.5 mg/m^3 exposure concentrations for any BAL fluid endpoints. About 60-70% (0.5 mg/m^3) to 90% (2.5 or 25 mg/m^3) of the lavaged pulmonary macrophages contained nanofibres. Cell proliferation endpoints at the high dose were increased in the terminal bronchiole (no longer after recovery), in lung parenchymal or subpleural regions (both still some increase after 90-days recovery) but not mesothelial regions. In contrast, no increases in cell proliferation indices vs. air-exposed controls were measured at the 0.54 or 2.5 mg/m^3 exposure concentrations for any measured endpoints. No effects on cell proliferation in the heart were found at any concentration. Based upon anatomical histopathological assessments, the NOAEL was determined to be 0.54 mg/m^3, because at 2.5 mg/m^3 only minimal inflammation of the terminal bronchiole and alveolar ducts was found by the study pathologist. In contrast, however, none of the more sensitive pulmonary biomarkers such as BAL fluid endpoints or cell proliferation indices of the respiratory tract at this exposure level (2.5 mg/m^3) were different from control values after 90-day exposures in male or female rats. In addition, minor translocation of inhaled fibres to extrapulmonary organs was observed without any adverse systemic histopathological effects. Numerous cardiovascular and coagulation parameters were measured and did not show any effects of inhalation exposure. Local (respiratory) effects were found, but no systemic (cardiovascular) effects, which mean there was no connection between local and systemic effects. Given the apparent discrepancy in this study between the minimal (inflammation) histopathology observations vs. the lack of any significant quantitative differences measured between control values and rats exposed to 2.5 mg/m^3 with respect to more sensitive measured endpoints (BAL fluid analyses and cell proliferation), it is recommended that there should be a discussion regarding guidance on 1) the establishment of toxicological endpoint criteria, 2) quality of effects measured, and 3) weight-of-evidence approach requirement for establishing No Adverse Effects Levels in subchronic inhalation studies with nanoparticles. Alternatively, some findings/observations may be considered as biological adaptations.
  The relative surface area of these CNF particles was discussed, which is rather small (13.8 m^2/g compared to CNT (> 250 m^2/g), and may, in part, help explain the limited/minor respiratory effects measured in this study. In the discussion afterwards, the potential importance of kinetic information and/or translocation was mentioned to provide important insights and relevance for facilitating mechanistic assessment of the study findings. With regard to the importance of developing methodologies for assessing kinetics, it was noted, however, that the specific/individual methodolog(ies) for assessing nanoparticle kinetics in inhalation studies currently are not available and should be considered a high priority research need. It should also be clearly recognized that kinetic studies will require specific methodologies and corresponding validation procedures for each nanoparticle-type being tested (e.g., demonstrating the selective measurement of nanoparticle burden (vs. background) for each tissue being evaluated). In the discussion on the sensitivity of cardiovascular parameters, the use of compromised animals was mentioned. It was generally acknowledged the noncompromised rat is not a very sensitive model for detection of cardiovasculair effects. **Recommendations:**

  i. The inclusion of bronchoalveolar lavage studies as essential complementary components to traditional subchronic inhalation toxicity studies (OECD 413 studies) with particles and nanoparticles;

ii. A strong consideration of cell proliferation studies (of various components of the respiratory tract) as recommended supplementary/ complementary components to traditional subchronic inhalation toxicity studies (OECD 413 studies) with particles and nanoparticles;

iii. Development of better guidance criteria for establishing No Adverse Effect Levels in subchronic inhalation toxicity studies (OECD 413 studies) with particles and nanoparticles.

