

Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2)

Final Project Report

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Document name	Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2) – Final Project Report
Document reference	RNC/RIP-oN2/FPR/1/FINAL
Author(s)	Hankin S.M., Peters S.A.K., Poland C.A., Foss Hansen S., Holmqvist J., Ross B.L., Varet J. and Aitken R.J.
Date	01 July 2011

EXECUTIVE SUMMARY

The REACH Implementation Projects on Nanomaterials (RIP-oNs) seek to provide scientific and technical advice on key aspects of the implementation of REACH with regard to nanomaterials. The objectives of the RIP-oN 2 project, undertaken by a consortium led by the Institute of Occupational Medicine, were to develop specific advice on i) how REACH information requirements on intrinsic properties of nanomaterials can be fulfilled, including the appropriateness of the relevant test methods (and dosimetry) for nanomaterials and outline, when relevant, possible specific testing strategies and ii) the information that is needed for safety evaluation and risk management of nanomaterials and, in particular, if information is needed beyond or in addition to the current information requirements listed in REACH Annexes VI-X.

The project was implemented through a series of specified and linked tasks (A, B1-B5, and C). The project identified and reviewed relevant information sources (Task A) for carrying out: an analysis of the current REACH guidance on information requirements and testing and whether these requirements are applicable for nanomaterials (Task B1); identification of additional relevant specific intrinsic properties for which an adaptation of the information requirements and testing and other information generation methods/strategies might be needed for nanomaterials (Tasks B2 & B3); identification of needs for further research and development of test methods and other information generation methods/strategies in regard to nanomaterials (Tasks B4 & B5); an analysis of the needs and options for metrics/parameters in the hazard assessment compatible with the exposure assessment parameters/metrics in order to prepare a meaningful risk characterisation (Task C). Where relevant, additional information requirements beyond current REACH requirements have been identified when it is considered that they are needed to adequately address the properties of nanomaterials.

Comprehensive discussions of the findings from each stage of the project are provided in the individual Task Reports. This Final Project Report summarises the key specific issues related to nanomaterials in a REACH context and recommends updates to the Guidance in a form compatible with the possible future integration into the existing REACH Guidance on Information Requirements and Chemical Safety Assessment. Clear reference to the existing REACH Guidance Part and Chapter and sub-chapter is provided. For issues which are not currently technically/scientifically mature for developing detailed guidance, the need for further

research and development is indicated. All Task Reports were subject to review by the project's European Commission Steering Group (constituting representatives of JRC, DG Environment, DG Enterprise and ECHA) and by a Stakeholder Consultation Group (SCG) consisting of the members of the REACH Competent Authorities Sub-Group on Nanomaterials (CASG-Nano) and other relevant experts from Member States, industry and NGOs nominated by the REACH and CLP Competent Authorities (CARACAL)s. The draft Task Reports were opened for consultation with the above mentioned groups, discussed at meetings of the SCG, revised by the Project Consortium and re-opened for comment before being finalised.

Summary of Findings

Of the existing Information Requirements reviewed in Task B1, in general the guidance on physico-chemical properties is considered to be applicable to nanomaterials, with the exceptions of the limited relevance and applicability of the property and methods for surface tension, flash point and viscosity. Further evaluation of the suitability of existing methods for water solubility, partition coefficient, adsorption/desorption has also been recommended.

The existing guidance on toxicological data Information Requirements is considered applicable for the assessment of nanomaterials, although it has been highlighted that attention needs to be given to measuring, dosing, delivery and tracking of nanomaterials in the test system. In general, the basic ecotoxicological properties and endpoints described in OECD Test Guidelines for the determination of potential effects of test substances in relevant environmental compartments (aquatic, terrestrial, sediment) after acute or chronic exposure are considered adequate and relevant for nanomaterials. However, OECD acknowledge that the Test Guidelines were not specifically designed for the testing of nanomaterials, and the guidance provided on the preparation, delivery of test substances to test system, exposure quantifications, dose metrics, measurement, and metrology in all of these test guidelines is considered to be insufficient for testing of nanomaterials.

The potential additional relevant specific intrinsic properties, which have been *identified* in Task B2 from an objective review of published scientific sources of information, include:

<u>Physico-chemical properties</u>	<u>Toxicological endpoints</u>	<u>Ecotoxicological endpoints</u>
Particle shape	Cell uptake	Ventilation rate
Surface area	Cell viability	Gill pathologies
Surface energy	Oxidative stress	Mucus secretion
Surface chemistry	Inflammation	Brain pathology
Surface charge	Fibrosis	Animal behaviour
Redox potential	Immunotoxicity	Oxidative stress biomarkers
Cell-free ROS/RNS production capacity	(sensitisation)	(CAT, SOD, GPX, GST)
State of dispersion	Cardiovascular toxicity	
State of agglomeration		

Consideration of the value and feasibility of incorporating the *identified* potential additional relevant specific intrinsic properties into the REACH Guidance, has been undertaken by considering the scientific evidence in Tasks B3 and the gap analysis of Task B4.

Notably, the published scientific evidence reviewed and summarised in Task B3 demonstrates a consensus that representative sample preparation and thorough and accurate physico-chemical characterisation using multiple techniques is an essential component of assessing the potential (eco)toxicity of nanomaterials. With regard to toxicity, a range of endpoints have been examined relating to some of the existing Information Requirements under REACH, including: acute toxicity, repeated dose toxicity, toxicokinetics, mutagenicity, carcinogenicity & reproductive toxicity. However, no information regarding other REACH Information Requirements (such as dermal and respiratory sensitisation and irritation) was identified. Frequently, studies considered endpoints of cell viability, oxidative stress, and pro-inflammatory effects *in vitro*. Studies undertaken and reported to date have highlighted a number of key issues or gaps in existing testing strategies which may influence the outcome of studies, and thus should be observed closely in the assessment of nanomaterials within the context of REACH. Factors such as the exposure method, dose selection, species used, cell type under investigation, all have the potential to impact on the assessed toxicity of nanoparticles, indicating the importance of how experimental design can influence the resulting toxicological profile. A limited body of scientific evidence is available to inform the provision of specific practical advice with regard to

the ecotoxicological Information Requirements in REACH, but a number of issues have been found to influence the ecotoxicologic responses observed in the study of nanomaterials. These include: 1) coating/functionalisation of the surface and particle impurities, 2) suspension preparation methods, 3) release of free metal ions, 4) particle aggregation and 5) relevance of dose [concentration] - response for ecotoxicological studies of nanomaterials. The extent of influence of these factors on the ecotoxicological impact of nanomaterials is still emerging. Although at the time of writing this report, results from the OECD-WPMN Sponsorship Programme had not emerged, the limited information currently available from OECD-WPMN has been considered. ISO/CEN documents published or classified as being at Final Draft International Standard (FDIS), Draft International Standard (DIS) stage were reviewed and commented upon. Those at an earlier stage of development (i.e. Committee Draft stage or lower) were reviewed and commented upon, to the extent possible.

The gap analysis undertaken in Task B4 of relevant intrinsic properties for nanomaterials assembled and further developed the findings from the examination of existing REACH Guidance (Task B1), the identification of additional relevant specific intrinsic properties for nanomaterials (Task B2), and the assessment of relevance and applicability of testing, endpoints and methods described in the scientific literature and on-going international work relevant to the fulfilment of the data requirements under REACH (Task B3). The framework used for the gap analysis considered physico-chemical properties, toxicological and ecotoxicological endpoints, and integrated the existing and *identified* additional properties/endpoints to identify those which may and may not be addressed by standard test guideline methods and where further development of *in vitro*, *in vivo* or other methodologies is required. The gap analysis was structured by property/endpoint and systematically assessed whether a property/endpoint is relevant and methods applicable to nanomaterials. Commentary is provided on aspects including whether the property/endpoint is applicable to substances, particles, or nanomaterials only; the method type (standard, non-standard method or widely-accepted in R&D); the applicability and limitations of the method; information on the type of data provided by the method; and identification of research and development needs. The outcomes of the gap analysis have informed the development of specific guidance updates and recommendations for research and development.

In Task B5, the relevance and applicability of the current Integrated Testing Strategies (ITS) to nanomaterials for properties and endpoints in the existing Guidance have been reviewed and any limitations identified. For each ITS, the relevance and applicability to nanomaterials has been indicated along with the need for any update to the existing Guidance text, where this is considered feasible given the current state of knowledge. In general, only minor updates are necessary to some of the existing ITS for the properties/endpoints considered, including the general testing strategy for physico-chemical properties, water solubility (reflecting a need to distinguish between solubilisation and dispersion), and the need to justify scientifically the use of QSAR and/or read-across in the toxicological endpoints. A substantive update to the ITS for granulometry is recommended, reflecting the recommended substantive update to the guidance for this property. Advice is provided on the scientific basis for the categorisation of nanomaterials and application of *in silico* methods, read-across and category approaches for deriving hazard information for nanomaterials from the information on bulk substances or from comparison between nanomaterials. Whilst the lack of data across a wide range of structural and compositionally different nanomaterials precludes a fully prescribed category-based approach being developed, the suggested approaches for possible development indicate where such groupings may be applied.

Task C identified the critical items on exposure/dose descriptors and outlined needs for adequate metrics/parameters as appropriate for exposure assessment compatible with those used for hazard assessment. The metrics currently used in risk assessment (both regulatory and otherwise) across the three elements of exposure, (eco)toxicology and risk are based on mass or particle number. The most prominent emerging alternative or additional metric identified for use in relation to the risk assessment of nanomaterials is surface area. This is based primarily on toxicological evidence relating particle surface area to inflammation, an indicator of toxicity. There are currently no definitive conclusions on the best metric. However, there is consensus that there should be sufficient characterisation of the forms of a substance to allow the dose-response to be expressed in the different metrics discussed - number, surface area and mass. It is important to note that there are other parameters which can act as modifiers of the (eco)toxicity, including particle size, size distribution, density, aggregation and shape, but these parameters would not generally be considered as scalable quantities and do not appear to conform to the current use of the term "metric" under REACH, and were therefore not considered further.

On the basis of the activities undertaken in each of the aforementioned Tasks, recommendations have been proposed for guidance updates in the following documents/chapters:

Physico-chemical Properties

Additional relevant specific intrinsic properties of Shape and Surface Area are recommended as physico-chemical Information Requirements and consequently new guidance chapters are recommended. Text has been developed in accordance with the structure and nature of current guidance and compatible with incorporating the properties either as new Information Requirements or subordinate to the existing Granulometry Information Requirement.

Updates to guidance have been recommended for:

- **Guidance on Information Requirements and Chemical Safety Assessment Part B: Hazard Assessment, B.6.1.4 Other physico-chemical properties**
- **Guidance on Information Requirements and Chemical Safety Assessment Part B: Hazard Assessment, Appendix to Part F – CSR Template with explanation**

A new section, in the introduction of “Guidance on Information Requirements and Chemical Safety Assessment Chapter R7a: Endpoint Specific Guidance” is recommended to address **sample preparation** issues applicable to the determination of all properties and endpoints.

Updates to specific chapters in R7 have been recommended for:

- **R.7.1.1 Introduction**
- **R.7.1.7 Water solubility**
- **R.7.1.8 Partition coefficient**
- **R.7.1.14 Granulometry**
- **R.7.1.15 Adsorption/desorption**

Toxicological Endpoints

No new additional relevant specific intrinsic properties are recommended for toxicological Information Requirements, however, *Advisory Notes* on the following aspects have been recommended and developed:

- **Prior to R.7.2**
 - Rat lung overload within inhalation toxicity assessment
 - Assay inhibition/enhancement (interference)
- **R7.7: Mutagenicity & carcinogenicity (*in vitro* data section)**
 - Bacteria assay interference

Updates to guidance have been recommended for:

- **R7.2 Skin & eye irritation/corrosion**
- **R.7.3 Skin & respiratory sensitisation**
- **R.7.4 Acute toxicity**
- **R.7.5 Repeated dose toxicity**
- **R7.6 Reproductive & developmental toxicity**
- **R.7.7 Mutagenicity & carcinogenicity**

Ecotoxicological Endpoints

No new additional relevant specific intrinsic properties are recommended for ecotoxicological Information Requirements, however, updates to guidance have been recommended for:

- **R7.8.4 Evaluation of available data on aquatic pelagic toxicity**
- **R.7.9.3 Information on degradation/biodegradation and its sources**
- **R.7.10.3.2 Non-testing data on bioaccumulation**
- **R.7.11.3.1 Laboratory data for effects on terrestrial organisms**

On the basis of the activities undertaken in each of the Tasks, recommendations for research & development have been indicated for the following aspects, properties or endpoints:

Physico-chemical properties

- **General aspects (e.g. characterisation, standards, protocols etc)**
- **Existing Information Requirements**

Relative density	Partition coefficient	Granulometry
Surface tension	Flammability	Adsorption/desorption
Water solubility	Explosive properties	Dissociation constant

- **Additional specific intrinsic properties**

Shape	Surface chemistry	Redox potential
Surface area	Surface acidity	Cell-free ROS/RNS production capacity
Porosity	Surface charge	
Surface energy		

Toxicological endpoints

- **Non-testing *in silico* approaches**
- **Study design**

Dispersion	Using physico-chemical data to inform experimental design	Adopting standardised controls
Selection of dose		Interferences
Selection of exposure route & duration	Target-organ toxicity considerations	
Interactions		

- **Endpoint-specific or ‘mechanism’-associated R&D**
- **Study design**

Utilising Bronchio Alveolar Lavage (BAL)	Reproductive toxicity	Genotoxicity
Acute toxicity	Inflammation & cytotoxicity	Particle translocation
Repeated dose toxicity	Oxidative stress	Cardiovascular toxicity

Ecotoxicological endpoints

- **General considerations**

- Aquatic testing
- Soil testing

- **Data on aquatic pelagic toxicity (under R.7.8.4.1)**

Growth inhibition in aquatic plants	Short-term toxicity testing on fish	Short-term testing on fish embryo and sac-fry stages
Short-term toxicity testing on invertebrates	Long-term toxicity testing on fish	Fish, juvenile growth test
Long-term toxicity testing on invertebrates	Fish early-life stage test	

- **Data on toxicity to sediment organism (under R.7.8.9.1)**

- Long-term toxicity to sediment organism

- **Information requirements for toxicity to STP microorganisms**

- Activated sludge respiration inhibition testing

- **Ready biodegradability (under R.7.9.2.1)**

- **Hydrolysis as a function of pH (under R.7.9.2.2)**

- **Data on aquatic bioaccumulation (under R.7.10.3.1)**

- Bioaccumulation in aquatic species

- **Data on avian toxicity (under R.7.11.3.1)**

- Long-term or reproductive toxicity to birds

- **Laboratory data (under R.7.11.3.1)**

Effects on soil micro-organisms	Long-term toxicity on soil invertebrates	Long-term toxicity on plants
Soil short-term toxicity to invertebrates	Short-term toxicity to plants	

- **Possible R&D on possible additional specific intrinsic properties (under R.7.8.4.1)**

Fish ventilation rate	Animal behaviour	Trojan-horse effect of nanomaterials
Fish gill pathology	Oxidative stress	
Fish mucous secretion	Daphnia heart rate, hopping frequency, appendage movement	
Fish brain path		

The RIP-oN 2 project has been performed as an objective scientific review based on an informed, objective and systematic gathering and consideration of evidence by experts who have used their knowledge and professional judgement when considering the relevance and contribution of the scientific evidence towards delivering the project's objectives.

A comprehensive synthesis of findings, implications, issues and advice has been developed and integrated through the Task Reports and the Final Project Report. Where considered relevant, feasible and justified, specific advice for updating guidance has been provided. For issues which are not currently technically/scientifically mature for developing detailed guidance, the need for further research and development has been indicated.

The assessment of the scientific evidence and subsequent recommendations are the considered opinion of the authors and are submitted for consideration by the European Commission.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
8-OH-dG	8-Oxo-2'-deoxyguanosine
AAN	Average Agglomeration Number
AFM	Atomic Force Microscopy
AM	Alveolar Macrophage
AP-1	Activator Protein 1
ApoE	Apolipoprotein E
APS	Aerosol Particle Sizer
ARDS	Acute Respiratory Distress Syndrome
ASTM	American Society for Testing and Materials
ATM	Atomic Force Microscopy
ATP	Adenosine triphosphate
AUC	Analytical Ultracentrifuge
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BCF	Bioconcentration Factor
BET	Brunauer, Emmet and Teller
BS	British Standard
CARACAL	Competent Authorities for REACH and CLP
CASG Nano	Competent Authorities Sub-group on Nanomaterials
CAT	Catalase
CE	Capillary electrophoresis
CEFIC	European Chemicals Industry Council
CEN	European Committee for Standardization
CLP	Classification, Labelling & Packaging
CMR	Carcinogenic, Mutagenic, or Reproductive toxin
CNT	Carbon Nanotube
CPC	Condensation Particle Counter
CSA	Chemical Safety Assessment
CSR	Chemical Safety Report
DCF	2', 7'-Dichlorodihydrofluorescein
DCFH	2', 7'-Dichlorodihydrofluorescin
DDP	Dossier Development Plans
DEMC	Differential Electrical Mobility Classifier
DFT	Discrete Fourier Transform
DHE	Dihydroethidium
DIS	Draft International Standard
DLS	Dynamic Light Scattering
DMAS	Differential Mobility Analyzing System
DNA	Deoxyribonucleic acid
DNEL	Derived No Effect Level
DSC	Differential Scanning Calorimetry
E. coli	Escherichia coli
EC	European Commission
EC50	Half Maximal Effective Concentration
ECB	European Chemicals Bureau
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals

Abbreviation	Definition
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EDXA	Energy Dispersive X-ray Analyzer
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
ELPI	Electrical Low Pressure Impactor
ENRHES	Engineered Nanoparticles – Review of Health & Environmental Safety
EPA	Environmental Protection Agency
ES	Embryonic Stem Cells
ESR	Electron Spin Resonance
EST	Embryonic Stem Cell Test
EU	European Union
FCS	Fluorescence Correlation Spectroscopy
FDIS	Final Draft International Standard
FELS	Fish Early-Life Stage
FFF	Field Flow Fractionation
FISH	Fluorescence In Situ Hybridisation
FMPS	Fast Mobility Particle Sizer
FP	Framework Programme
FRAP	Ferric-Reducing Antioxidant Power
GGT	Gamma Glutamyl Transferase
GHS	Global Harmonized System
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GPX	Glutathione Peroxidase
GRO	Growth Regulated Oncogene
GSD	Geometric Standard Deviation
GST	Glutathione-S-Transferase
HARN	High Aspect Ratio Nanoparticle
HDF	Hydrodynamic Fractionation
HO-1	Heme oxygenase-1
HRTEM	High Resolution Transmission Electron Microscopy
HSE	Health and Safety Executive
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
IHCP	Institute for Health and Consumer Protection
IL	Interleukin
ILSI	International Life Sciences Institute
IOM	Institute of Occupational Medicine
IR	Information Requirement
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
ITS	Integrated Testing Strategy
JRC	Joint Research Centre
Kd	Distribution Coefficient
Koc	Organic Carbon-Water Partition Coefficient
Kow	Octanol-Water Partition Coefficient
LC50	Half Maximal Lethal Concentration
LDH	Lactate Dehydrogenase
LLNA	Murine Local Lymph Node Assay
LOEC	Lowest Observed Effect Concentration

Abbreviation	Definition
LSLPC	Light Scattering Liquid-Borne Particle Counter
M1dG	N-1,N2 malondialdehyde-2'-deoxyguanosine
MAP	Mitogen-activated protein
MMAD	Mass Median Aerodynamic Diameter
MS	Mass Spectroscopy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCNT	Multi-walled Carbon Nanotubes
NFκB	Nuclear Factor Kappa B
NGO	Non-Governmental Organisation
NIA	Nanotechnology Industries Association
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute for Standards Technology
NM	Nanomaterial
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
NOM	Natural Organic Matter
NP	Nanoparticle
NPL	National Physical Laboratory
NRF2	NF-E2-related factor 2
NSAM	Nanoparticle Surface Area Monitor
NTA	Nanoparticle Tracking Analysis
OECD	Organisation for Economic Co-operation and Development
OECD-WPMN	Organisation for Economic Co-operation and Development Working Party on Manufactured Nanomaterials
OPC	Optical Particle Counter
ORAC	Oxygen Radical Anti-oxidant Capacity
PBPK	Physiologically-Based Pharmacokinetic
PCCS	Photon Cross Correlation Spectroscopy
PCS	Photon Correlation Spectroscopy
PEC	Predicted Environmental Concentration
PM	Particulate Matter
PMN	Polymorphonuclear Leukocytes
PNEC	Predicted No Effect Concentration
PSLT	Poorly Soluble, Low Toxicity
PZC	Point of Zero Charge
QSAR	Quantitative Structure Activity Relationship
QSPR	Quantitative Structure Property Relationship
R&D	Research and Development
RCR	Risk Characterisation Ratio
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals Regulation
RIP-oN	REACH Implementation Projects on Nanomaterials
RIVM	National Institute for Public Health and the Environment
RMM	Risk Management Measures
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAR	Structure Activity Relationship
SAXS	Small-Angle X-ray Scattering
SCENHIR	Scientific Committee on Emerging and Newly Identified Health Risks
SCG	Stakeholder Consultation Group

Abbreviation	Definition
SEM	Scanning Electron Microscopy
SI	Substance Identification
SMPS	Scanning Mobility Particle Sizer
SOD	Superoxide Dismutase
SOP	Standard Operating Procedure
STAT	Signal Transducers and Activators of Transcription protein
STEM	Scanning Transmission Electron Microscopy
SWCNT	Single-walled Carbon Nanotubes
t/y	Tonnes per year
TEAC	Trolox Equivalent Anti-oxidant Capacity
TEER	TransEpithelial Electrical Resistance
TEM	Transmission Electron Microscopy
TG	Test Guideline
TGA	Thermogravimetric Analysis
TGF- β	Transforming growth factor-beta
TH	T helper cell
THF	Tetrohydrofuran
TNF- α	Tumour Necrosis Factor-alpha
UV	Ultraviolet
VCI	Verband der Chemischen Industrie
Vis	Visible
VSSA	Volume Specific Surface Area
WHO	World Health Organisation
WoE	Weight of Evidence
WST-1	Water Soluble Tetrazolium salt-1
XRD	X-Ray Diffraction

1 INTRODUCTION

This document constitutes the Final Report provided by the contractor to the JRC on the project "Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH" (RIP-oN 2).

1.1 PREFACE

1.1.1 The implementation of the European Union's Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation (EC) No 1907/2006, represents a fundamental shift in the regulation of manufactured and imported chemicals in the European Union. Having entered 'into force' on 1 June 2007 and 'into operation' on 1 June 2008, the new regime's overriding objective, is 'to ensure a high level of protection of human health and the environment while including the promotion of alternative methods for assessment of hazards of substances, as well as the free circulation of substances on the internal market while enhancing competitiveness and innovation.' and is underpinned by the precautionary principle.

1.1.2 REACH effectively shifts responsibility from authorities to industry to gather information on chemical substances and assess their safety. The provisions of REACH refer to substances (in whatever size or forms) and also apply to nanomaterials, that are considered either as distinct substances or forms of a substance (CA/59/2008rev1). However, a degree of uncertainty exists concerning the adequacy of the regulation and the accompanying guidance for the emerging and rapidly developing nanomaterials industry.

1.1.3 Therefore the Commission launched the REACH Implementation Projects on Nanomaterials (RIP-oNs) with the objective to provide scientific and technical advice on key aspects of the implementation of REACH with regard to nanomaterials, namely:

- i) Substance Identification (SI) (RIP-oN 1)
- ii) Information Requirements (IR) (RIP-oN 2)
- iii) Chemical Safety Assessment (CSA) (RIP-oN 3)

1.1.4 The Institute for Health and Consumer Protection (IHCP) of the Joint Research Centre (JRC) was asked to perform and coordinate the activities aimed at developing advice for possible future REACH guidance improvement. The advice should be based on the scientific and technical state of the art information,

experience and methodology regarding nanomaterials (NM). It should provide concrete proposals that could be implemented directly, and indicate the possible way forward for any issues and methods that need further work and could be implemented in the short and medium term. The main focus should be on issues and methods that could be included in the REACH guidance and possibly implemented in the short term, after the pertinent further development and consultation process. These recommendations would contain practical proposals for how and based on which information this update could take place. The outputs are to be developed in such a way that the advice on specific issues related to nanomaterials can be integrated into the existing REACH guidance documents. The actual inclusion of any of the advice into the guidance documents is the responsibility of the European Chemicals Agency (ECHA) and is not part of the commissioned projects. The work is performed in close collaboration with DG Environment, DG Enterprise and ECHA who constitute the steering group for these activities.

1.1.5 JRC let competitive tenders and commissioned two REACH Implementation Projects on Nanomaterials (RIP-oN 2 and RIP-oN3), with the purpose of respectively providing "Specific advice on fulfilling information requirements for nanomaterials under REACH" (RIP-oN 2) and "Specific advice on exposure assessment and Hazard/Risk Characterisation for nanomaterials under REACH" (RIP-oN 3).

1.1.6 In this document, the consortium that was awarded the tender of the project "Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH" (RIP-oN 2) provides its final overall report to the JRC.

1.2 CONSIDERATION OF THE PURPOSE, SCOPE AND FINDINGS OF THE RIP-ON 2 PROJECT IN THE CONTEXT OF SUBSTANCE IDENTIFICATION AND REGISTRATIONS ADDRESSING SEVERAL FORMS, INCLUDING NANOFORMS

1.2.1 Assisted by the 'Guidance for identification and naming of substances in REACH'¹, the registrant shall decide whether different forms of a (nano-)material shall be registered in their own right or together with other forms, e.g. the micron or bulk (non-nanoscale) form. It should be noted that the ongoing REACH Implementation Project on Nanomaterials 1 (RIP-oN 1) is addressing how the guidance on

¹ http://guidance.echa.europa.eu/docs/guidance_document/substance_id_en.htm?time=1301642719

identification and naming could be updated to reflect in more detail how to address nanoforms. The results of RIP-oN 1 will eventually be handed over to ECHA and ECHA might in turn decide to update the guidance for identification and naming of substances.

- 1.2.2 Until a possible update of the identification and naming guidance, the registrant is referred to the document 'Nanomaterials in REACH' (CA/59/2008 rev. 1)², specifying, between others:

"REACH is based on the principle that it is for manufacturers, importers and downstream users to ensure that they manufacture, place on the market or use such substances that do not adversely affect human health or the environment (Article 1(3) of REACH). This principle is applicable to substances in whatever size or form and for all their identified uses. Thus, a registration of a nanomaterial has to include all relevant information on the nanomaterial as manufactured or imported, covering the properties, uses, effects and exposure related information as well as the relevant classification and labelling, safety assessment and any relevant exposure scenarios " (p. 6), and

"For substances at nanoscale that are phase-in substances, the registration can be more complex, especially when the same substance exists in the nanoform as well as in the bulk form. In such a case not only the information of the substance in the bulk form should be included in the registration dossier, but also any information regarding intrinsic properties where the properties of a substance in the nanoform differs from the bulk form, any different classification and labelling, any different chemicals safety assessment as well as all identified uses (see also Annex VI.3 of REACH) and relevant exposure scenarios for the nanoform of the substance." (p.8).

- 1.2.3 Until more concrete guidance is provided by ECHA, it is suggested that the registrant follows this line. This has a direct influence on the generation of hazard data, e.g. any read-across from one form to the other (being from a bulk form to a nanoform or between nanoforms) should be scientifically justified. It also has influence on the information in the supply chain, which has to be appropriate to the

² <http://ec.europa.eu/environment/chemicals/reach/pdf/nanomaterials.pdf>

form(s) passing down the supply chain and the Chemical Safety Assessment should support this. The suggested guidance updates from the RIP-oN 2 project need to be seen in this light.

1.3 PROJECT OBJECTIVES

1.3.1 The objectives of the RIP-oN 2 project were to:

- Develop specific advice on how REACH information requirements on intrinsic properties of nanomaterials can be fulfilled. This should address and advise on the appropriateness of the relevant test methods (including dosimetry) for nanomaterials and outline, when relevant, possible specific testing strategies.
- Develop advice on the information that is needed for safety evaluation and risk management of nanomaterials and in particular if information is needed beyond or in addition to the current information requirements listed in REACH Annexes VI-X.

1.4 THE PROJECT CONSORTIUM

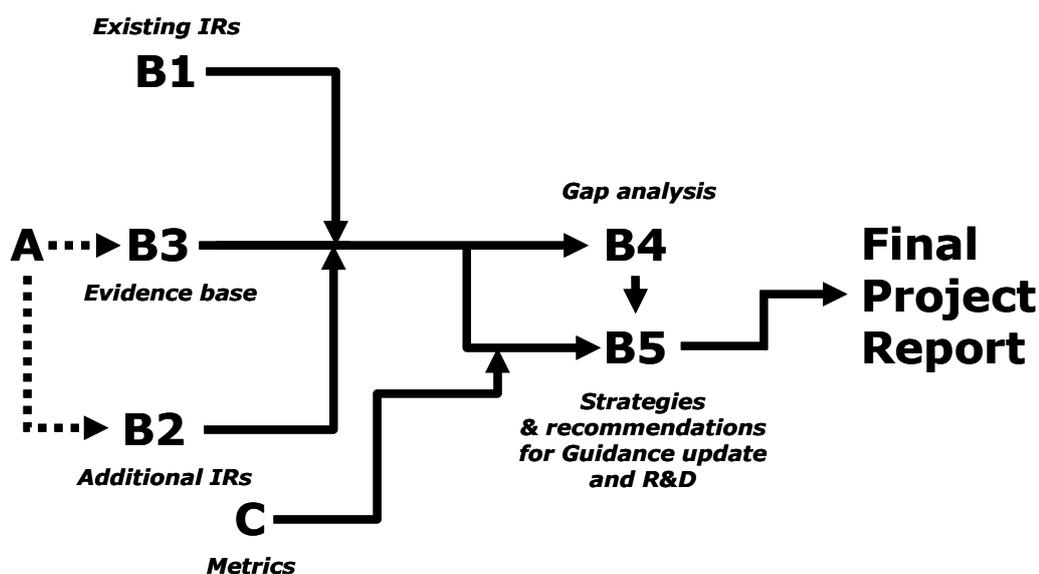
1.4.1 The consortium awarded the tender for RIP-oN 2 comprises the Institute of Occupational Medicine (IOM) through its SAFENANO unit, the Nanotechnology Industries Association (NIA) and the European Chemicals Industry Council (CEFIC).

1.4.2 IOM/SAFENANO, with an established reputation for independent scientific work, led the consortium and carried out majority of the technical activities. NIA facilitated and provided a transparent interface between the project and the stakeholder group, as well direct access to industry and industrial knowledge. CEFIC contributed a breadth of experience and expertise on REACH activity as well direct access to industry and industrial knowledge.

2 DESCRIPTION OF THE PROJECT

2.1 OVERVIEW

2.1.1 The project was implemented through a series of specified and linked tasks (A, B1-B5, and C). The relationship between each task is illustrated in the scheme below, with specific details of the task description provided in the table of Deliverables overleaf.



2.1.2 The project commenced with the identification and review of all relevant information sources (Task A) for carrying out the subsequent tasks (B1-B5 and C), which included: an analysis of the current REACH information requirements and testing and whether these requirements are appropriate for nanomaterials (Task B1); identification of additional relevant specific intrinsic properties for which an adaptation of the information requirements and testing and other information generation methods/strategies might be needed for nanomaterials (Tasks B2 & B3); identifying needs for further research and development of test methods and other information generation methods /strategies in regard to nanomaterials (Tasks B4 & B5); an outline of the needs and options for metrics/parameters in the hazard assessment compatible with the exposure assessment parameters/metrics in order to prepare a meaningful risk characterisation (Task C). Where relevant, additional information requirements beyond current REACH requirements have been identified when it is considered that they are needed to address adequately the properties of nanomaterials.

2.1.3 The Final Project Report (Task D) compiles and summarises all the previous Deliverables in a single comprehensive document in such a form that advice on specific issues related to nanomaterials can be considered for integration into the REACH guidance documents and further research and development on relevant issues can be initiated.

2.2 DELIVERABLES

2.2.1 A series of reports were developed for the specified tasks, as summarised below.

Task	Deliverable
A	<p>A Short Report containing:</p> <ol style="list-style-type: none"> 1. A brief description of the approach/methodology used to identify relevant information sources; 2. A list of identified information sources with clear indications of which ones are relevant for the subsequent tasks; 3. For relevant information sources, a brief summary of relevant content and timelines for final outputs (in the case of on-going projects).
B1	<p>An Analysis Report making direct reference to the existing Guidance on IR & CSA for REACH (Part and (sub)Chapter) of the general information requirements and testing(/information generation) strategies for nanomaterials as applied today, based, among others, on the three case example materials.</p>
B2	<p>A Scientific Report and table/grid on "Identification and overview of additional relevant specific intrinsic properties for nanomaterials".</p>

Task	Deliverable
B3	<p>A Summary Analysis Report containing sections on:</p> <ol style="list-style-type: none"> 1. Practical advice on the relevance and applicability of the experience reported in finalised and on-going FP6/7 projects on nanomaterials characterisation and hazard identification and assessment for workers, consumers and environment into the REACH context; 2. Practical advice on the relevance and applicability of the experience reported in the scientific literature on nanomaterials characterisation and hazard identification and assessment for workers, consumers and environment into the REACH context; 3. Practical advice on the use of information from e.g. the Organisation for Economic Co-operation and Development Working Party on Manufactured Nanomaterials (OECD-WPMN) and other sources on the appropriateness of existing testing methods and results from the sponsorship programme in fulfilling the REACH data requirements; 4. Practical advice on the basis of on-going work in ISO and CEN (and, as identified, other harmonization bodies) in relation to whether relevant methods for substance characterisation could be used in fulfilling REACH data requirements.
B4	<p>A Summary Analysis Report on the "Gap analysis of relevant intrinsic properties for nanomaterials, which may not be addressed by standard test guideline methods and for which further development of in vitro, in vivo or other methodologies is required".</p>

Task	Deliverable
B5	<p>Scientific Report on Test Methods and Strategies for Nanomaterials, including chapters on:</p> <ol style="list-style-type: none"> 1. Advice on integrated testing strategies relevant to specific nanomaterials properties and how the specific intrinsic properties of nanomaterials might affect the need for adaptations to the testing regime; 2. Advice on the scientific basis for the categorisation of nanomaterials and application of in silico methods, read-across and category approaches for deriving hazard information for nanomaterials from the information on bulk substances or from comparison between nanomaterials; 3. Proposals for further amendment of the REACH guidance documents in regard to information requirements, test methods or testing strategies for nanomaterials, where appropriate, taking into account the provisions to minimise use of animals for testing. These should also consider whether additional information requirements beyond the standard REACH requirements would be appropriate for nanomaterials e.g. for addressing the gaps identified in B4; 4. Proposal for further research and development of test methods and other data generation methods/strategies in regard to nanomaterials.
C	<p>A working document on identification of critical items on dose descriptors and related parameters, outlining needs for adequate metrics/parameters as appropriate for hazard assessment compatible with the ones used for exposure assessment as well as for the read-across from bulk substances and from other nanomaterials. This document has been developed in close collaboration with RIP-oN 3.</p>
D	<p>A Compiled Summary Report on the overall project, describing:</p> <ol style="list-style-type: none"> 1. Specific issues related to nanomaterials that can be integrated into the existing REACH Guidance on Information Requirements and Chemical Safety Assessment with reference to the corresponding Part and (sub)Chapter; 2. Needs for further research and development of test methods and other methods of information generation.

2.2.2 The present report, as already indicated in the introduction, constitutes the Final Project Report (Task D) and provides advice for updating the guidance and on research and development needs. This takes the form of specific recommendations or options for consideration by the Commission. For issues which are not currently

technically/scientifically mature for developing detailed guidance, the need for further research and development is indicated.

- 2.2.3 The focus of the RIP-oN 2 has been on nanomaterial relevant issues. Nevertheless, due to the nature of some nanomaterials, some proposals may have implications for other substances that are not nanomaterials. These would have to be considered if reshaping of the REACH guidance would take place.

2.3 REVIEW AND CONSULTATION

- 2.3.1 All Task Reports were subject to review by the project's Steering Group (constituting representatives of JRC, DG Environment, DG Enterprise and ECHA) and a Stakeholder Consultation Group (SCG) consisting of the members of the REACH Competent Authorities Sub-Group on Nanomaterials (CASG-Nano) and other relevant experts from Member States, industry and NGOs nominated by the REACH and CLP Competent Authorities (CARACAL). The draft Task Reports were opened for consultation with the above mentioned groups, discussed at meetings of the SCG, revised by the Project Consortium and re-opened for comment before being finalised.

2.4 TECHNICAL APPROACH

- 2.4.1 The project has been performed as an objective review of the existing guidance and available scientific evidence pertinent to the specified tasks. Existing Information Requirements across all tonnage levels have been considered and potential additional Information Requirements for nanomaterials identified, prior to conducting the gap analysis of properties and methods to facilitate the identification of recommendations and research & development requirements.
- 2.4.2 The conduct of this scientific review is based on an informed, objective and systematic gathering and consideration of evidence by experts who have used their knowledge and professional judgement when considering the impact and contribution of a source document to the task objective. It is important to note the inherent limitations of a review activity. Reviews are conducted at a fixed point in time which precludes the inclusion of information becoming available after a cut-off date. Information sourced may be incomplete or, on closer inspection, the content of a source document bears no relevance to the issues being considered. Information may also change in revisions of the sources considered.

- 2.4.3 Based on the objective and informed assessment of published reports constituting the evidence-base available, a synthesis of findings, implications and/or issues distilled from the sources has been developed and integrated into the task reports. The review of source reports has identified the key findings and gaps to establish a technical basis facilitating the development of advice pertinent to the project.
- 2.4.4 **Identification and review of information sources (Task A)**
- 2.4.5 Relevant information was collected, assessed, categorised and made available to the project team. There is a range of relevant information and information types. The information included background information from organisations such as CASG Nano, OECD WPMN, SCENIHR, Standards organisation such as ISO and CEN, FP6/7 projects, other ongoing national projects, other international regulatory organisations such as NIOSH and EPA and from the peer reviewed literature. Reports and papers were assessed for specific relevance to the project.
- 2.4.6 The report from Task A comprises a brief description of the approach/methodology used to identify relevant information sources, the list of identified information sources with clear indications of their relevance to the respective tasks and comment of the relevant content.
- 2.4.7 **REACH Information requirements for nanomaterials (Task B)**
- 2.4.8 Task B comprised five sub-tasks (B1-B5). Each sub-task was conducted using data available from the public domain, in the broadest context of nanomaterials, and used case-example materials (MWCNT, Ag, TiO₂ and ZnO for ecotoxicology) as appropriate, to exemplify specific aspects necessary for these types of nanomaterials. Each of the five sub-task reports built on the previous task(s) and its associated report, and comprise multiple components addressing physico-chemical properties, toxicology, and eco-toxicology aspects, as well as cross-cutting issues.
- 2.4.9 For Task B1, a thorough analysis was carried out of the endpoint specific guidance, R7a-c. Chapter-by-chapter, the current guidance text has been analysed to establish if there are any differences in application between what could be called conventional substances and those at nanoscale. The report is organised by REACH Guidance headings or groups of headings (in bold and labelled with their

corresponding section number from the current REACH Guidance document) and comments provided in the context of the task objective.

- 2.4.10 The approach undertaken to identify candidate additional relevant specific intrinsic properties for nanomaterials in Task B2 utilised key reports from different organisations, scientific opinions and working documents of OECD and ISO/CEN. Previously suggested properties and endpoints from these reports for use in nanomaterials risk assessment are highlighted. The task report (RNC/RIP-oN2/B2/2/FINAL) identifies candidate additional relevant specific intrinsic properties and provides a contextual or principle-based overview of their relevance to nanomaterials and REACH.
- 2.4.11 In Task B3, a comprehensive review of information sources identified in Task A was carried out. Detailed consideration of supporting information pertaining to intrinsic properties was undertaken in Task B3 in the context of i) the relevance and applicability of the experience reported in the scientific literature and gained in several finalised and on-going FP6/7 projects; ii) the use of information from OECD-WPMN; and iii) the basis of on-going work in ISO and CEN. The review of information sources provides a basis for the gap analysis in Task B4 of relevant intrinsic properties which may not be addressed by standard test guideline methods and for which further development of in vitro, in vivo or other methodologies is required. The associated Task report (RNC/RIP-oN2/B3/2/FINAL) is organised first into Sections in accordance with the sequence of objectives (the peer-reviewed literature is however reported separately from FP6/7 projects), and then by relevance to physico-chemical information, toxicological information and ecotoxicological information. Where possible, it has been identified where OECD test guidelines and ISO/CEN (or equivalent) standards have been utilised on the basis of the information provided in the publications reviewed. Thus, in the absence of any statement to the contrary, the protocols utilised in the FP7/FP6 projects and scientific literature are either non-standardised or there is insufficient information provided by the publication's authors to determine whether a standardised test method has been used. Insufficient information was often stated to describe the detail of the non-standardised methods and protocols used.

- 2.4.12 **Gap analysis of relevant intrinsic properties for nanomaterials possibly not addressed by standard test guideline methods and requiring further development of in vitro, in vivo or other methodologies (Task B4)**
- 2.4.13 The gap analysis table provided in the task report (RNC/RIP-oN2/B4/2/FINAL) is the outcome of an informed, objective and systematic consideration of the evidence by the Project Consortium, who have used their knowledge and professional judgement when considering the relevance of the existing and additional intrinsic properties and the applicability of methods for nanomaterials.
- 2.4.14 The gap analysis has assembled and further developed the findings from the examination of existing REACH Guidance related to information requirements and testing (information generation) strategies (Task B1), the identification of additional relevant specific intrinsic properties for nanomaterials (Task B2), the assessment of relevance and applicability of testing, endpoints and methods described in the scientific literature and on-going international work relevant to the fulfilment of the data requirements under REACH (Task B3).
- 2.4.15 The structural framework used for the gap analysis considers physico-chemical properties and toxicological and ecotoxicological endpoints, and integrates the existing and additional properties/endpoints to identify those which may and may not be addressed by standard test guideline methods and where further development of in vitro, in vivo or other methodologies is required.
- 2.4.16 The gap analysis tables are structured by property / endpoint, with tables presenting systematically an assessment of methods using the following format:
- Method name;
 - Supporting information from conclusions of the preceding B1, B2, and B3 reports;
 - A judgement on whether the property / endpoint is applicable to nanomaterials and the need for guidance amendment, according to the following categories outlined in the table below:

Category	Judgement	Outcome
1	Property / endpoint not applicable to nanomaterials.	No change to guidance.
2	Property / endpoint applicable to nanomaterials, but no difference between nano and non-nano in terms of the applicability of methods.	No change to guidance.
3	Property / endpoint applicable to nanomaterials, with differences between nano and non-nano in terms of the applicability of methods	Change(s) to guidance to be suggested.
4	Property / endpoint applicable to nanomaterials, with suspected important differences between nano and non-nano in terms of the applicability of methods, but an insufficient basis for guidance to be provided.	No change to guidance proposed at this time, but R&D requirements to be stated as needed.
5	Property / endpoint applicable to nanomaterials, with no suspected important differences between nano and non-nano, but an insufficient evidence basis to warrant acknowledgement in guidance.	No change to guidance at this point in time.