- Juergen Pauluhn from BIAC presented data of a subchronic 13-week inhalation studies with MWCNT in rats using a modular directed-flow nose-only inhalation system. The study design (duration of post exposure period of 6 months) and the selection of exposure concentrations utilized kinetic simulation of cumulative lung particle-volume burdens of MWCNT. The mechanistic basis of simulation was that lung inflammation is dependent on the cumulative displacement volume of MWCNT within the population of phagocytic cells as well the adaptive increase of these cells at overload conditions. The hypothesis of study was that onset of particle overload and the increase in particle elimination half-time are tightly associated. The outcome of study, both in regard to kinetic endpoints and biomarker of pulmonary inflammation, confirmed the PBPK simulations. The cumulative MWCNT dose that did not exceed an elimination half-time of t1/2 = 70-90 days at the end of the 13-week exposure period represented the NOAEL of study whereas the increase of t1/2 to approximately one year was considered to be as evidence that the Maximum Tolerated Cumulative Dose (MTD) has been attained or exceeded. JP concluded that pulmonary inflammation was best characterized by kinetic changes indicative of lung overload and neutrophilic granulocytes in Bronchoalveolar lavage. Moreover in regard to dosimetry and dose metric the apparent density of the particle (agglomerate or assemblage) appeared to be the most decisive dosimetric endpoint. This conclusion appears to be logical because the particle-displacement volume determines the degree of lung overload and inflammogenic potency accordingly. In other words, the most important dose metric in inhalation studies is the cumulative mass concentration which can readily converted to the cumulative retained particle volume by using the known relationship of particle volume = mass divided by agglomerate density. Of note is that the method used for density determination addresses pyknometrically the void space between particles (e.g., mercury pyknometry). Examples were presented which showed that the NOAELs correlated well with the particle densities of various types of particles (MWCNT, aluminum oxyhydroxide, AlOOH; iron oxide, Fe_3O_4, carbon black, and toner). The approximately 50-times lower density of MWCNT was considered to be the primary cause of their approximately 50-times higher inflammogenic potency relative to iron oxide. Based on computational toxicology, JP demonstrated further that the NOAELs and OELs for different kinds of particulates (from nano to micronsized) can be predicted and that the predictions match empirical data and existing workplace standards. It was concluded that for these types of particles, following dosimetric, morphometric, and exposure duration-related adjustments, a unified OEL of 0.5 microliter particle volume/m^3 (8 hours exposure/day/5 days per week, chronic exposure) is scientifically founded and defensible. This volumetric concentration can readily be converted to mass by multiplication with the particle density. The related derivation and conclusions were substantiated by multiple inhalation studies which can be summarized as follows: The subchronic exposure to respirable solid aerosols of MWCNT was tolerated without effects suggestive of systemic toxicity. Kinetic analyses demonstrated a markedly delayed clearance of MWCNT from lungs at overload conditions. Translocation into LALNs occurred only at dose levels where frank pulmonary inflammation occurred. Aluminum oxyhydroxide (AlOOH), a poorly soluble powder, with a primary particle size of either 10 or 40 nm, was examined to study as to how the agglomerate size and primary particle size modulate lung toxicity (6 hr/day, 5 days/week, 4 week exposure period followed by 3 months postexposure period). The findings support the hypothesis that deposited particles retained in BAL-cells exert toxic and kinetic properties similar to the agglomerated, micronsized particles than to

disintegrated nanoparticles. The degree of pulmonary effects appears to be better associated with the particle volume metric than a particle mass or a particle surface area metric. The particle-volume related overload was dependent on the 'agglomerate density', a particle characteristic commonly inappropriately defined in conventional inhalation studies. Rats nose-only exposed to pigment-sized iron oxide dust (Fe_3O_4, magnetite) in a subchronic 13-week inhalation study according to the OECD testing guidelines TG#413 and GD#39 were subjected to kinetic analyses made during a 3 months postexposure period. It was demonstrated that the kinetic factors were causative for lung inflammation occurring. Reversibility at cumulative exposure levels exceeding the lung overload threshold were dependent on kinetic factors. **Recommendations**: The apparent agglomerate density of inhaled particles appears to be amongst the most important characteristics which differentiate nanoparticle structures (low density) from granular micron sized particles (high density). The most robust exposure dose metrics appears to be the mass concentration (external, internal). Both the study design (dose selection) and the selection of the post exposure recovery period should take into account the kinetic behavior at non-overload and overload conditions. Animal numbers and study costs can be significantly reduced by executing hypothesis-based (PBPK-modeling) repeated exposure inhalation studies. Bronchoalveolar lavage and specialized approaches differentiating extravasated collagen (inflammatory) and that produced locally by myofibroblasts (fibrogenic) are important adjuncts worthwhile to consider. A combination of 4-week repeated inhalation studies accompanied by PBPK-based study design was considered to be the most powerful means to identify and assess nano-/micronsized pulmonary toxicity. Such an approach is self-validating in itself and delivers implicitly the information required for risk characterization and prioritization of testing (mode of action and associated critical metrics, reversibility, estimation of OEL).The duration of the recovery period is determined by the retention time of the particles. For each study, the outcome should be predicted and serve as a basis for a study. If the prediction is not correct, there is particle specific toxicity. GD39 should differentiate between acute and repeated dose toxicity and focus on cumulative lung burden. No nano-specific paradigms are needed, but overload occurs earlier for nanomaterials.

It was discussed whether there is a threshold for density, as this may be changed due to aerosolization, and whether an upper or lower limit applies to this concept. This concept is applicable for the particles in the respiratory range. Macrophage overload is the basis of this concept. Inhalation studies should be done to assess specific effects.

- Craig Poland from United Kingdom talked about the fibre paradigm and how it relates to other, non-fibrous nanoparticles that meet the same criteria of a low aerodynamic diameter yet one or more high aspects (e.g. plates, low density bundles). Fibrous shape can result in enhanced toxicity if the fibres are sufficiently long, durable and present in a location where their length prevents effective clearance such as the alveolar region and serosal cavities which may lead to inflammation, fibrosis and/or cancer. If not biopersistent, the dissolution of fibres will lead to breakage/dissolving resulting in their effective clearance and hence, reduction of dose. Short fibres are phagocytosed and cleared, again leading to a reduction in dose. Aerodynamic diameter (determining deposition), physical size and durability determine the fate of high aspect nanoparticles such as fibres (e.g. nanotubes or nanowires) or plates (e.g. grapheme platelets) and how they are dealt with in the lung and if they elicit frustration of phagocytosis.

  The pleura have been shown to be a target organ for pathogenic fibres such as asbestos. This would suggest a presentation of dose in this cavity and evidence from human autopsy show the presence of air pollution particles in the pleural space (black spots) suggesting that a fraction of inhaled particles transits through the pleural space. A recent study has also shown the transit of lung instilled carbon nanotubes into the pleural space of rats. Whilst low aspect (nano)particles can exit the pleural space to the draining lymph nodes via pore-like structures (stomata) on the parietal pleural wall, high aspect (nano)particles may be retained and elicit as response. This

presentation of dose and genesis of a response is shown in the staging of mesothelioma with the earliest stage involving the parietal pleura only, not the visceral pleura. This retention and effect was shown experimentally whereby fibrous and platelet nanoparticles injected in the pleural cavity resulted in their retention leading to an acute inflammatory response, and fibrosis which did not occur after injection of non-fibrous nanoparticles and these were detected in the outlying lymph nodes. However it was stressed that further work needs to be done to assess if such acute and sub-acute effects are likely to lead to mesothelioma or a benign lesion.
Some questions were asked about the preparation of the dispersions and about the next steps to assess the pleura. It was suggested that the effect should be studied, not just the presence of particles and also the retention over time. **Recommendations**: Inflammatory cells (differential cell count for total and percentage cells) in BAL should be a requirement in the test guidelines for all particles (and relevant to other substances) as a sensitive measure of respiratory effects. It is suggested that examining pleural effects and guidance on how to do this should be included in the test guidelines as a useful addition, but not compulsory and this would only be relevant to high aspect particles (an aspect >~10µm) such as fibres, plates and low density bundles.

- Shoji Fukushima from Japan presented data on a toxicity study with MWCNT. As for a carcinogenicity study, whole body exposure was preferred, this was also used for shorter term studies. Dry aerosols were generated up to 5 mg/m^3 by spin air sieve method. Lung tissue was dissolved to use for SEM and showing similar picture of MWCNT as for the aerosol. After the exposure, particles were found in the bronchiolar and alveolar space. On 28 days after exposure, all was phagocytozed and significant increases were found in total protein, albumin and alkaline phosphatase in BAL. A 14-day study again showed deposition of particles in the bronchiolar and alveolar spaces from 0.2 mg/m^3 in phagocytosed form and 1 mg/m^3 in free form, which were consistent up to a 4-week recovery period. At 5 mg/m^3 also deposition was found in the lung associated lymph nodes, which was higher after a 4-week recovery period. Histopathology showed granulation at 5 mg/m^3, which aggravated after a 4-week recovery and multinuclear giant cells were found after recovery. Total protein, albumin, alkaline phosphatase and inflammation were increased after exposure and showed less increase after 4 weeks. Neutrophil infiltration was found at 5mg/m^3. A 13-week study is ongoing at present.
  Some questions were asked about physicochemical characteristics, which was done on the bulk material, dose selection for the upcoming 2-year study, and about particle deposition in the nose where pathological changes were found.