- Where specific methods for a relevant property are considered, a prioritisation based on whether the method is applicable to nanomaterials and the need for guidance amendment was assessed according to the following categories outlined in the table below:

Category	Judgement	Outcome
1	Method not applicable to nanomaterials.	No change to guidance.
2a	Existing method will work with nanomaterials, with evidence of no difference of applicability between nano and non-nano.	No change to guidance.
2b	New method will work with nanomaterials, with evidence of no difference of applicability between nano and non-nano.	Change(s) to guidance to be suggested.
3a	Existing method will work with nanomaterials, but with evidence of differences in applicability between nano and non-nano.	Change(s) to guidance to be suggested.
3b	New method will work with nanomaterials, but with evidence of differences in applicability between nano and non-nano.	Change(s) to guidance to be suggested.
4a	Existing method will work with nanomaterials, but with suspected important differences in applicability between nano and non-nano, but an insufficient evidence basis for guidance to be provided.	No change to guidance, but R&D requirements to be stated as needed.
4b	New method will work with nanomaterials, but with suspected important differences in applicability between nano and non-nano, but an insufficient evidence basis for guidance to be provided.	No change to guidance, but R&D requirements to be stated as needed.
5b	New method will work with nanomaterials, but with no suspected important differences in applicability between nano and non-nano but an insufficient evidence basis to warrant acknowledgement in guidance.	No change to guidance, but R&D requirements to be stated as needed.

- (For applicable methods (i.e. categories 2-5), a differentiation is made between existing methods (category suffixed with 'a') and new methods (category suffixed with 'b'). The absence of a category 5a is self-evident,

as an ‘existing method that will work with nanomaterials, with no suspected serious differences in effectiveness between nano and non-nano’ is equivalent to category 2a and does not have an insufficient evidence basis to warrant acknowledgement in guidance.)

- A comment on the method type, according to the following categories:
 - Standard (e.g. ISO, OECD TG method);
 - Non-standard method;
 - Widely-accepted R&D method.
- A commentary, providing further detail on the applicability, limitations, and R&D needs, where considered necessary;
- A comment on whether the property / endpoint is applicable to substances, particles, or nanomaterials only;
- Information on the type of data (kind of information) provided by the method;
- Suggested Guidance amendments, where appropriate, identifying the relevant Guidance documents, sections, and figures and the basis of the change to be suggested.

2.4.17 Where a consensus opinion could not be reached within the Project Consortium on any aspect of the data considered, the different positions are stated.

2.4.18 On the basis of the gap analysis, the relevant intrinsic properties for nanomaterials which may not be addressed by standard test guideline methods (and others) and for which further development of *in vitro*, *in vivo* or other methodologies is required have been identified.

2.4.19 **Scientific Report on Test Methods and Strategies for Nanomaterials (Task B5)**

2.4.20 The Scientific Report on Test Methods and Strategies for Nanomaterials (RNC/RIP-oN2/B5/2/FINAL) comprises four aspects: i) integrated testing strategies relevant to specific nanomaterials properties; ii) categorisation of nanomaterials for deriving

hazard information for nanomaterials; iii) proposals for further amendment of the REACH guidance documents for nanomaterials; and iv) proposal for further research and development of test methods and other data generation methods/strategies in regard to nanomaterials.

- 2.4.21 The assessment of the integrated testing strategies relevant to specific nanomaterials properties, reported in full in RNC/RIP-oN2/B5/2/FINAL, presents the current ITS for each of the existing properties and endpoints in the REACH guidance, its relevance and applicability for nanomaterials, and any indicated recommendations for alteration.
- 2.4.22 The latter two objectives (amendment of the REACH guidance documents for nanomaterials, and proposal for further research and development) have been developed from the comprehensive series of tables that constitute the gap analysis undertaken in Task B4, in which the available evidence from Tasks B1, B2 and B3 has been gathered together and analysed. Where consensus has not been reached on any aspect, this is indicated in the report and requires further consideration by the European Commission.
- 2.4.23 The philosophy adopted for the development of specific recommendations for guidance updates and for research & development related to nanomaterials is based on the following aspects:
- The content of a recommendation for a specific update to guidance is consistent with the focus of current REACH Guidance document, its level, and language, such that:
 - where the need is for 'strategic-level' guidance applicable to nanomaterials (i.e. high-level or overarching principles), succinct contextual information and reference(s) to primary sources of information are provided;
 - where the need is for updated detailed pragmatic information on, for example methods, a synopsis of specific guidance with appropriate reference(s) are provided;

- where there is simply a need identified to acknowledge an important relevance or limitation in existing guidance to nanomaterials, a simple wording clarification may be proposed.
- Recommendations for updates to Guidance are made on the basis of the findings of the RIP-oN 2 tasks, and where there is a recognised case for doing so. Wide-scale acknowledgement confirming the *general* applicability of Guidance to nanomaterials has not been made.

2.4.24 **Metric(s) to compare in the risk characterisation (Task C)**

2.4.25 Critical aspects concerning descriptors and related parameters, outlining available and adequate metrics and needs have been identified based on consideration of the published positions of the OECD-WPMN, SCENIHR, and peer-reviewed scientific literature. A key issue considered is the possibility of using metrics which link toxicological effects and exposure assessment, based on a relationship between measured parameters (e.g. number of particles, alone or in combination) and existing metrics used for dosages (mass of substance per kg bodyweight).

2.4.26 A perspective of metrics used historically and currently in risk assessment and the positions of OECD-WPMN and SCENIHR are presented, followed by discussion of toxicological (in the context of inhalation, dermal and ingestion exposure routes) and ecotoxicological aspects. The report discusses metrics, measurement methods and epidemiological aspects in occupational and environmental settings, and the conversion between metrics.

3 SUMMARY OF FINDINGS

3.1 PREAMBLE

3.1.1 A comprehensive discussion of the findings is provided in the individual Task Reports, which were refined using input from consultation with the project's Steering Group and the Stakeholder Consultation Group, and from knowledge gained from preceding tasks.

3.1.2 This Final Project Report compiles findings from the previous deliverables into a single document, summarising the key specific issues related to nanomaterials in a REACH context and a form compatible with the possible future integration into the existing REACH Guidance on Information Requirements and Chemical Safety Assessment, with clear reference to the existing REACH Guidance Part and Chapter and subchapter.

3.1.3 The Summary of Findings is presented, with cross-referencing, according to the key outcomes from each task undertaken.

3.1.4 List of Task Reports:

- Final Report on Task A: Identification and Review of Information Sources (RNC/RIP-oN2/A/1/FINAL)
- Final Report on Task B1: Evaluation of the applicability of existing information requirements under REACH for Nanomaterials (RNC/RIP-oN2/B1/2/FINAL)
- Final Report on Task B2: Identification and Overview of Additional Relevant Specific Intrinsic Properties for Nanomaterials (RNC/RIP-oN2/B2/2/FINAL)
- Final Report on Task B3: Practical advice on relevance and applicability of existing information in fulfilling REACH information requirements (RNC/RIP-oN2/B3/2/FINAL)
- Final Report on Task B4: Gap analysis of relevant intrinsic properties for nanomaterials possibly not addressed by standard test guideline methods and requiring further development of in vitro, in vivo or other methodologies ((RNC/RIP-oN2/B4/2/FINAL)
- Final Report on Task B5: Scientific report on test methods and strategies for nanomaterials (RNC/RIP-oN2/B5/2/FINAL)
- Joint Final Report on RIP-oN2 Task C & RIP-oN3 Task D: Metric(s) to compare in the risk characterisation (RNC/RIP-oN2/C/2/FINAL)

3.2 IDENTIFICATION & REVIEW OF INFORMATION SOURCES (TASK A)

- 3.2.1 The identification and review of information sources (Task A) in RIP-oN 2 have identified screened (for relevance) and then categorised the sources of information to compile a resource for use in the subsequent tasks of the project.
- 3.2.2 Key organisations, FP6/7 projects and other national projects of relevance to the scope of the project were identified by the project team and through consultation with the European Commission, via JRC. Publically-available reports and outputs of relevance for the project from these sources were then identified and obtained directly from their associated websites and/or through web-based searching.
- 3.2.3 In relation to the OECD WPMN, it is recognised that there are three levels of accessible documents which can be used and referenced:
- Published documents available on the public OECD WPMN website;
 - Documents approved for declassification but not yet published;
 - OECD documents developed by the Steering Groups and presented at meetings of the WPMN.
- 3.2.4 With regard to ISO and CEN publications, only published documents and those classified as being at Final Draft International Standard (FDIS) or Draft International Standard (DIS) stage were assessed and utilised where appropriate. Documents in development but not possible to be cited at the time of carrying out the RIP-oN 2 project have been identified; recommendations for them to be considered as soon as they become available have been made.
- 3.2.5 A substantial resource of peer-reviewed literature references was constructed. Literature from the recently completed FP7 Coordination & Support Action entitled Engineered Nanoparticles – Review of Health & Environmental Safety (ENRHES) (Stone et al., 2009), provided an initial comprehensive listing of literature published up to 31st December 2008. The ENRHES literature search was updated for the period 1st January 2009- 3rd March 2010 and supplemented with additional literature of specific relevance to the RIP-oN 2 project through a non-date-limited Boolean search strategy similar to that of ENRHES using PubMed and Web of Knowledge. In cases where excessively large numbers of references were

obtained, the searches were refined by incorporating material-specific terms (e.g. silver, titanium dioxide, zinc oxide).

- 3.2.6 This search strategy provided a comprehensive bibliography of references across the topic areas of physico-chemical characterisation, production, use and exposure, toxicology, epidemiology, ecotoxicology, and environmental fate and behaviour.
- 3.2.7 The criteria upon which judgements were made for tagging a reference as relevant for a task are outlined in the table below:

Task	Task Name	Criterion for Inclusion
B1	Analysis of current REACH information requirements and testing and information-generation strategies for nanomaterials.	Reports and publications highlighting or commenting generally or specifically on the existing information requirements and testing approaches for nanomaterials or other relevant substances.
B2	Identification and scientific overview of additional relevant specific intrinsic properties of nanomaterials.	Reports and publications which discuss physico-chemical, toxicological and ecotoxicological information concerning nanomaterials or other relevant substances, which may propose or highlight or identify potential additional relevant specific intrinsic properties.
B3	Advice on relevance and applicability of existing information in fulfilling REACH information requirements.	Reports and publications which discuss physico-chemical, toxicological and ecotoxicological properties of nanomaterials or other relevant substances.
B4	Gap analysis of relevant intrinsic properties for nanomaterials, which may not be addressed by standard test guidelines methods and for which further development of in vitro, in vivo or other methodologies is required.	Reports and publications which themselves provide a property-test gap analysis for nanomaterials or extensive discussion of knowledge gaps.

Task	Task Name	Criterion for Inclusion
B5	Scientific advice and proposals for incorporation of (categorised) nanomaterial-specific requirements into REACH: (research on) integrated testing/(information generation) strategies.	Reports and publications which themselves make specific recommendations for dealing with nanomaterials under REACH.
C	Metric(s) to compare in the risk characterisation.	Reports and publications dealing specifically with metrics relevant to risk characterisation.

3.2.8 The number of information sources identified and categorised for RIP-oN 2 are as follows:

- 89 published reports and standards from key organisations;
- 54 reports and standards under development from key organisations;
- 161 reports and publications from EU FP6/7 and other relevant international projects;
- 557 reports and publications reviewed in the ENRHES report;
- 931 additional publications from the peer-reviewed literature.

3.2.9 An appendix in the corresponding task report provides the complete listing of the sources of information.

3.3 APPLICABILITY OF EXISTING INFORMATION REQUIREMENTS (TASK B1)

3.3.1 The applicability of the existing Information Requirements to nanomaterials under REACH has been considered and summarised for physico-chemical properties, toxicological endpoints and ecotoxicological endpoints below. Tabular summaries of the assessment are also provided.

3.3.2 Conclusions Concerning Physico-chemical Properties

3.3.3 Of the physico-chemical properties, the guidance to assess melting/freezing point (R.7.1.2), boiling point (R.7.1.3), relative density (R.7.1.4) and vapour pressure (R.7.1.5), flammability (R.7.1.10), explosive properties (R.7.1.11), self-ignition temperature (R.7.1.12) and oxidising properties (R.7.1.13) are all considered to be applicable to nanomaterials. Stability in organic solvent and degradation products (R.7.1.16) and dissociation constant (R.7.1.17) are also applicable to nanomaterials. Dissociation constant is indicated to be likely applicable to nanomaterials in OECD_15 Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials, ENV/JM/MONO(2009)21). Stability in organic solvent is not covered by the OECD review.

3.3.4 Surface tension (R.7.1.6) is not in general relevant for nanomaterials, except for the special sub-classes of Janus particles which may exhibit domains of differing hydrophilicity. Flash point (R.7.1.9) is not considered relevant for nanomaterials; the current Guidance states appropriately that “Flash Point is only a relevant property for liquids, thus it does not need to be done for substances that are solids or gases at room temperature.” Viscosity (R.7.1.18) is also not considered relevant for nanomaterials; the current Guidance states appropriately that “Viscosity is relevant only to liquids, therefore for many substances this determination is not required.”

3.3.5 Water solubility (R.7.1.7) is a significant relevant property for nanomaterials, and in many cases its determination is compatible with the determination of the state of agglomeration, ideally by the method of simple sedimentation. At strong agglomeration, the material would be considered 'not dispersible', which is the nanomaterial analogue of 'not soluble'. The state of agglomeration is determined by the surface modification / functionalisation shell, not necessarily by the particulate core of the nanomaterial. The same applies to the water solubility in the sense of dispersability. Water Solubility may change between as-produced and as-tested

materials (which could incorporate an as dosed / as exposed stage, and at the point(s) of interaction with the organism), so *in situ* characterisation is required. REACH guidance takes that into account already. We noted that SCENIHR (Risk Assessment of Products of Nanotechnologies; 28th plenary on 19 January 2009) devotes a major part of the 2009 report to the spontaneous changes of properties and strongly supports the need for *in-situ* characterisation, in the sense of characterisation in the biological test environment and indicated that currently available standard methods for measuring dissolution may not be applicable. However, OECD concluded that the test guideline relevant to characterising the water solubility (OECD TG 105) might be applicable under some circumstance or to some classes of manufactured nanomaterials. It stated that this TG is applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary (ENV/JM/MONO(2009)21).

- 3.3.6 For the methods to determine the partition coefficient n-octanol/water (R.7.1.8) to be applicable to nanomaterials, the OECD methods acknowledged in the Guidance would need to be revised. SCENIHR (Risk Assessment of Products of Nanotechnologies; 28th plenary on 19 January 2009) finds that the octanol-water partition coefficient K_{ow} is likely to have a limited role in predicting water-solids partitioning. All OECD guidelines on this property are marked as *applicable under some circumstances or to some classes of manufactured nanomaterials* in OECD_15 Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials, ENV/JM/MONO(2009)21). The property is determined by the presence of the substance in an oil-water interface. The possibility of a particle's surface changing with age and exhibiting different partitioning behaviour may result in the measurement being somewhat artificial with limited predictive value for environmental fate.
- 3.3.7 Granulometry (R.7.1.14) is, without doubt, the central issue for any nanomaterial. This property may change from as-produced, hence as-tested (or *in situ*) characterisation is required. Consideration of the adequacy of the definition of granulometry used in the Guidance and the appropriateness of incorporating properties distinct from purely size distribution characterisation, has been undertaken as part of Task B2. Methods specifically mentioned (Cascade impaction

"A well established techniques to measure the distribution of particles of respirable or inhalable size particles of all kind, size range: 0.1-20 and 0.5-80 microns", Laser scattering/diffraction "The method is suitable to determine the distribution of particles of respirable and inhalable size. Particles of all kind Size range: 0.1-100 microns"; Rotating drum method (BS EN 15051) do not cover the range below 100nm. Hence they are not adequate to generate data on nanomaterials. The important properties of surface area, state of agglomeration, shape, size & length (particle / platelet / fibre etc) are already acknowledged in the REACH Guidance under granulometry (Table R.7.1-30 Methods to determine particle size distribution of the material as it is), but there is a need of explicit guidance for granulometry of nanomaterials.

3.3.8 For adsorption/desorption (R.7.1.15), the definitions used can be applied with minimal changes also to a dispersed (not dissolved) substance such as a non-soluble nanomaterial. However, the guidance document states that the methods may not be suitable for: i) substances that react with the column, ii) solvent or other test system components, iii) surface active substances; iv) substances that interact in a specific way with inorganic soil components such as clay minerals; v) inorganic compounds; and vi) moderate to strong acids and bases. Nanomaterials may be close in their properties to clay minerals, surface active substances and inorganic compounds. All methods require a quantitative analytical method for the substance, reliable over the range of test concentrations. This presents an issue for many nanomaterials, if identification both by chemical nature and physical structure is to be performed. A practical solution for most cases is the elemental analysis, e.g. by ICP-MS of fractions, since most nanomaterials contain inorganic elements. Similar techniques (FFF-ICP-MS) have been developed for ecotoxicology sample characterisation, but are far from ISO or OECD standardisation (compare Hasselöv et al, *Ecotoxicology* (2008) 17:344–361).

3.3.9 **Conclusions Concerning Toxicological Information**

3.3.10 The toxicological data requirements under REACH that are further described in the corresponding guidance documents R.7a and R.7c are also relevant for the assessment of nanomaterials (summarised in Annex 2 of RNC/RIP-oN2/B1/2/FINAL). According to OECD WPMN and SCENIHR, the described and preferred OECD test guidelines to fulfil these data requirements are basically also

applicable for the assessment of nanomaterials (SCENIHR, 2007 and 2009; OECD WPMN, ENV/JM/MONO(2009)21).

- 3.3.11 Concerning toxicological tests, special attention needs to be given to measuring, dosing, delivery and tracking of nanomaterials in the test system. Furthermore, concerning toxicological endpoints it is also important to consider the physicochemical characteristics of the nanomaterial, including in the dosing vehicle. Therefore, there is a need for guidance on sample preparation and in situ characterisation for the toxicological assessment of nanomaterials.
- 3.3.12 For all toxicological tests, an adequate characterisation of the tested nanomaterial should be carried out and appropriate consideration of the actual exposure of the test system (e.g. allowing for possible agglomeration/disagglomeration) and the appropriate dose metric should be given.
- 3.3.13 With regard to non-testing data, it should be noted that non-testing approaches such as (Q)SAR, read-across etc are currently not applicable to nanomaterials, because available models and understanding of issues such as similarity are not trained/developed to properly address nanomaterials. As such their use will require scientific justification. However, the possibility to extrapolate certain information from studies conducted with bulk forms of the substance or modifications of the respective nanoforms which could influence the decision on testing or the testing strategy of the nanomaterial should be addressed. Guidance on such a grouping approach for nanomaterials based on toxicological test results is currently not available and therefore needed. Research is needed on these issues so that appropriate guidance can be developed.
- 3.3.14 According to OECD WPMN, studies on toxicokinetics of nanomaterials are important for the assessment of their potential health effects (OECD WPMN, ENV/JM/MONO(2009)21). Due to the current lack of knowledge concerning toxicokinetic behaviour of nanomaterials, especially concerning absorption, distribution and excretion, we propose to include newly developed methods in the guidance on toxicokinetics, e.g. barrier transfer methods (ENRHES review, Stone et al., 2009), to assess, if appropriate, the toxicokinetic behaviour of certain nanomaterials.

3.3.15 **Conclusions Concerning Ecotoxicological Information**

- 3.3.16 The current ECHA guidance document on Chemical Safety Assessment ECHA (2008) refers specifically to a number of OECD Test guidelines. The appropriateness of these OECD Test guidelines as well as other guidelines have been reviewed by the OECD project 'Safety Testing of a Representative Set of Manufactured Nanomaterials' (ENV/JM/MONO(2009)21). OECD stated that: "For 24 OECD ecotoxicity test guidelines, the subgroup for biotic effects section concluded that the guidance on preparation, delivery, measurement, and metrology is currently insufficient for testing of manufactured nanomaterials" (ENV/JM/MONO(2009)21, p. 13).
- 3.3.17 Currently 31 OECD guidelines exist for the determination of potential ecotoxicological effects of test substances in relevant environmental compartments (aquatic, terrestrial, sediment) after acute or chronic exposure. Endpoints determined in these studies, with a diverse set of species representing different taxa and environmental compartments, are mortality and non-lethal endpoints like growth, respiration, reproduction and development. The parameters determined in these studies generally reflect responses of complete organisms covering several modes of toxicity and often also several routes of exposure. Thus, the basic toxicological properties as well the endpoints described and determined in these guidelines are adequate and relevant also for nanomaterials.
- 3.3.18 At the moment, 18 OECD test guidelines exist to determine environmental fate covering methods evaluating e.g. ready biodegradability (OECD 301 A-F) to further guidelines on physico-chemical properties which will influence substance behaviour in the environment (e.g. OECD 105 Water Solubility, OECD 107/117/123 Octanol-water Partition Coefficient; OECD 111 Hydrolysis; OECD 106/121 Adsorption to soil or sediment). OECD test guidelines also exist for bioconcentration (OECD 305) and bioaccumulation methods (OECD 315, 317). For the environmental fate assessment, a pre-requisite for biodegradation is that the test material is based on organic carbon chemistry (for bulk chemicals as well as for nanomaterials). Furthermore, the test strategy and studies used in this assessment may need to be adapted to the particulate nature of nanomaterials dispersed in water or the test-specific test media.

- 3.3.19 Within the RNC/RIP-oN2/B1/2/FINAL report, and in the conclusions of the OECD WPMN document ENV/JM/MONO(2009)21, it was noted that many of the majority of the OECD test guidelines are applicable (in some cases with conditions). However, the guidance given on preparation, delivery of test substances to test system, exposure quantifications, dose metrics, measurement, and metrology in all of these test guidelines is currently insufficient for testing of nanomaterials. Therefore preliminary guidance notes have been developed in the OECD-WPMN for practical testing (ENV/JM/MONO/201025 Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials).
- 3.3.20 Currently, research also suggests that particle number, size distribution, surface area, charge and other surface characteristics might be better predictors of toxicity, and more accurate metrics to be used in statistical determinations of dose-response relationships than (mass) concentration. Given the historical and established use of (mass) concentration as a dose metric, it continues to be important in assessing dose-response relationships and modelling toxic effects, however, its usefulness in particle toxicology depends not least on knowledge of the nature of the substance under test. Sample preparation and delivery issues are complicated by the stability and consistency of the properties of nanomaterials in the various exposure media used. Due to the current uncertain situation, a combination of dose metrics is recommended for nanomaterial testing. In general, the current test guidelines do not provide adequate direction for monitoring the characteristics of nanomaterials over the duration of tests. (ENV/JM/MONO(2009)21 Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials).
- 3.3.21 (Q)SAR analyses are mentioned in the guidance documents R.7b and R.7c as another way to fulfil the information requirements, but models currently used are not applicable because they were not designed for specific nanomaterial characteristics.
- 3.3.22 Field studies in ecotoxicity and environmental fate can contribute significantly to the environmental hazard and risk assessment of nanomaterials and are therefore also listed as a possible source of information. Due to the present uncertainties regarding analytical characterisation (e.g. distinction between background concentration and nanomaterial), exposure quantifications, dose metrics and a generally low environmental concentration, field studies are not recommended.

- 3.3.23 The solubility of a test substance is a key parameter in defining the test strategy for determining ecotoxicity and environmental fate for bulk material; for nanomaterials the stability of a dispersion is the equivalent key parameter. A soluble nanomaterial would lose its particle characteristics when dissolved and exposure conditions would not be discernible from dissolved bulk material.
- 3.3.24 **Conclusions Concerning Classification, Labelling & Packaging (CLP)**
- 3.3.25 The Regulation on Classification, Labelling and Packaging of substances and mixtures (CLP Regulation) (EC) No 1272/2008 does not contain specific provisions for nanomaterials nor for particulate materials but some guidance on classification of metal powders can be found in its Annex IV. However, nanomaterials are covered, as in REACH, by the definition of substance and therefore the provisions of CLP apply to nanomaterials as well. Moreover, the CLP recognises the potential impact a change in physicochemical properties might have on the intrinsic properties of a substance, see Articles 5 and 9.5. In doing so the CLP requires the manufacturer or importer to first ensure that the information used to classify relates to the forms or physical states in which the substance is placed on the market and in which it can reasonably be expected to be used. Secondly, CLP requires additional testing relating to physical hazards (explosivity, flammability, etc) to be performed if such information is missing or not adequate to conclude on a classification.
- 3.3.26 With regard to nanomaterials, if information only exists for coarser materials it should be assessed whether this information is also applicable to nanomaterials, due to the impact of the increased surface area on physicochemical properties. Information derived by registrants to fulfil the registration requirements in REACH, according to Annexes VII-IX and following the methods in the Test Methods Regulation (EC/440/2008), may not be sufficient to determine all physical hazards in accordance with CLP. Any evaluation of particulate (nano)materials in the context of CLP regulation should be conducted in accordance with the principle of using the worst case scenario where the finest relevant fraction of the form and physical states as placed on the market, should be used when testing for physicochemical hazards, Article 33.4.1.4.3.5 in the Manual of Test and Criteria (the Orange Book) (United Nations New York and Geneva, 2009).
- 3.3.27 Based on the opinions expressed by OECD (ENV/JM/MONO(2009)20, ENV/JM/MONO(2009)21, ENV/JM/MONO(2010)25) and the SCENIHR (2007), it is

considered that in general there are no significant differences in the applicability of the standard test methods themselves between nanomaterials and conventional substances. However, this report has highlighted a number of considerations in relation to sample preparation, dosing vehicle and the actual exposure of the test system and these consideration remains the same also for any test method preformed under CLP. Nevertheless, the impact of an increased surface area on physicochemical properties should be given thorough consideration when determining the physical hazards.

3.3.28 Summary table of physico-chemical data requirements in REACH and guidance documents

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested	OECD methods	REACH Guidance reference number
Annex VII (required for substances manufactured or imported above 1 t/y):				
Flash point	-		-	R.7.1.9
Flammability	O		Apply methods stated	R.7.1.10
Explosive properties	O		Apply methods stated	R.7.1.11
Self-ignition temperature	O		Apply methods stated	R.7.1.12
Oxidising properties	O		Apply methods stated	R.7.1.13
Boiling point	O		Apply methods stated	R.7.1.3
Melting/freezing point	O		Apply methods stated	R.7.1.2
Vapour pressure	O		Apply methods stated	R.7.1.5
Relative density	O		Apply methods stated	R.7.1.4
Surface tension	-		-	R.7.1.6
Partition coefficient n-octanol/water	O		Modify TG107, TG117, TG123	R.7.1.8
Water solubility	+	+	Modify TG105	R.7.1.7
Granulometry	+	+		R.7.1.14
Annex VIII (required for substances manufactured or imported above 10 t/y):				
Adsorption/desorption screening	+		Modify TG106, TG108, TG121	R.7.1.15
Annex IX (required for substances manufactured or imported above 100 t/y):				
Stability in organic solvents	O			R.7.1.16
Dissociation constant	O		Modify TG112	R.7.1.17
Viscosity	-		-	R.7.1.18

3.3.29 Summary table of toxicological data requirements in REACH and guidance documents

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	Test methods / OECD test guidelines	REACH Guidance reference number
Annex VII (required for substances manufactured or imported above 1 t/y):				
Toxicokinetics	+	1)	OECD TG 427/ EU B.44: (Skin Absorption: In Vivo Method) OECD TG 428/EU B.45: (Skin Absorption: In Vitro Method) OECD TG 417/EU B.36: (Toxicokinetics)	R.7.12
Acute toxicity	o (increased solubility?)	1)	Oral: OECD TG 420 (EU B.1 bis) (Acute oral toxicity – Fixed dose procedure) OECD TG 423 (EU B.1 tris) (Acute oral toxicity – Acute toxic class method) OECD TG 425 (Acute oral toxicity – Up-and-down procedure)	R.7.4
Skin Irritation	O	1)	In vitro: OECD TG 430/EU B.40 (Transcutaneous Electrical Resistance (TER using rat skin) test) OECD TG 431/EU B.40 bis (Human Skin Model tests (EPISKIN™, EpiDerm™)) OECD TG 435 (<i>In vitro</i> Membrane Barrier test method, Corrositex®) OECD TG 439 (Human Skin Model tests (EPISKIN™, EpiDerm™))	R.7.2

Properties with REACH data requirements		Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	Test methods / OECD test guidelines	REACH Guidance reference number
Annex VII (required for substances manufactured or imported above 1 t/y):					
Eye irritation		O	1)	In vitro: Isolated rabbit eye (IRE) test OECD TG 438: Isolated chicken eye (ICE) test OECD TG 437: Bovine corneal opacity & permeability (BCOP) test Hen's egg test – chorio-allantoic membrane (HET-CAM) test	R.7.2
Skin Sensitisation		O	1)	OECD TG 429/EU B.42: Murine Local Lymph Node Assay (endorsed method) OECD TG 406/EU B.6: Guinea pig maximisation test (GPMT) and Buehler test OECD TG 442A: Local Lymph Node Assay: DA OECD TG 442B: Local Lymph Node Assay: BrdU-ELISA	R.7.3
Genetic Toxicity	In vitro gene mutation in bacteria	O	1)	OECD TG 471/EU B.13/14: Bacterial reverse mutation test	R.7.7

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	Test methods / OECD test guidelines	REACH Guidance reference number
Annex VIII (required for substances manufactured or imported above 10 t/y, in addition to requirements according to Annex VII):				
Eye irritation	o	1)	In vivo: OECD TG 405, Acute Eye Irritation/Corrosion Commission Directive 2004/73/EC, EU B.5, Acute Toxicity: Eye Irritation/Corrosion	R.7.2
Repeated dose toxicity, short-term (28 days) (either oral, dermal or inhalation depending on what is most appropriate based on exposure)	o o +	1)	Oral: OECD TG 407 / EU B.7 Dermal: OECD TG 410 / EU B.9 Inhalation: OECD TG 412 / EU B.8 Short-term inhalation test (5 days; Ma-Hock L <i>et al.</i> , 2008; certified by OECD WPMN SG7)	R.7.5

Properties with REACH data requirements		Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	Test methods / OECD test guidelines	REACH Guidance reference number
Annex VIII (required for substances manufactured or imported above 10 t/y, in addition to requirements according to Annex VII):					
Genetic Toxicity	chromosomal aberration	o	1)	In vitro: OECD TG 473/EU B.10: In vitro mammalian chromosome aberration test. OECD TG 487: In vitro mammalian cell micronucleus test.	R.7.7
	mammalian cell gene mutation			OECD TG 476/EU B.17: In vitro mammalian cell gene mutation test – HPRT test OECD TG 476/ EU B.17: In vitro mammalian cell gene mutation test – Mouse lymphoma assay	
	chromosomal aberration	O		In vivo: Somatic cells: OECD TG 475/EU B.11: In vivo mammalian bone marrow chromosome aberration test OECD TG 474/EU B.12: In vivo mammalian erythrocyte micronucleus test	
	DNA damage and/or repair			OECD TG 486/EU B.39: Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo Germ cells (only on very rare occasions)	

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	Test methods / OECD test guidelines	REACH Guidance reference number
Annex VIII (required for substances manufactured or imported above 10 t/y, in addition to requirements according to Annex VII):				
Toxicity to reproduction	0	1)	OECD TG 421: Reproductive screening study OECD TG 422: Combined repeated dose toxicity / reproductive screening study	R.7.6

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	OECD methods	REACH Guidance reference number
Annex IX (required for substances manufactured or imported above 100 t/y, in addition to requirements according to Annexes VII and VIII):				
Repeated dose toxicity, sub-chronic (90 days) (either oral, dermal or inhalation depending on what is most appropriate based on exposure)	o o +	1)	Oral: OECD TG 408/409 / EU B.26/B.27 in rodent/non-rodent species, respectively OECD TG 422: Combined repeated dose toxicity / reproductive screening study Dermal: OECD TG 411/EU B.28 OECD TG 422: Combined repeated dose toxicity / reproductive screening study Inhalation: OECD TG 413/EU B.29 OECD TG 422: Combined repeated dose toxicity / reproductive screening study	R.7.5
Toxicity to reproduction	O	1)	One- or two- (or multi-) generation studies (such as B.35, OECD TGs 415 or 416, or EU B.34 or a 'F1-extended one-generation study')	R.7.6
Developmental Toxicity / teratogenicity	O	1)	OECD TG 414/EU B.31: Prenatal developmental toxicity test	R.7.6

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	OECD methods	REACH Guidance reference number
Annex X (required for substances manufactured or imported above 1000 t/y, in addition to requirements according to Annexes VII, VIII, and IX):				
Repeated dose toxicity, long-term (either oral, dermal or inhalation depending on what is most appropriate based on exposure)	o (only if indicated)	1)	OECD TG 452/EU B.30: Chronic toxicity study OECD TG 453/EU B.33: Combined chronic toxicity / carcinogenicity study	R.7.5
Carcinogenicity	o (only if indicated)	1)	OECD TG 451: Carcinogenicity studies OECD TG 453/EU B.33: Combined chronic toxicity / carcinogenicity study US-EPA 870.4200: Carcinogenicity studies	R.7.7

1) Concerning toxicological endpoints important considerations need to be taken into account which particularly relate to the physicochemical characteristics of the nanomaterial, including in the dosing vehicle. For all toxicological tests, an adequate characterisation of the tested nanomaterial, appropriate consideration to the actual exposure of the test system, (e.g. allowing for possible agglomeration/disagglomeration) and consideration to the appropriate dose metric (if known) should be given.

3.3.30 Summary table of ecotoxicological data requirements in REACH and guidance documents

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	OECD methods	REACH Guidance reference number
Annex VII (required for substances manufactured or imported above 1 t/y):				
Aquatic toxicity	O	+	OECD TG 202 Short-term toxicity testing on invertebrates (preferred species Daphnia) OECD TG 201 Growth inhibition study aquatic plants (algae preferred)	R.7.8
Degradation	O	+	OECD TG 301 Biotic A-F 310 Ready biodegradability	R7.9
Annex VIII (required for substances manufactured or imported above 10 t/y, in addition to requirements according to Annex VII):				
Aquatic toxicity	O	1)	OECD TG 203 Short-term toxicity testing on fish OECD 209 Activated sludge respiration inhibition testing	R.7.8
Degradation	o	1)	OECD TG 302/303/305/306 Biotic OECD TG 111 Abiotic. Hydrolysis as a function of pH	R.7.9
Fate and behaviour in the environment	+	1)	OECD TG 121 Absorption/ desorption screening	R.7.1.15

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	OECD methods	REACH Guidance reference number
Annex IX (required for substances manufactured or imported above 100 t/y, in addition to requirements according to Annexes VII and VIII):				
Aquatic toxicity	+	1)	OECD TG 211 Long-term toxicity testing on invertebrates (preferred species Daphnia) unless already provided as apart of Annex VII requirements OECD TG 210 Fish early-life stage (FELS) toxicity test OECD TG 212 Fish short-term toxicity test on embryo and sac-fry stages OECD TG 215 Fish juvenile growth test	R.7.8
Degradation	O	1)	OECD TG 309 Simulation testing on ultimate degradation in surface water OECD TG 307 Soil simulation testing (for substances with a high potential for adsorption to soil) OECD TG 308 Sediment simulation testing (for substances with a high potential for adsorption to sediment) [No test method specified] Identification of degradation products	R.7.9
Fate and behaviour in the environment	O	1)	OECD TG 305 Bioaccumulation in aquatic species, preferably fish OECD TG 106 Further information on adsorption/desorption depending on the results of the study required in Annex VII	R.7.10 R.7.1.15

Properties with REACH data requirements	Property relevant for nanomaterials? - redundant or not relevant o as for any substance + specifically relevant	Property may change from as-produced to as-tested ¹⁾	OECD methods	REACH Guidance reference number
Annex IX (required for substances manufactured or imported above 100 t/y, in addition to requirements according to Annexes VII and VIII):				
Effect on terrestrial organisms	o	1)	OECD TG 207 Short term toxicity to vertebrates OECD TG 216/217 Effects on soil micro-organisms OECD TG 208 Short-term toxicity to plants	R.7.11
Annex X (required for substances manufactured or imported above 1000 t/y, in addition to requirements according to Annexes VII, VIII, and IX):				
Degradation	o	1)	OECD TG 302/303/304/306 Biotic e.g, further simulation testing	R.7.9
Fate and behaviour in the environment	o	1)	[No test method specified] Further information on the environmental fate and behaviour of the substance and/or degradation products	R.7.10 R.7.1.15
Effect on terrestrial organisms	O	1)	OECD TG 220/222 Long-term toxicity testing on invertebrates unless already provided as part of Annex IX requirements	R.7.11

1) Concerning toxicological endpoints important considerations need to be taken into account which particularly relate to the physicochemical characteristics of the nanomaterial, including in the dosing vehicle. For all toxicological tests, an adequate characterisation of the tested nanomaterial, appropriate consideration to the actual exposure of the test system, (e.g. allowing for possible agglomeration/disagglomeration) and consideration to the appropriate dose metric (if known) should be given.

3.4 ADDITIONAL RELEVANT SPECIFIC INTRINSIC PROPERTIES FOR NANOMATERIALS (TASK B2)

3.4.1 It is important to acknowledge that in identifying additional relevant specific intrinsic properties for nanomaterials, consideration has been given to how the existing Information Requirements included in REACH (considered in Task B1) could reflect any new or refined (i.e. improved or clarified description in the guidance) intrinsic properties specific for nanomaterials, without unnecessarily duplicating the effort and repeating content developed for Task B1. This has involved acknowledgement of the definitions provided in the legal text and guidance (and in nomenclature standards as necessary) and current regulatory status of some intrinsic properties, so that acceptable and pragmatic recommendations can be made regarding whether additional relevant specific intrinsic properties of nanomaterials could and should be addressed under the existing Information Requirements or as new ones.

3.4.2 It is arguable, from a technical/scientific definitions perspective, whether some of the candidate additional relevant specific intrinsic properties identified may be considered logically under the term 'granulometry' (used to describe an already existing Information Requirement (IR)). The only ISO definition of granulometry (sourced from the ISO Concept Database; <http://cdb.iso.org>) is a "measure of the particle (grain) content of irrigation water, as characterized by size dispersion and total amount of solids". No definition of the term 'granulometry', including the one purported (but not explicitly stated) to be a definition in the current REACH Guidance (section R.7.1.14), extends beyond the consideration of a size distribution of grain sizes. Hence, the logic of including intrinsic properties technically unrelated to characterising particle size distribution under the existing Information Requirement 'granulometry' (even in the context of them being particle-associated properties), may be questionable and has led the Consortium to suggest the option of a limited amendment of the title of an existing Information Requirement. With a more appropriate choice of term (in a similar vein to those adopted for the toxicology and ecotoxicity Information Requirements), this could facilitate the inclusion of a range of particle-associated properties as subordinate IRs, with provisions in Column 2 for the introduction of specific rules for adaptation from Column 1 in Annexes VI to X, as appropriate. Alternatively, a robust definition of the term 'granulometry' is required to be developed and adopted to facilitate the inclusion of additional particle-associated subordinates to the existing Information Requirement for Granulometry.

3.4.3 However, it is noted that Annex II to Regulation (EC) No 1907/2006 has been amended and now explicitly states in Section 9.1 that “The physical state (solid (including appropriate and available safety information on granulometry and specific surface area if not already specified elsewhere in this safety data sheet), liquid, gas) and the colour of the substance or mixture as supplied shall be indicated.” The inclusion of specific surface area as a separate term distinct from granulometry in the amendment of Annex II is observed. For specific surface area at least, confusion (or even a suggestion of heightened importance) may arise, albeit minor, when the property is seen to be stated separately from granulometry in Annex II but as subordinate in the guidance, if this were to be the case. This ambiguity is simply highlighted, for resolution by regulators in their future considerations.

3.4.4 **Identifying Potential Additional Relevant Specific Intrinsic Properties for Nanomaterials**

3.4.5 The debate over which parameters to use to characterise nanomaterials has been ongoing for some years. The task report’s discussion highlights that in many instances the suggestions are generic (e.g. surface chemistry). Furthermore, they are made in the absence of a) any detailed understanding of the characteristic, b) the relevance and applicability of any interpretable data on the characteristic (should it be available), and c) the availability of a technique to gather such data.

3.4.6 Of the numerous sources considered and commented on in RNC/RIP-oN2/B2/2/FINAL (including reports from ILSI, ECETOC, SCENIHR, VCI, OECD, and RIVM amongst others and more generally in the literature), the ‘pathfinding’ body identifying, developing and establishing properties and endpoints for nanomaterials hazard assessment is the OECD WPMN and its Sponsorship Programme on the Testing of Manufactured Nanomaterials. In 2008, a list of endpoints was recommended by the OECD-WPMN (ENV/JM/MONO(2010)46) for the first phase of testing that is intended to take into account ‘human health and environmental safety’ and ‘ensure consistency between the various tests to be carried out on specific nanomaterials’. OECD stated that it should also lead to the development of dossiers for each selected nanomaterial describing basic characterization, fate, ecotoxicity and mammalian toxicity information. It was acknowledged that the list of endpoints should be refined based on the practical results obtained through the testing programme and as such, phase one testing was expected to be of an exploratory

nature, science-based and without any consequences for existing regulatory datasets. (It is anticipated that incorporation of such refined recommendations and methods into appropriate OECD Guidance Documents will be forthcoming.)

- 3.4.7 A particular complex issue identified, including by the OECD-WPMN, concerns the stability of nanomaterials in the context of i) sample preparation for the determination of properties and ii) the behaviour of nanomaterials in the environment. Information on these two aspects is absent from REACH Guidance, and has been determined to be of crucial importance in the context of gathering characterisation data for Information Requirement purposes.
- 3.4.8 A number of suggested physico-chemical 'properties' have been identified as having a greater bearing on the quality of determining a characteristic or (eco)toxicological test outcome, due to its influence on sample preparation considerations. These include dispersion stability and state of agglomeration, which may also be important phenomena, in a physico-chemical sense, which influence the behaviour of nanomaterials in environmental media. These have been highlighted before being subsequently addressed in recommendations for Guidance amendment as part of Task B5. We suggest that their importance as supplementary information to the data gathered for the Information Requirement is acknowledged in the Guidance, but not necessarily as additional Information Requirements in their own right, due to their supplementary nature (i.e. non-intrinsic), their dependence upon other primary properties, and the need for further research and development of applicable methods as well as the interpretation of the primary data for these properties (and how it may inform the interpretation of data on existing Information Requirements) in the context of risk assessment. Nevertheless, it is acknowledged that such supplementary information may importantly advance and facilitate the use of responsible waiving, read-across, and QSARs, and the ability to select appropriate metrics that in turn widens the relevance and applicability of test methods to nanomaterials.
- 3.4.9 Furthermore, many biological effects may be only indirectly or non-causatively associated with a property or properties being measured. The potential for confounding in the assessment of a nanomaterial's hazard and exposure is particularly pertinent in the context of sample preparation and the limitations of

experimental techniques for characterisation and assessment of hazard and exposure.

3.4.10 **Conclusions Concerning Additional Relevant Specific Intrinsic Properties For Nanomaterials**

3.4.11 Potential additional relevant specific intrinsic properties have been identified on the basis of an objective review of published scientific sources of information, and a pragmatic and rationalised approach to their incorporation into REACH has been suggested. By virtue of their inclusion in this final report they are considered to be relevant to nanomaterials. For more in-depth considerations and details of the additional relevant specific intrinsic properties and how they have been identified in Task B2 and considered further in subsequent Tasks, the reader is referred to the relevant reports for Task B2, B3, B4 and B5.

3.4.12 Moreover, some properties or endpoints recommended in the sources considered in Task B2 for nanomaterials testing are not strictly intrinsic properties or the proxy-effect of a single intrinsic property, but are influenced by the material's 'conditions' or 'environment' in which the characterisation or testing is being done, and as a result can significantly impact on the determination, relevance and quality of other intrinsic properties or endpoints. These properties, indicated with an asterisk in the summary table below, have not been formally considered in the gap analysis conducted in Task B4, but are considered to warrant acknowledgement in updated guidance and recommendations have been made. It is also recognised that other aspects, such as assay interferences, also influence the determination, relevance and quality of intrinsic properties or endpoints, but as these cannot be defined as properties or be justified as a specific Information Requirement. Recommendations on issues related to sample preparation and assay interference were identified in Task B2 and subsequently have been further elaborated in the recommendations for Guidance update in Chapter 4.

Candidate property / endpoint	Suggested IR 'incorporation' status		
	Possible New IR	Subordinate to an existing (E) or updated (U) IR	Guidance recommendation (with or without an update to the appropriate Column 2 rule)
Particle shape		U/E (7.14)	
Surface area		U/E (7.14)	
Surface energy		U/E (7.14)	
Surface chemistry		U/E (7.14)	
Surface charge		U/E (7.14)	
Redox potential	✓		
Cell-free ROS/RNS production capacity	✓		
State of dispersion*			✓ (7.14 & 9.3)
State of agglomeration*			✓ (7.14 & 9.3)
Cell uptake*			✓ (8.3, 8.4, 8.8)
Cell viability			✓ (8.1, 8.2)
Oxidative stress			✓ (8.4, 8.5, 8.6)
Inflammation			✓ (8.1, 8.2, 8.5, 8.6)
Fibrosis			✓ (8.6)
Immunotoxicity (sensitisation)			✓ (8.3)
Cardiovascular toxicity		E (8.6)	
Ventilation rate [#]			✓ (9.1)
Gill pathologies [#]			✓ (9.1)
Mucus secretion [#]			✓ (9.1)

Candidate property / endpoint	Suggested IR 'incorporation' status		
	Possible New IR	Subordinate to an existing (E) or updated (U) IR	Guidance recommendation (with or without an update to the appropriate Column 2 rule)
Brain pathology [#]			✓ (9.1 & 9.6)
Animal behaviour [#]			✓ (9.1 & 9.6)
Oxidative stress biomarkers (CAT, SOD, GPX, GST) [#]			✓ (9.1 & 9.6)

*Properties or endpoints recommended in the sources considered in Task B2 for nanomaterials testing are not strictly intrinsic properties or the proxy-effect of a single intrinsic property, but are influenced by the material's 'conditions' or 'environment' in which the characterisation or testing is being done, and as a result can significantly impact on the determination, relevance and quality of other intrinsic properties or endpoints.

[#]In relation to these endpoints and proposed biological markers it should be noted that consensus has not been reached within the project consortium and further discussions can be found in section 4.3.157 onwards.

3.4.13 For the additional relevant specific intrinsic properties identified, an assignment has been made in the summary table above according to:

- whether a new formal Information Requirement is recommended, or;
- whether the property can be considered to be subordinate to an existing (E) Standard Information Requirement of Annex VI-X or following an update (U) to an existing Information Requirement, or;
- whether, acknowledging that the information should be gathered as part of good practice, this may be ensured through an update to existing Guidance (with or without a statement in Column 2 of the REACH annexes alongside an existing Information Requirement).

3.4.14 Where an existing Information Requirement has provisionally been considered to be appropriate for gathering of additional property/endpoint information, a cross reference to the existing IR in Annex VI-X of the legal text has been provided in parentheses.

- 3.4.15 In summary, on the basis of the evidence considered and extensively described in the Task B2 report, the additional relevant specific intrinsic properties *identified* to offer valuable supplementary information to interpreting and using other characterisation or endpoint data, in the REACH context, are highlighted in the table above.
- 3.4.16 The gap analysis conducted under Task B4 has assessed the *identified* additional relevant specific intrinsic properties regarding whether they can be determined using a) standard test guideline methods, b) non-standard test methods already in use, or through methods already being developed or that need to be developed through further research.
- 3.4.17 Proposals for updates to the Guidance on Information Requirements and Chemical Safety Assessment have been developed under Task B5 and provided in the corresponding report and in this Final Project Report. Suggestion of specific text to update the legal text is out with the remit of the project.

3.5 PRACTICAL ADVICE ON THE RELEVANCE AND APPLICABILITY OF EXISTING INFORMATION IN FULFILLING REACH INFORMATION REQUIREMENTS (TASK B3)

3.5.1 A comprehensive review of information sources identified in Task A was carried out and presented in the task report (RNC/RIP-oN2/B3/2/FINAL), first into four Sections in accordance with the sequence of objectives / sub-tasks (the peer-reviewed literature is reported separately from FP6/7 projects), and then by relevance to physico-chemical information, toxicological information and ecotoxicological information. Concluding summaries of the practical advice, distilled from across each of sources and organised by property/endpoint, are reproduced below.

3.5.2 B3 Sub-Task I: Summary of Practical Advice on the Relevance and Applicability of the Experience Reported in FP6/7 Projects in the REACH Context

3.5.3 PHYSICO-CHEMICAL PROPERTIES INFORMATION

3.5.4 Key characterisation issues and properties

3.5.5 It is evident from the review of the FP6/7 project outputs that there is now a consensus that thorough and accurate particle characterisation is an essential component of assessing the potential (eco)toxicity of nanomaterials, and it is generally indicated that the characterisation of test materials should be broad in scope and as thorough as possible.

3.5.6 Within the FP6/7 projects undertaken to date, the following properties of nanomaterials were deemed necessary for characterisation prior to (eco)toxicological and/or exposure investigations: elemental composition, purity, primary particle size/particle size distribution, aggregation/agglomeration state, surface area, surface charge, crystallinity/crystal phase, surface chemistry (primarily in terms of surface functionalisation and coatings), solubility and dustiness. The explosivity and flammability of nanopowders, and methods for its determination, has also been investigated in a single FP6 project (NANOSAFE2). Thus, in terms of the existing physico-chemical REACH Information Requirements and guidance, the FP6/7 outputs may be of use for informing any necessary changes to the guidance on Water Solubility (R.7.1.7), Flammability, Explosive Properties and Self-Ignition Temperature (R.7.1.9-R7.1.12) and Granulometry (R.7.1.14). No information has

been identified pertaining to the other existing REACH physico-chemical Information Requirements and associated guidance.

3.5.7 Within the FP6/7 project outputs, several publications have been identified which provide useful overviews and comparisons of the most common methods for the physico-chemical characterisation of nanomaterials, citations for and details from which are of relevance for further consideration into relation to the updated REACH Guidance (ECHA, 2008. Chapter R.7a), specifically:

- Chapter 2 of the ENRHES review (Stone et al., 2009);
- The NanoCap guide on “Measurement Techniques for Nanoparticles” (University of Essex, undated);
- The NANOSH “NanoAtlas of Selected Engineered Nanoparticles” (Vippola et al., 2009);
- Reviews by Fadeel & Garcia-Bennett (2010) and Gwinn & Tran (2009).

3.5.8 An important general conclusion with regards nanomaterials characterisation that has emerged from the findings of the several of the reviewed FP6/7 project outputs (e.g. Stone et al., 2009; Tran et al., 2008; University of Essex, undated) is that no individual technique can satisfy a meaningful characterisation of nanomaterials such that multiple techniques should be used where possible in order to formulate a complete understanding of the nanomaterial properties. It is highlighted that different techniques will suit different sample forms (e.g. aerosol, suspension etc.) and the optimum set of required techniques should be selected based on the specific nanomaterial type and form under investigation. The need for multi-method characterisation and material-specific selection of techniques applies across a range of nanomaterial properties will be stressed within the updated REACH Guidance documents.

3.5.9 Zuin et al. (2010) developed an evidence-based approach for undertaking a preliminary ranking of nanoparticles in terms of their hazard potential. This involves assigning a hazard rating of high, moderate or low to a specific nanoparticle across a range of “indicators” (physico-chemical or toxicological endpoints), according to a ranking table.

- 3.5.10 The WoE approach developed in PARTICLE_RISK has the potential to inform the choice of “triggers” for hazard testing based on nanomaterial type in the context of REACH. However, the current ranking values were based on measurement data which differed in their uncertainty and reliability, and it is unlikely that sufficient data will be available for some time to enable a robust system to be developed, and further research data is required to enable a robust system to be developed.
- 3.5.11 It has also been highlighted that further data is required (regarding the toxicity and mode of action of nanomaterials, as well as descriptors for characterising their structure) before the traditional (Q)SAR paradigm can be extended to nanomaterials (Burello & Worth, 2009), and this is therefore recognised as a further development need.
- 3.5.12 **Sample preparation**
- 3.5.13 The preparation of stable nanoparticle dispersions has been highlighted within the FP6/7 project outputs as an important and cross-cutting issue spanning across toxicological and ecotoxicological testing and this issue should be further considered in relation to recommended updates to the REACH Guidance (ECHA, 2008. Chapter R.7). Of key concern is that ions in the physiological media may influence the zeta potential and thus destabilise the nanoparticle dispersion resulting in agglomeration. This therefore alters the particle size distribution which may then bias the (eco)toxicological test result (NanoCare, 2009).
- 3.5.14 In relation to this issue, it has been suggested that it is not sufficient to simply characterise the intrinsic properties of the nanomaterial (i.e. chemical composition, primary particle size, surface chemistry etc) but that many facets of the preparation of nanoparticle dispersions must be taken into account before physiological assays with nanoparticles can be correctly interpreted including: a suitable dispersion protocol; agglomerate size distribution and agglomeration state; zeta-potential; wettability and tendency to agglomerate/deagglomerate due to absorption of solvent compounds and; desorption of solvent compounds with possible influence on passivation, solubility and molecular recognition (NanoCare, 2009; Schultze et al., 2008).
- 3.5.15 A number of projects have published protocols/SOPs that provide useful guidance for the preparation and characterisation of stable nanoparticle dispersions in

physiological media, specifically Meißner et al. (2009a), NanoCare (2007, 2008 and 2009), Bihari et al. (2008) and Vippola et al. (2009b). Citations to and information from these documents are useful in relation to updating the REACH Guidance. However, these procedures cannot be universally applied to all nanomaterials and certain modifications to the procedures may be required for different nanomaterials. It is therefore important to stress that such procedures should be carefully examined to determine if they are adequate for the test material under consideration.

3.5.16 **Particle size/size distribution**

3.5.17 The review of the outputs of FP6/7 projects has allowed identification of several commonly applied methods for determining the particle size/size distribution of nanomaterials in powder, suspension and aerosol form.

3.5.18 For *powders*, the following methods have been identified as common methods for determining the particle size of nanoparticles: transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray diffraction (XRD) and atomic force microscopy (AFM). For determining the particle size distribution of nanomaterials in powder form, TEM, SEM, scanning mobility particle sizer (SMPS), Field Flow Fractionation (FFF), Laser Diffraction Particle Size Analyser and Photon Correlation Spectroscopy (PCS) have been identified as available methods.

3.5.19 For nanomaterials dispersed in *suspension*, the particle size has been commonly determined using SEM and atomic force microscopy (AFM) in the FP6/7 projects. Less commonly applied methods include cryo-TEM, cryo-SEM and Fluorescence Correlation Spectroscopy (FCS). For determining the particle size distribution of nanomaterials in suspension, Dynamic Light Scattering (DLS), PCS, and nanoparticle tracking analysis (NTA) are commonly applied methods, whilst Analytical Ultracentrifuge (AUC), Hydrodynamic Fractionation (HDF) and cryo-TEM have also been utilised but to a lesser extent. However, it is important to note that Fadeel & Garcia-Bennett (2010) suggest that SEM/TEM often do not reflect the average particle size values that may be measured in solutions or biological media containing dispersed particles, with the authors suggesting that DLS offers a more routine approach for measuring the average particles sizes in different media.

3.5.20 In addition to the particle size/size distribution of dispersed nanomaterials, the effective hydrodynamic diameter (which takes into account the layer of biological

molecules on the surface of the nanoparticle) has also been suggested as an important parameter to characterise for biological applications, as bigger particles may not be able to enter pores of a certain size (Sperling et al., 2007). This parameter may be important to consider in relation to the update of the REACH guidance and requires further investigation. However Sperling et al. (2007) conclude that the more molecules attached to the particle surface and the bigger the particles become, the more unreliable the measurements are. Thus, depending on the actual particle nature, the adequate method for measurement has to be chosen with great care. Different measurement methods to measure effective sizes of colloidal nanoparticles are based on different physical principles, resulting in deviations of the resulting particle diameters between the different methods. These difficulties should be taken into consideration.

- 3.5.21 For *aerosols* of nanomaterials, the particle size may be determined using aerosol time of flight microscopy. Commonly utilised methods for determining the particle size distribution of nanoaerosols include the differential mobility analyzer and scanning mobility particle sizer (SMPS). Methods such as the electrical low pressure impactor (ELPI), Optical Particle Counter (OPC), Aerosol Particle Sizer (APS), Fast Mobility Particle Sizer (FMPS) have also been used, although somewhat less frequently.
- 3.5.22 The cascade impactor may also be used to determine the mass median aerodynamic diameter (MMAD) of aerosols. Ma-Hock et al. (2007) have highlighted that, although this method is well established for fine particles with diameters in the micrometer range, it fails to describe the dimension of nanoparticles as they no longer follow aerodynamic rules. However, this method is still valuable for inhalation studies with nanomaterials, as the cascade impactor can be used to determine the MMAD of agglomerates. In addition, it is noted that assembling the data of the measurements from different methods (e.g. OPC, SMPS) to provide a whole picture of the particle size distribution may not be appropriate due to the different principles employed by the methods (Ma-Hock et al., 2007). However, data from both methods are still valuable in providing information about particle size distribution relative to the amount of aerosol in their respective measurement ranges.
- 3.5.23 Also important to take into account is that considerable evolution of nanoaerosols has been demonstrated to occur over time, with average particle size increasing (as

a result of agglomeration) and concentration decreasing, such that nanoparticles will be present in size classes other than those in which they were originally emitted (Wu et al., 2008). Thus, in workplace studies, it is not sufficient to look for nanoparticles in the nano size range in which they may have been originally emitted, but that the size range the agglomerates formed over time also need to be considered.

3.5.24 Information regarding a potential link between particle size/size distribution and the toxicological effects of nanomaterials has been identified as part of the ENRHES review (Stone et al., 2009). In addition, Zuin et al. (2010) outlined the following relationship between primary particle size and deposition potential:

- Particles with size < 10 nm were considered indicative of high deposition, as more than 80% of the particles may be retained in three regions of the human respiratory tract (i.e. extrathoracic, tracheobroncal and alveolar) if inhaled;
- Particles with size between 10-30 nm were considered indicative of moderate deposition, as between 80% and 60% of inhaled particles are deposited in the human respiratory tract;
- Particles with size > 30 nm were considered indicative of low deposition, as less than 60% of the inhaled nanoparticles may be retained.

3.5.25 Zuin et al. (2010) have also defined the following relationship between primary particle size and translocation potential:

- Particles with sizes < 2.5 nm were considered indicative of high translocation potential;
- Particles with sizes between 2.5 – 5 nm were considered indicative of moderate translocation potential;
- Particles with sizes > 5 nm were considered indicative of low translocation potential.

3.5.26 Within the toxicokinetic studies undertaken as part of the PARTICLE RISK project, translocation to other organs beyond portal of entry following intratracheal instillation or intravenous injection in rats was determined to be strongly dependent on both the size and surface area of gold nanoparticles (Kreyling, undated). Particle size was

also suggested to be a critical factor in the assessment of toxicological and biological responses of WC-Co materials (Ding et al., 2009).

3.5.27 **Aggregation/agglomeration state**

3.5.28 The review of the outputs of FP6/7 projects has allowed identification of several commonly applied methods for determining the aggregation/agglomeration state of nanomaterials in powder and suspension form.

3.5.29 Commonly applied methods for determining the aggregation degree of nanomaterial powders include SEM and TEM. For determining the agglomeration state and dispersion stability of nanoparticles in solution, zeta potential measurements are often employed. However, Meißner et al. (2009a, 2009b and 2010) conclude that zeta potential measurements alone are not sufficient for predicting the stability of nanoparticles in physiological suspensions, recommending that measurements of the particle size distribution are also undertaken using DLS. It has been suggested that a method for measuring the strength of the agglomerates is needed (Wu et al., 2008), and this will be considered as a potential development need.

3.5.30 Several projects have suggested that the behaviour and effects of agglomerated nanoparticles are important to assess in relation to their potential toxicity (Ma-Hock et al., 2007; Wu et al., 2008; Stone et al., 2009), such that studies focusing on the toxicity of agglomerates and their stability should be considered as a potential development need. In addition, in relation to nanoaerosols, agglomeration over time has been demonstrated in the NANOTRANSPORT project to be of key importance to consider in relation to their particle size distribution and in the exposure assessment.

3.5.31 **Surface area**

3.5.32 The review of the outputs of FP6/7 projects has allowed identification of the Brunauer, Emmet and Teller (BET) method as the most commonly applied technique for determining the specific surface area of nanomaterials in powder form (providing also providing additional information on average pore size and pore volume) information about which may be of relevance for inclusion into the updated REACH Guidance. The surface area of nanoparticle aerosols has been

demonstrated to be determined using the nanoparticle surface area monitor (NSAM), which is a similar method to the Electrical Aerosol Detector.

3.5.33 Surface area has been highlighted as an important property in relation to the toxicity of nanoparticles, as increased surface area relates to increased potential for biological interaction (Stone et al., 2009). The potential relationship between surface area and (eco)toxicological effects has been discussed further in Section 4 of RNC/RIP-oN2/B3/2/FINAL, taking into account further information available in the peer-reviewed literature.

3.5.34 **Surface charge**

3.5.35 Based on the review of FP6/7 project outputs, it is evident that various instruments are available for determining the zeta potential of dispersed nanomaterials, such as the Zetasizer 3000 HAS.

3.5.36 It has been suggested by Zuin et al. (2010) that it is essential to measure zeta potential as a function of pH, as this allows the determination of the point of zero charge (PZC; the pH value where the zeta potential equals zero) where a dispersion of engineered nanomaterials exhibits the highest propensity to aggregate.

3.5.37 Several FP6/7 projects have suggested that the zeta potential is an important parameter to characterise, usually in relation to assessing whether engineered nanomaterials in suspension remain stable during exposure in toxicity studies (e.g. Zuin et al., 2010; Stone et al., 2009). Based on evidence available in the peer-reviewed literature, Zuin et al. (2010) outlined the following relationship between zeta potential and stability:

- Nanoparticles with zeta potential at pH 7 of $> +30$ mV or < -30 mV were considered to have high water stability (i.e. no aggregation over time);
- Nanoparticles with zeta potential at pH 7 of between -30 mV and $+30$ mV were considered to have a low water stability (i.e. tendency to aggregate over time).

3.5.38 The surface charge of nanoparticles has also been reported as being an important factor influencing particle uptake and interaction with exposed cells (Stone et al., 2009). The potential relationship between surface charge and (eco)toxicological

effects has been discussed further in Section 4 of RNC/RIP-oN2/B3/2/FINAL, taking into account further information available in the peer-reviewed literature.

3.5.39 **Shape/aspect ratio**

3.5.40 The shape of nanoparticles has been suggested to be an important property to characterise in relation to nanoparticle toxicity and fate within a number of literature reviews identified as outputs from FP6/7 projects (Stone et al., 2009; Burello & Worth, 2009). However, no studies investigating the relationship between shape and (eco)toxicity have been identified as outputs from the reviewed FP6/7 projects.

3.5.41 **Surface chemistry**

3.5.42 The most commonly characterised property in the FP6/7 project outputs reviewed in relation to surface chemistry is determination of the nature of surface functionalisation and coatings. Spectroscopic techniques such as Nuclear Magnetic Resonance (NMR), Infrared, UV-Vis and Raman Spectroscopy have been utilised for this purpose. In a single project (PARTICLE RISK), the “surface activity” was also characterised using electron spin resonance (ESR) (Marcomini, 2007).

3.5.43 In addition, three studies have been identified in which the surface reactivity of TiO₂, gold and ferrihydride nanoparticles has been modelled using discrete Fourier transform (DFT) (Burello & Worth, 2009). However, significant differences between the experimental data and the calculated values were seen and thus this method is concluded to be of limited value/reliability at present for aiding prediction of the surface chemistry of nanomaterials in the context of REACH.

3.5.44 The influence of coating and surface functionalisation of various nanomaterial types on toxicological effects has been highlighted as part of the ENRHES review (Stone et al., 2009). In addition, it has been suggested that the presence of hydrophilic surface groups on the surface of nanoparticles may be used as an indicator of their reactivity (in relation to their potential uptake and bioaccumulation) according to the following relationship (Zuin et al., 2010):

- Particles possessing hydrophilic surface groups were considered to be highly reactive, as the reactive groups of the particle surface are likely to modify the exposure;

- Particles without reactive surface groups were considered to be of low reactivity.

3.5.45 **Dustiness**

3.5.46 The review of the FP6/7 outputs reveals that the measurement of the dustiness of the nanomaterial powders has been carried out using the rotating drum and continuous drop methods currently suggested in the REACH Guidance chapter for granulometry for the measurement of airborne dispersed or nebulised particles (ECHA, 2008. R.7.1.14, Table R 7.1-31) (Vippola et al., 2009; Jensen et al., 2009). However, studies undertaken as part of the NanoCare project suggest a need for modified versions of these tests to be used for determining the dustiness of nanomaterials (NanoCare, 2009; Stahlmeche et al., 2009).

3.5.47 Results of agglomerate stability studies under various shear forces has suggested that the deagglomeration of nanoparticles depends on a multitude of factors, including the nanoparticle material and its modification, such that general conclusions regarding the propensity of nanomaterials to release particles smaller than 100 nm cannot be made (NanoCare, 2009). Thus, in assessing the dustiness behaviour of nanomaterials, each material must be investigated on a case by case basis. This is important to highlight in the updated REACH Guidance on granulometry, and may have negative implications for the use of read across in relation to dustiness data for nanomaterials.

3.5.48 **Explosivity/flammability**

3.5.49 Within the FP6/7 projects outputs, two dissemination reports have been identified to provide valuable information and guidance for assessing and characterising the explosivity and flammability of powder-based nanomaterials, citations for and information from which should be used to inform the updated REACH Guidance.

3.5.50 It is highlighted that most nanopowders display high reactivity characteristics that can lead to fire or explosion accidents, providing support to suggest that the “Explosive Properties” Information Requirement is as relevant for nanomaterials as for bulk materials (Bouillard et al., 2008). The following properties have been defined as important for estimating the explosivity risk of nanomaterials (Bouillard, 2008):

- Particle size, size distribution and shape;
 - Surface area and surface charge;
 - Particle and surface composition.
- 3.5.51 Investigations of the oxidation of CNT have been demonstrated using differential scanning calorimetry (DSC) and isothermal kinetic studies in order to determine the onset combustion temperature. These methods are in accordance with those suggested in the guidance (ECHA, 2008. R7.1.11.2) for providing supplementary data to support an explosivity assessment for bulk materials.
- 3.5.52 However, several commonly applied methods for explosivity studies have been highlighted as unsuitable for nanopowders (Bouillard et al., 2008), namely:
- current modified, open-ended Hartmann tubes (used to visualise ignition of powders and measure the minimal ignition energy), due to the potential release of nanoparticles during the experiment;
 - current falling hammer equipment used to measure mechanical stability with regards to shock/impact.
- 3.5.53 The limitations and unsuitability of these methods for nanomaterials will be considered further in relation to the updated REACH guidance, and any details of potential replacement method(s) that can be obtained from Bouillard et al. (2008).
- 3.5.54 According to Bouillard et al. (2008), the explosion sensitivity and severity of carbon black powders, aluminium nanoparticles and carbon nanotubes can be assessed using a 20 L explosion sphere in accordance with standards including the American Society for Testing and Materials Methods E 1226 and the National Fire Protection Association Standard 68 or German Society of Engineers (VDI) Method 3673.
- 3.5.55 They suggested that the oxide layer on aluminium nanopowders may render them less explosive than micropowders, CNT may exhibit similar explosion severities and sensitivities as coals, food flours and carbon black, and nanopowders which tend to agglomerate exhibit explosion characteristics of the same order of magnitude as micropowders of the same substance (Bouillard et al., 2008). However, there is not enough supporting evidence available in the publication to judge whether there may be the potential for read-across of explosivity data from bulk equivalents for certain

nanomaterials. In addition, it is suggested by others that read-across of explosivity data from bulk materials to nanomaterials is not possible, since nanomaterials may have explosive properties which are solely due to the small particle size (RIVM, 2009).

3.5.56 **Solubility and release of metals ions**

3.5.57 ICP-MS has been demonstrated as a useful method for studying the solubility of powdered nanomaterials in various solvents (NanoCare, 2009).

3.5.58 The use of linear regression and multi-linear regression to predict the solubility of carbon nanotubes and fullerene in a range of organic solvents has been described (Burello & Worth, 2009). However, many of the studies showed significant differences between the experimental data and the calculated values and these methods are therefore of limited value/reliability at present for supporting the fulfilment of REACH information requirements.

3.5.59 The water solubility of the fullerenes has been suggested to be related to anti-oxidant/cytoprotective or pro-oxidant/cytotoxic properties, such that the greater the water solubility of the fullerene sample the lower the toxicity (although this situation is complicated by the presence of residual solvents which also appeared to contribute towards the observed toxicity) (Stone et al., 2009). The release of silver ions from nanoparticulate silver has also been suggested to be a realistic prospect, responsible for their antibacterial properties and potentially linked to the observed toxicity (Stone et al., 2009).

3.5.60 **TOXICOLOGICAL INFORMATION**

3.5.61 Within the FP6/7 projects undertaken to date, a range of endpoints have been examined relating to standard information requirements for human toxicity within REACH. These include: acute toxicity, repeated dose toxicity, toxicokinetics, mutagenicity, carcinogenicity & reproductive toxicity. No information regarding other REACH Information Requirements (such as dermal and respiratory sensitisation and irritation) was identified. Almost all projects also consider the non-standardised endpoints of cell viability, oxidative stress, pro-inflammatory effects *in vitro*.

3.5.62 Within the FP6/7 project outputs, several publications have been identified which provide useful overviews and comparisons of the most common methods for the

human health hazard testing of nanomaterials, citations for and details from which are of relevance for incorporation into the updated REACH Guidance (ECHA, 2008. Chapter R.7a), specifically:

Chapter 6 of the ENRHES review (Stone et al., 2009);

- Nanocare review on the *in vivo* and *in vitro* assessment of nanotoxicology (Kroll, 2009)
- Nanocare review on the issues related to genotoxicity assessment of NMs (Landsiedel et al, 2009).
- Review by Gwinn & Tran (2009).

3.5.63 The outputs and recommendations of the FP6/7 projects indicate that both *in vitro* and *in vivo* findings of projects have identified clear variations in fate and biological effect according to nanomaterial type.

3.5.64 In addition, it is notable that for many NM, any toxicity exhibited may result from multiple mechanisms. For example, the ENRHES project notes that metal oxide nanoparticle toxicity is thought to be inflammogenic, oxidative, and genotoxic in mechanism; with all endpoints considered to be inherently linked. Testing for NM toxicity within REACH should therefore consider this complexity of mechanism in the experimental approach adopted.

3.5.65 In addition, there also exists a strong consensus from projects that thorough and accurate particle characterisation of nanoparticles prior to commencing toxicological testing is an essential component of understanding their potential toxicity.

3.5.66 **R.7.2 Skin- and eye irritation/corrosion and respiratory irritation, & R.7.3 Skin and respiratory sensitisation**

3.5.67 To date, no information of specific relevance to this information requirement has been identified within the outputs of those FP 6&7 projects reviewed. However, the Nanoderm project, which aimed to develop new techniques and methodologies to complement high resolution transmission microscopy (HRTEM), undertook a number of studies to examine dermal penetration by NMs, and *in vitro* testing to investigate damage at the cellular level. This included monitoring for cell proliferation, apoptosis, necrosis, expression of adhesion molecules, and differentiation).

- 3.5.68 The project concluded that there was no indication that diffusive transport of nanoparticles occurred, thus suggesting that existing techniques such as static Franz-diffusion cell (OECD 428) might not be suitable to study nanomaterial permeation into the skin. Moreover, the project consortium suggested that standardised protocols to study mechanical flexion need to be developed to study dermal penetration of nanomaterials.
- 3.5.69 Within the NANOSH project, exposure in healthy and immune-compromised mice (asthmatic model) using a 28 day repeated-dose testing assay (discussed in detail within the repeated-dose IR summary), it was found healthy animals expressed an immune and cytokine mediated response to TiO₂, whereas compromised (asthmatic) animals showed suppression of most mediators of allergic asthma when exposed. Its results suggest that TiO₂ modulates airway inflammation depending on allergic status and coating of the nanoparticles.
- 3.5.70 **R.7.4 Acute toxicity**
- 3.5.71 A number of FP6/7 projects undertook short term exposure studies both in vivo and in vitro. Although the exposure periods were not within a 24hour period, their results are nonetheless which are relevant to the acute toxicity IR. Notable examples are detailed below:
- 3.5.72 ***In vivo***
- 3.5.73 The Nanocare developed a short term inhalation study in the rat (short inhalation exposures over 5 days, followed or not by a recovery period of 21 days), in order to provide an earlier screening of particle toxicity compared to the typical 90-days study. Endpoints of cytotoxicity & inflammation were monitored, via analysis of broncho alveolar lavage fluid (BALF). The results of this study indicated that the effects observed in the lung with TiO₂ were similar in this short term inhalation study as in typical sub-chronic inhalation studies (such as that outlined within OECD TG412). From this pilot study, the authors recommended that analysis be carried out at day 3 of exposure, and at 21 days after the end of exposure. Their proposed minimal selection of endpoints for inclusion was BALF differential cell count, total protein, LDH, GGT and glucosaminidase activities. The authors also suggested that haematology and respiratory tract histopathology, which are required in OECD studies, were not very sensitive in this short-term inhalation study.

- 3.5.74 The NANOSH project undertook 5 day inhalation exposure testing in mice using TiO₂, silica coated TiO₂, SWCNT, MWCNT and ZnO NPs in order to investigate endpoints of genotoxicity & pulmonary inflammation.
- 3.5.75 The PARTICLE_RISK project undertook an acute *in vivo* assessment of inflammation and genotoxicity via intra-tracheal instillation in compromised ApoE^{-/-} mice (Jacobsen et al. 2009).
- 3.5.76 The NanoKem project undertook work to develop an *in vivo* murine model for screening lung inflammation following intra-tracheal instillation (Roursgaard et al., 2010). Acute inflammation was assessed by BALF analysis. The authors also suggested that at 3 months BALF analysis and histopathology were sufficient to assess sub chronic lung inflammation.
- 3.5.77 It is notable that *in vivo* acute inhalation exposures are not generally encouraged within REACH due to their animal welfare implications, and the general move towards weight-of-evidence approaches utilising pre-existing information and supported by QSAR and other non-animal data sources. However, as there is little pre-existing information on nanomaterial toxicity any information generated by projects to date it is likely that the results of those *in vivo* investigations outlined above are of relevance to their evaluation within the context of REACH.
- 3.5.78 ***In vitro***
- 3.5.79 Work is underway in a number of projects to develop *in vitro* assays to study relevant endpoints of acute toxicity. For example, the NANOSH project demonstrated that TiO₂, silica coated TiO₂, SWCNT, MWCNT and ZnO NPs were dose-dependently cytotoxic in macrophages and dendritic cells, and that the most significant induction of inflammatory mediators was in macrophages following nanoparticle exposure. The PARTICLE_RISK project also investigated pulmonary inflammation and genotoxicity within pulmonary, endothelial and immune cell lines.
- 3.5.80 Although at the current time no validated tests exist for *in vitro* acute toxicity, it is recognised that *in vitro* approaches would be important for the replacement of *in vivo* acute toxicity testing. Therefore, assays such as those utilised here may be both relevant and applicable after they will have gone through the appropriate validation and regulatory adoption processes. In addition, such approaches will

support the 3Rs principle in relation to the number of animals required for acute toxicology testing.

3.5.81 **R.7.5 Repeated dose toxicity**

3.5.82 In relation to repeated dose toxicity, the ENRHES project observed that in many published peer-reviewed studies a single dose was administered and toxicity assessed at a number of post exposure time points. However, it notes that repeated dose studies over a long time period are likely to be of greater relevance in consideration of the potential risk of fullerenes within occupational or consumer settings.

3.5.83 Of those projects completed to date, the following outcomes are of particular relevance to consideration of repeated dose toxicity testing within REACH.

3.5.84 ***In vivo***

3.5.85 The Nanocare project undertook a 28 day inhalation study within rats (followed by a 90 day recovery period) using a protocol similar to that of OECD TG412 (Pauluhn, 2009). Endpoints of cytotoxicity & inflammation were monitored. The protocol produced findings similar to those produced when carrying out repeated dose testing according to OECD TG413.). The authors stressed the importance of having at least 3 months recovery post exposure in the study design, to allow assessment of pulmonary toxicity related to biopersistence of nanomaterials. In particular, the recovery period is essential in order to avoid misinterpretation of the results about retention-specific effect due to lung overloading.

3.5.86 The NANOSH project also developed a (non-standard) 28 day repeated dose protocol. Following inhalation exposure in healthy and immune-compromised mice (asthmatic model), the consortium found that particles accumulate in alveolar macrophages, and that of the nanomaterials tested, silica-coated TiO₂ nanoparticles elicited a clear-cut pulmonary neutrophilia in healthy mice. Healthy animals expressed an immune and cytokine mediated response to TiO₂, whereas compromised (asthmatic) animals showed suppression of most mediators of allergic asthma when exposed. Its results suggest that TiO₂ modulates airway inflammation depending on allergic status and coating of the nanoparticles. The project therefore provides a repeated-dose testing protocol which may be of

relevance to NM toxicity within the context of REACH, and also information which is relevant to considering immune-modulated sensitisation.

3.5.87 Projects underway with relevance to both acute and repeated toxicity testing

3.5.88 Several of those FP6/7 project reviewed which are underway but yet to provide significant outputs include investigations which, once completed, are likely to be of relevance to both acute and repeated dose toxicity testing considerations for nanomaterials within REACH.

3.5.89 Of those FP6/7 projects reviewed, the NANOMMUNE project is the only to focus solely on the immune aspects of NM toxicity. The project is undertaking an array of *in vitro* (murine and human cell lines, as well as primary and more complex co-cultures of cell lines and/or primary cells) and *in vivo* (following exposure via the lung and skin) investigations, as well as transcriptomic and oxidative lipidomic profiling strategies to determine specific nanotoxic profiles (signatures) of these materials.

3.5.90 In addition, as part of the NANOTEST project, investigations of immune toxicity are also underway. These include monitoring of lymphocyte proliferation, phagocytic activity, adhesion molecules and interleukins/cytokines.

3.5.91 The outcomes of this work, once completed and published, should provide relevant information to developing testing strategies for consideration of immune toxicity within acute and repeated-dose testing paradigms.

3.5.92 Also of relevance to both acute and repeated dose toxicity IRs, the NANOTEST project is working to develop alternative testing strategies and high-throughput toxicity-testing protocols using *in vitro* and *in silico* methods. *In vitro* screening tests being undertaken by the consortium are intended to provide alternatives to animal testing to a number of major target organs (blood, vascular system, liver, lung, placenta, digestive system and central nervous system), via three main exposure routes (intravenous, respiratory and digestive). *In vivo*, validation of *in vitro* testing protocols which investigate toxicity to the heart/aorta, liver, lung, brain, blood, spleen, and bone marrow, following a maximum of three doses by either intravenous injection, intratracheal instillation or *per os* administration route will be selected according to the medical use of the nanoparticles under consideration.. In

addition, *in silico*, development of quantitative structure-activity relationships (QSARs) and physiologically-based pharmacokinetic (PBPK) modelling for nanomaterials is underway. The outcomes of this work, once completed and published, should provide relevant information to further developing NM testing strategies within the context of REACH.

3.5.93 **R.7.6 Reproductive and developmental toxicity**

3.5.94 To date, there exists a limited body of work on the reproductive and developmental toxicity of nanomaterials. Of the preliminary work undertaken, the following experiments and results may be of use when considering testing of nanomaterials within the context of REACH.

3.5.95 A study undertaken as part of the NanoInteract project (Park et al, 2009), examined the *in vitro* effects of silica nanoparticles on stem cell differentiation, with a focus on the uptake of particles by the cells and at their cytotoxic effect, using the WST-1 assay. One of the important features of this study is that it tested for potential interference of the material tested in the WST-1 assay. Based on the results, the authors suggested that this *in vitro* embryonic stem cell differentiation test might be a valuable tool to test embryotoxicity of nanomaterials.

3.5.96 In vivo work undertaken within the NanoKem project used a non-standard protocol to assess the effect of prenatal exposure to TiO₂ nanoparticles in mice via inhalation (Hougaard et al. 2010). The study focused both on maternal and embryonic effects, and investigated endpoints including lung inflammation, quantification of Titanium in tissue and milk, and several behavioural tests.

3.5.97 One published output of the NANOTEST project considers reproductive toxicity in vitro. Bhabra et al (2009), details results of *in vitro* work on the translocation and potential for genotoxic effects to be elicited by nanoparticles across the placental barrier. However, whilst the results of this work are interesting to the field from a mechanistic perspective, no results are of direct relevance to the consideration of NM within the context of REACH.

3.5.98 **R.7.7 Mutagenicity & Carcinogenicity**

3.5.99 The endpoint of mutagenicity/genotoxicity was frequently studied within the FP6 & 7 projects using a variety of different assays and techniques. Amongst these were:

- The Comet assay – NanoInteract, NANOSH, PARTICLE_RISK, NANOTEST
- micronucleus assay – NanoInteract, NANOSH, NANOTEST
- lacZ assay – NanoInteract
- FISH (fluorescence in situ hybridisation) – NANOSH

3.5.100 Of the *in vitro* test methods outlined, the micronucleus assay (OECD test guideline 474), comet assay, and chromosome aberration tests are specifically recommended as Information Requirements under REACH guidance. However it is recognised within the guidance that many protocols for mutagenicity are modified following expert judgement, or alternatives used as appropriate.

3.5.101 As part of the Nanocare project, Landsiedel et al. (2009) published a review focusing on the issues related to genotoxicity assessment of NMs. This recommended that as the mechanisms triggering genotoxicity are numerous, the use of several assays in order to study the potential genotoxicity of any substance should be considered. It also highlighted that the most commonly used assay to test genotoxicity of nanomaterial was the comet assay, which at this time is not an OECD test guideline, but is undergoing validation by ECVAM. This was followed by the micronucleus assay, which was recently included in the OECD test guidelines collection.

3.5.102 **R.7.12 Guidance on Toxicokinetics**

3.5.103 With reference to toxicokinetics, the ENRHES project considered the toxicokinetics of uptake (ingestion, inhalation, dermal adsorption and injection), distribution, metabolism and excretion of for four key types of manufactured unfixed nanoparticles and nanotubes in and by the body (CNT, fullerenes, metals and metal oxides). Toxicokinetic aspects specifically considered included persistence and bioaccumulation potential of nanoparticles and nanotubes in the body; differences in toxicokinetics and any subsequent toxicity posed by variations in nanoparticle size, physical structure, chemical composition; mechanisms of interaction of nanoparticles with cells and their components, and partitioning within and between tissues in organisms.

- 3.5.104 Although the project did not itself generate any novel data, the body of evidence reviewed in ENRHES summarises a wealth of knowledge relating to the toxicokinetics of NM within the body, and draws conclusions on this. In particular, the review focussed on *in vitro* and *in vivo* studies using the lungs, skin, gastrointestinal tract (GIT), or blood as routes of entry, with the inclusion of sub-sections which examine toxicity at a number of target organs following the realisation that nanomaterials can distribute from their exposure site within the blood or even nerves. As such, ENRHES represents one of the most comprehensive reviews of toxicokinetic data available to date, and is thus valuable to the consideration of NM within the context of REACH.
- 3.5.105 The Nanocare project developed new *in vitro* models to assess the translocation of particles well as their interactions with the cells, both of which provide relevant input to the building of a toxicokinetic profile for nanomaterials.
- 3.5.106 Using a range of cell types, the INOS project undertook *in vitro* studies which considered cell-particle interactions and translocation into the cellular compartments, outcomes of which may be of relevance to establishing toxicokinetic profiles of NM within the context of REACH.
- 3.5.107 The NANOTEST project is undertaking *in vitro* investigations of uptake and intracellular distribution of NPs Uptake and intracellular distribution of NPs (using labelled NPs and endpoints of Phagocytic activity), and Transport of NPs across biological barriers (using TEER and diffusion). This work is underway thus there are no significant results to date.
- 3.5.108 The PARTICLE_RISK project undertook investigation of translocation and effects of nanoparticles *in vivo* via three different routes of administration: (1) intratracheal instillation into the lungs (IT) or (2) intravenous injection into the tail vein or (3) intra-oesophageal instillation (gavage) into the gastro-intestinal-tract (GI-tract). The results indicated that the pattern of nanoparticles biodistribution differed according to both administration route and physico-chemical characteristics.
- 3.5.109 To investigate the potential neurotoxicity of NPs, the Neuronano project is undertaking a series of investigations relating to the physico-chemical properties of NM dictating their translocational potential and fate *in vivo*. To date, *in vivo* dosimetry studies have suggested that route of exposure, size and protein corona

around the nanoparticle are all influencing factors. However, to date, no significant translocation of nanoparticles into the brain has been shown. *In vitro* results show that transcytosis of nanoparticles is an energy-dependent mechanism which is a function of the cell polarisation of the system, and depends on many parameters such as the surface, size and protein corona.

3.5.110 It is worth noting that some of the projects stress that the concept of “protein corona” associated with NMs forms one of the major differences between chemical and particle toxicity. According to these projects (NanoInteract, PARTICLE_RISK), such nanoparticle-protein is likely to be important in determining the “identity” of the nanomaterial and therefore its subsequent interaction with the environment, such as cellular uptake.

3.5.111 **Additional endpoints of specific relevance to NMs**

3.5.112 The following endpoints, although not specific Information Requirements, are considered to be of particular relevance to establishing toxicity of NMs and as a result are included in many of the FP6/7 projects reviewed. Relevant testing outcomes for each are discussed in detail within the B2 report. However, commonly utilised assays for each are highlighted below.

3.5.113 **Cell Viability**

3.5.114 The endpoint of cell viability was frequently studied within the FP6 & 7 projects using a variety of different non-standardized assays and techniques, and forms an important endpoint which should be further considered in relation to recommended updates to the REACH Guidance for toxicity testing (ECHA, 2008. Chapter R.7). Amongst those assays most commonly utilised were:

- Cell Morphology - NANOTEST
- BALF cell count & protein analysis (in vivo) - Nanocare
- Cell metabolic activity (MTT) – Nanocare, Nanoderm, PARTICLE_RISK, NANOTEST
- Cellular membrane integrity (LDH release) – Nanocare , PARTICLE_RISK
- Lung cell damage (gamma glutamyl transferase; GGT) - Nanocare
- TransEpithelial Electrical Resistance (TEER) - Nanocare

- Apoptosis – NanoCare, Nanoderm
- Necrosis - NANOTEST

3.5.115 **Oxidative Stress**

3.5.116 The initiation of oxidative stress has been frequently studied within the FP6/7 project outputs, and forms an important endpoint which should be further considered in relation to recommended updates to the REACH Guidance for toxicity testing (ECHA, 2008. Chapter R.7). Amongst the non-standardised assays most commonly utilised were:

- ROS Production – Nanocare, NANOTEST
- Glutathione Status – Nanocare, PARTICLE_RISK, NANOTEST
- NO Generation – NANOTEST

3.5.117 Within the ENRHES project, the mechanism of toxicity associated with metal oxides was thought to be inflammogenic, oxidative, and genotoxic in nature; with all endpoints considered to be inherently linked. In addition, ENRHES highlighted that it appears fullerene toxicity also involves an oxidant driven response, thus suggesting that toxicity evaluations should evaluate the potential of fullerenes to cause oxidative stress and related consequences such as inflammation or genotoxicity (Johnson et al. 2010). CNT pathogenicity was noted to be most likely linked to their ability to elicit oxidative stress and inflammation.

3.5.118 The INOS project undertook *in vitro* assays for oxidative stress, the outputs of which may be of relevance to supporting consideration of this outcome in its recommendation as a specific endpoint for consideration of nanomaterials within the context of REACH (justification is outlined within the B2 report).