- Wolfgang Kreyling from Germany talked about study designs for biokinetics. Nanoparticles deposit by diffusion and as the distance between air and circulation is small in alveoli (1-2µm), particles may have easy access to the circulation and can accumulate in organs. An intratracheal intubation study was done with radiolabelled spark generated iridium particles (CMD is 20nm, primary particle size 2 nm). The aerosol was inhaled within 5 seconds, allowing high (number) concentrations (107 /cm³). Particles were deposited in the complete lung and the fractions deposited in airways were excreted through the feces within 24 hours. Only little (0.1%) translocation in the liver, which increased over the first week and remained constant up to six months after exposure, and also similar translocation in the brain, spleen and heart. Experiments with 20nm and 80nm iridium and iridium particle labelled carbon nanoparticles (20nm, spark-generated) showed that translocation is dependent on the size of the material. Intratracheal intubation / ventilation versus nose-only exposure showed that ¾ of the deposition in the brain goes via the olfactory nerves and ¼ via systemic translocation. Further inhalation studies using 20nm gold, silver and TiO_2 nanoparticles clearly showed further substantial material differences in translocation of nanoparticle. Just after inhalation, most particles are deposited in the lungs. A rapid translocation occurs, resulting in long term retention (28 day) at low levels. All the nanoparticle aerosols used were freshly generated without any surface coating. Quantitative

biokinetics can teach us about differences in translocation between particles. It was discussed whether coatings or surface modifications particularly used to obtain stable nanoparticle suspensions may affect translocation, which is unknown, whether more studies should be done with radiolabelled materials, which may be helpful to get information on distribution, and whether an overload concept applies, which may for aggregates but not single particles. **Recommendations**: Include biokinetics in the test since different distribution patterns in the whole organism are likely dependent on physicochemical characteristics of nanoparticle aerosols and the dose at the target site will therefore be different. This will allow the assessment of accumulation of nanomaterials in the body.

ANNEX III: PARTICIPANTS LIST

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ANNEX IV:SUGGESTED REVISION FOR THE OECD TEST GUIDELINES AND GUIDANCE DOCUMENTS

Suggested revision for OECD Test Guidelines and Guidance Documents Drafted by Hans Muijser (TNO Triskelion bv) and Flemming R. Cassee (RIVM)

A. TG 403/412/413/436 on Inhalation Toxicity in light of the use for testing nanomaterials

- **Add to par 15 of TG 403** (and as such applies to other inhalation TGs): "The individual nano-sized particles of nanomaterials (or the elements of nanostructured materials) are smaller than the particle size ranges mentioned above. Nanomaterials should be dispersed as small as reasonably possible. This means that the aerosol preparation has to be optimized for respirability (< 3 μm MMAD) as well as being representative for worst-case exposure scenarios. However, aggregation and agglomeration seriously hamper generation of an aerosol consisting of nano-sized particles from a powder. Maximum reasonable effort is to aerosolize a powder by a venturi (Cheng et al., 1989) and further deagglomerate the particles in the aerosol with a jet-mill (Cheng et al., 1985; Castranova et al., 1996). If human exposure data are available adjust the size distribution of the aerosol such that it reflects realistic exposure scenarios. Attainable aerodynamic particle size distributions are generally expected to be in the 0.3 – 3 μm range, but aerosols consisting of single nanoparticles cannot be expected. If relevant, aerosols with smaller particles can be generated by atomizing a sufficiently diluted dispersion of nanoparticles (preferable in water; Mahurin and Cheng, 2007) or by evaporation and subsequent condensation of e.g. metals."
- **Add to par 25 of TG 403** (and as such applies to other inhalation TGs): For nanoparticles mass based dose metrics may not be the most appropriate and therefore also CMAD (count median aerodynamic diameter) may be relevant to characterize exposure to nano particles. This implies that instruments based on counting individual particles should be used (e.g. SMPS, ELPI).

TG 436

Delete the last sentence of paragraph 4:

"This Test Guideline is not specifically intended for the testing of specialized materials, such as poorly soluble isometric or fibrous materials or manufactured nanomaterials."