3.5.119 **Pro-Inflammatory effects *in vitro***

3.5.120 The investigation of inflammation has been commonly studied within the FP6/7 project outputs, and forms an important endpoint which should be further considered in relation to recommended updates to the REACH Guidance (ECHA, 2008. Chapter R.7). Amongst those assays most commonly utilised were:

- TNF- α – Nanocare, PARTICLE_RISK
- Trypan Blue assay – NANOSH
- Cytokine production – NANOTEST, Nanocare, PARTICLE_RISK
- Signalling pathways (NF κ B, AP-1 etc) - NANOTEST

3.5.121 The PARTICLE_RISK project also investigated pulmonary inflammation and genotoxicity within pulmonary, endothelial and immune cell lines.

3.5.122 Within the ENRHES project, the mechanism of toxicity associated with metal oxides was thought to be inflammogenic, oxidative, and genotoxic in nature; with all endpoints considered to be inherently linked. CNT pathogenicity was also noted to be most likely linked to their ability to elicit oxidative stress and inflammation.

3.5.123 The Nanocare project utilised a co-culture system to study pro-inflammatory response *in vitro* (via monitoring for IL-8 release). Its results indicate that *in vitro* the use of a co-culture system as well as the use of lipopolysaccharide stimulation increases the sensitivity of the assay, in detecting pro-inflammatory potential of a nanomaterial.

3.5.124 **Mechanistic Aspects / Experimental Design**

3.5.125 All projects were in consensus that the physico-chemical characterisation of NMs to be tested for human health endpoints was imperative to enable a full interpretation of test results. This is discussed in detail within the physico-chemical characterisation section of RNC/RIP-oN2/B3/2/FINAL.

3.5.126 In relation to study design, the ENRHES review noted that exposure method, dose administered, species used, cell type under investigations and light conditions also have the potential to impact on the toxicity of metal oxide particles. In addition, the reviewers highlighted that experimental quality (including the concentrations used & model selected), of conducted studies is of vital importance when considering the risk associated with metal oxide exposure.

3.5.127 The INOS project emphasised the importance of preliminary characterisation of the materials tested, in the media used, when assessing the hazard *in vitro* (Meissner

et al., 2009b). It also stressed that comparing toxicity test results from the salts of nanoparticles that contained metals to the particles themselves to establish whether the effect produced was linked to the NP or its salt.

3.5.128 The Nanocare project developed several standard operating protocols (SOP) for bioassays, dispersion of the nanomaterial tested in relevant media and for cell exposure. This need to develop SOPs highlights the fact that current standard guidelines need to be adjusted for the testing of nanomaterials and/or that additional tests have to be carried out to avoid misinterpretation of results.

3.5.129 In addition, the Nanocare project (Kroll, 2009) published a number of reviews on the *in vivo* and *in vitro* assessment of nanotoxicology, which highlights specific properties of nanomaterials which make them more prone to interfere with assays, and suggests that new standardised *in vitro* methods might need to be developed to assess the toxicity of nanomaterials which overcome such drawbacks.

3.5.130 Finally, the IMPART project highlighted undertook a large review of the literature about nanotoxicity and highlighted several key issues related to the assessment of nanomaterial hazard. Specifically in relation to experimental design and conduction, the project highlighted the need to consider interference of nanomaterials with toxicity assays, the importance of verification of oxidative stress as a marker for potential toxicity, modification of dosimetrics by particle aggregation, development of new strategies to determine the mechanisms of action for toxicity, and formation of models to predict potential impacts over their whole life-cycle (McCormack et al., 2008). All of these considerations are particularly relevant to hazard identification and characterisation within REACH.

3.5.131 **ECOTOXICOLOGICAL INFORMATION**

3.5.132 The key experience relevant to REACH from the FP6/7 projects is summarised below. The advantages, limitations and applicability of which are being considered further within the gap analysis (Task B4) with a view to informing the updated REACH guidance.

3.5.133 Only a few FP6/7 projects i.e. ENRHES, INOS and Nanointeract have reported results and/or used methods (i.e. fish cytotoxicity, growth inhibition of algae and

acute toxicity of crustaceans and fish embryos) that are relevant in regard to providing practical advice in the REACH context.

- 3.5.134 As noted previously, it is evident from the FP6/7 projects with relevance for the ecotoxicity of NM that a thorough and accurate particle characterisation is an essential component of assessing the potential ecotoxicity of nanomaterials.
- 3.5.135 Considered specifically, ENRHES, INOS and Nanointeract have underlined the importance of considering agglomeration behaviour over time in various form of medium and hence underline the importance of measuring the state of agglomeration both at the beginning and the end of the experiment (Kuhnel et al. 2009, van Hoecke et al., 2009, Stone et al. 2009).
- 3.5.136 An FP6/7 funded review of the scientific literature (ENRHES) has highlighted that changes in the metal speciation (association of a nanomaterial with a molecular or ionic dissolved chemical substance) can occur depending on redox conditions, salt content, etc. and this need to be determined and reported in order to enable to interpretation of the reported studies in a REACH context (Stone et al. 2009).
- 3.5.137 It has furthermore been highlighted how the use of any solvents (for instance THF which was used in some of the first ecotoxicological studies on C60) should be avoided since, as for the mammalian toxicology studies, it has been demonstrated that not only nanoparticle/solvent interactions may affect ecotoxicity, but also solvent degradation products may be responsible for some of the observed effects. Instead of using solvents, extensive stirring and sonication can be used as alternatives to bring some nanoparticles in dispersion.

3.5.138 B3 Sub-Task II: Practical advice on the Relevance and Applicability of Experience Reported in the Scientific Literature on Nanomaterial Characterisation, Hazard Identification and Assessment for Workers, Consumers and Environment in the REACH Context

3.5.139 PHYSICO-CHEMICAL PROPERTY INFORMATION

3.5.140 Key characterisation considerations

3.5.141 Based on a review of the scientific literature, it is evident that it is now widely acknowledged that adequate characterisation of a nanomaterial is necessary to accompany any toxicity study, particularly in cases where nanomaterials (e.g. carbon nanotubes) can be produced by different processes yielding notionally the same material, but which exhibit quite different properties (Zuin et al., 2007; Boverhof & David, 2010).

3.5.142 Within the scientific literature, several publications have been identified which provide useful overviews and comparisons of the most common methods for the physico-chemical characterisation of nanomaterials, further details from which are of relevance for consideration in relation to the updated REACH Guidance (ECHA, 2008. Chapter R.7a), specifically Nanoforum (2006) and Tiede et al. (2008)

3.5.143 A number of key general points have been noted from the review of the scientific literature for further consideration in the gap analysis and subsequent guidance amendments:

- Whilst characterisation of nanomaterials as-produced or as-supplied is the most direct and currently realistic approach to obtaining physico-chemical information about the material being studied, this data may not appropriately represent the properties of the material when in contact with the environment in which it is being observed, for example in air or physiological environments of *in vivo* or *in vitro* assays;
- It has been suggested that adequate particle characterisation should be performed in three distinct phases, primary, secondary, and tertiary, where: primary characterisation is performed on particles as-synthesised or as-received in its dry native state; secondary characterisation is performed on particles in the wet phase as a solution or suspension in aqueous media;

tertiary characterisations are performed on particles following interactions with cells under *in vivo* or *in vitro* conditions (Sayes and Warheit, 2009).

- Characterisation after administration is particularly advantageous where the possibility of physico-chemical changes in the material before and after administration exists;
- It is recognised that in many cases characterisation at the point of administration will remain to be essential for the comparison of studies;
- The limitations of each analytical method for nanoparticle characterisation can lead to inconsistent results and, therefore, to inaccurate predictions of material properties and structure (Carter et al., 2005);
- Nanoparticle sizing standards, as well as standardised methods for sampling and measurement, are urgently required to overcome the problem of inconsistent data (Borm et al., 2006);
- The lack of consistent reference materials and standards further exacerbates this problem (Lead and Wilkinson 2006). Some progress has been made recently regarding reference materials for characterisation, but standardised nanoparticles are not yet widely available and researchers have to rely on commercially available, often not well-characterised, nanoparticles.

3.5.144 **Sample preparation**

3.5.145 Within the review of the scientific literature, sample preparation has been highlighted as one of the most critical steps towards successful characterisation of nanoparticles, in which there are many variables to consider when designing a method for preparation. This issue is of key importance for further consideration in the gap analysis (Task B4) and proposals for amendments to the guidance. In relation to this issue, key points to note are:

- the need to have “reliable” sampling, such that a test aliquot is collected from a defined sample of particulate material that can be considered to be representative of the entire sample (NIST 960-1);

- Powder sampling is more difficult than sampling from suspension, but some general guidelines on powder sampling are available (Allen, 2001a and 2001b);
- Steps in the sample preparation will be governed largely by the requirements of individual measurement methods;
- Ideally, samples for analysis should be free from the inherent aggregation problems associated with nanoparticles and other contaminants not associated with the said nanoparticles. However, to achieve such goals are not trivial;
- The use of sonication to disperse nanoparticles in solution has the potential to change the size distribution of the nanotubes and introduce defects (Islam et al., 2001);
- It is important to establish the 'state' of the sample required for analysis i.e. whether the nanoparticles should be fixed on to a solid substrate, suspended in liquid media or aerosolised (solid or liquid aerosols).

3.5.146 A number sample preparation protocols and guidance for nanomaterials such as carbon nanotubes (Decker et al., 2009) have been emerging in the scientific literature. However, these procedures cannot be universally applied to all nanomaterials and certain modifications to the procedures may be required for different nanomaterials. It is therefore important to stress that such procedures should be carefully examined to determine if they are adequate for the test material under consideration.

3.5.147 **Particle size/size distribution**

3.5.148 The current REACH guidance on particle size measurement (albeit with a focus on micron-sized particles) refers to the TG110 document published in 1981, with limited recognition of more modern particle sizing techniques. The literature review has identified that there are many methods available for detecting and accurately characterising the size/size distribution of nanomaterials in powder form, suspension and aerosols.

3.5.149 A number of important points have been noted and will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the guidance, specifically:

- It has been highlighted that different methods, based on different measurement principles, may yield different results when measuring the same nano-object or structural feature (Lövestam et al., 2010);
- It has been recommended that measured particle size values should be regarded as 'method-dependent', and should be reported with sufficient detail on the analytical technique used to acquire the data and the applied protocol used to deduce the size from the measured raw data (Lövestam et al., 2010). This also applies to other physico-chemical properties in addition to particle size/size distribution measurement;
- It is noted that no single technique can be considered to be without artefacts or can be employed in all cases when determining nanoparticle sizes, and it is thus recommended that the multi-analytical techniques and/or multiple preparation techniques should be used when characterising nanoparticles (Domingoes et al., 2009);
- It is noted that Dynamic Light Scattering (DLS) does not provide a full particle size distribution. DLS measures fluctuations in the intensity of scattered light caused by Brownian motion, from which the hydrodynamic diameter is calculated, enabling estimation of the particle size distribution. Thus, even though DLS does not measure particle size distribution directly, this method provides a good background for the estimation of the full particle size distribution. The method also provides a number (the 'polydispersity index') indicating the polydispersity of the particle population. There are software routines available that facilitate the calculation of a particle size distribution from DLS data, but the adequacy and the comparability of these routines needs to be further evaluated (Lövestam et al., 2010).
- Drying samples under vacuum for analysis using electron microscopy may alter the size and shape of the particles being characterised. Plasma sputter-coating the surface-adhered particles with a layer of a conducting material may modify the sample being characterised;

- The analysis of particles in solution has been advanced through the development of Environmental Scanning Electron Microscopy; this offers the potential for dispersed samples prepared for exposure/toxicological experiments to be characterised as well as limiting the need to dry samples which may influence the observed size distribution;
- The quality of the images to be analysed using electron microscopy techniques is of critical importance and it should also be noted that electron microscopy normally provides only two-dimensional images, so care must be taken to avoid bias introduced by orientation effects. High-resolution microscopy may be subject to artefacts caused by sample preparation or special analysis conditions;
- In aerosol physics, the most commonly used methods for particle sizing are differential electrical mobility (from about 10 – 1000 nm) and light scattering aerosol spectrometry (from 60 nm – 45 µm). These methods allow determination of the equivalent aerodynamic diameter which may be different from the geometric diameter measured with microscopy techniques (Lövestam et al., 2010);
- Relatively few toxicological studies published in the scientific literature have directly compared nanoparticulate and microparticulate forms of metals, which may limit establishing, at this time, the validity of read-across in the REACH context;
- Overall, the size at which genuinely nanoscale properties are observed depends strongly on the material, and although most of these effects appear at sizes of 30 nm and below, no general limit can be given (Lövestam et al., 2010). As such, case-by-case studies are necessary for every material, since there is no direct, material-independent relationship between size and novel effects or functions.

3.5.150 **Aggregation/agglomeration state**

3.5.151 The aggregation/agglomeration state of nanoparticles affects the stability of nanoparticle dispersions prepared for (eco)toxicological experiments. The size of aggregate particles can be determined using many of the same methods described

above for 'particle size/size distribution' analysis, an overview of which is provided earlier in the "particle size/size distribution" and "aggregation/agglomeration state" sub-section of chapter 4.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.152 In addition, a number of other important points have been noted and will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the guidance, specifically:

- The state of dispersion (i.e. level of (de)agglomeration) is typically estimated using comparative particle size measurements, with shaking, sonication, and/or surfactants commonly used to disperse nanoparticles in solution. However, these tools may damage cells and interfere with toxicity testing if used in living systems (Powers et al., 2006);
- The combined use of dielectrophoretic assembly and Raman spectroscopy has been suggested to be a more sensitive measure of the aggregation state of carbon nanotubes than either one of these methods alone (Kumatani and Warburton, 2008);
- Surface energy, charge and solvation have been suggested to be relevant parameters to consider in relation to nanoparticle-nanoparticle interactions (SCENHIR, 2006).
- The propensity of particles to aggregate has prompted researchers to prevent against its occurrence through the use of sonication, or inclusion of dispersants within particle suspensions, particularly in the case of CNTs. However, the relevance of considering monodispersed CNT also requires consideration, since, if this is so difficult to achieve experimentally, the relevance to human and environmental exposure may be questionable. It might be more useful to achieve a CNT suspension with limited or controlled aggregation, promoting uniform, more easily characterisable exposure conditions for the model under investigation. In addition, the exposure route is likely to impact on the aggregation and agglomeration of CNT. For example, limiting the aggregation of CNT when generating aerosols is difficult, whereas a number of dispersants can be employed to improve the dispersion of CNT suspensions;

- The use of grinding to increase dispersion has been demonstrated to impact on both length and surface properties, and therefore toxicity of the MWCNT (Muller et al., 2005).
- There is a difficulty in testing CNT toxicity due to their high propensity to aggregate and achieving a suspension or aerosol where individual CNT are contained is difficult. It is therefore important to consider how interactions between CNT, which promote the formation of larger structures, impact on the toxicity of CNT;
- Preliminary studies on the inclusion of commonly used surfactants (namely Pluronic L61, Pluronic L92, Pluronic F127, Tween 20, and Tween 60, at concentrations of 0.1-10%) in dispersions of MWCNT has illustrated that all surfactants (with the exception of Pluronic F127) reduced cell viability at all concentrations, and were therefore deemed inappropriate to use despite their ability to reduce CNT aggregation (Monteiro-Riviere et al., 2005);
- Filtering can be used as a technique to reduce the presence of aggregates within the dispersing solution of SWCNT (Raja et al., 2007);
- It has been suggested that the degree of particle aggregation and agglomeration associated with metal oxide administration is also likely influencing the resultant toxicity of these particle types, in addition to particle size. However the nanoparticles that make up the agglomerates are within the nano size range, and this appears to be fundamental to driving their toxicity;
- Nanoparticle aggregation is also general issue that has been reported in a wide range of the published environmental studies. The extent of particle aggregation has been reported to be influenced by particle type and differences in test media as well as procedures to prepare test suspensions as note before;
- Only a few studies have systematically explored the influence of aggregation on the ecotoxicity of various nanomaterials;
- From the research published to date, it is not yet clear how various levels of aggregation of nanoparticles influence their ecotoxicity, and the derivation of LC50, EC50 and NOEC also remains uncertain. Adding to the complexity of

this issue, it has recently been found that aggregation behaviour in the media might follow a non-linear concentration-aggregation relationship (Baalousha et al., 2009; Baun et al., 2009).

3.5.153 **Surface area**

3.5.154 The reduction in size to the nanoscale is accompanied by an inherent increase in the surface-to-volume ratio, and therefore a greater proportion of entities at the surface compared to the bulk (non-nanoscale) material. For particle-based substances, the surface plays an important role in influencing the physical and chemical interactions between the substance and the receptor (i.e. cell, tissue, organism, media etc). The influence of surface area on toxicity is obviously intrinsically linked to particle size. Powers et al. (2006) highlight that it has been established in several toxicity studies that effects correlate with surface area (Powers et al. reference Brown et al., 2001; Donaldson et al., 1998, 2002; Oberdorster et al., 1992; Tran et al., 2000) to a greater extent than mass as a dose metric.

3.5.155 Several methods have been identified for measuring the surface area of nanoparticles. A description of the techniques identified has been provided along with their advantages and limitations. Emerging methods such as diffusion charging have begun to provide a more viable approach to measuring aerosol surface area in situ. Implications of a number of issues, however, remain to be considered including the effect of initial aerosol charge, the composition of the material, presence of aggregates and the effect of particle shape. The advantages and disadvantages of measuring *deposited* particle surface area, rather than *aerosol* surface area, also need to be considered further.

3.5.156 **Shape/Aspect ratio**

3.5.157 Length has been accepted to influence fibre clearance, because it dictates the ability of phagocytic cells to completely internalise CNT. Longer fibres promote the development of frustrated phagocytosis, reduced clearance and hence the potential to persist to increase their propensity for damage (Brown et al., 2007; Poland et al., 2008). The shape of nanoparticles is also likely to influence their uptake by cells, as has been demonstrated for metal nanoparticles (Chithrani et al., 2006; Chithrani and Chan, 2007; Pal et al., 2007). Only a few studies have documented links between physico-chemical characteristics and ecotoxicity. In regards to shape, only three published studies have been identified. It is evident that much more research is needed before specific properties, or combinations of properties, can be linked to the effects observed in ecotoxicity tests (Stone et al., 2009).

3.5.158 A number of methods have been identified for measuring the surface area of nanoparticles, primarily microscopy based. A description and comparison of the techniques identified is readily accessible in the “shape/aspect ratio” sub-section of Chapter 4.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.159 In addition, a number of other important points have been noted and will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the guidance, specifically:

- A method for processing SPM images in order to estimate nano-object positions and dimensions, using dictated fits based on the least-squares method and the matrix operations has been published (Silly, 2009);
- UV-visible spectroscopy, commonly used to confirm the presence of nanoparticles in a liquid, can be used to indicate the shape of the nanoparticles, but confirmation is usually performed using imaging methods such as TEM (Weir et al., 2008);
- An extension of thermogravimetric analysis (TGA), a method principally used for determining chemical composition and purity, to analyse the specific properties of SWCNT including length and diameter has recently been reported. However additional characterisation methods (such as SEM or

Raman Spectroscopy) may be needed to support and fully interpret the TGA results (Mansfield et al., 2010).

3.5.160 **Surface Charge**

3.5.161 In relation to toxicity testing, the major influence of surface charge is on the stability of the administered dispersion (see, for example, Jiang et al. (2009)). However, size and charge can also influence the adsorption of ions, contaminants, and biomolecules, and the way cells react when exposed to them (e.g. Goodman et al., 2004). Particle uptake by cells has also been observed to be influenced by the particle's charge, particularly for metal oxides (Hankin et al., 2008). It is evident that much more research is needed before specific properties, such as surface charge, can be related to the effects observed in ecotoxicity tests (Stone et al., 2009).

3.5.162 A number of methods have been identified for determining the zeta potential of nanoparticles, as described in the “surface charge” sub-section of chapter 4.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.163 **Surface chemistry**

3.5.164 The term surface chemistry is often used in the context of surface chemical composition, and is somewhat a broad and non-specific term which does not predispose itself to ‘quantitative’ characterisation according to a single comparable metric or measurand. Surface chemistry includes elements of solubility equilibrium, catalytic properties, surface charge, and surface adsorption and desorption of molecules from solution, amongst others. Most of these properties are functions of the atomic or molecular composition of the surface and the physical surface structure. Chemical purity, functionalisation and surface coating are also important aspects to take into account.

3.5.165 Modification of the surface of CNTs has been demonstrated to both enhance and reduce toxicity. It is therefore unreasonable to make definite conclusions about how the modification of CNT affects their toxicity, as it is driven by the particular modification employed, which is generally undertaken for a specific purpose. The modification of the surface of TiO₂ particles has also been demonstrated to influence its toxicity. However, this is likely to be dependent on the modification, and cell type in question. The impact of functionalisation on fullerene toxicity has also been the

focus of investigation since functionalisation of the surface of fullerenes is often completed for a specific purpose, such as improving water solubility. Fullerene functionalisation has also been observed to promote the appearance beneficial properties such as antioxidant, or anti-inflammatory activity. The influence of surface attachments on fullerene toxicity may be dependent upon the target cell/organ and/or the fullerene type under investigation.

3.5.166 Although some evidence is available in the literature to suggest that surface functionalisation may influence the ecotoxicity of nanomaterials, the number of studies is, at present, too limited to draw general conclusions on the influence of functionalisation on ecotoxicity, speciation, and accumulation.

3.5.167 A number of methods have been identified for analysing the surface properties of nanoparticles, as described in the “surface chemistry” sub-section of chapter 4.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.168 In addition, a number of other important points have been noted and will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the guidance, specifically:

- Atomic Force Microscopy (AFM) can provide 3D imaging/visualisation of nanoparticles distributed on a flat surface, allowing access to qualitative and/or quantitative information about the physical properties of nanoparticles including size, morphology, surface texture, and roughness. However, the influence of the AFM tip size and shape on the acquired images must be properly accounted and corrected for;
- Another property related to surface chemistry that may be of relevance to characterise is porosity. It has been suggested that there is no all-encompassing analytical technique available to study porosity and a synergistic approach involving application of a combination of various techniques is necessary (Heo et al., 2006);
- The nano scale chemical and structural environment, including factors such as surface roughness, may also be important properties to consider in relation to bacterial activity.

3.5.169 Redox activity

3.5.170 Redox potential may be useful in determining how active a given nanomaterial (and in principal any chemical substance) would be in human and environmental oxidation-reduction processes, potentially also generating reactive oxygen/nitrogen species (ROS, RNS). Redox reactions can occur abiotically or biologically, and may alter a nanomaterial's physico-chemical properties including surface area, surface charge, and chemical composition, which in turn can affect the material's potential to aggregate, size, toxicity and mobility. Redox reactions are the basis of chemical transformations of inorganic and organic species and the precipitation and dissolution of inorganic substances that influences their sequestration and mobility. Hence measurement of the redox potential would be potentially meaningful for nanomaterials which can participate in electron transfer or uptake.

3.5.171 It has been suggested that chemically stable inorganic nanomaterials in physiological redox conditions do not appear to exhibit cytotoxicity in vitro, whereas nanomaterials with strong oxidative (e.g. CeO_2 , Mn_3O_4 and Co_3O_4) or reductive powers (e.g. Fe^0 , Fe_3O_4 , Ag^0 and Cu^0) can be cytotoxic and genotoxic towards biological targets in vitro (Auffan et al., 2009). Standard electrochemical methods, such as cyclic voltammetry, may be used to study the redox activity of nanomaterials.

3.5.172 ROS generation potential

3.5.173 The ability to generate ROS and oxidant injury is one paradigm that may be used to compare the toxic potential of nanomaterials (Xia et al., 2006; Auffan et al., 2009). However, the relationship between ROS generation and ecotoxicity has been studied to a lesser extent.

3.5.174 Whilst it has been demonstrated that ROS generation and oxidative stress can be used as a paradigm to assess nanomaterial toxicity, not all nanomaterials exhibit the electronic configurations or surface properties that allow spontaneous or acellular ROS generation; particle interactions with cellular components could generate ROS during these interactions.

3.5.175 A number of methods have been identified for detecting ROS generation from nanomaterials, under both abiotic conditions and in cells, as described in the “ROS generation potential” sub-section of chapter 4.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.176 **Photocatalytic activity**

3.5.177 The ability of UVA or visible light to increase the toxic potency of metal oxide particles, through increased ROS production, has been a focus of a number of toxicology studies (e.g. Dunford et al., 1997; Zhang and Sun, 2004; Dufour et al., 2006), but does not always transpire (Linnainmaa et al., 1997; Theogaraj et al., 2007). Results of ecotoxicology studies to date also do not allow any firm conclusions to be drawn regarding the influence of photocatalytic activity on ecotoxicity.

3.5.178 A number of methods for studying the photocatalytic activity have been described in the “Photocatalytic activity” sub-section of Chapter 4.1 (RNC/RIP-oN2/B3/2/FINAL). However, it is currently unclear what distinct measurand for photocatalytic activity could be standardised to the extent that would be meaningful to risk assessment. The lack of clarity in the relationship between photoactivity and (eco)toxicity, combined with the lack of a clear measurand, may not provide sufficient justification for the inclusion of photocatalytic activity as a new Information Requirement.

3.5.179 **Dustiness**

3.5.180 Measurement of the ‘dustiness’ of a material provides information relating to the propensity of that material to produce airborne dust, and is important to consider from an exposure perspective. Dustiness is also a key parameter for assessing the risk of dust explosions. This property is covered to some extent under the current REACH guidance chapter on Granulometry.

3.5.181 Rotating drum and continuous drop methods are currently suggested in the REACH Guidance chapter for granulometry for the measurement of airborne dispersed or nebulised particles (ECHA, 2008. R.7.1.14, Table R 7.1-31). However, rotating drum dustiness tests are usually performed as three replicate tests and need quite large amounts of test material, typically 300–600 g. The EN 15051 continuous single-drop method requires a total amount of 500 g for the required five single-test runs. It has been highlighted that such large amounts of test material may not be

practical if very toxic and/or costly materials are to be tested and there is a need for test systems that can be operated under controlled atmospheric environments using much smaller amounts of material (Schneider & Jensen, 2008). Several advances towards this have been reported in the scientific literature, specifically:

- A fluidisation (vortex shaker) method has been developed for testing nanosize powders (Maynard, 2002; Baron et al., 2003; Maynard et al., 2004). This method is included in ISO's forthcoming technical document entitled "Nanomaterials – General framework for determining nano-object release from powdered nanomaterials by generation of aerosols". However, sufficient methodological detail has yet to be incorporated into the document being developed;
- A dustiness test that uses only 6 g of material per test run and that characterises the test material by both a single-drop and a rotating drum type of challenge. The test apparatus is based on a downscaled version of the EN 15051 rotating drum, whilst maintaining important test parameters, and was demonstrated to provide very reproducible results both in terms of amount and size distribution of the generated particles (Schneider & Jensen, 2008).

3.5.182 **Explosive properties**

3.5.183 No studies have been identified in the literature relating to the explosive properties (an existing REACH Information Requirement) of nanomaterials, over and above the outputs from the NANOSAFE2 project discussed in the FP6/7 section of RNC/RIP-oN2/B3/2/FINAL, therefore the ability to prepare practical advice in the context of REACH is limited.

3.5.184 However, it has been suggested that read-across of explosivity data from bulk materials (non-nanoscale) to nanomaterials is not possible, since nanomaterials may have explosive properties which are solely due to the small particle size (RIVM, 2009). This issue will be considered further in the gap analysis (Task B4) and used to inform any subsequent amendments to guidance.

3.5.185 Boiling/Melting/Freezing Point

3.5.186 No studies have been identified amongst the extensive literature sourced during Task A in which the boiling, melting or freezing point (existing REACH Information Requirements) of nanomaterials is specifically addressed, therefore the ability to prepare practical advice in the context of REACH is limited.

3.5.187 However, in undertaking a hypothetical registration of nanosilver under REACH, RIVM (2009) concluded that these properties will be similar to those of metallic bulk (non-nanoscale) silver. Data for these parameters was effectively read across from the bulk form in their hypothetical registration of nanosilver. However, they conclude that “the ‘sameness’ analysis can, as yet, not be properly tackled under REACH, and thus the read-across from data on the bulk form of a substance to its nanoform will be very troublesome”. Furthermore, they concluded that “a nanomaterial cannot be properly characterized with the data normally required under REACH, and it is unclear how to address different sizes of a nanomaterial in substance identification”.

3.5.188 Relative Density

3.5.189 No studies have been identified amongst the extensive literature sourced during Task A in which the relative density (an existing REACH Information Requirement) of nanomaterials is specifically addressed, therefore the ability to prepare practical advice in the context of REACH is limited.

3.5.190 However, RIVM (2009) concluded that the relative density of nanosilver will be similar to that of metallic bulk (non-nanoscale) silver. Data for this parameter was effectively read across from the bulk form in their hypothetical registration of nanosilver. However, RIVM (2009) highlight that, for relative density, caution needs to be taken when performing read-across of data from the bulk form. Overall, they conclude that “the ‘sameness’ analysis can, as yet, not be properly tackled under REACH, and thus the read-across from data on the bulk form of a substance to its nanoform will be very troublesome”. Furthermore, they concluded that “a nanomaterial cannot be properly characterized with the data normally required under REACH, and it is unclear how to address different sizes of a nanomaterial in substance identification”.

3.5.191 Solubility & release of metal ions into solution

3.5.192 As stated in the Task B1 report, the property of water solubility is considered to be very relevant and applicable to nanomaterials. The release of ions into solution (including through an active electrochemical 'corrosion' process) may confound the interpretation of results perceived to be the result of solubilised substances. It is anticipated that the release of silver ions from nanoparticulate silver is a realistic prospect, responsible for their antibacterial properties and potentially linked to the observed toxicity. It is postulated that Ag^+ mediates these effects, although the mechanism by which this occurs is unknown at this time but likely to involve particle oxidation to enable their release. Further investigations are necessary to confirm the contribution of particles and/or ion release to their toxicity. In relation to ecotoxicity of nanosilver, the issue of dissolution may also be crucial to understanding the mechanisms involved. A number of studies have reported that the observed ecotoxicity of these nanomaterials towards various organisms may be partly or fully attributed to the release of dissolved metal ions, covering zinc oxide as well as nanosilver. However, the influence of dissolved metal ions on ecotoxicity is not clear based on current literature and requires further investigation.

3.5.193 In undertaking a hypothetical registration of metallic silver (in bulk and nanoform) under REACH, RIVM (2009) highlighted that there is no information available on the kinetics of dissolution in dependence of nanosilver particle properties (such as (time-dependent shifts in) size distribution, shape) and properties of the medium (like pH, dissolved organic carbon, silver-complexing ions and recommended the inclusion of 'Dissolution Kinetics' as a sub-information requirement under the existing REACH Information Requirement for 'Solubility'.

3.5.194 TOXICOLOGICAL INFORMATION**3.5.195 Considerations for study design**

3.5.196 Investigations undertaken and reported to date have highlighted a number of key issues or gaps in existing testing strategies which may influence the outcome of studies, and thus should be observed closely in the consideration of nanomaterials within the context of REACH.

3.5.197 In relation to the manner in which experiments are set up, factors such as the exposure method, dose selected, species used, cell type under investigation and in the case of photo-reactive nanomaterials such as some metal oxides, light conditions (Warheit *et al.* 2005) all have the potential to impact on the toxicity of nanoparticles, indicating that the experimental set up is very influential.

3.5.198 A summary and discussion of those issues considered to be of importance in this respect is provided below.

3.5.199 **Dispersion**

3.5.200 As discussed at various points within this B3 report, it is clear that dispersion impacts upon the potential toxicity of NMs in animals or cells. A multitude of dispersing processes have been utilised by investigators to improve the dispersion including solvents (such as acetone), surfactants (such as pluronic), proteins (albumin and serum) or mechanical processes (such as centrifugation, or sonication). At this time it is not possible to conclude which techniques are most appropriate, but in designing experimental protocols for consideration of nanomaterials in the context of REACH, it is essential that such techniques should try to mimic realistic exposure scenarios, routes and avoid interference by dispersants.

3.5.201 **Selection of Dose**

3.5.202 Many investigations reported have used very high doses, raising the question of whether the toxic effects observed are likely to derive from dose used or the material. In particular, at high doses, the aggregation of CNT is promoted, and so the toxicity that transpires *in vivo* is potentially a result of the blockage of airways and blood vessels, rather than to a specific toxic effect.

3.5.203 In order to inform selection of relevant nanomaterial exposure concentrations to be used within *in vitro* and *in vivo* experiments, information regarding the human exposure levels is required as a reference. However, at the current time this is severely lacking and thus exposure assessment is of key importance to developing sound dose selection for future studies

3.5.204 In relation to dosing regime, many investigations of NM toxicity have used a single dose administered to animals or cells. Within occupational or consumer settings, it is

more likely that normal NM exposures (non-accidental) will occur over a period of time, depending on their application.

3.5.205 **Selection of exposure route and duration**

3.5.206 Testing methods for initial *in vivo* toxicity assessment normally use the oral route of exposure. However for the testing of nanomaterials, this may not be the ideal first candidate. As a general observation, repeated dose studies with lower doses over a long time period and use of a route of exposure appropriate to the end use of the nanomaterial in question, are likely to be of greater relevance in consideration of the potential risk of nanomaterials within occupational or consumer settings, than extremely short exposures at high doses. In addition, the use of chronic studies will also allow for the more relevant identification of the potential carcinogenic consequences of NM exposure.

3.5.207 **Interaction of nanoparticles with biological molecules**

3.5.208 Attention should be directed to consideration of the role that interaction of NPs with biological molecules plays in altering their behaviour within biological systems. It is known that on entering the body, particles immediately become coated in biological molecules, including proteins. It is hypothesized that this coating can influence particle behaviour and toxicity, with different particles having different capacities to bind different molecules. Furthermore, there is a possibility that the particles can alter the protein structure and function (and thus behaviour), which again may contribute to toxicity. Further research is required to generate a greater understanding of this complex area, and it is advised that consideration should be given in future experimentation to the role that such interactions may play in toxicity.

3.5.209 **Use physico-chemical data to inform experimental design**

3.5.210 It has been highlighted within this report, but also more fully within the toxicology and physico-chemical sections of RNC/RIP-oN2/B3/2/FINAL that the physico-chemical characteristics of particles used such as size, crystallinity, functionalisation, contamination, solubility etc. can impact on their toxicity. Thus, as

these factors are able to influence the findings obtained, a thorough physico-chemical characterisation should be undertaken and used as justification of the relevancy of the experimental approach used in future toxicological investigations undertaken.

3.5.211 In addition, a specific consideration in relation to metal nanomaterials is determination of whether any toxicity observed derives from their small size, is mediated through the release of ions from particles, or perhaps a combination of both. Elucidation of this is essential for the hazard characterisation of metal NPs within the context of REACH

3.5.212 **Target Organ Toxicity considerations**

3.5.213 At the current time, there is a paucity of data relating to the systemic transfer of particles following exposure via the lungs, skin and gut, and this should be a focus of future experiments. Studies have focused on dermal and pulmonary toxicity of particles, but there is an absence of data on the consequences of exposure to the gastrointestinal tract, and within damaged/diseased skin. Other relevant target organs include the liver, kidney, cardiovascular system and brain which are necessary due to the fact that nanoparticles are likely to become systemically or neuronally available. The liver could be highlighted as a priority due to the propensity of particles to accumulate in this organ. Based on this, it has been suggested that toxicokinetics and certain additional target organ specific effects be considered as specific information requirements for hazard identification and characterisation of nanomaterials within the context of REACH

3.5.214 ***Adoption of standardised 'controls' to assist assessment of toxicity***

3.5.215 The use of both nanoparticulate and non-particulate controls (such as carbon black or asbestos within CNT studies, or zymosan within inflammation studies) has been reported on numerous occasions. The choice of controls can, to some extent be driven by the hypothesis being tested (and links to determination of the physico-chemical characteristics responsible for toxicity). The use of such benchmark controls (those for which extensive background information is available) provides a useful indication of the relative toxicity of the nanomaterial under investigation versus other particles or reagents of known toxicity (see for example, Warheit *et al.* 2004, Shvedova *et al.* 2005). Thus, it should be encouraged as standard practise

for the hazard identification and characterisation of nanomaterials within the context of REACH.

3.5.216 ***Interference of nanomaterials with toxicity assays***

3.5.217 Nanomaterials have been on occasion found to interfere with some of the assays utilised to determine their cellular or toxic effects. For example, some nanoparticles may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanoparticles may bind to assay components including the substrates (such as CNT with the reagent in MTT assays; Belyanskaya *et al.* 2007) or the biomarker being measured, (such as LDH and cytokine proteins, see for example Davoren *et al.* 2007). Kroll *et al.* (2009) undertook a review of cytotoxicity assays commonly used to investigate nanoparticle toxicity, and provided a discussion of interference reported for each.

3.5.218 All of these factors can contribute to the production of inaccurate, misleading results that make nanoparticles appear more or less toxic than they actually are. In many of the studies reported it is not possible to ascertain whether the assays were adequately controlled to assess for interference. Thus, as a general precaution, it is advisable to use more than one assay to assess the endpoint or effect in question. Further investigation of this area is required, as is integration of known interferences into any guidance developed for the toxicology testing of nanomaterials under REACH.

3.5.219 **ECOTOXICOLOGICAL INFORMATION**

3.5.220 Given the current state of the published science, it has not been possible to provide specific practical advice with regard to the ecotoxicological Information Requirements in REACH, but a number of factors have been found to influence the ecotoxicologic responses observed in the study of nanomaterials. These include: 1) particle impurities, 2) suspension preparation methods, 3) release of free metal ions, and 4) particle aggregation and 5) relevance of dose [concentration] - response for ecotoxicological studies of nanomaterials. The extent of influence of these factors on the ecotoxicological impact of nanomaterials is unknown and, even, the scientific evidence for them have an influence in the first place is contradictory and varies

from nanoparticle to nanoparticle (Baun et al., 2009). This impedes the reliability and interpretation of the available ecotoxicity data as well as the direct use of the reported LC50, EC50 and NOEC for PNEC derivation.

- 3.5.221 As noted by Stone et al. (2009) traditional predictions of fate and transport are based on inherent properties such as phase transfer properties (e.g. boiling point, vapour pressure, partition coefficients), reactivity (e.g. photo-reactivity and hydrolysis) and biological degradation behaviour (Mackay and Hendry 2009). Many of these inherent properties are reported on for regular chemicals under REACH. However, we know at this point that these properties are not adequate to understand and predict the fate and behaviour of nanomaterials. This is further complicated by our current lack of understanding of the novel physico-chemical properties exhibited by many nanomaterials and the effect these have on particle behaviour. In addition, it is most likely that those nanomaterials released into the environment will also exist as modified forms of their primary counterpart (SCENIHR. 2009).
- 3.5.222 The fate and behaviour of nanomaterials in the environment is dependent on type, form and physico-chemical characteristics of the nanomaterial in question, as well as those of the receiving environment (Chen et al., 2008; Chen and Elimelech 2008; Saleh et al., 2008). Nanomaterial transport and distribution are influenced by a number of factors, such as Brownian diffusion, inertia effects, gravitational influences, thermal influences, pH, ionisation, and presence/absence of Natural Organic Matter (NOM). These interactions ultimately affect the processes the nanomaterial consequently undergoes in its transport and subsequent fate.
- 3.5.223 Nevertheless, the lack of actual measured data in the available public domain in relation to the environmental fate and behaviour of nanomaterials in water represents a major gap in developing realistic prediction of fate and transport of nanomaterials in the aquatic environment.
- 3.5.224 Most of our current knowledge stems from colloid science, which provides preliminary information, but there is a need for systematic studies on different types of nanomaterials within aquatic bodies using a range of physico-chemical parameters (e.g. size, shape, form, surface area) to generate data and to support development of reliable models. Predictive modelling of emission scenarios and

subsequent transport pathways will also play an important role in furthering understanding in this area (Stone et al., 2009).

- 3.5.225 Knowledge to date indicates that in many cases rather than remaining intact, nanomaterials will tend to aggregate, agglomerate or become associated with other dissolved, colloidal or particulate matter present in the environment (SCHENIR 2009). However, the novel physico-chemical characteristics which make nanomaterials desirable also present a challenge for determining how they interact with the environment, how, when and where they are distributed, and in what form they ultimately end (Darlington et al., 2009).
- 3.5.226 Appropriate metrics for measuring engineered nanomaterials in the environment are still subject to much discussion, and in particular those pertaining to exposure concentrations, or dose, are considered to be of high importance. Differences in behaviour across different physical and chemical species of the same nanoparticulate material must also be considered. In addition, their tendency to aggregate/agglomerate, adsorb to NOM and, in the case of wastewater treatment, potentially associate with the solid phase, must be taken in consideration, as all of these processes could lead to environmental 'hot spots' where concentrations of nano-particulates are particularly high.

3.5.227 **B3 Sub-Task III: Summary of practical advice on the use of information from OECD-WPMN in fulfilling REACH data requirements**

3.5.228 **PHYSICO-CHEMICAL PROPERTY INFORMATION**

3.5.229 The results from the OECD-WPMN sponsorship programme for exploratory testing of nanomaterials are expected to become available in 2012 and will provide further information for the further development of OECD test guidelines and ISO standards. It is anticipated that this work will contribute to the REACH implementation for nanomaterials and to the assessment of the REACH Information Requirements. The forthcoming results from OECD-WPMN Sponsorship Programme are expected to shed further light on practical testing issues and provide a common knowledge basis on key nanomaterials. However, at the time of writing this report, no testing results have yet emerged from the Sponsorship Programme. It is important to acknowledge that this limits the ability to identify and critically assess the appropriateness of existing test methods and results from the sponsorship programme in fulfilling the REACH data requirements for physico-chemical properties at this time. However, any practical advice that can be extracted from the information currently available from OECD-WPMN is summarised below.

3.5.230 **Octanol-water partition coefficient (K_{ow})**

3.5.231 OECD has concluded that the three TGs relevant to characterising the partition coefficient (OECD TG 107, 117, 123) might be applicable under some circumstances or to some classes of manufactured nanomaterials, although further work is required to determine this and modify the TGs, if necessary (ENV/JM/MONO(2009)21). This issue has been considered further in the Task B1 report, and will be taken forward into the gap analysis (B4).

3.5.232 **Water solubility**

3.5.233 The important points noted from the OECD publications in relation to water solubility which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the guidance, are:

- It has been suggested that the measurand of interest (beginning with a pre-determined unit of particles in a standardised solution and temperature) is to measure the mass proportion of nanomaterials which

are held in solution, and whether this mass diminishes after a set period of time, or; determine the amount of time required for mass to diminish by X% (ENV/JM/MONO(2009)20/REV);

- OECD concluded that the test guideline relevant to characterising the water solubility (OECD TG 105) might be applicable under some circumstance or to some classes of manufactured nanomaterials. It stated that this TG is applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary (ENV/JM/MONO(2009)21).

3.5.234 Particle size/size distribution

3.5.235 The important points noted from the OECD publications in relation to particle size/size distribution which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the REACH guidance, are:

- It has been suggested that the conditions to which a substance is subjected may affect the size of the discrete form of a substance (ENV/JM/MONO(2009)20/REV);
- It has been highlighted that the measured size of a particle is always dependent on the particular method that is being used to examine, measure or visualize it such that the size of a particle reported by one technique might not be the same as the size when measured with another technique (ENV/JM/MONO(2009)20/REV);
- The appropriate measurands have been suggested to be, for a representative sample of nanoparticles, both the average size of individual particles and the size distribution of the sample of particles (ENV/JM/MONO(2009)20/REV);
- OECD concluded that method A (designed to provide information on the transportation and sedimentation of insoluble particles in water and air) of the test guideline for determining particle size distribution/fibre length and diameter distributions (OECD TG 110) "is not applicable to nanomaterials" whilst method B (used in the special case of materials which can form fibres,

involving microscopic examination) "would, with some modification (the inclusion of fibres of less than 5 microns in length and less than 100 nm in diameter), be applicable to nanoparticles as well as nanotubes and nano fibers". It is suggested that studies should be carried out in order to extend its range of applicability to fibres with nano-scale dimensions. It is known that alternative methods for (nano)particle size distribution already exist, which OECD suggest should be taken into account if such studies are undertaken (ENV/JM/MONO(2009)21).

3.5.236 The alternative methods suggested by OECD to be used in the Sponsorship Programme are clearly detailed in the sub-section "Particle size/size distribution" in chapter 5.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.237 **Agglomeration/aggregation**

3.5.238 The important points noted from the OECD publications in relation to agglomeration / aggregation which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the REACH guidance, are:

- The measurands of interest, beginning with a pre-determine unit of particles, have been suggested to be (ENV/JM/MONO(2009)20/REV):
 - a) The effective mean particle size in a given medium and its evolvement over time (including the standard deviation); and/or;
 - b) Qualitative assessment of state of aggregation and estimation of the primary particle size by TEM pictures; and/or
 - c) Indirect confirmation of the estimated primary particle size by BET measurements, for materials with low/no internal porosity as is typical for pyrogenic oxides.
- Predictions of agglomeration in natural waters will be limited to homo-aggregation of the particles since the data needed to predict the deposition with a heterogeneous set of natural surfaces (e.g. Hamaker constants and zeta potentials) is often not available (ENV/JM/MONO(2010)25).

3.5.239 The methods suggested by OECD to be used in the Sponsorship Programme for the determination of aggregation/agglomeration state are clearly detailed in the sub-section "Agglomeration/aggregation" in chapter 5.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.240 **Crystallite and grain size**

3.5.241 Crystallite size is included in OECD's list of testing endpoints of importance for (eco)toxicological evaluation (ENV/JM/MONO(2010)46), although no specific reason/evidence is provided for this. This limits our ability to provide practical advice on this property and its test methods in the REACH context.

3.5.242 **Specific surface area**

3.5.243 The important points noted from the OECD publications in relation to specific surface area which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the REACH guidance, are:

- It has been suggested that the specific surface area will dictate the surface charge density in cases where nanomaterials are surface functionalized and that this has direct consequences on (ENV/JM/MONO(2009)20/REV):
 - (a) nanomaterial interaction (i.e., agglomeration) with other naturally occurring particulate matter (i.e., contaminant vectors);
 - (b) route of exposure as a function of surface ligand-biological interface (i.e., bioaccumulation pathway, bioavailability); and
 - (c) mechanisms of toxicity (e.g., dose response curves normalized for surface area may indicate different results compared to results presented on a per mass basis)
- It has been highlighted that *in many cases specific surface area measurements are derived quantities that depend on the nature of the probe molecule. Nevertheless, in comparison with some of the other characterisation procedures, measurement of the specific surface area of a given sample is relatively straightforward*" (ENV/JM/MONO(2010)25);
- It has been suggested that it may be appropriate to evaluate whether the particle size distributions (and surface areas) of sparingly soluble

manufactured nanomaterials are altered through ripening and/or phase alteration phenomena (ENV/JM/MONO(2010)25);

- It has also been highlighted that the measurement of the specific surface area might most efficiently be conducted concurrently with measurements of pore size, pore size distribution, porosity and perhaps even particle density as these properties will most probably have an important influence on the (eco)toxicological properties of the material (ENV/JM/MONO(2010)25).

3.5.244 OECD propose the Brunauer, Emmett, and Teller method as a possible method for determining the specific surface area of nanomaterials within the Sponsorship Programme (ENV/JM/MONO(2009)20/REV).

3.5.245 **Surface charge**

3.5.246 The important points noted from the OECD publications in relation to surface charge which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the REACH guidance, are:

- It has been noted that the zeta potential is not measurable directly but can be calculated using theoretical models and an experimentally-determined electrophoretic mobility or dynamic electrophoretic mobility (ENV/JM/MONO(2009)20/REV);
- OECD highlighted that the significance of zeta potential is that its value can be related to the stability of nanoparticle dispersions, in that (ENV/JM/MONO(2009)20/REV):
 - a) The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in a dispersion;
 - b) For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist aggregation;
 - c) When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. This has serious implications for

the interpretation of toxicological affects with a particle's physical or chemical characteristics no longer in effect.

3.5.247 **Surface chemistry**

3.5.248 The important points noted from the OECD publications in relation to surface chemistry which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the REACH guidance, are:

- It has been highlighted that various modifications of the surfaces of nanomaterials will lead to numerous potential interactions and will play a key role in determining: i) fate in natural aqueous systems; ii) colloidal stability; iii) exposure (ENV/JM/MONO(2009)20/REV);
- It has been suggested that a given modification to surface chemistry can affect other physical-chemical properties, such as agglomeration, dustiness, zeta potential, surface area, water solubility (ENV/JM/MONO(2009)20/REV).

3.5.249 **Dustiness**

3.5.250 The methods suggested by OECD to be used in the Sponsorship Programme for the determination of dustiness of nanomaterials are clearly detailed in the sub-section "Dustiness" in chapter 5.1 of RNC/RIP-oN2/B3/2/FINAL. The advantages and limitations of these methods will be further compared and assessed in Task B4 (gap analysis), and information on the most appropriate methods will be recommended to be added to the REACH Guidance accordingly in Task B5.

3.5.251 **Porosity**

3.5.252 The important points have been noted from the OECD publications in relation to porosity which will be taken forward for further consideration in the gap analysis and used to inform proposed amendments to the REACH guidance, are:

- It has been suggested that porosity data should be taken into account as it is relevant to the indirect confirmation of the estimated primary particle size by BET measurements, for materials with low/no internal porosity (ENV/JM/MONO(2009)20/REV);

- An additional consideration while exploring this endpoint is that, in addition to the basic large surface area provided by nanomaterials, a high porosity may permit the nanomaterials to act as vectors for other contaminants, such as heavy metals (ENV/JM/MONO(2009)20/REV).

3.5.253 The methods suggested by OECD to be used in the Sponsorship Programme for the determination of porosity of nanomaterials are clearly detailed in the sub-section “Porosity” in chapter 5.1 of RNC/RIP-oN2/B3/2/FINAL.

3.5.254 **Pour density**

3.5.255 On the basis of the limited information identified in OECD-WPMN publications pertaining to pour density, a conclusion on the relevance of characterising this property for nanomaterials and its inclusion under REACH cannot be determined at this time.

3.5.256 **Photo-catalytic activity**

3.5.257 The important points noted from the OECD publications in relation to photo-catalytic activity, are:

- It is suggested that photocatalytic activity may also lead to the generation of excited state species on a material’s surface, which have the potential to directly and indirectly lead to potential toxicity;
- OECD states that measuring photocatalytic activity will give an indication of the potential for transformations in the environment which in turn represents an important point of concern when evaluating the full life-cycle of the nanomaterial (ENV/JM/MONO(2009)20/REV);
- It has been highlighted that, due to absorption of UV-B radiation by water, impurities and substances in the environment, and the different masses of these at different locations, evaluation of UV activation in the environment is likely to be very difficult (ENV/JM/MONO(2009)20/REV).

3.5.258 However, as highlighted in the literature section of RNC/RIP-oN2/B3/2/FINAL, the current lack of clarity in the relationship between photoactivity and (eco)toxicity, combined with the lack of a clear measurand, may not provide sufficient justification for the inclusion of photocatalytic activity as a new Information Requirement.

3.5.259 Radical formation potential

3.5.260 OECD WPMN have highlighted that the potential to induce free radicals in organisms has been demonstrated for a number of nanomaterials and may have relevance to the toxicity of a manufactured nanomaterial (ENV/JM/MONO(2009)20/REV).

3.5.261 However, whilst it has been demonstrated that ROS generation and oxidative stress can be used as a paradigm to assess nanomaterial toxicity, not all nanomaterials exhibit the electronic configurations or surface properties that allow spontaneous or acellular ROS generation; particle interactions with cellular components could generate ROS during these interactions.

3.5.262 OECD WPMN have not suggested any specific methods for the determination of radical potential, noting that it can be measured by various means for different biological systems.

3.5.263 Redox potential

3.5.264 OECD WPMN have highlighted that redox reactions are the basis of chemical transformations of inorganic and organic species and the precipitation and dissolution of inorganic substances that influences their sequestration and mobility (ENV/JM/MONO(2010)46). Hence, OECD suggest that measurement of the redox potential would be potentially meaningful for nanomaterials which can participate in electron transfer or uptake.

3.5.265 Flash point

3.5.266 OECD concluded that the test guideline relevant to characterising flashpoint (OECD TG 113) is considered applicable to nanomaterials (ENV/JM/MONO(2009)21). This TG is not currently referenced in the REACH Guidance (ECHA, 2008. R.7.1.9) and its inclusion will be considered as part of the gap analysis (Task B4).

3.5.267 Boiling Point

3.5.268 OECD has concluded that the test guideline relevant to characterising boiling point (OECD TG 103), though applicable for determining the boiling point of manufactured nanomaterials, is probably not relevant to existing solid nanomaterials (ENV/JM/MONO(2009)21). This guideline is currently referenced in the REACH

Guidance although not explicitly cited in the text and a number of other methods have been suggested (ECHA, 2008. R.7.1.3).

3.5.269 **Melting/freezing point**

3.5.270 OECD has concluded that the test guideline relevant to characterising melting point/melting range (OECD TG 102) is considered to be applicable to nanomaterials (ENV/JM/MONO(2009)21),.

3.5.271 **Relative density**

3.5.272 OECD has concluded that the test guideline relevant to characterising relative density is (OECD TG 109) might be applicable under some circumstances or to some classes of manufactured nanomaterials, although further work is required to determine this and modify the TG, if necessary (ENV/JM/MONO(2009)21). This guideline is currently referenced in the REACH Guidance although not explicitly cited in the text (ECHA, 2008. R.7.1.4).

3.5.273 **Surface tension**

3.5.274 In its preliminary review of OECD Test Guidelines and their applicability to nanomaterials OECD has concluded that the test guideline relevant to characterising surface tension (OECD TG 115) might be applicable under some circumstance or to some classes of manufactured nanomaterials. It stated that this TG is applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary (ENV/JM/MONO(2009)21). However, as stated in the Task B1 report, surface tension is not in general a relevant property for nanomaterials.

3.5.275 **Adsorption/desorption screening**

3.5.276 OECD has concluded that the three test guidelines relevant for adsorption/desorption screening (OECD TG 106, 108, 121) might be applicable under some circumstances or to some classes of manufactured nanomaterials. It stated that this TG is applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the

sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary.

3.5.277 **Dissociation constant**

3.5.278 OECD have highlighted that surface acidity (related to dissociation constants of surface ionisable sites) is an aspect of surface chemistry that may be particularly relevant, noting that:

- ionisable sites may influence the surface charge which has been considered significant in toxicological studies; and
- surface ionisation may also play a major role in colloidal particle stability and may even inhibit migration into hydrophobic phases (e.g., octanol/water partition coefficients).

3.5.279 OECD has concluded that the test guideline relevant to characterising dissociation constant (OECD TG 112) might be applicable under some circumstances or to some classes of manufactured nanomaterials. It stated that this TG is applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary (ENV/JM/MONO(2009)21). This test guideline is currently referenced in the REACH Guidance (ECHA, 2008. R.7.1.17), however a number of other methods are also suggested.

3.5.280 **Viscosity**

3.5.281 OECD has concluded that the test guideline relevant to characterising viscosity (OECD TG 114) is only applicable to liquids and does not refer to solutions, suspensions or emulsions (ENV/JM/MONO(2009)21). Although the viscosity of a solution can be measured, standardised preparation procedures would need to be included but are not given in TG 114. Additionally, it is not known what impact a colloidal suspension would have on the results. It is not clear yet what the importance of this property might be for the behaviour of nanomaterials, both in the environment and in living organisms. At the same time, there would be the need to define the medium.

3.5.282 **Sample preparation**

3.5.283 OECD has published a report focusing on providing guidance for nanomaterial sample preparation and dosimetry, of high value for further consideration in the context of the updated REACH Guidance (ENV/JM/MONO(2010)25). It highlighted that:

- Due to the wide variety of nanomaterials, it is difficult to develop specific or detailed advice applicable to all nanomaterials;
- The Guidance Notes should be used with some degree of expert judgement on a case-by-case basis;
- The Guidance Notes refer and apply to the water insoluble manufactured nanomaterials as the OECD considered that soluble nanomaterials are unlikely to need different sample preparation techniques than other chemicals, other than precautions dictated by the specific reactivity of each material. However their size will still affect where they are being deposited e.g. in the lung;
- As few, if any, standard testing approaches have been developed for nanomaterials, the OECD stress such guidance cannot be considered a “cookbook” for preparing samples and administering doses, but rather an outline (often in a general or descriptive manner) of considerations based on early results with nanomaterials or other experience with chemicals and particulates.

3.5.284 Common issues regarding sample preparation and dosimetry have been outlined in the document of key importance for further consideration in Task B4 and B5, including:

- storage and stability of the test material;
- the chemical composition of the test media, with the following parameters recommended to be measured for ecotoxicology studies or salines used in mammalian studies:
 - a) ionic strength, calcium concentration and hardness, pH, dissolved organic matter, alkalinity, dispersing agents;

- characterisation of stock dispersions; characterisation of samples (prepared from stock dispersions) prior to administration/testing.

3.5.285 It is also recommended that, when a procedure for generating nanomaterial preparations intended for (eco)toxicological studies is employed, attention should be paid to minimising any alteration of the physical, chemical or (eco)toxicological properties of the substrate.

3.5.286 OECD has also outlined a number of media considerations for both airborne particles and particles in aqueous solutions (ENV/JM/MONO(2009)20/REV).

3.5.287 **TOXICOLOGICAL INFORMATION**

3.5.288 As previously mentioned, the results from the OECD-WPMN sponsorship programme for exploratory testing of nanomaterials have yet to be made available, which limits the ability to identify and critically assess the appropriateness of existing test methods and results from the Sponsorship Programme in fulfilling the toxicological data requirements for REACH at this time.

3.5.289 It is however notable that 'ENV/JM/MONO(2009)10: EHS Research Strategies on Manufactured Nanomaterials: Compilation of Output' provides a summary of research status documents, and includes: i) a list of research themes relevant to EHS of nanomaterials; and ii) an overview on completed, current or planned research activities as well as urgent and medium/long term research priorities. As with the opening statement to this summary, the document also recognises that current available information on existing research projects and research strategies is too limited and heterogeneous to adequately address identifying common research needs and undertaking to meet those research needs.

3.5.290 One set of publications from the OECD sponsorship programme which are likely to be of particular importance are the Draft Dossier Development Plans. These are the output of a sponsorship programme for testing a set of manufactured nanomaterials using appropriate test methods (which include OECD Test Guidelines or other internationally agreed methods). No testing results have yet emerged from the Sponsorship Programme, however a number of draft dossier development plans (DDP) have been published, namely for Zinc Oxide, Cerium Oxide, Silicon Dioxide, Single Walled Carbon Nanotubes, Multi-Walled Carbon Nanotubes and Fullerenes.

This is of particular value, as many of the FP6/7 studies and much of the published literature do not (unless specifically stated) utilise OECD or ISO standardised methods. Thus, upon publication, their results are likely to be of particular relevance to consideration of nanomaterial human toxicity within the context of REACH.

3.5.291 **ECOTOXICOLOGICAL INFORMATION**

3.5.292 The current ECHA guidance document on Chemical Safety Assessment ECHA (2008) refers specifically to a number of OECD Test guidelines. The appropriateness of these OECD Test guidelines as well as other guidelines have been review by the OECD project Safety Testing of a Representative Set of Manufactured Nanomaterials (ENV/JM/MONO(2009)21). The reviewers stated that: "For 24 OECD ecotoxicity test guidelines, the subgroup for biotic effects section concluded that the guidance on preparation, delivery, measurement, and metrology is currently insufficient for testing of manufactured nanomaterials". (ENV/JM/MONO(2009)21, p. 13).

3.5.293 As previously mentioned, the results from the OECD-WPMN Sponsorship Programme for exploratory testing of nanomaterials have yet to be made available, which limits the ability to identify and further critically assess the appropriateness of existing test methods and data generated in fulfilling the ecotoxicological data requirements under REACH at this time.

3.5.294 **B3 Sub-Task IV: Summary of practical advice in relation to whether relevant ISO/CEN methods for substance characterisation could be used In fulfilling REACH data requirements**

3.5.295 **PHYSICO-CHEMICAL PROPERTIES INFORMATION**

3.5.296 **Water solubility**

3.5.297 Although no published standards of relevance for assessing the water solubility of nanomaterials have been identified, ISO/AWI TR 13014 (currently at Committee Draft stage), which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing, should be reviewed in further detail when the document reaches FDIS stage in order to assess its relevance to determining water solubility in the REACH context.

3.5.298 **Particle size/ size distribution**

3.5.299 **CEN EN 481:1994**, which currently forms the backbone of the existing REACH Guidance on Granulometry (ECHA, 2008. R7.1.14), defines sampling conventions for particle size fractions which are to be used in assessing the possible health effects resulting from inhalation of airborne particles in the workplace. A number of key issues have been highlighted:

- For particles of aerodynamic diameter less than 0.5 µm (i.e. including nanoparticles < 100 nm), CEN EN 481:1994 states that the particle diffusion diameter should be used instead of the particle aerodynamic diameter, defined as "the diameter of a sphere with the same diffusion coefficient as the particle under the prevailing conditions of temperature, pressure and relative humidity";
- Thus, the common methods used to determine particle size ranges of aerosols may not be appropriate for nanoparticles.

3.5.300 These issues are acknowledged briefly in the current REACH Guidance chapter on Granulometry (ECHA, 2008. R7.1.14) but no information on the availability of alternative methods for handling these issues is currently provided. This has been addressed through recommendations to update guidance in Task B5.

3.5.301 In **ISO/TS 27687:2008** several key issues are highlighted in relation to particle size measurement, including:

- the measured size of a particle is always dependent on the particular method that is being used to examine, measure or visualise the particle;
- interaction with the environment will differ between particle types, and thus the particle size reported by one technique may not be the same as that reported with another technique;
- even with a single detection method, the results depend upon how the information is processed and length parameters used for particle size characterisation should always be indicated;
- when reporting particle size measurement results, the method used to determine the particle size should be reported.

3.5.302 **ISO/TR 27628:2007** contains guidelines on characterising occupational nanoaerosol exposures against a range of metrics (mass, number, surface area) with a view to forming a basis for extending knowledge on how occupational exposure to nanoaerosols should be most appropriately measured. Specific information is provided within this technical report on methods for aerosol characterisation and single particle analysis, of relevance to consider in relation to the REACH Guidance chapter on Granulometry. ISO/TR 27628:2007 outlines the principles, advantages and disadvantages of each of these methods, of relevance for incorporation into the amended REACH Guidance chapter on Granulometry. In addition, valuable guidance on aerosol sample collection and preparation for SEM and TEM analysis is also provided. However, this technical report does not provide specific methodology or protocols for undertaking nanomaterials characterisation using these methods.

3.5.303 **ISO 10808:2010** highlights that:

- measurement of number-weighted particle size distribution and measurement of total particle mass concentration are essential parameters to characterise for inhalation toxicity testing of nanoparticles;
- conventional methods of fine or coarse particle monitoring (such as weight based mass dose monitoring) are considered insufficient for nanoparticles,

since nano-specific parameters such as particle surface area, particle number etc. might be critical determinants and thus should also be monitored.

3.5.304 The FDIS currently suggests a battery of inhalation toxicity testing chamber monitoring to include a Differential Mobility Analyzing System (DMAS) to measure particle size, distribution, number, surface area and estimated mass dose, as well as morphological examination using Transmission Electron Microscopy (TEM) or Scanning Electron Microscopy (SEM) equipped with an Energy Dispersive X-ray Analyzer (TEM-EDXA) for chemical composition. This method thus allows evaluation of nanoparticle distribution, surface area, mass dose, composition and dispersion to support effective analysis of inhalation toxicity testing results.

3.5.305 For the purposes of inhalation toxicity testing, measurement with DMAS is the only currently available method that meets all of the following requirements in the size range below 100 nm:

- measurement of particle size distribution during particle exposures in a continuous manner with time resolution appropriate to check stability of particle size distribution and concentration;
- measurement range of particle sizes and concentrations covers those of the nanoparticle aerosols exposed to the test system during the toxicity test;
- particle size and concentration measurements are sufficiently accurate for nanoparticle toxicity testing and can be validated by ways such as calibration against appropriate reference standards;
- resolution of particle sizing is sufficiently accurate to allow conversion from number-weighted distribution to surface area-weighted or volume-weighted distribution.

3.5.306 However, it is noted within the FDIS that, for non-spherical particles (e.g. carbon nanotubes), estimation of diameter and mass concentration by DMAS can result in significant error.

3.5.307 In relation to this, published standard ISO/15900:2009 provides guidelines on the determination of aerosol particle size distribution using a DMAS. This standard is not currently referenced in the existing REACH Guidance (ECHA, 2008. R.7a). It is highlighted in the standard that it is important to know the stability of the source, since rapid changes of the size distribution, particle concentration, or both, can affect measurement of the size distribution. This is relevant to consider for nanomaterials, which have a high tendency to agglomerate in the atmosphere.

3.5.308 ISO 28439:2011 provides further supplementary information regarding the use of DMAS to determine the particle size distribution on nanomaterial aerosols. It is noted that:

- the size range 3-1000 nm in electrical mobility diameter can be partly covered by other instruments (e.g. nanometer aerosol differential mobility analyser). However, DEMC has the advantage that the electrical mobility diameter is approximately equivalent to the project-diameter of the particles (defined as the diameter of a sphere with the same projected area as the particles being sized) with compact geometries;
- particle size calibration is possible with the use of polystyrene reference particles, available in the size range 20-900 nm. However, the smallest polystyrene particle that can be used for DMAS is approximately 100 nm. This means that the performance of a DMAS cannot be easily tested by the user in the size range of ultrafine and nanoparticles smaller than 100 nm.

3.5.309 Thus the DMAS method and associated standards are of importance for consideration in relation to the updated REACH Guidance on Granulometry, and will be further assessed in the gap analysis (Task B4).

3.5.310 **ISO/21501-1:2009** specifies characteristics of a light scattering aerosol spectrometer (LSAS) which is used for measuring the size, number concentration and number/size distribution of particles suspended in a gas. Due to its coverage of the nano-size range, the LSAS method outlined in ISO/21501-1:2009 may be relevant to consider in relation to informing the characterisation of particle size distribution under the REACH Granulometry Information Requirement. However,

based on the reviewed literature, there is limited evidence of the application of this method for characterising nanomaterials at present.

- 3.5.311 **ISO/13318-1:2001** outlines general methods for determining the particle size distribution of particulate materials, typically in the size range 0.1 - 5 µm by centrifugal sedimentation in a liquid. The methods described are applicable to slurries, particulate materials that can be dispersed in liquids and some emulsions. It is acknowledged in the standard that no single method of size analysis can be specified to cover the many different types of materials encountered, but that general procedures can be recommended that are applicable to the majority of cases.
- 3.5.312 **ISO/13322-1:2004** provides a standardised description of static image analysis methods for particle size analysis. An exact standard method is not provided by ISO due to the wide variety of applications of the technique. ISO/13322-1:2004 highlights that this method is essentially limited to narrow size distributions of less than an order of magnitude, requiring over 6, 000 particles to be measured in order to obtain a repeatable volume-mean diameter heightening to 61, 000 particles for the mass median diameter. The second part of ISO 13322 (**ISO/13322-2:2006**) provides guidance for measuring and describing particle size distribution, using image analysis where particles are in motion (dynamic image analysis) i.e. in a gas or a liquid. Neither standard comments on its applicability to nanomaterials, however ISO has listed these standards as potentially relevant to nanoscale measurement or observation (NIST, 2008). There is also evidence in the peer-reviewed literature and FP6/7 outputs of the application of image analysis alongside electron microscopy methods (such as TEM and SEM) for determining the particle size distribution of nanomaterials.
- 3.5.313 These standards are not currently referenced in the existing endpoint specific guidance (ECHA, 2008. R.7a). In relation to the counting procedures of static image analysis, in ISO 13322-1 it is stated in the standard that "it is a prime requirement of the method that measurements shall be made on isolated particles. There should be as few particles as possible touching each other". This may have implications for nanomaterials, which have a high tendency to agglomerate.
- 3.5.314 **ISO/TS 13762:2001** outlines the principles of the small-angle X-ray scattering (SAXS) technique, applicable to particle sizes ranging from 1 - 300 nm, and

provides guidance for sample preparation (including preparation of dry powder samples and colloidal solutions), the experimental procedure and calculation and expression of results (pointing to ISO 9276-1 and ISO 9276-2 on the representation of the results of particle size analysis).

3.5.315 Given its coverage of the nano-size range, ISO/TS 13762:2001 and the SAXS method are therefore relevant to consider in relation to determination of particle size under REACH. The success of this technique is mainly based on the fact that SAXS effect results from the difference in electron density between particles and their surroundings such that the measurement always indicates the size of a primary particle rather than the internal crystallite or external agglomerate size. Thus, the requirement of particle dispersion of a sample for SAXS analysis is not as strict as that for other methods. However, SAXS does have limitations:

- The method cannot distinguish pores from particles and the interference effect between particles will arise as the sample is available only in concentrated form.
- In the data analysis for this method, it is assumed that particles isotropic and spherically shaped and it is stated that this technical specification does not apply to powders containing particles whose morphology is far from spherical.
- This method would therefore not be relevant to consider for non-spherical nano-objects such as carbon nanotubes.
- The method cannot be used for powders consisting of porous particles, which may also limit its applicability to the measurement of certain nanomaterials.

3.5.316 **ISO/13320:2009** and the method of laser diffraction is relevant to consider in relation the characterising the particle size distribution, although no specific advice for sub-100 nm particles is provided. It is important to highlight that this technique assumes a spherical particle shape. It is stated in the standard that test products should have no extreme aspect ratios, with a restriction of 1:3 for non-spherical particles. This method is therefore unlikely to be applicable to the measurement of high-aspect-ratio nanomaterials such as carbon nanotubes. Another major source of error arises

from incomplete deagglomeration of the particles, due to an improper dispersion procedure. This highlights the importance of establishing suitable dispersion protocols for nanomaterials.

- 3.5.317 **ISO/22412:2008**, which specifies a method for the application of Dynamic Light Scattering (DLS) to the estimation of an average particle size and the measurement of the broadness of the size distribution of mainly sub micrometre-sized (approx 1 - 1000 nm) particles or droplets dispersed in liquids, is not currently referenced in the existing endpoint specific guidance (ECHA, 2008. R.7a). As outlined in the literature section of RNC/RIP-oN2/B3/2/FINAL, the method of DLS does have several limitations in relation to the characterisation of nanomaterials (e.g. lack of discrimination between agglomerates of nanoparticles and larger particles) which would need to be acknowledged in the amended guidance. It is also likely that the DLS method would need to be used in combination with other techniques.
- 3.5.318 Related to this standard is **ISO/13321:1996** which provides more detailed information on the procedures to allow the determination of the correct particle size using photon correlation spectroscopy (PCS). It is highlighted that samples should consist of well-dispersed particles in a liquid medium, which may have implications for nanomaterials with a tendency to agglomerate and further highlights the need for reproducible dispersion protocols. Published standard **E56 WK8705** from the American Society for Testing and Materials (ASTM) also deals with the measurement of particle size distribution of suspended particles, which are solely or predominantly sub-100 nm, using PCS. It is highlighted within E56 WK8705 that PCS measurement is hydrodynamically based and therefore provides size information in the suspending medium (typically water). Thus the hydrodynamic diameter will almost certainly differ from other size diameters isolated by other techniques and users of the PCS technique need to be aware of the distinction of the various descriptors of particle diameter before making comparisons between techniques.
- 3.5.319 **ISO/22412:2008**, **ASTM E2490-09** and **ISO/13321:1996** are thus also relevant to consider with regards characterising the particle size distribution of nanoparticles in suspensions, potentially in relation to the REACH Granulometry Information Requirement as well as investigating the preparation of stable dispersions for (eco)toxicological testing.

- 3.5.320 **ISO/20998-1:2006**, which describes ultrasonic methods for determining the size distributions of one or more material phases dispersed in a liquid, is also relevant to consider in relation to determining the particle size distribution of nanoparticles in suspension, potentially in relation to the REACH Information Requirement on Granulometry. Ultrasonic methods can be used to monitor dynamic changes in the size distribution, including agglomeration or flocculation in concentrated systems and may therefore be of use in the preparation of stable dispersions of nanomaterials for (eco)toxicological testing. However, there is limited evidence of the application of this method in relation to nanomaterials in the scientific literature at present, with methods such as DLS preferred.
- 3.5.321 **ISO/21501-2:2007**, describes a calibration and verification method for a light scattering liquid-borne particle counter (LSLPC), which is used to measure the size and particle number concentration of particles suspended in liquid. The typical size range of particles measured by this method is between 0.1 µm and 10 µm in particle size. This standard and the method of LSLPC may be relevant to the characterisation of the particle size distribution of nanomaterials in suspension, potentially in relation to the REACH Granulometry Information Requirement. However, as with acoustic methods, there is limited evidence of the application of LSLPC in the scientific literature at present.
- 3.5.322 **BS EN 13925-1:2003** describes the general principles of X-ray diffraction (XRD) for polycrystalline and amorphous materials, but does not go as far as to define a specific or detailed standard for each field or type of analysis. XRD allows estimation of the average particle size by mathematical adaption of a simulated diffractogram, with sensitivity down to 1 nm. **BS EN 13925-1:2003** outlines the general principles of the method, characteristics of powder diffraction line profiles, types of analysis and experimental conditions. This standard is further supported by **BS EN 13925-2:2003**, which specifies the basic procedures of the XRD method, and **BS EN 13925-3:2005**, which sets of the characteristics of instruments used for XRD as a basis for their control and hence quality assurance of the measurements made by this technique. **BS EN 13925-1:2003**, **BS EN 13925-2:2003** and **BS EN 13925-3:2005** and the method of XRD are relevant to consider in the context of the REACH Granulometry Information requirement for nanomaterials.

- 3.5.323 **ISO/AWI TS 10797** (which describes the characterisation of SWCNT using TEM, ISO/AWI TS 10798 (which describes the characterisation of SWCNT using SEM and energy dispersive X-ray spectrometry analysis) are currently at Committee Draft stage, while work on ISO/NP TS 10812 (which outlines the use of Raman Spectroscopy for the characterisation of SWCNT) has just recently commenced. It is recommended that these documents are reviewed in detail when they reach FDIS stage in relation to their potential to inform the determination of particle size distribution in the REACH context.
- 3.5.324 **ISO/NP TS 10868** (currently at FDIS stage) provides guidelines for the characterisation of compounds containing SWCNTs by using optical absorption spectroscopy. Although this standard does not address particle size/size distribution *per se*, the information provided in relation to diameter determination may be relevant to consider under the REACH Granulometry Information Requirement, depending on the coverage of this term.
- 3.5.325 **ISO/AWI TR 13014** (which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing) and **ISO/DTR 10929** (which outlines a collection of measurement methods for the characterisation of multi-walled carbon nanotubes (MWCNT)), currently at Committee Draft stage, may offer information of relevance to determining the particle size/geometrical properties (e.g. length/diameter) of nanomaterials. It therefore recommended that these documents are reviewed for their relevance in the REACH context when they reach FDIS stage.
- 3.5.326 In addition, **ISO/CD 12025** (which aims to provide a general framework for determining nano-object release from powdered engineered nanoparticles into the gaseous surroundings by means of analysis of the generated aerosols particles) and the methods it advocates, is relevant to consider in relation to REACH Granulometry Information Requirement, in terms of measurement related to dustiness as well as methods for the generation of nanoaerosols for studying parameters such as particle size distribution. The release of nano-objects from nanomaterials into the surrounding air is an important consideration in relation to the hazard potential of nanomaterials. However, this document is currently at Committee Draft stage with the content still under development. It is recommended that it should be reviewed in further detail when the document reaches FDIS stage.

3.5.327 **Agglomeration/ aggregation state**

3.5.328 A number of the methods and standards described above for determining the particle size distribution of particles will also apply to determining the level of agglomeration/aggregation of particles in powder and suspension form, of relevance to consider in the context of REACH. Of key importance are **ISO/20998-1:2006** (which describes ultrasonic methods for determining the size distributions of one or more material phases dispersed in a liquid), **ISO/13322-1:2004** (which describes static image analysis for determining the size distribution of particles in both powder and suspension form) and **ISO/TS 13762:2001** (which describes the small angle X-ray scattering method, also applicable to both powders and suspensions).

3.5.329 A number of the standards are currently at Committee Draft level may also offer information of value to consider in relation to characterising the agglomeration/aggregation state of nanomaterials, specifically **ISO/AWI TS 10797** (which describes the characterisation of SWCNT using transmission electron microscopy), **ISO/AWI TS 10798** (which describes the characterisation of SWCNT using scanning electron microscopy (SEM) and energy dispersive X-ray spectrometry analysis) and **ISO/AWI TR 13014** (which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing). It is recommended that these standards should be reviewed with regards their relevance for informing characterisation under REACH they reach FDIS stage.

3.5.330 **Crystallite or grain size**

3.5.331 A number of the methods and standards described above for determining the particle size distribution of particles will also apply to determining the crystallite or grain size of nanomaterials, of possible relevance to consider in the context of REACH. Of particular relevance is **BS EN 13925-1:2003** which describes the general principles of X-ray diffraction (XRD) for polycrystalline and amorphous materials. XRD can be used to study single crystal or polycrystalline materials in powder or dispersion form, with sensitivity down to 1 nm, providing a wealth of information, including crystallite size, as well as crystalline phase composition, lattice strain and defects, crystallographic orientation and charge distribution. **BS EN 13925-1:2003** outlines the general principles of the method, characteristics of powder diffraction line profiles, types of analysis (including analysis of crystallite size) and experimental conditions. This standard is further supported by **BS EN**

13925-2:2003, which specifies the basic procedures of the XRD method, and **BS EN 13925-3:2005**, which sets of the characteristics of instruments used for XRD as a basis for their control and hence quality assurance of the measurements made by this technique.

3.5.332 In addition, **ISO/AWI TS 10797** on the use of TEM for charactering SWCNT, currently under development, will also be relevant to consider in this context. It is recommended that this document be fully reviewed when it reaches FDIS status.

3.5.333 **Aspect ratio/ shape**

3.5.334 The static image analysis methods described in **ISO/13322-1:2004** and the FDIS standard **ISO/NP TS 10868** which provides guidelines for the characterisation of compounds containing SWCNTs by using optical absorption spectroscopy, covering determination of the mean diameter may be particularly useful for studying the shape of nanomaterials.

3.5.335 It is recommended that the following draft standards under development should be reviewed when they reach FDIS status in regards to their relevance to inform the measurement of the aspect ratio/shape of nanoparticles in the context of REACH: **ISO/AWI TS 10797** (which describes the characterisation of SWCNT using TEM), **ISO/AWI TS 10798** (which describes the characterisation of SWCNT using SEM and energy dispersive X-ray spectrometry analysis), **ISO/AWI TR 13014** (which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing), **ISO/DTR 10929** (which outlines a collection of measurement methods for the characterisation of multi-walled carbon nanotubes (MWCNT)) and **ISO/DTS 11888** (which addresses the determination of mesoscopic shape factors of MWCNT).

3.5.336 **Specific surface area**

3.5.337 **ISO/9277:2010** (which outlines a method for determination of the total specific external and internal surface area of disperse or porous solids by measuring the amount of physically absorbed gas according to the BET method) and **ISO/18757:2005** (which provides guidelines for the determination of the total specific external and internal surface area of disperse or porous (pore diameter > 2 nm) fine

ceramic materials by the BET method) are relevant to consider in the context of characterising the specific surface area of nanomaterials.

- 3.5.338 Although the BET method assumes a mono-dispersed spherical system, it is commonly applied to determine both the surface area and average particle size (not size distribution) of nanomaterials. **ISO/9277:2010** highlights that, in order to ensure proper working conditions and correct data evaluation, the apparatus performance should be monitored periodically using a surface-area reference material, with certified reference materials listed in an annex. This highlights the importance of defining suitable reference nanomaterials.
- 3.5.339 **ISO/TR 27628:2007** (which contains guidelines on characterising occupational nanoaerosol exposures against a range of metrics) provides general information of relevance to consider in relation to characterising the surface area of aerosolised nanoparticles. However, no specific methodological guidance or protocols are detailed. It is important to note that within this standard it has highlighted that the BET method has been used with some success for measuring aerosol surface area, but that it suffers the disadvantages of requiring the collection of relatively large amounts of material, and measurements are influenced by particle porosity and collection/support substrate.
- 3.5.340 **ISO/TS 13762:2001** (which specifies a method for determining particle size distribution of ultra-fine powders by the small-angle X-ray scattering (SAXS) technique, applicable to particle sizes ranging from 1 - 300 nm) can also be used to derive the specific surface area of a powder sample from the measured particle size distribution and the mass density of the powder particle.
- 3.5.341 Similarly, the static image analysis techniques described in **ISO/13322-1:2004** enable determination of the specific surface area of particles from the particle size measurements.
- 3.5.342 The DMAS system described within **ISO 10808:2010** can be used to calculate the surface area based on the determined particle diameter. However, it is noted that for non-spherical particles (e.g. carbon nanotubes), estimation of diameter and mass concentration by DMAS can result in significant error.

3.5.343 Three published ISO standards which address determination of the pore size distribution of solid materials by mercury porosimetry and gas adsorption using related methods (*ISO 15901-1:2005; ISO 15901-2:2006; ISO 15901-3:2007*) may enable indirect determination of the specific surface area of the characterised material. Indeed, ISO 15901-1:2005 provides an equation to calculate a specific surface area of the intruded pores. However, these methods are quite specific and unlikely to obtain widespread use for the determination of specific surface area alone.

3.5.344 It is recommended that *ISO/AWI TR 13014* (which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing) should be reviewed in further detail when the document reaches FDIS stage in the context of identifying methods for the determination of surface area under REACH.

3.5.345 **Surface charge/zeta potential**

3.5.346 No published standards of relevance for determining the surface charge of nanomaterials have been identified.

3.5.347 It is recommended that draft standards under development *ISO/CD 13099-1* and *ISO/CD 13099-2* (which both relate to the measurement of zeta potential) as well as *ISO/AWI TR 13014* (which aims to provide guidance on physicochemical characterisation for manufactured nano-objects for toxicological testing), should be reviewed when the reach FDIS stage in relation to their relevance in providing methods for the characterisation of surface charge of nanomaterials

3.5.348 **Surface chemistry**

3.5.349 ISO has published a wide-range of standards related to surface chemical analysis using three principle techniques: X-ray photoelectron spectroscopy (XPS), Auger electron spectroscopy (AES), and secondary-ion mass spectrometry. However, these documents are highly specific and they most relevant to consider in the context of determining the surface chemical composition of materials.

3.5.350 It is highly recommended that *ISO/WD TR 14187* (which looks specifically at the surface chemical analysis of nanostructured materials), as well as *ISO/NP TS 10812* (which outlines the use of Raman Spectroscopy for the characterisation of SWCNT)

and *ISO/AWI TR 13014* (which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing), should be reviewed when they reach FDIS stage to assess their relevance for informing the characterisation of the surface chemistry of nanomaterials in the REACH context.

3.5.351 **Dustiness**

3.5.352 The standard current referenced in the REACH Guidance on Granulometry for determining the dustiness of bulk materials is *EN 15051:2006*. The current limitation of these methods in relation to nanomaterials is that they measure the mass, rather than e.g. the particle number, of the resultant dust and provide no size-based information. In addition, rotating drum dustiness tests are usually performed as three replicate tests and need quite large amounts of test material, typically 300–600 g. The EN 15051 continuous single-drop method requires a total amount of 500 g for the required five single-test runs. Modified methods for measuring the dustiness of nanomaterials have been described in the FP6/7 section and the literature section of RNC/RIP-oN2/B3/2/FINAL.

3.5.353 It is highly recommended that draft standard under development *ISO/CD 12025* (which aims to provide a general framework for determining nano-object release from powdered engineered nanoparticles into the gaseous surroundings by means of analysis of the generated aerosols particles, using adapted procedures for determining dustiness) should be reviewed in further detail when the document reaches FDIS stage as it will likely contain information of importance to consider in relation to REACH guidance updates relating to dustiness measurements for nanomaterials,

3.5.354 **Porosity**

3.5.355 The three published ISO standards which describe methods for determination of the pore size distribution of solid materials by mercury porosimetry and gas adsorption (*ISO 15901-1:2005; ISO 15901-2:2006; ISO 15901-3:2007*) may be applicable to determining the porosity of nanomaterials.

3.5.356 **Photocatalytic activity**

3.5.357 *ISO 22197-1:2007* (which specifies a test method for determination of the a test method for the determination of the air-purification performance of materials that

contain a photocatalyst or have photocatalytic films on the surface) may be applicable for the determination of the photocatalytic activity of nanomaterials. However it has not been established whether the interpretation of data gathered by this method, and used for hazard assessment purposes, will be meaningful. Whilst it is being investigated under the OECD Sponsorship Programme, it is stated that the method does not apply to powder or granular photocatalytic materials, thus restricting its use for some forms of nanomaterials.

3.5.358 **Sample preparation**

3.5.359 **ISO/14887:2000** (which provides general guidance to assist in the preparation of good dispersions from various powder/liquid combinations) covers procedures for wetting a powder into a liquid; deagglomerating the wetted clumps; selecting dispersing agents to prevent reagglomeration; evaluating the stability of the dispersion against reagglomeration. This standard is applicable to particles ranging in size from approximately 0.05 to 100 µm, and is therefore relevant to consider for informing the preparation of nanomaterials dispersions in the REACH context. For examining the resultant dispersion, ISO 14887:2000 states that, for particles smaller than 1 µm, the standard method of optical microscopy should be replaced with some other form of evaluation.

3.5.360 The characterisation of particle properties like size, form and specific surface area requires very careful sampling and sample splitting practices to be followed. It is considered vital that the test aliquot used for measurement is representative of the sample of particulate material. While this standard does not specifically address procedures for nano-sized materials, **ISO 14488:2007** (which specifies methods for obtaining a test aliquot from a defined sample of particulate material that can be considered to be representative of the entire sample with a defined confidence level) offers general information and good practice of relevance to powder sampling of a range of materials of value for further consideration in relation to the updated REACH Guidance.

3.5.361 It is highly recommended that ASTM draft standard E56 WK10417 (which outlined standard practice for the preparation of nanomaterial samples for characterisation) is reviewed when published.

3.5.362 Other information of relevance

3.5.363 Although *ISO/DTR 13121* (which describes a process for evaluating, addressing, making decisions about, and communicating the potential risks of developing and using engineered nanoscale material) does not address specific methods for characterising the physico-chemical properties of nanomaterials, it highlights a number of key issues of importance to consider further in development of recommendations for guidance updates, specifically:

- The importance of characterising the physico-chemical properties of a nanomaterial over its entire life cycle is highlighted;
- It is recommended that any anticipated changes in relevant physical and chemical properties across the lifecycle of the material should be noted. It may be necessary to characterize the material at multiple points unless there is good reason to expect that the material will remain unchanged;
- It is recommended that the properties of the nanomaterial should be compared to those of the corresponding bulk (non-nanoscale) materials, where appropriate, to determine the nature and extent to which the properties are different.

3.5.364 It is highly recommended that the following draft standards *ISO/AWI TS 11931-1* and *ISO/AWI TS 11937-1* which describe characteristics and measurement methods for nano-calcium carbonate and nano-titanium dioxide, respectively currently under development are reviewed when published to assess their relevance in the context of the updated REACH guidance.