TG412 and 413

Delete in par 2: "This Test Guideline is not specifically intended for the testing of nanomaterials"

B. ENV/JM/MONO(2009)28 Guidance Document on Acute Inhalation Toxicity Testing

On Page 44 include an extra item between 74 and 75

"Although by definition nano-sized particles do not seem to fit into the 1-3 μm size range, the MMAD of an aerosol generated from a powder of nano-sized particles is invariably much larger than the primary particle size. This is because the aggregates or agglomerates of primary particles are hardly broken apart into primary sized nanoparticles when applying the usual methods of aerosol generation (e.g. venturi, fluidized bed). While inhalation toxicity tests with aggregates or agglomerates of nano-sized particles seem adequate for risk assessment for handling of powders, it may be relevant for risk assessment of nanoparticles in the production phase to include inhalation toxicity of aerosols consisting only of single nanoparticles. This can only be accomplished using special techniques e.g. electrospray, spark generators, atomizing a sufficiently diluted dispersion (Mahurin and Cheng, 2007), evaporation and subsequent condensation of e.g. metals alternatively, the original production process should be used in a small scale version. However, it should be taken into consideration that primary particles in an aerosol readily aggregate, in particular at high concentrations ($>10^5$ cm^{-3}) and that the aggregation velocity and size is dependent on the concentration. Hence, it may not always be technically feasible to keep (aggregated) particle size constant for different concentrations at the port of entry of the test animals (nose). In this case, C x t may not be constant. As indicated above (see 33), mass based dose metrics may not be the most appropriate and therefore also CMAD (count median aerodynamic diameter) may be relevant to characterize exposure to nano-sized particles. This implies that instruments based on counting individual particles should be used (e.g. SMPS, ELPI) such that representative measures are taken for animal exposures. Given the impact of the shape of fibres and particles, information on morphology is recommended to be collected from the test atmospheres."

Item 76 on Page 44: The third sentence should read:

"They should be designed to collect and classify the entire range of particle sizes present in the inhalation chamber. "

C. ENV/JM/MONO(2010)25 Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials

The following modifications are suggested for ENV/JM/MONO(2010)25 Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials:

Section D.4

i) "Exposure by inhalation of an aerosol of nano-sized particles which will in most cases be aggregated or agglomerated with substantial larger dimensions than the primary particles."

Section D.4.1 Respiratory tract exposures

"Although much research on nanoparticle toxicity have used saline to deliver test material to the lung via intra-tracheal instillation/aspiration, inhalation is the physiological process during which (nano)particles are deposited in the respiratory tract and the lungs, allowing for a slow build up of the dose and for normal clearance processes to occur. Therefore, inhalation is the highly preferred route of exposure prescribed in standard OECD Test Guidelines and this is the only way to determine the NOEL for the airborne concentration of suspended (nano-sized) particle dust."

"All technical aspects of inhalation toxicology studies including the use of dynamic nose-only inhalation systems are addressed in the OECD guidelines 403, 436, 412 and 413 and the associated guidance document GD 39 [OECD (2009) Guidance Document on Acute Inhalation Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No, 39].

The following specific aspects for studying the inhalation toxicity of nano-sized particles should be taken into consideration:

1: Characterization of the test atmosphere

The toxicity of aerosols in the 1-3 μm particle size range (MMAD) is expressed in mass based concentrations (e.g. mg/m^3). In most cases, a test atmosphere generated from a powder consisting of nano-sized particles will contain aggregates or agglomerates of nano-sized particles. Therefore, in general, the aerosol can be characterised with the usual instruments. ie. cascade impactor or other instruments based on mass and inertial forces, However, for the toxicity of nano-sized particles mass based dose metrics may not be optimal and the number of particles or the total surface area may be more relevant. Mass based instruments are insensitive to nano-sized particles because of their low weight (e.g. 1 million 10 nm particles may have the same weight as one 1μm particle). Therefore, if separate nano-sized particles can be expected to be present, mass based instruments to characterize the

concentration and particle size distribution should be supplemented with instruments based on counting individual particles (e.g. Scanning Mobility Particle Sizer (SMPS), Electrical Low Pressure Impactor (ELPI)). The concentration and the size distribution of surface area can be estimated from the particle size number distribution. In addition, separate instruments are available to assess the deposited particle surface area per part of the respiratory system. BET surface area only applies to the powder material and not necessarily to characterize the generated aerosol.

2: "If test atmospheres with separate nano-sized particles are generated, the use of material (expressed in mass) will often be too low to be measured accurately. Hence, it may not be possible to estimate the nominal concentration (mass of test material used divided by the volume of the test atmosphere used)."