3.5.365 TOXICOLOGICAL INFORMATION

3.5.366 Those ISO/CEN documents published or classified as being at Final Draft International Standard (FDIS), Draft International Standard (DIS) stage or at an early stage of development (i.e. Committee Draft stage or lower) were reviewed and commented upon. It is clear that whilst there a number of standards at ballot level or under development, there is to date only one published document pertaining to the toxicity of nanomaterials (ISO 10801:2010). It is expected that this standard will provide a useful contribution to supporting good experimental design in the mammalian toxicity testing under hazard identification within the context of REACH. In summary, at the current time there exists only one fully published method from ISO which may be considered directly relevant for fulfilling the REACH Information Requirements relating to toxicity.

3.5.367 ECOTOXICOLOGICAL INFORMATION

3.5.368 On the basis of the review carried out, it is considered that no work has so far been published by ISO and CEN that is considered directly relevant for fulfilling the REACH Information Requirements in regard to ecotoxicity.

3.6 GAP ANALYSIS OF RELEVANT INTRINSIC PROPERTIES FOR NANOMATERIALS POSSIBLY NOT ADDRESSED BY STANDARD TEST GUIDELINE METHODS AND REQUIRING FURTHER DEVELOPMENT OF IN VITRO, IN VIVO OR OTHER METHODOLOGIES (TASK B4)

3.6.1 The gap analysis of relevant intrinsic properties for nanomaterials, which may not be addressed by standard test guideline methods and for which further development of in vitro, in vivo or other methodologies is required, has assembled and further developed the findings from the examination of existing REACH Guidance related to information requirements and testing (information generation) strategies (Task B1), the identification of additional relevant specific intrinsic properties for nanomaterials (Task B2), the assessment of relevance and applicability of testing, endpoints and methods described in the scientific literature and on-going international work relevant to the fulfilment of the data requirements under REACH (Task B3).

3.6.2 The structural framework used for the gap analysis considers physico-chemical properties and toxicological and ecotoxicological endpoints, and integrates the existing and additional properties/endpoints to meet the objective of identifying those which may and may not be addressed by standard test guideline methods and where further development of in vitro, in vivo or other methodologies is required.

3.6.3 The outcomes of the gap analysis, specifically addressing the aforementioned objective, are summarised below. (The information developed for the gap analysis has been used subsequently in the development of specific guidance updates and recommendations for research & development (two of the objectives of Task B5)).

3.6.4 The following intrinsic properties, identified from the gap analysis, are **relevant to nanomaterials, with suspected important differences between nano and non-nanomaterials, which may not be addressed by standard test guideline methods** and require further development of in vitro, in vivo or other methodologies. Those considered to be of low priority for further research and development are indicated using an asterisk. Those for which standards, or other important documents, are known to be in preparation are indicated with a .

3.6.5 Physico-chemical properties

Existing Information Requirements

- R.7.1.7 Water Solubility
- R.7.1.10 Flammability

- R.7.1.11 Explosive properties

Additional Relevant Specific Intrinsic Properties

- Porosity
- Surface energy
- Surface acidity
- Surface charge (zeta potential)
- Redox potential

3.6.6 Toxicological endpoints

- None identified.

3.6.1 Ecotoxicological endpoints

- #R 7.8.15 Activated sludge respiration inhibition testing
- R. 7.9.1 Hydrolysis as a function of pH
- #Daphnia heart rate*
- #Daphnia hopping frequency*
- #Number of cycles per minute of daphnia in appendage movement*

*In relation to these endpoints and proposed biological markers it should be noted that consensus has not been reached within the project consortium and further discussions can be found in section 4.3.157 and onwards.

3.6.2 The following intrinsic properties / endpoints are considered to be **relevant to nanomaterials, with no suspected important differences between nano and non-nanomaterials** in terms of the applicability of the standard test guideline methods, **but an insufficient evidence basis to warrant acknowledgement in the guidance**, and further research & development is required. Those considered being of lower priority for further research and development are indicated using an asterisk. Those for which standards, or other important documents, are known to be in preparation are indicated with a .

3.6.3 Physico-chemical properties

Existing Information Requirements

- None identified

3.6.4 Additional Relevant Specific Intrinsic Properties:

- Surface chemistry
- Cell-free ROS/RNS production capacity

3.6.5 Toxicological endpoints

- Acute toxicity (oral and inhalation: it is recommended that either the method or the guidance includes extended pathology/ histology)
 - OECD TG 420 (EU B.1 bis) (Acute oral toxicity – Fixed dose procedure)
 - OECD TG 423 (EU B.1 tris) (Acute oral toxicity – Acute toxic class method)
 - OECD TG 425 (Acute oral toxicity – Up-and-down procedure)
- Acute toxicity (inhalation: it is recommended that either the method or the guidance includes the requirement to use BAL as a measurand)
 - OECD TG 403 (EU B.2) (Acute inhalation toxicity)
 - Draft OECD TG 433 (“Acute Inhalation Toxicity, Fixed Dose Procedure”)
 - Draft OECD TG 436 (“Acute Inhalation Toxicity, Acute Toxic Class Method”)
- Repeated dose toxicity (it is recommended that either the method or the guidance includes the requirement to use BAL as a measurand)
 - OECD TG 412 / EU B.8 (Subacute Inhalation Toxicity: 28-Day Study)
 - OECD TG 413/EU B.29 (Subchronic Inhalation Toxicity: 90-day Study)
- Repeated dose toxicity (it is recommended to include inhalation as a route of exposure)
 - OECD TG 422: Combined repeated dose toxicity / reproductive screening study
 - OECD TG 424 (rodents)
- Respiratory Sensitisation
- Cardiovascular toxicity (focus on understanding mechanism and developing predictive standardised models)
- Reproductive Toxicity
 - Reproductive toxicity (nano-focussed, in vitro) – Embryonic stem cell test for embryotoxicity;
 - Reproductive toxicity (nano-focussed, in vitro) – Micromass embryotoxicity assay;

- Reproductive toxicity (nano-focussed, ex vivo) – Whole rat embryo embryotoxicity assay.
- Inflammation (with relevance to acute, repeated dose)
 - Pro-Inflammatory effects in vitro – Cytokine, chemokine and their receptor expression and transcription e.g. IL-8, GRO, TNF- α , IL-1 β
 - Pro-fibrogenic effects – Histopathological examination e.g. using Collagen staining (e.g. Sirius Red, Trichrome)
 - Pro-fibrogenic effects – Pro-fibrotic mediators e.g. TGF- β , IL-6, IL-10, EGF etc
 - Pro-Inflammatory effects in vitro - Cell signalling and Transcriptional activation e.g. AP1, NF κ B, STAT, NRF2, MAP Kinase*
 - Pro-fibrogenic effects – Collagen production, type and biochemical modifications e.g. Hydroxyproline, pro-collagen peptides*
- Cytotoxicity
 - Propidium Iodide
 - LDH release
 - Neutral Red uptake*
 - Trypan Blue*
 - Apoptosis - Annexin V
- Oxidative stress (with relevance to inflammation and genetic damage)
 - Oxidative Stress: Antioxidant capacity - Glutathione Depletion (intra- and extra-cellular)*
 - Oxidative Stress: Antioxidant capacity - vitamin C depletion*
 - Oxidative Stress: Antioxidant capacity - TEAC (trolox equivalent anti-oxidant capacity)*
 - Oxidative Stress: Antioxidant capacity - ORAC (oxygen radical anti-oxidant capacity)*
 - Oxidative Stress: Antioxidant capacity – FRAP (Ferric/Reducing Antioxidant Power)*
 - Oxidative Stress: Redox sensitive dyes e.g. DCFH, DHE etc.
 - Oxidative Stress: Antioxidant capacity – Mitochondrial dysfunction*
 - Oxidative Stress: Antioxidant capacity - Antioxidant gene/protein expression and transcription factors (HO-1 expression etc.)*
- Genotoxicity
 - DNA Repair reporter assays: e.g. GreenScreen assay*
 - Mutagenicity: Oxidative adducts of DNA e.g. 8-OH-dG
 - Mutagenicity: Lipid adducts e.g. N-1,N2 malondialdehyde-2'-deoxyguanosine (M1dG)

- Particle translocation
 - Barrier Transfer Models - Transwell - co culture system (using relevant cell types for barrier of interest, e.g. epithelial/ endothelial = blood-air barrier; endothelial/ astocyte = blood-brain barrier)

3.6.6 **Ecotoxicological endpoints**

3.6.7 None identified.

3.6.8 The following intrinsic properties / endpoints have been identified to be **relevant** to nanomaterials, but **with differences between nano and non-nanomaterials** in terms of the applicability of standard test guideline methods and for which **guidance updates have been recommended**:

3.6.9 **Physico-chemical properties**

3.6.10 Existing Information Requirements

- R.7.7.8 Partition coefficient N-octanol/water
- R.7.1.14 Granulometry

3.6.11 Additional Relevant Specific Intrinsic Properties

- Particle shape
- Surface area

3.6.12 **Toxicological endpoints**

3.6.13 Existing Information Requirements:

- R.7.3 Skin and eye irritation
- R.7.3 Skin and respiratory sensitisation
- R.7.4 Acute Toxicity
- R.7.5 Repeated dose toxicity
- R.7.6 Reproductive and developmental toxicity
- R.7.7 Mutagenicity and carcinogenicity

3.6.14 **Ecotoxicological endpoints**

In relation to the following parameters currently within guidance, it is noted that in relation to ecotoxicity testing there are issues surrounding sample preparation and characterisation. These include measuring, dosing, delivery and tracking of the substance in the testing

system and in particular aquatic effects issues surrounding dispersability vs. solubility which may lead to difficulties and inaccuracies in interpreting results. In addition, suitable analytical methods may need to be developed which are capable of characterising actual exposure concentration in a test system (e.g. in the soil) during the experimental duration.

- Short-term toxicity testing on invertebrates (preferred species Daphnia)
- Growth inhibition study aquatic plants (algae preferred)
- Ready biodegradability
- Short-term toxicity testing on fish
- Adsorption/desorption screening
- Long-term toxicity testing on invertebrates (preferred species Daphnia), (unless already provided as part of Annex VII requirements)
- Long-term toxicity testing on fish, (unless already provided as part of Annex VIII requirements)
- Fish early-life stage (FELS) toxicity test
- Fish short-term toxicity test on embryo and sac-fry stages
- Fish, juvenile growth test
- Bioaccumulation in aquatic species, preferably fish
- Soil short-term toxicity to invertebrates
- Effects on soil micro-organisms
- Short-term toxicity to plants
- Long-term toxicity testing on soil invertebrates, unless already provided as part of Annex IX requirements
- Long-term toxicity testing on plants, unless already provided as part of Annex IX requirements
- Long-term toxicity to sediment organisms

- 3.7 SCIENTIFIC REPORT ON TEST METHODS AND STRATEGIES FOR NANOMATERIALS (TASK B5)**
- 3.7.1 B5 Sub-Task I: Advice on integrated testing strategies relevant to specific nanomaterials properties and how the specific intrinsic properties of nanomaterials might affect the need for adaptations to the testing regime**
- 3.7.2 The relevance and applicability to nanomaterials of the Integrated Testing Strategies provided for each property and testing endpoint has been considered and identified any limitations and the need for updates to the current Guidance text. Any recommendation for updating the current Guidance considers the feasibility of doing so, given the current state of knowledge.
- 3.7.3 A summary of the findings from considering the Integrated Testing Strategies for Existing Information Requirements in terms of the relevance to nanomaterials is presented in the tables below. For each ITS, relevance and applicability to nanomaterials is indicated along with the need for any update to the existing Guidance text, where this is consider feasible given the current state of knowledge.

Integrated Testing Strategy	Applicable to nanomaterials	Update to Guidance proposed
Physico-chemical properties		
General testing strategy for physico-chemical properties (R.7.1.1.4)	✓	Minor - Update depending upon agreed definition of granulometry
Melting point / Freezing point (R.7.1.2.5)	✓	None
Boiling point (R.7.1.3.5)	✓	None
Relative density (R.7.1.4.5)	✓	None
Vapour pressure (R.7.1.5.5)	✓	None
Surface tension (R.7.1.6.5)	✓	None
Water solubility (R.7.1.7.5)	✓	Minor - Distinguish between solubilisation and dispersion
Partition coefficient (R.7.1.8.5)	✓	None
Flash point (R.7.1.9.5)	✓	None
Flammability (R.7.1.10.5)	✓ (with some limitations)	None
Explosive properties (R.7.1.11.5)	✓ (with some limitations)	None
Self-ignition temperature (R.7.1.12.5)	✓	None
Oxidising properties (R.7.1.13.5)	✓	None
Granulometry (R.7.1.14.5)	✓	Substantive - scope of update depends on the concept and regulatory status of granulometry and parameters included in it
Toxicological Endpoints		
Irritation / corrosion (R.7.2.6)	✓	Minor - Use of QSAR & read-across data
Skin & respiratory sensitisation (R.7.3.8)	✓	Minor - Use of QSAR
Acute toxicity (R.7.4.6)	✓	Minor - Use of QSAR, Route of Exposure phrasing
Repeated dose toxicity (R.7.5.6)	✓ (some TG limitations)	Minor - Use of QSAR and lung overload
Reproductive toxicity (R.7.6.6)	✓ (some TG limitations)	Minor - Use of QSAR and read-across
Mutagenicity (R.7.7.6)	✓ (some TG limitations)	Minor - Use of QSAR and use of multiple methods re: addressing interferences
Carcinogenicity (R.7.7.13)	✓	Minor - Use of QSAR and read-across
Ecotoxicological Endpoints		
Adsorption / desorption (R.7.1.15.5)	✓	None
Aquatic pelagic toxicity (WoE) (R.7.8.5)	✓ (with some limitations)	None
Sediment organisms (R.7.8.5)	✓ (with some limitations)	None
STP micro-organisms (R.7.8.5)	✓ (with some limitations)	None
Degradation / biodegradation (R.7.9.6)	✓ (with some limitations)	None
Aquatic bioaccumulation (R.7.10.6)	✓ (with some limitations)	None
Terrestrial bioaccumulation (R.7.10.6)	✓ (with some limitations)	None
Avian toxicity (R.7.10.19.3)	✓	None
Terrestrial organisms (R.7.11.6)	✓ (with some limitations)	None

3.7.4 Summary of findings on the Integrated Testing Strategies for Additional Relevant Specific Intrinsic Properties relevant to nanomaterials

ITS (Physico-chemical properties)	Issues identified which require clarification / consensus
Particle shape	Status as an additional IR or subordinate to Granulometry, will determine whether a new ITS is developed or included in the existing one for granulometry.
Surface area	Status as an additional IR or subordinate to Granulometry, will determine whether a new ITS is developed or included in the existing one for granulometry. (Note that specific surface area is mentioned separately from granulometry in the revised Annex II.)

3.7.5 Full details of the considerations and proposed updates to the Guidance text are provided in the final report for Task B5 (RNC/RIP-oN2/B5/2/FINAL) and in Chapter 4 of this report.

3.7.6 By way of illustration, three *examples* of the advice provided on the ITS for i) oxidising properties (no changes recommended) and ii) granulometry (updates recommended), and iii) skin & respiratory sensitisation, extracted from RNC/RIP-oN2/B5/2/FINAL, are provided below.

3.7.7 OXIDISING PROPERTIES

3.7.8 R.7.1.13.5 Integrated testing strategy (ITS) for oxidising properties

3.7.9 The following ITS for oxidising properties is outlined in the existing REACH Guidance (R.7.1.13.5):

The screening procedures above represent an intelligent testing strategy for oxidising properties. If applied correctly, only substances which, it is suspected, will give a positive result in one of the oxidising properties tests will need to be tested. Together with the choice of an appropriate test method, this constitutes the testing strategy.

3.7.10 **Relevance and applicability of the ITS for nanomaterials**

3.7.11 *The testing strategy for oxidising properties is considered to be relevant and applicable to nanomaterials. This conclusion is consistent with the conclusion in the gap analysis (Task B4) that no change to Guidance is required for this property.*

3.7.12 **Recommendations for alterations**

3.7.13 No change required.

3.7.14 **GRANULOMETRY**

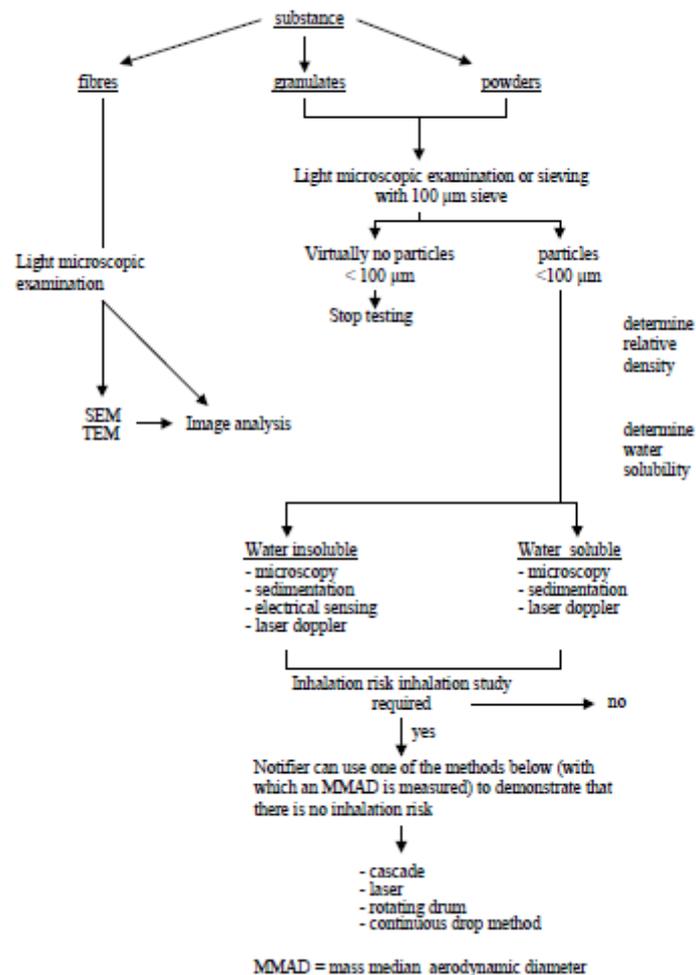
3.7.15 **R.7.1.14.5 Integrated testing strategy (ITS) for granulometry**

3.7.16 *The following ITS for granulometry is outlined in the existing REACH Guidance (R.7.1.14.5):*

An integrated testing strategy (ITS) detailing the appropriate methods for determination of particle size distribution of respirable and inhalable particles is shown in Figure R.7.1-7.

Testing for particle size analysis is not required for those substances which are marketed or used in a non solid or non granular form. A testing strategy detailing which methods to use to determine particle size distribution of respirable and inhalable particles is provided.

Figure R.7.1-7 Integrated testing strategy for granulometry



3.7.17 Relevance and applicability of the ITS for nanomaterials

3.7.18 The testing strategy for granulometry does not adequately represent the issues for nanomaterials and requires substantial amendment. It is recognised that testing for particle size analysis (and all other particle related properties) is not required for those substances which are marketed or used in a non-solid or non-granular form. The nature of the examples and case studies provided in the current ITS for granulometry (second paragraph of R.7.1.14.5) is considered irrelevant and inconsistent with the nature of examples and case studies provided for other properties in the current Guidance in that no detailed example of studying the granulometry of a specific substance is provided.

- 3.7.19 *With regard to Figure R.7.1-7 the use of light microscopy and sieving, whilst applicable to larger particles, are not suitable for nanomaterials. The ITS suggests that if there are virtually no particles < 100 µm (measurement methods unspecified) no further testing is required. This is clearly inappropriate for nanomaterials not suitable detection by light microscopy and sieving. Where particles < 100 µm are present, the determination of relative density and water solubility is suggested but these are Information Requirements in their own right with their own ITS and Tier/group allocations in the general testing strategy for physico-chemical properties. The current granulometry ITS then separates water soluble and water insoluble granulates/powders and recombines them for the assessment of inhalation risk through a demonstration using a limited number of methods mainly suited to micron sized particles. Moreover, posing the question of whether an inhalation study is required, whilst important, is considered not relevant to include in the granulometry ITS.*
- 3.7.20 **Recommendations for alterations**
- 3.7.21 *Firstly, Figure R.7.1-7 entitled “Integrated testing strategy for granulometry” currently resides in R.7.1.14.2 (Available information on granulometry; pg. 144, R.7a) and is required to be moved to R.7.1.14.5 (Integrated testing strategies for granulometry; pg. 152, R.7a). Secondly, the ITS requires significant updating. This should at least include referencing to revised versions of Tables R.7.1-30 and R.7.1-31 providing more information on methods. Specifically, additional methods other than solely light microscopic examination and sieving (as identified in RNC/RIP-oN2/B4/2/FINAL and RNC/RIP-oN2/B5/2/FINAL) need to be included at the screening level of granulates and powders to take into account testing of nanomaterials.*
- 3.7.22 *A greater acknowledgement of the sub-ordinate properties (e.g. particle size, size distribution; shape and surface area if not considered to be separate Information Requirements, and any others) with reference to available measurement methods needs to be incorporated into the ITS for granulometry.*
- 3.7.23 *With regard to examples and case studies on granulometry, a more appropriate description of the issues and typical data from granulometry is required to be developed, which will be dependant upon the accepted composition of the granulometry Information Requirement (i.e. whether shape and surface area are*

sub-ordinate to granulometry or included as additional relevant specific intrinsic properties).

3.7.24 SKIN & RESPIRATORY SENSITISATION

3.7.25 R.7.3.8 Integrated testing strategy (ITS) for skin and respiratory sensitisation

3.7.26 An integrated strategy for skin and respiratory sensitisation is presented in Section R.7.3.8. of the REACH Guidance. An overview of the ITS for skin sensitisation is given in Figure R.7.3-1, where LLNA stands for murine local lymph node assay (LLNA) (OECD TG 429).

3.7.27 Testing respiratory sensitisation is not required under REACH. An overview of the integrated evaluating strategy for respiratory sensitisation is given in Figure R.7.3-2.

Figure R.7.3-1 Integrated testing strategy for skin sensitisation

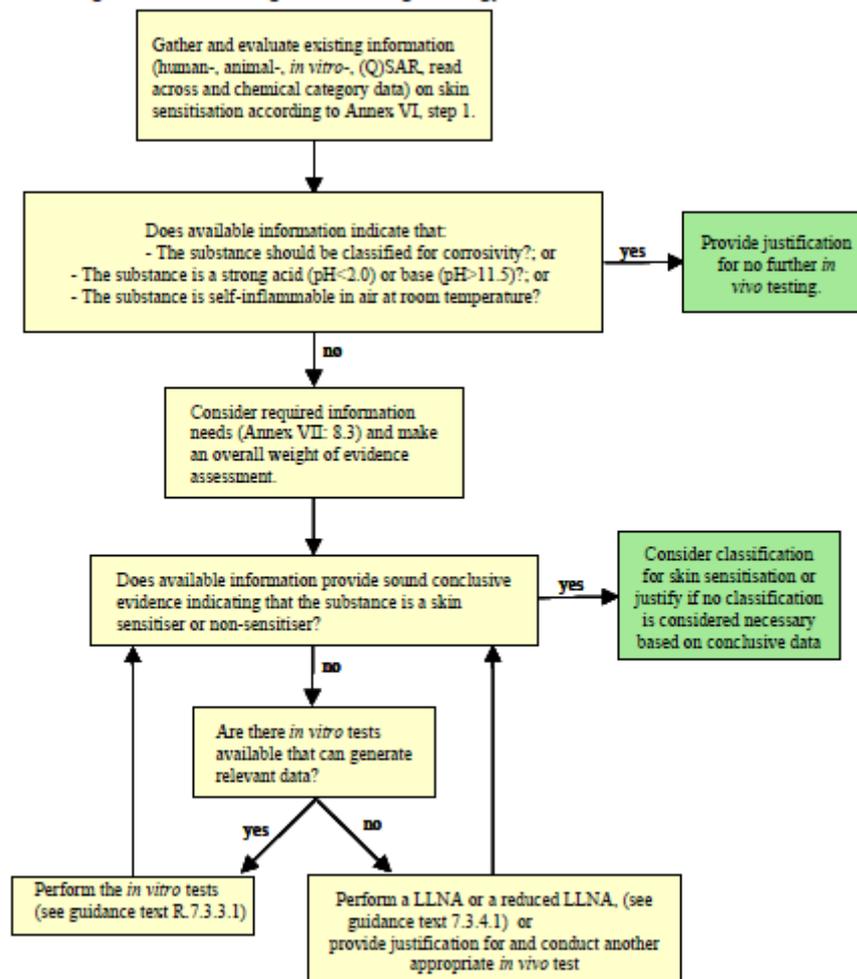
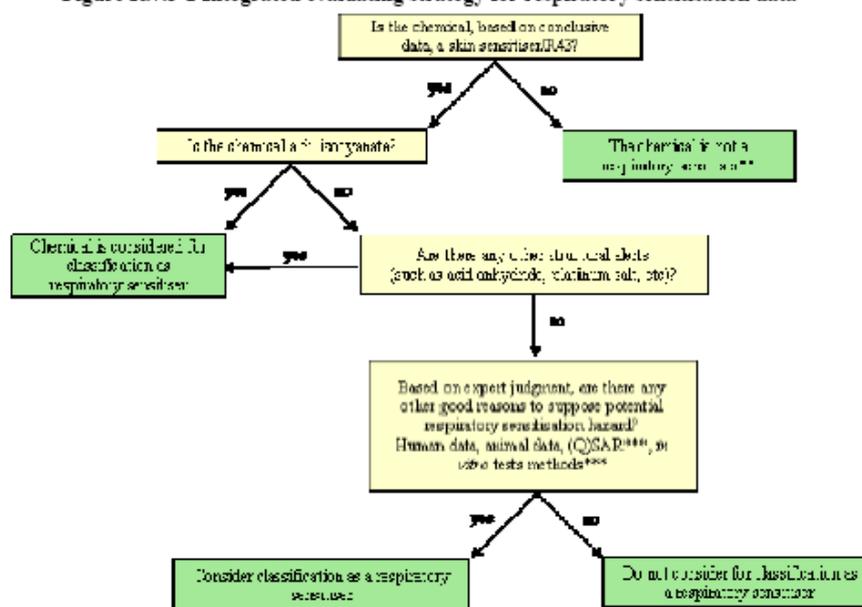


Figure R.7.3-2 Integrated evaluating strategy for respiratory sensitisation data*



* In contrast to tests for skin sensitisation, the performance of tests for respiratory sensitisation is currently not required under REACH. Therefore the present IES scheme depicts a strategy for evaluating existing data.

** This does not discount the possibility that the chemical may induce respiratory hypersensitivity through non-immunological mechanisms. Chemicals that act through such mechanisms are usually identified on the basis of evidence from human exposure.

*** not yet available

3.7.28 **Relevance and applicability of the integrated testing strategy for nanomaterials**

3.7.29 *The ITS for sensitisation is considered to be relevant and applicable to nanomaterials although a change should be made in relation to the use of in silico approaches towards data gathering as outlined below.*

3.7.30 *At present, testing for respiratory sensitisation is not required under REACH and methods to address respiratory sensitisation are not sufficiently developed to include an amendment to Guidance (although we do consider it a priority R&D area for both nanomaterials and other substances alike). The ITS referring to respiratory sensitisation (figure R.7.3-2) does outline the use of in silico approaches to data gathering but also includes a footnote specifying that such approaches are not yet available. As such this is accurate for nanomaterials as well as does not require alteration.*

3.7.31 **Recommendations for alterations**

3.7.32 *Page 279 figure R.7.3-1 Integrated testing strategy for skin sensitisation*

- 3.7.33 *The testing strategy whilst applicable for nanomaterials identifies the gathering of (Q)SAR, read across, and chemical category data. As previously mentioned such an approach is not yet appropriate for nanomaterials and is an R&D requirement. As such, a foot note should be added and linked to the first step of the ITS (box 1) so that the step reads:*
- 3.7.34 *“Gather and evaluate existing information (human-, animal-, in vitro-, (Q)SAR^a, read across^a and category^a data) on skin sensitisation according to Annex VI, step 1.”*
- 3.7.35 *And the following footnote inserted:*
- 3.7.36 *“^a For nanomaterials the use of in silico models has yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.”*

3.7.37 **B5 Sub-Task II: Advice on the scientific basis for the categorisation of nanomaterials and application of in silico methods, read-across and category approaches for deriving hazard information for nanomaterials from the information on bulk substances or from comparison between nanomaterials**

3.7.38 **CATEGORISATION SCHEME FOR HUMAN HAZARD ASSESSMENT**

3.7.39 Inclusion into the categorisation scheme for nanomaterials is based on the premise that the particle in question satisfies the criteria of a nanomaterial. By virtue of its small size, nanomaterials have a high external surface area/ volume ratio and as such a large, biologically available and hence relevant, surface area is taken as a basis within this categorisation framework. This concept of biologically relevant surface area relates to the actual surface which is accessible to the biological environment/ cells and as such is available for interaction. A particle surface which is not accessible by the biological environment, e.g. an internal structure or pore which via size etc excludes biomolecules, cannot contribute to a biological reaction and as such may be considered biologically irrelevant. As such, within the following scheme, the term surface area relates only to the biologically relevant surface area which may contribute to a biological response.

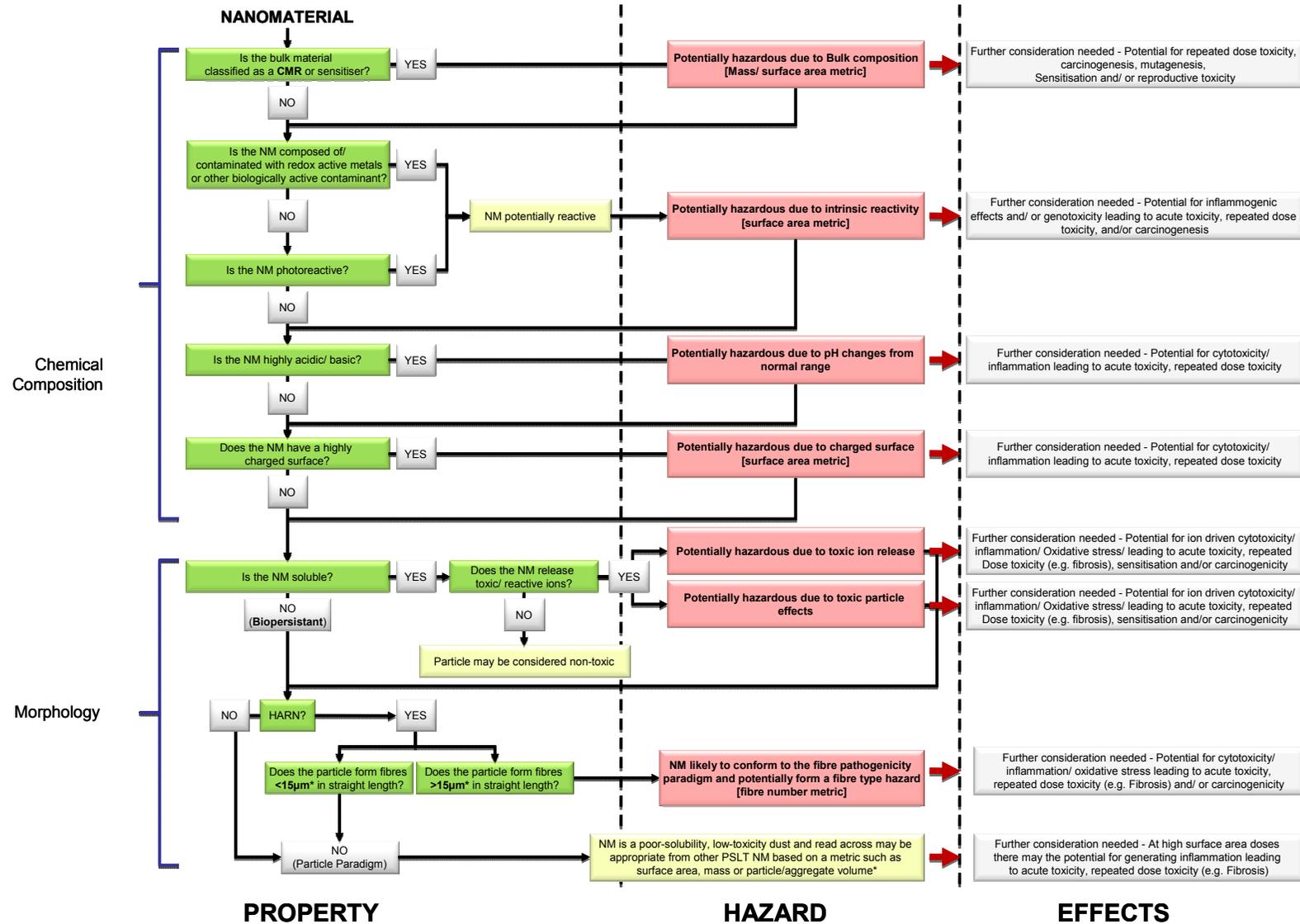
3.7.40 Within the following framework there exists the potential for variation in surface area based on those particles within the lower reaches of the nanomaterial size range (very high surface area) vs. those at the upper limits of the nanomaterial size range (moderately high surface area). In establishing a potential hazard, the greater the biologically relevant surface area the greater the hazard due to amplification of surface properties.

3.7.41 The approach taken herein could be described more accurately as an analogue approach because the groupings shown are based upon hypotheses, or limited particle data. Because of a lack of data across a wide range of structural and compositional different nanomaterials, a fully prescribed category approach is not yet possible. Instead, the approach applied allows a higher-level overview that suggests where such groupings may be applied. Further development, both of the approach itself and of testing methods is needed before a true category approach can be taken.

3.7.42 Mechanistic basis for hazard categories

3.7.43 A schematic process for the hazard identification and categorisation based upon physico-chemical criteria known to be drivers of toxicity within (nano)particle toxicology has been developed by the Consortium and is shown below. The process is broken down into a series of questions to be asked of every nanomaterial considered, each of them addressing a proposed key physicochemical property. A 'yes' answer to any of the attributes is associated with either a further question or a hazard attribute. As no question is ranked above or below the other in importance and all questions must be asked of particle under consideration, both 'yes' and a 'no' response lead to the next physico-chemical attribute. A hazard attribute is further explained with suggested effect(s) given within the context of REACH.

Hazard categorisation of nanomaterials based upon physico-chemical property, hazard and effect



3.7.44 The basis for properties examined are the scientific literature discussed in task RNC/RIP-oN2/B3/2/FINAL and the approaches taken to establish qualitative hazard characterisation discussed in RNC/RIP-oN3/C2/2/FINAL and a discussion of each question follows.

3.7.45 **Is the (nano)material classified as a CMR or sensitizer?**

3.7.46 The first question relates to the hazard characteristics of the bulk or parent version of a nanomaterial (if such exists) and if it is already classified as a carcinogen, mutagen, or reproductive toxin (CMR) or skin/ respiratory sensitizer. This enables a broad identification of potential hazard (and a form of read-across) from a previously identified hazard associated with the material.

3.7.47 For example both hexavalent chromium (VI) and cobalt dusts are known sensitizers. Nanomaterial forms of these compounds may also demonstrate this potential for sensitisation and as such a preferential starting point would be to consider that the nanomaterial is a sensitizer. It would not necessarily be considered to be any less active than the bulk compound and based on their high surface area may in fact be far more active than the bulk compound and should therefore be considered as potentially hazardous. The nature of CMR and sensitisation suggests that effects such as cancer; asthma and reproductive toxicity are likely to occur after repeated exposure so one would expect such effects to be covered by REACH repeated dose IR.

3.7.48 **Is the (nano)material composed of reactive metal(s)?**

3.7.49 The second question is part of a collection of 3 questions that relate to the potential reactivity of a nanomaterial. The first asks if the nanomaterial in question is composed of a redox active metal which would infer that the nanomaterial itself is likely to be redox active. For example a nanomaterial composed of copper oxide (II) is likely to be redox active which may pose a hazard, especially considering the nanomaterials large surface area (e.g. $23\text{m}^2/\text{g}$ for a 42nm powder vs. $1.5\text{m}^2/\text{g}$ for a $3\mu\text{m}$ powder) over which redox reactions can take place potentially meaning the nanomaterial is far more reactive than the parent compound (Karlsson et al. 2009). This combination of high surface area and high reactivity may lead to the formation

of a 'double hazard' in inflammatory potential (Karlsson et al. 2009; Duffin et al. 2007)

3.7.50 Is the (nano)material photoreactive?

3.7.51 The fourth questions relates to the photoreactivity of a particle. Photocatalytic nanomaterials such as certain forms of titanium dioxide could release oxygen radicals during exposure to light (Konaka et al. 1999) and as such could trigger various mechanisms of toxicity including inflammation (via activation of oxidant sensitive transcription factors), oxidative damage and genetic aberrations (Hirakawa et al. 2004; Carlotti et al. 2009). Any nanomaterial that is photoreactive should therefore be considered for further toxicological analysis for various endpoints such a carcinogenicity, irritation and inflammation during both acute and repeat dose exposure.

3.7.52 Taken together, these three attributes account for the surface activity and potential drivers of toxicity for surface active particles which may well be highly appropriate to nanomaterials. Again, when considering the extreme surface area of a nano-sized particle in relation to its micro-sized counterpart, any hazard depending on a biologically accessible surface area would be correspondingly increased whilst other associate with other metrics such as mass, may not.

3.7.53 Is the (nano)material highly acidic/ basic?

3.7.54 The fifth criteria for consideration are the acidic/ basic nature of the material. A substantial pH derivation away from the normal range of the biological environment at the site of deposition could cause local effects such as skin irritation/ corrosion, or cell death within the lungs leading to inflammation/ oedema/ fibrosis. In the same way that other highly acidic/ basic chemicals are assessed, nanomaterials that fit these criteria could also be categorised and risk assessed.

3.7.55 Does the (nano)material have a highly charged surface?

3.7.56 The surface charge of a material can influence several factors such as its propensity to agglomerate/aggregate, interaction with charged molecules such as proteins and cellular uptake. However it is hypothesised that particles with a highly charged

surface (e.g. aminated polystyrene particles) may also adversely affect cells by strongly binding and disrupting membranes.

- 3.7.57 Goodman et al. (2004) examined the effects of gold nanoparticle surface charge on cytotoxicity by studying cationic (amine) and anionic (carboxyl) gold nanoparticles on Cos-1 cells, red blood cells, and E. coli. It was concluded that cationic gold particles were moderately toxic and anionic particles were non-toxic, suggesting the initial electrostatic binding of the particles to the negatively charged cell membrane as a probable mechanism of toxicity and that electrostatic repulsion may limit anionic and neutral particle interaction with the cell surface (Goodman et al., 2004). As such within this experiment it can be seen that cationic gold nanoparticles can be expected to exhibit more toxic effects relative to anionic particles.
- 3.7.58 It is unknown to what extent surface charge may play upon the toxicity of nanomaterials within a complex environment such as that found *in vivo* but currently it is thought that surface charge can exert effects on various attributes of particle behaviour (e.g. agglomeration, uptake) which may affect toxicity.
- 3.7.59 **Is the (nano)material soluble?**
- 3.7.60 Particle solubility can have both positive and negative effects pertaining to a particles propensity to cause harm. A soluble particle that does not release toxic ions or other components could result in the overall progressive reduction/removal of dose as the particle dissolves ultimately removing any toxic stimulus (if caused) or be intrinsically non-toxic.
- 3.7.61 However if during dissolution, the particle releases reactive or cytotoxic components such as toxic ions it may cause toxic effects. It has been shown that soluble components such as the release of zinc ions from nanoparticulate zinc oxide can play a significant role in toxic effect of ZnO particles (Song et al., 2010).
- 3.7.62 An attribute of nanoscale materials is the potential for the alterations in physico-chemical characteristics such as the solubility. For example bulk silver is insoluble, but nanosilver releases free silver ions in aqueous solutions by dissolution and subsequent oxidation. It has been hypothesised that the toxic effects of silver

substances are proportional to the rate of release of free silver ions from them (see, for example, Wijnhoven et al., 2009).

- 3.7.63 As such when considering the hazardous nature of a material, it is pertinent to consider both the insoluble (particle) and soluble component in the hazard assessment.
- 3.7.64 **Is the nanomaterial a high aspect ratio nanomaterial (HARN)?**
- 3.7.65 Within the paper by Zuin et al. (2010), the authors outline that fibres which have an aspect ratio of $\geq 3:1$ (length-to-diameter ratio) or greater and are insoluble/biopersistent should have a rating value of 'high harmful'. However the derivation of an aspect ratio of $\geq 3:1$ and its role in classifying a fibre is based upon the definition of a fibre by the World Health Organisation which considers a fibre as having an aspect ratio of $\geq 3:1$ and a length greater than $5\mu\text{m}$. This length qualification is crucial when establishing nanomaterial as being 'a fibre' as a nanomaterial may have a length many times that of its diameter but could still be far shorter than the definition of a fibre and as such would be considered a particle. A nanomaterial by definition, (e.g. a single aspect 100nm or less in size) would require an aspect ratio of $50:1$ at minimum to be considered a fibre. Within fibre toxicology, the length at which a fibre begins to generate difficulties with normal clearance mechanisms is that length in which it cannot be fully enclosed by professional phagocytes such as alveolar macrophages which is considered to occur at between $15\text{-}20\ \mu\text{m}$ in length (Donaldson et al. 2010). As such, the cut-off within the proposed scheme is placed at $15\mu\text{m}$ although it should be stressed that further work is needed to confirm or refute this demarcation or a more conservative length adopted.
- 3.7.66 Within the framework shown, a particle that is not considered a HARN, or is a HARN but less than $15\mu\text{m}$ is considered in a similar way as a non-fibrous particle based on its biological relevance. A nanomaterial which exceeds this $15\mu\text{m}$ could be seen to be likely to frustrate clearance mechanisms within the lung if deposited past the ciliated airways and cause hazardous effects associated with other harmful fibres.
- 3.7.67 In RNC/RIP-oN2/B5/2/FINAL, use of the category approach to derive potential sources of hazard from a nanomaterial has been applied to two very different forms of nanomaterial to demonstrate the utility of the scheme.

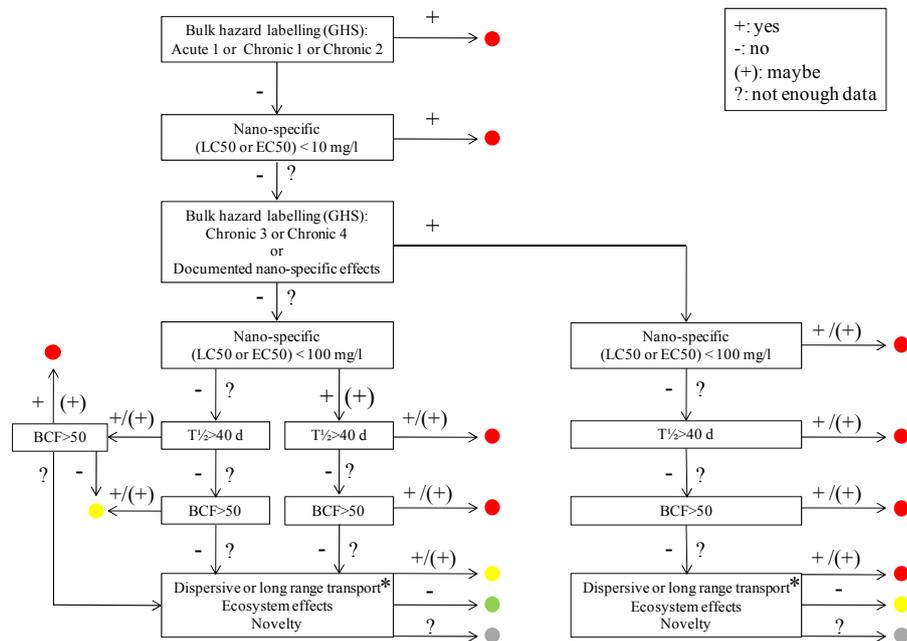
3.7.68 CATEGORISATION SCHEME FOR ENVIRONMENTAL HAZARD ASSESSMENT

3.7.69 The basis for the properties examined in this final report is the scientific literature discussed in Task B3 RNC/RIP-oN2/B3/2/FINAL as well as the comprehensive literature review performed by Stone *et al.* (2009) and published in the report Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES). The approaches taken to establish qualitative hazard characterisation discussed in RIP-oN 3 Task C2. The scheme proposed represents a work in progress and is proposed from Hansen, S.F., Baun, A., Jensen, K.A. 2010. *NanoRiskCat - draft. Copenhagen: Danish Environmental Protection Agency (forthcoming)*. The need to develop such an environmental hazard categorization scheme was only identified recently by the Danish EPA and its relevance is highlighted by its utility to RIP-oN2. As noted several times in the RNC/RIP-oN2/B5/2/FINAL, the suggested scheme is a draft and still subject to revisions in the light of constructive comments and suggestions. The categorisation scheme is one of the very few that exist in regard to nanomaterial environmental hazard evaluation and hence we acknowledge the Danish EPA for permission to include a substantial amount of early work associated with developing the framework and the information needed to make a sound judgement about the nature of the scheme as well as its strengths and weaknesses.

3.7.70 In the proposed scheme termed NanoRiskCat, a number of indicators/qualifiers should, as a minimum, be considered when providing an initial evaluation of the environmental hazards related to a given nanomaterial, its application and what is already known about the bulk form of the material. These include whether:

- the nanomaterial in question is reported to be hazardous to environmental species
- the nanomaterial in question is persistent
- the nanomaterial in question is bioaccumulative
- use of the nanomaterial in question could lead to potentially irreversible harm to the environment (e.g. ecosystem effects)
- the nanomaterial in question is readily dispersed

- the nanomaterial in question is novel (i.e. new materials, new form of existing material, new applications, new pathways (Royal Commission on Environmental Pollution 2008))
- 3.7.71 The task report (RNC/RIP-oN2/B5/2/FINAL) provides a more detailed discussion of each question.
- 3.7.72 For each of these questions, reasoning should be provided with proper referencing to the scientific and/or non-scientific literature and an answer of each of the questions should be provided in the form of either: yes, maybe, no, and no information. The answer “yes” implies that there is conclusive evidence for categorizing that indicates that the nanomaterial in question as having ir-/reversible effects (e.g. reproductive damage) or holding a given property (e.g. persistent, accumulates). “Maybe” implies that there is evidence that indicates that no categorization of the nanomaterial can be concluded whereas “no” implies that there is conclusive evidence that indicates that the nanomaterial can be categorized as not causing adverse ir-/reversible effects and/or hold the properties in question (Hansen et al., 2010).
- 3.7.73 While in principle none of these indicators are more important than others, the proposed scheme shown below gives a guidance on the order in which they may be evaluated and a short description of the criteria to be used.



*outcome will be based on a written evaluation

Road-map for assigning an environmental hazard colour code in NanoRiskCat (reproduced with permission from Hansen et al. 2010 – all rights reserved)

3.7.74 From the outset, consideration is taken of existing criteria for chemicals with due consideration to the uncertainty related to ecotoxicological hazard of nanomaterials e.g. by changing the cut-off values for LC50 or EC50. The cut-off values are deliberately made stricter in NanoRiskCat than the existing ones in REACH. Whilst this is not based on any scientific review of the literature, justification is made in Hansen et al. (2010) on the basis of expert judgment with reference to current uncertainty about the appropriateness of using mass as cut-off value for establishing nano-specific thresholds (Stone et al. 2009). Evidence is scarce, but from the reported EC50- and NOEC values (reviewed in RNC/RIP-oN2/B3/2/FINAL), it seems that there might be between a factor of 1-20 difference in the ecotoxicity of bulk size particles and nanosized particle (Templeton et al. 2006, Fujiwara et al. 2008, Arouja et al. 2008). Based on this, Hansen et al (2010) speculate that a 10-fold stricter cut-off value is adequate to make up for this uncertainty for most nanomaterials. If a nano-specific endpoint is not triggered even when using the stricter cut-off value, Hansen et al. (2010) believe that it is justified to categorise the nanomaterial as not causing adverse ir-/reversible effects and/or hold the properties

in question. Below follows a more detailed description of each indicator and the cut-off values chosen. It should be noted that also the classification according to the Global Harmonized System (GHS) for the bulk material is used in the environmental hazard categorization in NanoRiskCat.

- 3.7.75 The red colour code in the pictorial scheme indicates that adverse effects are expected in the environment, the yellow indicate that adverse effects are possible, and green that there is no environmental hazard documented. Grey should be used if there are numerous data gaps and unknowns to warrant any conclusion to be made about the environmental hazards of the nanomaterial toward. Transparency in the assigning of a colour code is key and very important. Therefore, all categorisations made must be accompanied by an explanatory text on how the conclusion was reached.
- 3.7.76 It is important to note that NanoRiskCat is a tiered approach in the sense that once a higher-tier colour code has been triggered (e.g. bulk materials GHS classified as Chronic 1 which would trigger “red”) the nanomaterial cannot get a lower-tier colour code (yellow, green or grey) even though the LC50 or EC50 might be > 100 mg/l and the persistency might be < 40 days and the BCF < 50 (Hansen et al. 2010).
- 3.7.77 **Use of read-across for environmental hazard characterisation of nanomaterials**
- 3.7.78 The read-across approach is based upon the identification of a source material or analogue for which environmental hazard information exists and using that information to make predictions about the target material. The sourcing of an analogous material is based upon categorisation of existing materials with similar physico-chemical and ecotoxicological properties which may follow a similar pattern to structural similarity. For nanomaterials this may be done by employing read-across from the parent or bulk compound to the nano-form. For example, the utilisation of the ecotoxicological information pertaining to bulk silver for accessing nano-silver ecotoxicity. As it has been well-established that bulk silver is toxic to the aquatic environment, it would be justified to assume that the same is the case for nano-silver (Stone et al. 2009).
- 3.7.79 It is known that certain factors such as surface area and particle size alter as we move into the nano-scale and these are scalable (although may be affected by other

parameters such as aggregation). However other properties such as solubility and reactivity may change in unpredictable ways as a material becomes nano-sized. As such, it could be regarded that a lack of ecotoxicity in the bulk form, does not necessarily mean that a nanoparticle of the same material is inactive. There is however validity, with due care, in perhaps extrapolating certain positive data on the ecotoxicity from bulk to nano. For example a bulk material classified as Acute 1 and Chronic 1-4 according to GHS should inform the hazard assessment of a nanomaterial as this one is likely to have a similar activity (although perhaps enhanced due to a larger surface area to volume ratio). It is important to underline that if the bulk compound is not classified as Acute 1 and Chronic 1-4, it cannot be assumed that a nanomaterial is not potentially hazardous for the environment.

3.7.80 Generating environmental hazard information based upon read-across from analogous materials is also not without its problems but may offer greater reliability if such linkages are made based on physico-chemical similarities rather than simple bulk chemistry.

3.7.81 **Use of in silico methods for environmental hazard characterisation of nanomaterials**

3.7.82 With REACH, there exists guidance on the regulatory use of quantitative structure activity relationships (QSAR) in hazard assessment of substances. Within section R.6.1.5.1, there is a scheme to enable the determination of the suitability of (Q)SAR results to replace a test result which is shown below:

1. An evaluation of the scientific validity (relevance and reliability) of the model
2. An assessment of the applicability of the model to the chemical of interest and the reliability of the individual model prediction
3. An assessment of the adequacy of the information for making the regulatory decisions, including an assessment of completeness, i.e. whether the information is sufficient to make regulatory decision, and if not, what additional (experimental) information is needed.

3.7.83 The Guidance on the regulatory use of quantitative structure activity relationships (QSAR) in hazard assessment of substances requires that, for a full replacement of

experimental tests, all of the conditions outlined above are met. In situations where there are elements missing, the (Q)SAR information may still be used within a weight of evidence approach.

- 3.7.84 Based on these criteria, currently, *in silico* approaches for environmental hazard assessment of nanomaterials are not validated as there is yet to exist solid quantitative structure activity relationships for nanomaterials on which to base such models. Several studies have highlighted the need for new interpretative descriptors for the development of nanomaterial QSARS and predictive *in silico* modelling but none have yet suggested a validated approach (Hansen et al. 2007, RCEP 2008, Stone *et al.* 2009). There are several on-going studies as summarised in RNC/RIP-oN 2/B3/2/FINAL such as that of Ennsatox. This project intends to develop a comprehensive theoretical model describing the environmental system as a series of biological compartments and following optimisation of the transfer functions a generic predictive model will be derived for the environmental impact of each class of nanoparticles in aqueous systems. Biological membrane models will be used to increase the understanding the interaction of nanoparticles with cell membranes from an organism health point of view but also to develop suitable nanoparticle screening procedures, which can substitute for the more lengthy *in vivo* tests. However, this project is at a very early stage and no information has been identified as to which kind of nanoparticles will be subject for the modelling as well as it remain unclear what the (eco)toxicological endpoints will be and which kind of species will be investigated. It is therefore not possible to comment at this stage whether this project will produce outputs of relevance in the context of REACH.
- 3.7.85 As in the case of hazard characterization, it should be stressed however that where modelling is possible, it should not be performed on simple classes of nanomaterials (e.g. TiO₂) as within such a class there exists a very high potential for variability making conclusion based on a single arbitrary material class virtually meaningless. Instead, it has been suggested that, due to high variability in the molecular structures and different mechanisms of toxicity, individual nanoparticles should be modelled separately (Puzyn et al., 2009).

3.7.86 **B5 Sub-Task III: Proposals for further amendment of the REACH guidance documents in regard to information requirements, test methods or testing strategies for nanomaterials**

3.7.87 The following summary of findings identifies the *nature* of suggested updates to Guidance and the location(s) within the suite of Guidance documents constituting the Guidance on Chemical Safety Assessment under REACH. Specific text for Guidance update, developed on the basis of this identification, is recommended in the Chapter 4 of this report.

3.7.88 **PHYSICO-CHEMICAL PROPERTIES**

3.7.89 The following section outlines proposals for further amendment of the REACH Guidance documents in regards to physico-chemical information requirements, test methods or testing strategies for nanomaterials, based on the outcomes of the gap analysis (Task B4).

3.7.90 **GENERAL CONSIDERATIONS**

3.7.91 **Multi-method analysis**

3.7.92 An important general conclusion with regards nanomaterials characterisation that has emerged from the findings of the project is that no individual technique can satisfy a meaningful characterisation of nanomaterials, such that multiple techniques should be used where possible in order to formulate a complete understanding of the nanomaterial's properties. It is highlighted that different techniques will suit different sample forms (e.g. aerosol, suspension etc.) and the optimum set of required techniques should be selected and justified based on the specific nanomaterial type and form under investigation. The need for multi-method characterisation and material-specific selection of techniques applies across a range of nanomaterial properties and would facilitate the gathering of data on multiple metrics.

3.7.93 R.7.1.1 (R.7a, pg. 17) – A new fourth paragraph should be added to the introduction which stresses the need for multi-method characterisation and material-specific selection of techniques as well as some information on the status of the applicability and validation of the methods.

3.7.94 **Sample Preparation**

- 3.7.95 Information on sample preparation for the determination of properties has been determined to be of crucial importance in the context of gathering data to fulfil Information Requirement ((RNC/RIP-oN2/B1/2/FINAL), RNC/RIP-oN2/B2/2/FINAL; RNC/RIP-oN2/B3/2/FINAL), regarding physico-chemical, toxicological and ecotoxicological (including environmental fate and behaviour) properties for nanomaterials, and is currently missing from the REACH Guidance (RNC/RIP-oN2/B1/2/FINAL). Within this context, dispersion stability and state of agglomeration are recognised as important parameters to study and characterise as supplementary information to the data gathered for the formal IRs (RNC/RIP-oN2/B2/2/FINAL). These properties are not necessarily additional IRs in their own right, due to their secondary nature (i.e. non-intrinsic) and dependence upon other primary properties. Techniques available for monitoring the state of agglomeration and state of dispersion will be covered in property-related aspects of the updated Guidance (i.e. Granulometry). These include electron microscopies and Dynamic Light Scattering techniques.
- 3.7.96 R.7 (R.7a) - Additional sub-section entitled "Sample preparation" should be added into the Introduction, providing general guidance on Sample Preparation, highlighting the importance of characterising dispersion stability and agglomeration state and including information on available methods. This section will not propose a sample preparation protocol (as generic procedures cannot be applied universally to all nanomaterials) but should provide useful guidance and references to relevant resources.
- 3.7.97 It is anticipated that this section will be informed by OECD's "Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials" (ENV/JM/MONO(2010)25) and "Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme; First Revision" (ENV/JM/MONO(2009)20/REV), ISO/14887:2000 which provides general guidance to assist in the preparation of good dispersions from various powder/liquid combinations and ISO 14488:2007 which specifies methods for obtaining a representative test aliquot from a defined sample of particulate material, as well as

further resources identified and discussed in RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B3/2/FINAL.

3.7.98 **Reference materials**

3.7.99 R.7.1.1.3 - An acknowledgement of the importance of reference materials is recommended for inclusion in this section.

3.7.100 **EXISTING INFORMATION REQUIREMENTS**

3.7.101 **Water solubility**

3.7.102 R.7.1.7.5 - The difference between solubilisation and dispersion and a recommendation to elucidate between the two for nanomaterials is recommended to be included in the ITS, including Figure R.7.1-5. However, as highlighted in ENV/JM/MONO(2009)20/REV, specific methods to determine dispersion stability remain to be determined.

3.7.103 **Partition coefficient N-octanol/water**

3.7.104 R.7.1.8.3 - A caveat that highlights the limitations of OECD TGs 107, 117 and 123 for nanomaterials should be included at end of paragraph headed "Experimental data on partition coefficient n-octanol/water" (R.7a, p. 100)

3.7.105 Include acknowledgement of general aspects relating to differences between dispersion and solubility.

3.7.106 **Granulometry**

3.7.107 In order to finalise amendments to the REACH Guidance on Granulometry, agreement regarding the scope and definition of this term in the context of the REACH Regulation is required.

3.7.108 *Part B: Hazard Assessment. B.6.1.4* - Paragraph on Granulometry should be added.

3.7.109 R.7.1.1.1 - The phrase "Granulometry (particle size distribution)" in Table R.7.1-1 should be updated to remove "(particle size distribution)".

- 3.7.110 R.1.1.4 – Regarding the general testing strategy for physico-chemical properties, the label of “particle size distribution” under Tier 3 should be amended to state “Granulometry”, consistent with the Regulation and Guidance. All other sub-ordinate or recommended additional relevant specific intrinsic properties are appropriate to include in Tier 3. Figure R.7.1-1 should also be amended to incorporate sub-ordinate or additional properties in Tier 3.
- 3.7.111 R.7.1.1.5 - Depending on the agreed definition and regulatory scope of the term "Granulometry", the scope of the information requirement on Granulometry in Table R.7.1-5 may need updating.
- 3.7.112 R7.1.14 - The existing text on Granulometry requires substantial updating and enhancement, to acknowledge standards and informative sources published since the last revision of the chapter. The definition and regulatory scope of the term granulometry needs to be clarified. Further detail and clarity in this section is required to include and describe methods for i) powders; ii) suspensions and iii) aerosols.
- 3.7.113 Additional introductory text is required in R.7.1.14.2 highlighting that measured particle size values are method dependent and should be reported with sufficient detail on the analytical technique employed to derive data. A recommendation for the use of multi-analytical techniques for the characterisation of nanoparticles should also be included.
- 3.7.114 A caveat which highlights the limitations of OECD TG 110 for nanomaterials should be included. Information on available modern and standardised equipment (i.e. centrifugal sedimentation, ultracentrifuge) should be provided, which can be used to provide data in accordance with this method.
- 3.7.115 The following alterations are recommended to be made in a revised version of Table R.7.1-30:
- More information on Transmission Electron Microscopy (TEM) should be incorporated, including reference for further informative information for nanoparticles in ISO 27628:2007 and a note indicating importance of image

quality. ISO/13322-1:2004 and ISO/13322-2:2006 which provide general image analysis guidance could also be referenced;

- More information on Scanning Electron Microscopy (SEM) should be incorporated, including reference for further informative information for nanoparticles in ISO 27628:2007 and a note indicating importance of image quality. ISO/13322-1:2004 and ISO/13322-2:2006 which provide general image analysis guidance could also be referenced;
- Information on Centrifugal Sedimentation should be incorporated, including reference to available standards (ISO/13318-1:2001; ISO/13318-2:2007; ISO/13318-3:2004) and an acknowledgement that this is a new method to facilitate the measurement of size under TG 110;
- Information on Ultrasonic spectroscopy should be incorporated, including reference to available standard (ISO/20998-1:2006).
- Information on Small Angle X-ray Scattering (SAXS) should be incorporated, including reference to available standard (ISO/TS 13762:2001), as an alternative to the preferred methods of TEM and SEM;
- Information on X-ray Diffraction (XRD) should be incorporated, including reference to available standards (BS EN 13925-1, BS EN 13925-2 and BS EN 13925-3). It should be acknowledged that particle size does not equal crystallite size, and that other factors can influence the peak width (e.g. microstrain, lattice defects, temperature factors).
- Information on Dynamic Light Scattering (DLS)/ Photon Correlation Spectroscopy (PCS) should be incorporated, including reference to available standards (ISO/22412:2008; ISO/13321:1996; ASTM E2490-09) and an acknowledgement of some of the limitations associated with this method;
- In addition, Capillary Hydrodynamic Fractionation (CHDF), Field Flow Fractionation (FFF), Capillary electrophoresis (CE), Hydrodynamic chromatography (HDC) and Ultracentrifuge could be included as alternative methods, but no published reference methods are available for these

techniques. In the case of Ultracentrifuge reference to available standards for Centrifugal Sedimentation could be used but with a note that higher centrifugal forces can be employed specifically to cover smaller sizes. Accordingly, this should link to Method A of OECD TG 110.

3.7.116 The following alterations are recommended to be made in a revised version of Table R.7.1-31:

- Details of Scanning Mobility Particle Sizer (SMPS) should be incorporated, including reference to available standards (ISO/15900:2009; plus ISO 10808:2010, ISO 28439:2011);
- Details of Fast Mobility Particle Sizer (FMPS) should be incorporated;
- Limitations of cascade impaction for HARN should be highlighted, including reference to informative description in ISO/TR 27628:2007;
- Electrical Low Pressure Impactor (ELPI) should be included as an example of a cascade impactor, including reference to informative description in ISO/TR 27628:2007;
- Information on diffusion batteries as an alternative method should be incorporated, including reference to informative description in ISO/TR 27628:2007;
- Details of Optical Particle Counter (OPC) should be incorporated, including reference to informative description in ISO/TR 27628:2007;
- Information on Light scattering aerosol spectrometer (LSAS) should be incorporated to complement existing information of light scattering methods, including reference to available standard (ISO/21501-1:2009). The current stated size range for light scattering should be extended to 0.06 μm as allowed by LSAS;
- A caveat highlighting the limitations of Laser Diffraction for the sub-100nm range and high aspect ratio nanoparticles (HARN) should be included, and

the size range widened to up to 3 mm. A reference to the available standard (ISO/13320:2009) should be included;

- A caveat highlighting the limitations of Rotating Drum and Continuous Drop methods for nanomaterials should be included. References to modified methods available in the literature could be included at this stage (i.e. Maynard et al., 2004; Schneider & Jensen et al., 2008; Boundy et al., 2006), but it is considered more appropriate to wait until they are standardised in ISO/CD 12025 before including in the Guidance.

- 3.7.117 With regards to the ITS for granulometry (R.7.1.14.5), Figure R.7.1-7 entitled “Integrated testing strategy for granulometry” currently resides in R.7.1.14.2 (Available information on granulometry; pg. 144, R.7a) and is required to be moved to R.7.1.14.5 (Integrated testing strategies for granulometry; pg. 152, R.7a). Secondly, explanatory text is required to accompany the detailed sequence of steps in the ITS. This should at least include referencing to revised versions of Tables R.7.1-30 and R.7.1-31 providing more information on methods. Specifically, additional methods other than solely light microscopic examination and sieving need to be included at the screening level of granulates and powders to take into account testing of nanomaterials.
- 3.7.118 A greater acknowledgement of the sub-ordinate properties (particle size, size distribution, dustiness; shape and surface area if not considered to be separate Information Requirements) with reference to available measurement methods needs to be incorporated into the ITS for granulometry.
- 3.7.119 With regard to examples and case studies on granulometry, a more appropriate description of the issues and typical data from granulometry is required to be developed, which will be dependant upon the accepted composition of the Information Requirement for granulometry (i.e. whether shape and surface area are sub-ordinate to granulometry or included as separate additional specific intrinsic properties).
- 3.7.120 *Appendix to Part F CSR Template with explanation* – Granulometry’s sub-ordinate properties need to be added to the CSR template (Table 5: Overview of physico-chemical properties).

3.7.121 ADDITIONAL RELEVANT SPECIFIC INTRINSIC PROPERTIES**3.7.122 Particle Shape**

3.7.123 The specific guidance amendments for shape are subject to the decision of whether the property is sub-ordinate to granulometry or included as an additional Information Requirement.

3.7.124 *Suggested changes to Guidance if shape is included as an additional IR*

3.7.125 Part B: Hazard Assessment. B.6.1 – Shape and reference to a new chapter in R.7a would need to be included in the list of physico-chemical properties. B.6.1.4 – Paragraph on Shape would be required to be added.

3.7.126 R.7a - A new chapter would be required to be added to R7a entitled "SHAPE", on the basis of the properties inclusion as an additional IR. The section's content will follow the standard format of the existing Guidance: Definition; Information requirements on shape; Available information on shape; Evaluation of available information on shape; Conclusions on shape; Integrated testing strategy (ITS) for shape; References on shape. It is anticipated that this section will incorporate text from ISO 9276-6 (with a preliminary suggestion for inclusion of Figure 2 from the ISO document), as well as further text from resources identified and discussed in RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B3/2/FINAL and information on the selected methods:

3.7.127 TEM and SEM, including reference to informative description in ISO/TR 27628:2007. ISO/13322-1:2004 and ISO/13322-2:2006, which provide general image analysis guidance, could also be referenced. A note highlighting the importance of image quality should also be included;

3.7.128 Scanning Probe Microscopy (SPM), including reference to informative description in ISO/TR 27628:2007.

3.7.129 Should shape be a separate Information Requirement, as a minimum, we would suggest that the ITS for shape follows the tiered approach to testing (Section R.7.1.1.4) in conjunction with the choice of an appropriate test method. The

property would be included in Tier 3 of the general testing strategy for physico-chemical properties.

- 3.7.130 R.7.1.1.1 - Shape would be required to be included in Table R.7.1-1.
- 3.7.131 R7.1.1.6 - Shape, and details of its impact on other physico-chemical test, toxicology, ecotoxicology and risk assessment, would be required to be included in Table R.7.1-5.
- 3.7.132 R.7.1.14. – The limited existing references to requirements for shape information made within this chapter on Granulometry (R7.1.14.1, R7.1.14.2) would need to be removed and further clarified in a chapter on "Shape".
- 3.7.133 *Appendix to Part F CSR Template with explanation* – Shape would need to be added to Table 5: Overview of physico-chemical properties.
- 3.7.134 ***Suggested changes to Guidance if shape is included under an agreed definition of the term "Granulometry"***
- 3.7.135 R.7.1.14 - A sub-section on shape would be included under R7.1.14. It is proposed that, in the aims of clarity, this should be a distinct sub-section (rather than merged into the existing text which focuses primarily on particle size distribution) and follow the standard format outlined above. It is anticipated that this section will incorporate text from ISO 9276-6 (with a preliminary suggestion for inclusion of Figure 2 from the ISO document), as well as further text from resources identified and discussed in RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B3/2/FINAL and information on the selected methods:
- 3.7.136 TEM and SEM, including reference to informative description in ISO/TR 27628:2007. ISO/13322-1:2004 and ISO/13322-2:2006, which provide general image analysis guidance, could also be referenced. A note highlighting the importance of image quality should also be included;
- 3.7.137 SPM, including reference to informative description in ISO/TR 27628:2007.

- 3.7.138 It is evident where shape can feature in the ITS for granulometry: image analysis (currently for fibres, but requiring extension to granules and powders in a revised ITS).
- 3.7.139 **Surface area**
- 3.7.140 The specific guidance amendments for surface area are subject to the decision of whether the property is sub-ordinate to granulometry or included as an additional Information Requirement.
- 3.7.141 ***Suggested changes to Guidance if surface area is included as an additional IR***
- 3.7.142 *Part B: Hazard Assessment. B.6.1* – Surface area and reference to a new chapter in R.7a would need to be included in the list of physico-chemical properties. *B.6.1.4* – Paragraph on surface area may be required to be added.
- 3.7.143 R.7a - A new chapter would be required to be added to R7a entitled "SURFACE AREA", on the basis of the properties' inclusion as an additional IR. The section's content will follow the standard format of the existing Guidance: Definition; Information requirements on: Available information on surface area; Evaluation of available information on surface area; Conclusions on surface area; Integrated testing strategy (ITS) for surface area; References on surface area. It is anticipated that this section will incorporate text from ENV/JM/MONO(2009)20/REV, as well as further text from resources identified and discussed in RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B3/2/FINAL and information on the BET method, including reference to the available ISO standards (ISO/9277:1995; ISO/18757:2003) and details of potential limitations of this technique (e.g. assumes spherical system). Limitations of the indirect calculation of surface area of nanoaerosols from particle size using approaches such as SMPS/FMPS requires acknowledgement. Emerging techniques for measuring surface area of particles in dispersion (e.g. NMR) should also be acknowledged.
- 3.7.144 Should surface area be a separate Information Requirement, as a minimum, we would suggest that the ITS for surface area follows the tiered approach to testing (Section R.7.1.1.4) in conjunction with the choice of an appropriate test method.

The property would be included in Tier 3 of the general testing strategy for physico-chemical properties.

- 3.7.145 R.7.1.1.1 – Surface area would be required to be included in Table R.7.1-1.
- 3.7.146 R.7.1.1.6 – Surface area, and details of its impact on other physico-chemical test, toxicology, ecotoxicology and risk assessment, would be required to be included in Table R.7.1-5.
- 3.7.147 *Appendix to Part F CSR Template with explanation* – Surface area would need to be added to Table 5: Overview of physico-chemical properties.
- 3.7.148 ***Suggested changes to Guidance if surface area is included under an agreed definition of the term "Granulometry"***
- 3.7.149 R.7.1.14 - A sub-section on surface area should be included under R7.1.14. It is proposed that, in the aims of clarity, this should be a distinct sub-section (rather than merged into the existing text which focuses primarily on particle size distribution) and follow the standard format outlined above. It is anticipated that this section will incorporate text from ENV/JM/MONO(2009)20/REV, as well as further text from resources identified and discussed in RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B2/2/FINAL and information on the BET method, including reference to the available ISO standards (ISO/9277:1995; ISO/18757:2003) and details of potential limitations of this technique (e.g. assumes spherical system). Limitations of the indirect calculation of surface area of nanoaerosols from particle size using approaches such as SMPS/FMPS requires acknowledgement. Emerging techniques for measuring surface area of particles in dispersion (e.g. NMR) should also be acknowledged.
- 3.7.150 The ITS for granulometry does not currently acknowledge surface area and the present structure does not facilitate its simple inclusion. Should surface area be included under an agreed definition of the term 'granulometry, an updated ITS (including Figure R.7.1.7) would be required to include surface area, with reference to available measurement methods.

3.7.151 TOXICOLOGICAL ENDPOINTS & TESTING

3.7.152 The following section outlines proposals for further amendment of the REACH Guidance documents in relation to toxicological information requirements, test methods or testing strategies for nanomaterials.

3.7.153 GENERAL CONSIDERATIONS

3.7.154 Within RNC/RIP-oN2/B1/2/FINAL, RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B3/2/FINAL several aspects of guidance have been identified that, whilst not requiring a specific amendment, could benefit from the insertion of an advisory note. Specifically this was identified in situations such as the consideration of lung overload phenomena, assay interference and the suitability of bacterial mutation assays for assessing particles. Due to their applicability to several endpoints, advisory notes on lung overload and assay interference should be positioned at the beginning of Chapter R.7A or prior to the commencement of section R.7.2., which marks the beginning of the toxicology section of Guidance R.7a. The 'Advisory note on the consideration of bacterial assay interference' should be considered for insertion into Guidance section R.7.7 under the 'In vitro data' heading on page 380 prior to table R.7.7-2.

3.7.155 These advisory notes will not propose a protocol but aim to provide useful guidance and references to relevant resources. These advisory notes are outlined below.

3.7.156 EXISTING INFORMATION REQUIREMENTS

3.7.157 *R.7.3 Skin and eye irritation/ corrosion*

3.7.158 *Skin and eye irritation: Non-testing Data*

3.7.159 R.7.2.3.1 Non-human data on irritation/ corrosion

3.7.160 The use of non-testing is an attractive alternative to animal testing in the face of a lack of human epidemiological data on NM. However there does not exist sufficient robust evidence on the concept of sameness between non-nano and nano materials of the same material and nano to nano comparisons of differing materials.

3.7.161 Use of non-testing methods such as read-across may only be suitable in certain cases of NM and strong justification must be given for its use. In the context of NM, care must be taken when applying read-across from bulk to nano material forms taking into account particle size and surface area so that potential hazards are not underestimated. Thus far it may be possible to conduct read-across (nano-nano) on certain forms of NM such as low toxicity, low solubility particles but this still requires further study and validation.

3.7.162 As such we would propose the insertion of a statement to inform users of Guidance of the current ambiguity surrounding the use of non-testing data for nanomaterials, into the following sections of R.7.2.

- R.7.2.3.1 Non-human data on irritation/ corrosion - Page 203 appended to paragraph 3
- R.7.2.4.1 Non-human data on irritation/ corrosion - Page 215 appended to paragraph 4
- Also appendix R7.2.2-2 (p241 under the contents of Appendix 7.2-2 list), R7.2.2-3 (p245 under the contents of Appendix 7.2-3 list)

3.7.163 ***R.7.3 Skin and respiratory sensitisation***

3.7.164 *Skin and respiratory sensitisation: Non-testing Data*

3.7.165 As previously mentioned, the use of in silico approaches such as (Q)SAR, groupings and read across are not sufficiently developed for nanomaterials and statement to this effect should be made in the following sections:

- R.7.3.3.1 Non-human data for skin sensitisation – Page 259 appended to paragraph 7
- R7.3.4.1 Non-human data on skin sensitisation - Page 267 appended to paragraph 1
- R7.3.5.1 Non-human data on respiratory sensitisation - Page 271 appended to paragraph 1

3.7.166 **R7.4: Acute Toxicity**

3.7.167 *Acute Toxicity: Non-testing Data*

3.7.168 As previously mentioned, the use of in silico approaches such as (Q)SAR, groupings and read across are not sufficiently developed for nanomaterials and statement to this effect should be made in the following sections:

- R.7.4.3.1 Non-human data on acute toxicity – Page 291 appended to paragraph 3
- R.7.4.4.1 Non-human data on acute toxicity – Page 297 appended to paragraph 1
- R.7.4.5.1 Conclusions on suitability for Classification and Labelling – Page 301 appended to paragraph 2

3.7.169 **R7.5 - Repeated Dose Toxicity**

3.7.170 As previously mentioned, the use of in silico approaches such as (Q)SAR, groupings and read across are not sufficiently developed for nanomaterials and statement to this effect should be made in the following sections:

- R.7.5.3.1 Non-human data on repeated dose toxicity– Page 314 inserted between paragraphs 4 and 5
- R.7.5.4.1 Non-human data on repeated dose toxicity– Page 320 appended to paragraph 6

3.7.171 **R.7.6 Reproductive and developmental toxicity**

3.7.172 As previously mentioned, the use of in silico approaches such as (Q)SAR, groupings and read across are not sufficiently developed for nanomaterials and statement to this effect should be made in the following sections:

- R.7.6.4.1 Non-human data on reproductive toxicity– Page 355 inserted between paragraphs 4 and 5

3.7.173 ***R.7.7 Mutagenicity and Carcinogenicity***

3.7.174 As previously mentioned, the use of in silico approaches such as (Q)SAR, groupings and read across are not sufficiently developed for nanomaterials and statement to this effect should be made in the following sections:

- R.7.7.3.1 Non-human data on mutagenicity – Page 379 appended to paragraph 2
- R.7.7.4.1 Non-human data on mutagenicity – Page 383 appended to paragraph 6
- R.7.7.10.1 Non-human data on carcinogenicity – Page 407 appended to paragraph 1
- R.7.7.11.1 Non-human data on carcinogenicity – Page 412 appended to paragraph 5

3.7.175 ECOTOXICOLOGICAL ENDPOINTS & TESTING

3.7.176 As for many substances, the importance of material suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, characterization of samples (prepared from stock dispersions) has been identified, both prior to administration/testing and possibly during and at least at the end of the test. As such, and in line with the recommendations set out in the OECD Guidance Manual for testing (ENV/JM/MONO(2009)20/REV) and Preliminary Guidance Notes on Sample Preparation and Dosimetry for nanomaterials (ENV/JM/MONO(2010)25) it is proposed that a reference be made within the following sections referring Guidance users to a general section on “Sample Preparation” to be included in the introduction of R.7a.

3.7.177 This reference should be made in the following sections:

3.7.178 ***R.7.8 Aquatic toxicity; long-term toxicity to sediment organisms***

3.7.179 *R.7.8.4.1 Data on aquatic pelagic toxicity*

- Short-term toxicity testing on invertebrates (preferred species Daphnia)
- Growth inhibition study aquatic plants (algae preferred)
- Short-term toxicity testing on fish
- Long-term toxicity testing on invertebrates (preferred species Daphnia), (unless already provided as part of Annex VII requirements)
- Long-term toxicity testing on fish, (unless already provided as part of Annex VIII requirements)
- Fish early-life stage (FELS) toxicity test
- Fish short-term toxicity test on embryo and sac-fry stages
- Fish, juvenile growth test

3.7.180 *R.7.8.9.1 Laboratory data on toxicity to sediment organisms*

- Long-term toxicity to sediment organisms

3.7.181 ***R.7.11 Effects on Terrestrial organisms***

3.7.182 *R.7.11.3.1 Laboratory Data*

- Soil short-term toxicity to invertebrates
- Effects on soil micro-organisms
- Short-term toxicity to plants
- Long-term toxicity testing on soil invertebrates, unless already provided as part of Annex IX requirements
- Long-term toxicity testing on plants, unless already provided as part of Annex IX requirements

3.7.183 In addition to the important, but more general issue of sample preparation outlined above, more specific amendments are proposed for the following.

3.7.184 *R 7.9.3.1 Ready biodegradability*

3.7.185 It should be noted that the OECD ready biodegradability test guidelines have been developed and validated principally for assessment of organic compounds whereas many nanomaterials are principally inorganic and even carbon-based nanomaterials tend to behave as if they were inorganic in nature. However, surface coating and functionalisations might be organic and consist of biodegradable materials. Methods measuring carbon dioxide production or oxygen uptake are applicable, but they require large amounts of test material. If several conclusive aerobic degradation tests indicate very low or negligible degradation, then other aerobic degradation tests will most likely also be negative and it may be useless to proceed with additional tests. It may be better to decide to skip the more elaborate test, and conclude that the substance is not biodegradable (ENV/JM/MONO(2010)25).

3.7.186 *R.7.1.1.15 Adsorption/desorption screening*

3.7.187 It should be noted that the distribution coefficient K_d has to be based on actual testing using one of the methods for the measurement of adsorption outlined in Table 7.1-33 since estimations of K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}) have no merit when it comes to nanomaterials.

3.7.188 *R.7.10.3.1 Bioaccumulation in aquatic species, preferably fish*

3.7.189 It should be acknowledged that for bioaccumulation it is not possible to make $\log K_{ow}$ or solubility estimations since nanomaterials are dispersed and not in solution. Measured BCF values are required and stability and changes in e.g. aggregation and agglomerate size are of vital importance to consider. There is furthermore a need to emphasise that for nanomaterials under dissolution like Ag0 obtaining information, if possible, on the form of the substance present in the animal tissue may provide useful additional information.

3.7.190 It should be noted that estimates based on “partitioning” are limited to distribution of a substance in molecular form. However, substances may also be distributed in the environment as particles (caused by abrasion/weathering of anthropogenic materials) and extrapolation based on partitioning may not be relevant. In such a case the partitioning method may underestimate exposure of soil and sediment environments and overestimate the exposure of water. If the particle size is small also air distribution may occur. There are no estimation methods available for particle distribution so this has to be dealt with on a case-by-case basis. This issue is relevant to and could be expressed in the following sections:

3.7.191 **R.7.8 Aquatic toxicity; long-term toxicity to sediment organisms**3.7.192 *R.7.8.9.1 Laboratory data on toxicity to sediment organisms*

- Long-term toxicity to sediment organisms

3.7.193 **R.7.11 Effects on Terrestrial organisms**

3.7.194 R.7.11.3.1 Laboratory Data

- *Soil short-term toxicity to invertebrates*
- *Effects on soil micro-organisms*
- *Short-term toxicity to plants*
- *Long-term toxicity testing on soil invertebrates, unless already provided as part of Annex IX requirements*
- Long-term toxicity testing on plants, unless already provided as part of Annex IX requirements

3.7.195 **ADDITIONAL RELEVANT SPECIFIC INTRINSIC PROPERTIES**

3.7.196 In conducting the RIP-oN2 B2 and B3 tasks, a number of potential additional relevant specific properties have been identified. These include fish ventilation rates, fish gill pathologies, fish mucus secretion, fish brain pathology, animal behaviour, oxidative stress. Whilst these biological markers have been used within the peer reviewed literature, they are not considered suitably advanced for direct incorporation into guidance at this time as required endpoints. Indeed the consortium has identified that further research is required to assess both the relevance of the markers, their suitability for regulatory testing and the development and adoption of standardised testing methodologies. These candidate additional relevant specific properties have therefore been listed under B5 subtask IV: Proposals for further research and development with further detail given in section 4.3.157 of this report onwards.

3.7.197 B5 Sub-Task IV: Proposals for further research and development of test methods and other data generation methods/strategies in regard to nanomaterials

3.7.198 Research and development needs have been identified systematically during Tasks B1, B2, B3, B4 for each property, testing endpoints and methods. A summary listing of the aspects considered to warrant further research and development is provided below. Detailed recommendations are provided in Chapter 4 of this report.

3.7.199 Physico-chemical Properties

3.7.200 General aspects (characterisation practice, standards, and protocols)

3.7.201 Existing Information Requirements

- Relative density
- Surface tension
- Water solubility
- Partition coefficient
- Flammability
- Explosive properties
- Granulometry
- Dissociation constant

3.7.202 Additional Relevant Specific Intrinsic Properties

- Particle shape
- Surface area
- Porosity
- Surface energy
- Surface chemistry
- Surface acidity
- Surface charge
- Redox potential
- Cell-free ROS/RNS production capacity

3.7.203 (Particle shape and surface area have been recommended for incorporation into REACH Guidance at this time on the basis of the justification and practicability developed from the assessment of the available evidence.)

3.7.204 **Toxicological Endpoints**

3.7.205 Aspects relating to study design

- Dispersion
- Selection of dose
- Selection of exposure route & duration
- Interactions
- Using physico-chemical data to inform experimental design
- Target-organ toxicity considerations
- Adopting standardised controls
- Interferences

3.7.206 Existing Information Requirements

- Skin irritation
- Eye irritation

3.7.207 Additional Relevant Specific Intrinsic Properties

- Inflammation
- Pro-fibrogenic effects
- Barrier transfer models
- Cardiovascular toxicity

3.7.208 **Ecotoxicological Endpoints**

3.7.209 Within the following proposed research and development needs for ecotoxicological endpoints, test methods or testing strategies for nanomaterials those considered to be low priority are indicated with an asterisk.

3.7.210 General considerations

- Aquatic testing
- Soil testing

3.7.211 Existing IRs[#]

- Short- and long-term toxicity on invertebrates, fish and plants
- Growth inhibition in aquatic plants
- Ready biodegradability
- Short- and long-term toxicity on fish
- Activated sludge respiration inhibition
- Hydrolysis as a function of pH
- Adsorption / desorption screening
- Fish: early life stage, embryo & sac-fry, juvenile growth
- Bioaccumulation in aquatic species
- Soil micro-organisms
- Sediment organisms
- Long-term or reproductive toxicity to birds

3.7.212 Additional Relevant Specific Intrinsic Properties[#]

- Fish ventilation rate
- Fish gill path and mucous secretion
- Fish brain pathology
- Animal behaviour
- Oxidative stress
- Daphnia heart rate*, hopping frequency*, appendage movement*
- Trojan-horse effect of nanomaterials*

[#]In relation to these endpoints and proposed biological markers it should be noted that consensus has not been reached within the project consortium and further discussions can be found in section 4.3.157 onwards.

3.8 METRIC(S) TO COMPARE IN THE RISK CHARACTERISATION (TASK C)

- 3.8.1 The objective of Task C was to develop a working document on the identification of critical items on exposure/dose descriptors and related parameters, outlining needs for adequate metrics/parameters as appropriate for exposure assessment compatible with the ones used for hazard assessment. The underlying principal metrics is the number of molecules expected to participate in the process in question. Most commonly mass, but particle number or surface area are eventually chosen as the proxy for this number.
- 3.8.2 The question of what is the best metric to measure the risk of nanoparticles is a frequently posed question when attempting to assess the risks of nanomaterials. In practice there are many metrics, all of which include mass or number, which are currently used in the risk assessment (both regulatory and otherwise) across the three elements of exposure, toxicology and risk. The most commonly used are identified below:

Target	Route	Exposure metric (example units)	Toxicology /ecotoxicology dose metric (example units)	Risk evaluation metric (example units)
Human	inhalation	mass conc in air (mg/m ³)	mass per animal or per body part (m)	mass conc in air (mg/m ³)
	inhalation	fibre number conc in air (f/ml)	fibres per animal or per body part (#f)	fibre number conc in air (f/ml)
	dermal	mass per surface area of skin exposed (mg/cm ²)	mass per animal or surface area (m)	mass per surface area of skin exposed (mg/cm ²)
	dermal	mass per kg body wt per day (mg/kg/day)	mass per animal or surface area (m)	mass per kg body wt per day (mg/kg/day)
	ingestion	mass per kg body wt per day (mg/kg/day)	mass per animal (m)	mass per kg body wt per day (mg/kg/day)
Environment	air/water/soil	Environmental concentration (mg/L or mg/kg)	Predicted no effect concentration (mg/L or mg/kg)	Ratio between environmental concentration and predicted no effect concentration

- 3.8.3 The metrics can be units, concentrations, or ratios. They can be measured directly or modelled.
- 3.8.4 The most prominent alternative or additional metric identified for use in relation to the risk assessment of nanomaterials, is surface area. This is based primarily on toxicological evidence relating particle surface area to inflammation, an indicator of toxicity. The evidence for this has been assessed in the Task report.
- 3.8.5 Other parameters debated as possible metrics include surface reactivity and charge. Surface reactivity is clearly an important parameter although whether this could be considered as a potential metric or simply a unit to express the toxicological response is a matter for discussion. Its use as a metric (in toxicology) would be as “units of reactivity per body part”. This same is true of charge in which the metric would be coulomb/body part. It is considered that the basis of these two properties becoming “metrics” is not yet sufficiently advanced to a level at which use and guidance for REACH can be recommended.
- 3.8.6 It is important to note that there are other parameters which can act as modifiers of the toxicity. These include particle size, size distribution, density, aggregation and shape. These parameters would not generally be considered as scalable quantities and do not appear to conform to the current use of the term “metric” under REACH. Therefore they have not been considered further in this discussion.
- 3.8.7 Metrics in risk assessment need to be scalable quantities which may be used to express the levels of hazard, exposure or risk. To date, conversion between mass, number and surface area has largely been based on simple assumptions, treating particles as spheres and using mean particle diameters. It is considered advantageous to be able to provide for each nanomaterial functional conversions between the three metrics based on established and validated relationships. Conversion between the metrics of mass, number and surface area remains challenging both within and between exposure, hazard and dose. Measurement of surface area in relation to dose is still mostly indirect and is typically based on a mass assessment times a measure of specific surface area of the powdered material obtained by BET analysis or similar. Encouragingly, in relation to inhalation

exposure, measurement systems are available to measure mass, number and surface area concentrations.

- 3.8.8 For example, Wake et al. (2001) carried out a laboratory study to compare the performance of Matter LQ1-DC active surface area monitor, a TSI Model 3934 Scanning Mobility Particles Sizer and an R&P Tapered Element Oscillating Microbalance. Using the three instruments described above, experiments were carried out in the laboratory with polydisperse aerosols, containing ultrafine particles, to establish what relationships exist between the three measurement parameters mass, surface area and number as determined by each instrument and how these relationships may be influenced by particle composition and morphology. For each of the five aerosol types investigated, consistent relationships were found for mass and active surface area with increasing particle number concentrations for all the particle sizes investigated. However, these relationships were not consistent with particle size. Amongst Wake's conclusions were that no simple relationship was found for predicting active surface area and mass from the results of measurements made with the benchmark instrument the SMPS. This instrument, therefore, should not be used to calculate surface area and mass unless a detailed knowledge of the aerosol is known. In view of this, the use of all three instruments, measuring in parallel, should continue despite the difficulty in arranging this in the workplace. Moreover, Wake considered it unwise to make measurements in terms of just one parameter, be it mass, active surface area or number/size, when assessing the potential for engineered nanoparticles to cause ill health when the causal factor has not yet been established.
- 3.8.9 An advantage of mass over surface area (and virtually all other alternatives) is that the mass in a system is conserved i.e. remains constant (and could be assessed through mass balance), whereas surface area is not. In other words, the actual surface area can change due to aggregation/de-aggregation which may occur following deposition of the particles and influence the interpretation of data. The same is also true (to an even greater extent) for particle number.
- 3.8.10 However, there is nevertheless evidence that surface area is an important metric in describing the human health hazard potential of some types of nanoparticles. For low toxicity low solubility materials, surface area of particles administered rather than

mass burden of particles may be a more appropriate dose metric for pulmonary toxicity studies. The same type of relationship has also been demonstrated for higher toxicity nanoparticles. For dermal effects, any metric proposed to assess dermal exposure to nanoparticles should be biologically relevant and should relate to health effects. It may be that for local effects, inflammation is the key driver in which it could be speculated that surface area would be the important metric. Further work including workplace studies and in-vivo/vitro assessment of penetration is required. For the environment, it seems too early to tell whether a dose [concentration] - response relationship can be established as well as whether, for instance, size or surface area can be substituted with dose by mass. Too few studies have actually investigated alternative dose metrics at this point in time and correlated these with the observed effects.

- 3.8.11 In relation to the guidance which can be given now on hazard assessment, it is considered important to continue with mass based measurement. This is the basis of the current risk assessment process and the linkage to past work in both exposure and toxicology. Based on the evidence available, it seems justified to additionally express the data in terms of surface area. In practical terms, this would only require knowledge of BET results for the nanomaterial used. For exposure assessment, both surface area and number concentration data are achievable and provide useful information and addition to the standard mass data, and should be collected.
- 3.8.12 Further consideration of additional issues relating to metrics, as part of an ongoing international dialogue and subsequent to the acceptance of the Task C report, warrant acknowledgement in this final report:
- There is no general rule for the choice of metric as the relevance may depend on the exposure route and even the material itself (e.g. aspect ratio), so it should be decided on a case-by-case basis by the registrant. The mass metric may not always be the most appropriate or relevant metric. However, given the historical and established use of the mass metric, which is the case in most if not all elements of hazard, exposure and risk characterisation in a Chemical Safety Assessment (CSA) under REACH, it continues to be considered appropriate that even in cases where another

metric is relevant and has been used, the mass metric description/data/result should continue to be provided.

- It is clear that one, two or more metrics may be relevant to undertake the best possible CSA for different forms of a materials covered by one registration, including all exposure scenarios etc. This is to be encouraged, albeit with a clear justification and transparency to ensure that the CSA can be understood. The most relevant for determination of the Risk Characterisation Ratio (RCR) should be considered in such cases.
- At present, evidence of the emergence of new metrics is strong in some case (e.g. surface area), but this is acknowledged as an evolving field. It has been made clear in the RIP-oN 2 and 3 projects that there is evidence to recommend surface area as a metric appropriate in inhalation exposure but there is no conclusive evidence with regard to dermal exposure. This has already been reflected in the guidance recommendations. The choice of metrics is rightly left to the registrant, with the expectation that the choice is scientifically justified. As stated above, as exposure scenarios differ, so can the choice of metrics for the related form and the individual scenario.
- It should always be clear how different metrics have been used (in rare cases perhaps even from separate studies) or derived through transformation of final results of the same test etc. It should be ensured that the whole CSA (hazard, exposure, risk characterisation ratio including any required risk management measures) is performed consistently. If the Derived No Effect Level (DNEL) or Predicted No Effect Concentration (PNEC) are determined using one metric, so should the estimation of the Predicted Environmental Concentration (PEC) to characterise the risk ratio. The basis for selecting and assessing the efficiency of RMM employed should be expressed in the same metric. The same applies to the potential application of models and to the justification of any read-across. If there are transformations of metrics involved, they need to be transparent so the applicability of the data can be justified.

- It is clear that a consideration of Assessment Factors needs to be performed for different metrics, separately, and including the uncertainty potentially arising from the transformation of metrics and from the differences in the tests performed.
- There is more to the conversion between metrics than simply working in one metric and then express results in another. Adequate characterisation and the scope of applicability of the test is required, along with consideration of the design of the test (e.g. selected doses, sample preparation to minimise uncertainty/bias) and the selection of the most appropriate instrumentation/method.
- It remains that there are currently no definitive conclusions on the best metric. However, there is growing consensus that if new animal tests on nanoforms are performed, there should be a sufficient characterisation of those forms allowing the dose-response to be expressed in the different metrics discussed - number, surface area and mass.

4 RECOMMENDATIONS

4.1 UPDATES TO EXISTING GUIDANCE

4.1.1 Specific recommendations for updates to guidance, derived from the work undertaken in RIP-oN 2 project, are presented in this chapter on a section-by-section basis, using the numbering system of the existing guidance document, chapters and sections. Suggested updates to higher-level guidance (e.g. Part B: Hazard Assessment) are made at the time of presenting the Endpoint Specific Guidance updates for a particular property.

4.1.2 R.7 ENDPOINT SPECIFIC GUIDANCE

4.1.3 Introduction

4.1.4 It is recommended that the following sub-section is included in the overall Introduction for the R.7 Guidance after the sub-section entitled "Adequacy of methods for generating additional information" (R.7, pg. 15):

4.1.5 Sample preparation

4.1.6 Sample preparation is widely recognised as one of the most critical steps towards successful characterisation and subsequent (eco)toxicological testing of nanomaterials, in which there are many variables to consider when designing a method for preparation. Common issues regarding sample preparation include storage and stability of the test material; the chemical composition of the test media; characterisation of stock dispersions, and; characterisation of samples (prepared from stock dispersions) prior to administration/testing (OECD, 2010). Preliminary guidance on sample preparation for the physico-chemical characterisation of nanomaterials, covering properties including particle size distribution, shape, specific surface area, octanol-water partition coefficients, degree of agglomeration and dispersion behaviour, is available (OECD, 2010). ISO 14887:2000 outlines procedures for the preparation of good dispersions from various powder/liquid combinations for particle size analysis of substances in general. Suggested dispersion procedures for a range of nanomaterials are also emerging in the scientific literature. However, such procedures should be carefully examined

to determine if they are adequate for the test material under consideration and modifications may be required for different materials. With regards inhalation toxicity testing, standards are available that outline procedures for the generation of metal nanoparticles using the evaporation/condensation method (ISO 10801:2010) and support the characterisation of nanoparticles in inhalation exposure chambers (ISO 10808:2010).

4.1.7 An important component of sample preparation is the need to have “reliable” sampling, such that the test aliquot used for measurement represents the physical and chemical characteristics of the entire sample. The characterisation of particle properties like size, form and specific surface area requires very careful sampling and sample splitting practises to be followed. ISO 14488:2007 specifies methods for obtaining a test aliquot from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative with a defined confidence level and is of particular relevance to the measurement of particle size, size distribution and surface area.

4.1.8 Also in relation to sample preparation, it is necessary to be aware that aggregates and agglomerates of nanomaterials can form in solution, powder and aerosol forms, and their presence is influenced by a number of factors including the method of synthesis, storage, handling and environmental conditions. An agglomerate is defined as a collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components. An aggregate is a particle comprising of strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components (ISO 27687:2008).

4.1.9 In addition, it is known that the observations and interpretation of toxicity, and fate and behaviour, as a result of exposure to agglomerates may or may not be associated with the primary particle’s characteristics. The state of agglomeration or aggregation is recognised as an important parameter influencing the interpretation of characterisation and testing of nanomaterials

(“as received”, “as used”, “as dosed / as exposed”) and should therefore be considered during sample preparation. A number of measurands have been proposed for assessing agglomeration or aggregation state, including the effective cross-section, determined by measuring aerodynamic/light scattering properties or by electron microscopy (OECD, 2009). OECD (2009) suggest for nanomaterials with a non-zero width of the distribution of the degree of agglomeration should be characterised. Other measurands include the average agglomeration number (AAN), which is derived from the ratio of the volume based median particle size to the average equivalent spherical volume derived from BET gas adsorption.

4.1.10 In addition to aggregation and agglomeration, the behaviour of particles in solution presents some additional important aspects and challenges to recognise. In particular, it can be difficult to distinguish between when a nanomaterial is *dispersed* and when it is *dissolved* due to its small particle size. It is important to recognise that solubility and dispersibility are two distinct phenomena. Solubility is the degree to which a material (the solute) can be dissolved in another material (the solvent) such that a single, homogeneous, temporally stable phase (a suspension down to the molecular level) results, and is relevant to solids, liquids and gases. Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase). Historically, the term “dissolved” meant the component of a liquid sample that had passed through a 0.45µm (or similar) filter. However, as (colloidal) dispersions of nanoparticles might also pass through such filters, it is recommended that use of the term “dissolved” should be restricted to the formation of true solutions, and where both liquid and particulates are present the term “dispersed” should be used (OECD, 2010).

4.1.11 A dispersion is a suspension of discrete insoluble particles in a fluid, which may falsely have the visible appearance of a solution (i.e. the product of the conversion of a solid substance to liquid form by mixture with a solvent). A dispersion of an insoluble material may elicit a different response from that anticipated from the classical molecular or elemental toxicity expected from the

chemical composition. Dispersion stability is an important parameter to assess in the context of sample preparation.

- 4.1.12 The dispersion of particles is determined by intermolecular forces involving particle-particle interactions as well as those between the particles and their environment. Due to attractive forces (e.g. Van der Waals interactions) particles tend to agglomerate unless stabilised by surface charge or steric effects. As a result, the state of dispersion is dynamic and determined primarily by the environment of the nanoparticles. In solution, slight modifications in pH, ionic strength, and concentrations of molecular constituents can significantly alter the dispersion of particles. For aerosolised powders, the situation can be even more complex as the concentration and diffusion characteristics of the aerosol can cause the state of dispersion to change over time.
- 4.1.13 The state of dispersion is typically assessed using comparative particle size measurements and requires a reliable method of measuring the baseline particle size distribution of the material. By comparing changes in particle size distribution, a qualitative assessment or proxy measure of the state of dispersion can be made. Zeta potential measurement, combined with Dynamic Light Scattering (DLS) also enables the stability of nanoparticle dispersions to be monitored and a qualitative understanding of the agglomeration process.
- 4.1.14 If a nanomaterial is soluble in biological or environmental media, then it is likely to be presented to the test system in its molecular or ionic form and can therefore be expected to elicit the same response as bulk (non-nanoscale) solubilised substances. If, however, the nanomaterial under investigation is insoluble or sparingly soluble in biological or environmental media, then it will likely be presented to the test system in a particle form.
- 4.1.15 In addition, nanoparticles may interact with the liquid phase components, partially or totally yielding soluble or dispersed transformation products (as well as some solubilised nanomaterial itself) that may influence the overall toxicity and fate processes. This possibility needs to be taken into account when

selecting the media and procedures as well as in the assessment of the result of any experiment (OECD, 2010).

- 4.1.16 Other important considerations to take into account during sample preparation include the influence of contaminants and impurities on (eco)toxicological test results. Adverse effects on a number of species used in PNEC derivations for nanomaterials have been attributed to particle impurities (e.g. Cheng et al., 2007; Brayner et al., 2006).
- 4.1.17 Of particular concern is the influence of endotoxin on certain testing results. Endotoxin (lipopolysaccharide) is a constituent of the outer cell wall of Gram-negative bacteria and as such is found ubiquitously within the environment. Endotoxin however can generate a range of toxic effects either at the whole organism level causing responses such as fever, 'endotoxin shock' and death, or at the cellular level via the triggering of inflammatory cascades leading to the secretion of pro-inflammatory mediators.
- 4.1.18 Due to the potent response endotoxin can generate in biological assays, toxicity testing of a contaminated test sample would lead to a confounding of results (including a potential false positive). As such the establishment of the presence or level of endotoxin in a test sample is useful as a preliminary undertaking during the preparation of a sample for toxicological testing. International standards are available for the testing of nanomaterials (ISO 29701:2010) although issues regarding endotoxin contamination are not necessarily nano-specific and are equally relevant other particles or aqueous substances undergoing toxicological evaluation.
- 4.1.19 In order to eliminate potential confounding of the interpretation of results due to particle contaminants/impurities, data from the characterisation of the test material including its purity and, if technically feasible, quantities of identified contaminants and impurities should be considered prior to the start of a study, consistent with the substance identification requirement.

4.1.20 R.7.1.1 Introduction

4.1.21 The following text is recommended to be added as a new fourth paragraph in R.7.1.1. (R.7a, pg. 17), following the paragraph ending "...and be operating within its validity range":

4.1.22 With regards nanomaterials characterisation, it is important to note that different techniques will suit different sample forms (e.g. aerosol, suspensions etc.) and, in many cases, no individual technique can satisfy a meaningful characterisation of nanomaterials (Stone et al., 2009; Tran et al., 2008). Multiple techniques should therefore be used where possible in order to formulate an appropriate understanding of the nanomaterial's properties, and the optimum set of required techniques should be selected and justified based on the specific nanomaterial type and form under investigation. The need for multi-method characterisation and material-specific selection of techniques applies across a range of nanomaterial properties and would facilitate the gathering of data on multiple metrics.

4.1.23 R.7.1.1.3 Evaluation of available information on physico-chemical properties**4.1.24 Experimental data**

4.1.25 The last sentence of the second paragraph of R.7.1.1.3 sub-section on "Experimental data" (R.7a, pg. 27) is recommended to be updated with the following text to acknowledge the importance of reference materials:

4.1.26 Comparison of the experimentally determined physico-chemical property with a suitable reference material and a scientifically justified QSAR prediction is often, if not always, recommended to provide reassurance that the experimentally derived value is acceptable and has not been influenced by the presence of impurities in the product. A number of particle based reference materials are available from commercial sources and National or Community Measurement Standard Bureaus e.g. NPL, IRMM, NIST.

4.1.27 R.7.1.1.7 References for introduction of Physico-Chemical properties

4.1.28 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.1.1.7 (R.7a, pg. 45-47):

- 4.1.29 Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M.F. and Fievet, F. 2006, "Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium", *Nano Letters*, vol. 6, no. 4, pp. 866-870.
- 4.1.30 Cheng, J.P., Flahaut, E. and Cheng, S.H. 2007, "Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos", *Environmental Toxicology and Chemistry*, vol. 26, no. 4, pp. 708-716.
- 4.1.31 ISO 10808:2010. "Nanotechnologies -- Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing", ISO TC 229.
- 4.1.32 ISO 14488:2007. "Particulate materials – Sampling and sample splitting for the determination of particulate properties", ISO TC 24/SC 4.
- 4.1.33 ISO 14887:2000. "Sample preparation – Dispersing procedures for powders in liquids", ISO TC 24/SC 4.
- 4.1.34 ISO/TS 27687:2009 "Nanotechnologies - Terminology and definitions for nano-objects - Nanoparticle, nanofibre and nanoplate", ISO TTC/229.
- 4.1.35 ISO 28439:2011. "Workplace atmospheres -- Characterization of ultrafine aerosols/nanoaerosols -- Determination of the size distribution and number concentration using differential electrical mobility analysing systems", ISO TC 146/SC 2.
- 4.1.36 ISO 29701:2010. "Nanotechnologies – Endotoxin test on nanomaterial samples for in vitro systems – Limulus amoebocyte lysate (LAL) test", ISO TC/229.
- 4.1.37 OECD. 2009, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme. First revision", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV, Paris.

- 4.1.38 OECD. 2010, "Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2010)25, Paris.
- 4.1.39 Stone, V., Hankin, S., Aitken, R., Aschberger, K., Baun, A., Christensen, F., Fernandes, T., Hansen, S.F., Hartmann, N.B., Hutchison, G., Johnston, H., Micheletti, C., Peters, S., Ross, B., Sokull-Kluettgen, B., Stark, D., Tran, L. 2009, "ENRHES – Engineered nanoparticles: review of health and environmental safety", EC contract number 218433.
- 4.1.40 Tran, C.L., Hankin, S.M., Ross, B., Aitken, R.J., Jones, A.D., Donaldson, K., Stone, V., Tantra, R. 2008, "An outline scoping study to determine whether high aspect ratio nanoparticles (HARN) should raise the same concerns as do asbestos fibres", Defra Research Report CB0406, UK.

4.1.41 **R.7.1.7 WATER SOLUBILITY**

4.1.42 **R.7.1.7.3 Evaluation of available information on water solubility**

4.1.43 Remaining uncertainty on water solubility

4.1.44 It is recommended that the following text is added as a new third paragraph in R.7.1.7.3 sub-section "Remaining uncertainty on water solubility" (R.7a, pg 93):

4.1.45 Water solubility has the potential to increase in the nano-size range. For nanomaterials, it can be difficult to distinguish between when a substance is dispersed and when it is dissolved due to its small particle size. It is important to recognise that solubility and dispersibility are different and distinct phenomena, with different implications on testing and characterisation, and it is important to differentiate between them. Further information on these issues is provided in the Sample Preparation sub-section of the R.7a Introduction. It should also be ensured that no undissolved material contributes to what is being measured.

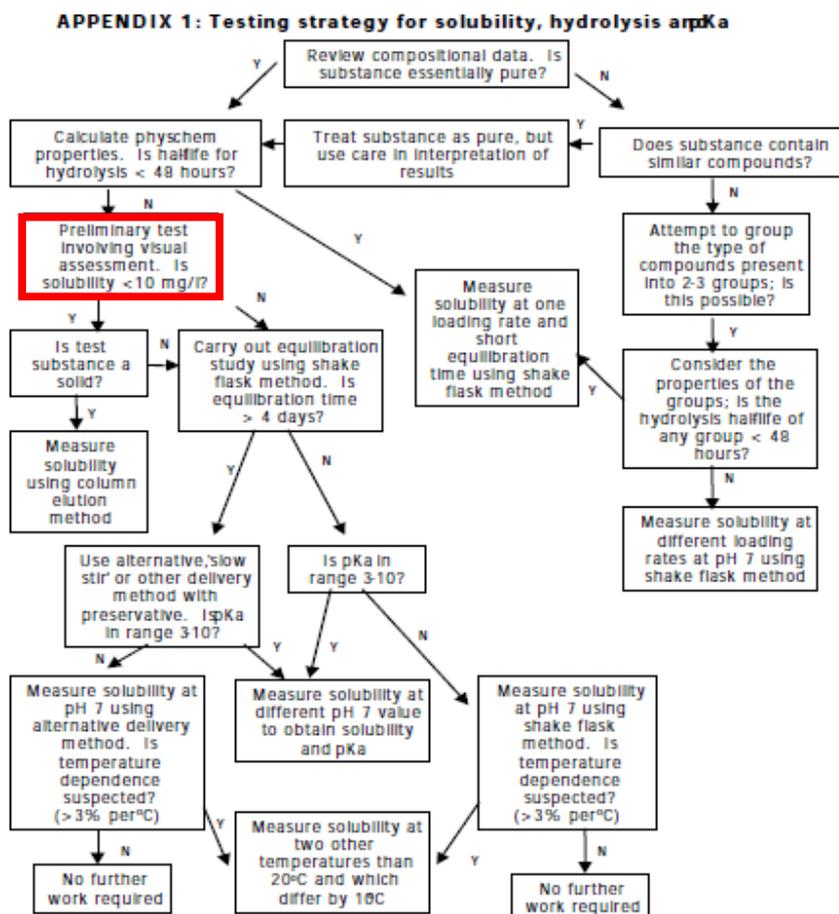
4.1.46 **R.7.1.7.5 Integrated testing strategy (ITS) for water solubility**

4.1.47 Figure R.7.1-5

4.1.48 It is recommended that, in Figure R.7.1-5 (R.7a, pg. 94), the qualifier in the box highlighted red below is replaced with the following text:

4.1.49 Preliminary test involving visual/instrumental assessment of solubilisation or dispersion. Is solubility < 10 mg/l?

Figure R.7.1-5 The three properties, solubility, hydrolytic stability and acid dissociation constant are inter-related. It is not possible to measure any of these without some knowledge of the other two



4.1.50 R.7.1.8 PARTITION COEFFICIENT N-OCTANOL/WATER

4.1.51 R.7.1.8.3 Evaluation of available information on partition coefficient n-octanol/water

4.1.52 Experimental data on partition coefficient n-octanol/water

4.1.53 It is recommended that the following caveat is added to the end of the second paragraph of R.7.1.8.3. sub-section "Experimental data on partition coefficient n-octanol/water" (R.7a, pg. 101), after the sentence ending "...should equally be accepted":

- 4.1.54 It is important to note that, following a review of the applicability of test guidelines to nanomaterials, OECD concluded that test guidelines 107, 117 and 123 might be applicable under some circumstances or to some classes of manufactured nanomaterials, although further work is required to determine this and modify the TGs, if it is considered necessary (OECD, 2009). Results might be impacted upon by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve (OECD, 2009).
- 4.1.55 Difficult to test substances
- 4.1.56 It is recommended that the following paragraph is added to the end of R.7.1.8.3. sub-section "Difficult to test substances" (R.7a, pg. 106), after the paragraph ending "...hydrolysis, oxidation, or biotic degradation":
- 4.1.57 For nanomaterials, it can be difficult to distinguish between when a substance is dispersed and when it is dissolved due to its small particle size. It is important to recognise that solubility and dispersibility are two distinct phenomena and it is important to differentiate between them. Further information on these issues is provided in the Sample Preparation sub-section of the R.7a Introduction.
- 4.1.58 **R.7.1.8.6 References on n-octanol/water partition coefficient**
- 4.1.59 Subject to acceptance of the recommended guidance changes above, the following additional reference would need to be included into R.7.1.8.6 (R.7a, pg. 114):
- 4.1.60 OECD. 2009, "Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)21, Paris.

4.1.61 **NB. The following guidance update for GRANULOMETRY is recommended if the scope of the term “Granulometry” and thus the corresponding information IS NOT considered to include additional specific intrinsic properties “shape” and “surface area”.** It is noted that Annex II to Regulation (EC) No 1907/2006 has been amended and now explicitly states in Section 9.1 that “The physical state (solid (including appropriate and available safety information on granulometry and specific surface area if not already specified elsewhere in this safety data sheet), liquid, gas) and the colour of the substance or mixture as supplied shall be indicated.” The inclusion of specific surface area as a separate term distinct from granulometry in the amendment of Annex II is observed. For specific surface area at least, confusion (or even a suggestion of heightened importance) may arise, albeit minor, when the property is seen to be stated separately from granulometry in Annex II. This ambiguity is simply highlighted, for resolution by regulators in their future considerations. In this circumstance, new chapters are recommended and provided in Section 4.2 of this report for Shape and Surface Area, which should accompany the updates made below for Granulometry. The alternative circumstance where the information requirement on “Granulometry” IS considered to include Shape and Surface Area is addressed in paragraphs 4.1.191 to 4.1.222 of this report.

4.1.62 **Part B: Hazard Assessment**

4.1.63 **B.6.1.4 Other physico-chemical properties**

4.1.64 It is recommended that the following paragraphs on Granulometry, Shape and Surface Area are added to the end of section B.6.1.4. (PART B – HAZARD ASSESSMENT, pg. 21), following the paragraph ending “...are preferred over other determinations of K_{ow} ”:

4.1.65 **Granulometry**, which can be defined as the determination of particle size distribution, is an important property to consider from a hazard assessment perspective. Determination of the particle size fractions are used to assess the possible health effects resulting from inhalation of airborne particles in the workplace. The inhalable size range of particles is important in determining not only if the situation poses an inhalation problem, but also where in the respiratory tract the particles may deposit. Therefore, the particle size

distribution can be used as an argument when considering inhalation testing. A number of methods covering different ranges of particle sizes are available, although none of them is applicable to the entire size range. The particle size distribution is needed in order to decide which route of administration is most appropriate for the acute toxicity and 28-day base set animal studies. Particle size is also a factor in environmental exposure assessment.

Shape is an important parameter in the characterisation of some particles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape.

Surface area is an important parameter in the characterisation of nanomaterials, with emerging evidence of quantitative value as an additional dose metric / descriptor for hazard assessment. The surface area will dictate the surface charge in cases where nanomaterials are surface functionalised, with direct consequences on nanomaterial interaction (i.e. agglomeration) with other naturally occurring particulate, route of exposure as a function of surface ligand-biological interface and mechanisms of toxicity.

4.1.66 **APPENDIX TO PART F – CSR TEMPLATE WITH EXPLANATION**

4.1.67 **1.3. Physico-chemical properties**

4.1.68 **Table 5: Overview of physico-chemical properties**

4.1.69 It is recommended that the following rows be added to Table 5 (Appendix to Part F, pg. 13):

Property	Value	Remarks
Shape	Idem	Idem
Surface area	Idem	Idem

- 4.1.70 **R.7.1.1.1 Information requirements on physico-chemical properties**
- 4.1.71 **Table R.7.1-1**
- 4.1.72 It is recommended that the phrase “Granulometry (particle size distribution)” in Table R.7.1-1 (R.7a, pg. 18) is clarified to the word “Granulometry”, consistent with the Regulation and Guidance.
- 4.1.73 It is recommended that the additional relevant specific intrinsic properties of “Shape” and “Surface Area” are included in Table R.7.1-1 (R.7a, pg. 18).
- 4.1.74 **R.7.1.1.4 General testing strategy for physico-chemical properties**
- 4.1.75 **Tier 3**
- 4.1.76 It is recommended that the term “Particle Size Distribution” under R.7.1.1.4 “Tier 3” (R.7a, pg. 36) be amended to state “Granulometry”, consistent with the Regulation and Guidance.
- 4.1.77 It is recommended that the additional relevant specific intrinsic properties of “Shape” and “Surface Area” are included under R.7.1.1.4 “Tier 3” (R.7a, pg. 36).
- 4.1.78 **Figure R.7.1-1 Tiered testing scheme on physico-chemical testing**
- 4.1.79 It is recommended that the “particle size distribution” box in Tier 3 of Figure R.7.1-1 (R.7a, pg. 37) is updated to state “Granulometry”, consistent with the Regulation and Guidance.
- 4.1.80 It is recommended that boxes for the additional relevant specific intrinsic properties of “Shape” and “Surface Area” are included under Tier 3 of Figure R.7.1-1 (R.7a, pg. 37).
- 4.1.81 **R.7.1.1.6 Overall consistency of the physico-chemical profile**
- 4.1.82 **Table R.7.1-5 Summary of use of physico-chemical properties**
- 4.1.83 It is recommended that the following rows on shape and surface area are inserted at the end of Table R.7.1-5 (R.7a, pg. 45):

Test	Impact on other physico-chemical test	Impact on toxicology	Impact on ecotoxicology	Impact on Risk assessment
Shape		<p>Knowledge of high aspect ratio particles and surface area may inform interpretation of some toxicity test results.</p> <p>Shape is an important parameter in the characterisation of nanoparticles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media.</p>		
Surface area		Knowledge of surface area may inform interpretation of some toxicity test results.		

4.1.84 R.7.1.14 GRANULOMETRY

4.1.85 It is recommended that the addition of the following paragraph as a new first paragraph in R.7.1.14 GRANULOMETRY (R.7a, pg. 142) be considered:

4.1.86 The potential release of particles into the workplace or environment is an important consideration in the design and operation of many industrial processes and safe handling of substances. Release of particles may present a safety hazard and may cause adverse health effects to humans and affect the environment. It is therefore important to obtain data about the propensity of substances to be released as particles, allowing risks to be evaluated, controlled and minimised. Measurement of the release of particles from powdered substances has similarities to the conventional measurement of the dustiness of a powder, but with significant differences in the methods and instrumentations suited to different particle size ranges. It is worth noting that the particle size distribution and the behaviour of the airborne fraction may be different to those determined for the powdered substance.

4.1.87 In order to bring the current first paragraph of R.7.1.14 GRANULOMETRY (R.7a, pg. 142) up to date, it is recommended that the substitution of the current text with the following updated text: is considered:

4.1.88 The CEN document, EN 481 “Workplace Atmospheres – size fraction definitions for measurement of airborne particles” (CEN 1993) provides definitions of the inhalable, thoracic and respirable size fractions, and target specifications (conventions) for sampling instruments to measure these fractions. This standard defines sampling conventions for particle size fractions which are to be used in assessing the possible health effects resulting from inhalation of airborne particles in the workplace. In addition, the following recommended documents provide background information and sampling guidelines, representing the current state-of-the-art, to effectively characterise and monitor exposures in the workplace:

- Method for Determination of Hazardous Substances MDHS 14/3 “General methods for sampling and gravimetric analysis of respirable and inhalable dust” (HSE, 2000)
- “Stationary source emissions – Determination of mass concentration of particulate matter (dust) at low concentrations – manual gravimetric method” (BS ISO 12141:2002)
- “Stationary source emissions – Manual determination of mass concentration of particulate matter” (BS ISO 9096:2003)
- “Ambient air quality – Standard gravimetric measurement method for the determination of the PM_{2.5} mass fraction of suspended particulate matter” (BS ISO 14907:2005)
- “Workplace atmospheres – Ultrafine, nanoparticle and non-structured aerosols – Inhalation exposure characterization and assessment” (ISO/TR 27628:2007)
- “Nanotechnologies – Health and safety practices in occupational settings relevant to nanotechnologies” (ISO/TR 12885:2008)

4.1.89 The different particle sizes defined in EN 481 are:

- inhalable fraction (the mass fraction of particles that can be inhaled by nose and mouth). Particles >100 µm are not included in the inhalable convention;
- thoracic fraction (the mass fraction of particles that passes the larynx). It has been shown that 50% of the particles in air with an aerodynamic diameter of 10 µm belong to the thoracic fraction;
- respirable fraction (the mass fraction of particles that reaches the alveoli). It has been shown that 50% of particles with an aerodynamic diameter of 4 µm belong to the respirable fraction.

4.1.90 The first sentence of the current third paragraph of R.7.1.14 GRANULOMETRY (R.7a, pg. 143) commencing “Methods capable of particle size distribution measurement...” is recommended to be updated with references to the two more recent ISO technical reports as follows:

4.1.91 Methods capable of particle size distribution measurement can determine the appropriate fractions as defined in EN481 (CEN 1993), ISO/TR 27628:2007 and ISO/TR 12885:2008, using the aerodynamic diameter of a particle, which is the measure of its behaviour in air, as the basis of the measurement.

4.1.92 **Definition of granulometry**

4.1.93 It is recommended that the current first sentence of R.7.1.14 sub-section “Definition of granulometry” (R.7a, pg. 143) - which states “Details of methods for determining particle size distribution and for fibre length and diameter distributions are outlined in OECD TG 110 and HSE Guidance document on methods for measuring particle size distribution (1996)” - is removed from this section and reinserted in R.7.1.14.2 as specified below.

4.1.94 It is recommended that the following definition of Granulometry is included as a new first paragraph in R.7.1.14 sub-section “Definition of granulometry” (R.7a, pg. 143):

- 4.1.95 Particle size is a fundamental attribute of disperse materials. When a group of particles are of differing sizes, they may be described by a particle size distribution. Granulometry can be defined as the determination of particle size distribution.
- 4.1.96 It is recommended that the following sentence is inserted after the last sentence of the current second paragraph in R.7.1.14 sub-section "Definition of granulometry" (R.7a, pg. 143) ending "...measured in micrometres ($=10^{-6}$ m)":
- 4.1.97 When a group of particles are of differing sizes, they may then be described by a Particle Size Distribution.
- 4.1.98 **R.7.1.14.1 Information requirements on granulometry**
- 4.1.99 No change needed.
- 4.1.100 **R.7.1.14.2 Available information on granulometry**
- 4.1.101 **Testing data on granulometry**
- 4.1.102 It is recommended that the first paragraph of R.7.1.14.2 sub-section "Testing data on granulometry" (R.7a, pg. 144) is amended to the following text:
- 4.1.103 The characterisation of particle requires very careful sampling and sample fractionation practises to be followed. ISO 14488:2007 specifies methods for obtaining a test aliquot from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative with a defined confidence level. Further is available in the Sample Preparation section of the Introduction to R.7.
- Many methods are available for particle size measurements, but none of them is applicable to the entire size range (see Tables R.7.1-30 to R.7.1-33). Multiple techniques should be used where possible in order to formulate a complete understanding of the particle properties, and the optimum set of required techniques should be selected based on the specific substance and form under investigation. Methods for determining particle size distribution are designed to provide information on the transportation and sedimentation of

insoluble particles in water and air. The OECD test guideline applicable to measuring the particle size distribution is OECD TG110. It is important to note that Method A of OECD TG 110 (sedimentation, or centrifugation) is not considered applicable to nanomaterials (OECD, 2009), as it is useful only in the range $2 \mu\text{m} < R_s < 100 \mu\text{m}$. However, alternative standardised equipment (e.g. centrifugal sedimentation) can be used in accordance with this method. Method B of OECD TG 110 (electron microscopy) requires a necessary but minor deviation in the data reporting for nanomaterials (i.e. particles/fibres of less than 5 microns in length and less than 100 nm in diameter). Details of methods capable of measuring nanoparticle size distributions are provided in ISO/TR 27628:2007 and ISO/TR 12885:2008.

- 4.1.104 It is recommended that the last sentence of the current second paragraph of R.7.1.14.2 sub-section "Testing data on granulometry" (R.7a, pg. 144) is updated to the following:
- 4.1.105 They are applicable to water insoluble (i.e. water solubility $< 10^{-6}$ g/l) substances and cover the range $\sim 5\text{nm} - 100 \mu\text{m}$.
- 4.1.106 It is recommended that the last sentence of the current third paragraph of R.7.1.14.2 sub-section "Testing data on granulometry" (R.7a, pg. 144) stating "Fibres of length $< 5 \mu\text{m}$ need not be considered." is removed. This is on the basis of the granulometry guidance being provided to registrants in the context of characterising particle/fibre properties as well as informing the likely impact on hazard assessment. Disregarding fibres of length $< 5 \mu\text{m}$ is only on the grounds of using data gathered in the assessment of a fibre in the context of the WHO (1997) guidelines.
- 4.1.107 It is recommended that the first two sentences of the current fourth paragraph of R.7.1.14.2 sub-section "Testing data on granulometry" (R.7a, pg. 144) are updated to remove reference to shape, as follows:
- 4.1.108 Image analysis of particle size can be used to determine the aspect ratios of fibrous particles. Image analysis generates data by capturing direct images of each particle. This provides users with the ultimate sensitivity and resolution as subtle differences in particle size can be accurately characterised.

- 4.1.109 It is recommended that the last sentence of the current fourth paragraph of R.7.1.14.2 sub-section "Testing data on granulometry" (R.7a, pg. 144) and its bullet points, are removed as it is duplication of text in the preceding sentences.
- 4.1.110 It is recommended that **Figure R.7.1-7 Integrated testing strategy for granulometry** is removed from R.7.1.14.2 and replaced with a new ITS in R.7.1.14.5, as specified later.
- 4.1.111 It is recommended that the third column of **Table R.7.1-30** (R.7a, pg. 146) entitled "MMAD" is removed and replaced with a column on "Data type".

4.1.112 It is recommended that the first row of Table R.7.1-30 (R.7a, pg. 146) is amended to the following:

Method and details	Material and size range	Data type
<p>Optical microscopic examination</p> <p>It is preferable to prepare samples directly in order not to influence shape and size of the particles.</p> <p>This method determines distribution of particles of respirable and inhalable size and does not refer to airborne dust or dispersed or nebulised particles.</p> <p>Optical microscopy can be used to examine likelihood of fibres present by comparing similarities to known fibrous or fibre releasing substances or other data. Extreme care required during sample preparation to avoid fibre breaking and clumping. Care should also be taken to avoid contamination by airborne fibres. Samples might be prepared by (a) producing suspensions in water by gentle hand agitation or vortex mixing or (b) transfer of dry material onto copper tape either directly or by spraying of the dry fibres by use of atomiser or pipette. Length and diameter distributions should be measured independently at least twice and at least 70 fibres counted. No two values in a given histogram interval should differ by > 50% or 3 fibres, whichever is larger. The presence of long thin fibres would indicate a need for further, more precise measurements. This method might be suitable to determine the distribution of fibres of respirable and inhalable size.</p>	<p>Particles of all kinds, including fibres</p> <p>Size range: 0.2–5000 µm.</p> <p>Fibre diameters as small as 0.2 µm and as large as 100 µm and lengths as small as 5 µm and as large as 300 µm</p>	<p>Particle size/size distribution, from which mass median aerodynamic diameter (MMAD) can be calculated with knowledge of the particle density.</p> <p>Fibre number as defined by WHO (1997): Aspect ratio > 3:1, fibre length > 5 microns</p>

4.1.113 It is therefore recommended that the last row of the current Table R.7.1-30 (R.7a, pg. 146) on “determination of fibre length and diameter distributions” by light microscopy is removed.

4.1.114 It is recommended that the following additional rows of methods are added to Table R.7.1-30 (R.7a, pg. 146):

Method and details	Material and size range	Data type
<p>Transmission Electron Microscopy (TEM)</p> <p>TEM can be used for samples collected from the air or prepared in suspension on a TEM grid, including those from separation and sampling instruments. Powder preparation is very easy and fast for this method. TEM enables qualitative assessment of size and form of particles, and differentiation between agglomerates and primary particles. Quantitative determination of size distribution of primary particles is achievable in cases where agglomeration is not significant. TEM has a very high local resolution (nm) and is capable of imaging lattice planes and individual rows of atoms with resolution better than 0.2 nm. Additions to TEM can provide further information e.g. Scanning Transmission Electron Microscopy (STEM), High-Resolution TEM (HRTEM) or in-situ measurements using Environmental TEM, which offers the potential for dispersed samples to be characterised.</p> <p>However, TEM is a highly work-intensive method and requires manual preparation of samples. Dispersions need to be diluted (to ca. 1%) or prepared into work-intensive cryo-sections. Drying samples under vacuum for analysis may alter the size and shape of the particles being characterised. An extremely small area of the sample is analysed, which might not be representative enough. The comparatively small share of evaluated particles (ca. 1,000) results in limited statistical precision. Only a two-dimensional projection of particles is visible and can be evaluated; and the interpretation of pictures is difficult. Picture analysis is impossible if agglomeration is significant. Contours of particles may not be clearly resolved in some samples. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects.</p> <p>Further informative information on this method is available in ISO/TR 27628:2007. ISO/13322-1:2004 and ISO/13322-2:2006 provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.</p>	<p>Particles in solid, powder and suspension form.</p> <p>Size range: < 0.1 – 10 µm. Particularly suitable for the particle size range of 1 - 500 nm.</p>	<p>Particle size/size distribution, from which number/mass median diameter can be calculated with knowledge of the particle density.</p>
<p>Scanning Electron Microscopy (SEM)</p> <p>SEM can be used for samples collected from the air or prepared in suspension on a SEM grid,</p>	<p>Particles in solid, powder and suspension form.</p>	<p>Particle size/size distribution, from which</p>

<p>including those from separation and sampling instruments. Sample preparation is easier than for TEM, and only a small quantity of sample needed. Testing possible with undiluted dispersions and emulsions. SEM enables non-destructive testing of samples, and provides an image of the sample structure with very precise size determination at high local resolution. This method can be used in-situ as Environmental SEM.</p> <p>A representative sample of the material must be used. Where samples are not electrically conducting, plasma sputter-coating the surface-adhered particles with a layer of a conducting material is often required. This process may modify the sample being characterised. Only a small section of the sample is pictured and imaging is limited to surface features. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects.</p> <p>Further informative information on this method is available in ISO/TR 27628:2007. ISO/13322-1:2004 and ISO/13322-2:2006 provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.</p>	<p>Size range: < 0.01 – 10 µm. Particularly suitable for the particle size range of 10 nm – 1 µm.</p>	<p>number/mass median diameter can be calculated with knowledge of the particle density.</p>
<p>Centrifugal Sedimentation (ISO 13318-1:2001; ISO 13318-2:2007; ISO 13318-3:2004)</p> <p>Measures the particle size distribution of particulate materials dispersed in a liquid by fractionation. Centrifugal sedimentation methods are based on the rate of settling, under a centrifugal field, of particles in a liquid. The relationship between settling velocity and particle size reduces to the Stokes equation at low Reynolds numbers. Thus, the calculation of particle size using this method is dependent on Stokes law. This technique can be used to supply data in accordance with Method A of OECD TG 110.</p> <p>When using optical turbidity detection, the measuring range depends on the density of the material, the viscosity of the medium and the number of revolutions of the centrifuge. High absolute precision of particle size through calibration with a particle standard, and high resolution compared with other methods. A small quantity of sample is sufficient. This method involves fewer artefacts and possible errors than integral methods (e.g. light scattering), which measure all fractions together without separation. However, the measuring concentration is very low and therefore significant dilution is necessary. The potential for agglomeration must be considered, and the suspension / emulsion must be stable for analysis. A sedimentation liquid suitable for the sample must be determined, in which a density gradient can be established for measuring. The measuring time for samples with</p>	<p>Particulate materials dispersed in a liquid</p> <p>Size range: 0.1 to 5 µm</p>	<p>Settling velocity ($m s^{-1}$), from which particle size can be calculated based on Stokes law.</p>

<p>small particles is long. For evaluation, the density and optical constants of particles must be known. Evaluation of a fine fraction in a wide distribution can be critical.</p> <p>When using x-ray detection, the measuring range depends on the density of material. Implementation and evaluation is simple, without the need for calibration, gradients, mie correction or optical information. A high resolution of distribution spectra is possible, and only a small quantity of sample is required. This method provides good statistics, with 10^{10} particles assessed in one measuring activity. However, dilution to ~ 5% necessary and, for evaluation, the density of particles must be known.</p>		
<p>Ultrasonic spectroscopy (ISO/20998-1:2006)</p> <p>Allows determination of the size distribution of one or more material phases dispersed in a liquid. Measurements can be made for concentrations of the dispersed phase ranging from 0.1-50% by volume. Enables dynamic changes in the size distribution to be monitored, including agglomeration or flocculation in a concentrated system.</p> <p>However, this method is air- and temperature-sensitive. Parameter adjustment is complex. Measurement results may vary with different vol%.</p>	<p>Particles in colloids, dispersions and emulsions</p> <p>Size range: 10 nm - 3 mm</p>	<p>Attenuation spectrum, from which the particle size distribution based on mass/number can be extracted via a model (which may be empirical or based on first principles)</p>
<p>Small Angle X-ray Scattering (SAXS) (ISO/TS 13762:2001)</p> <p>Allows determination of the particle size distribution of ultra-fine powders and suspensions. The requirement for particle dispersion of the sample is not as strict as for other methods.</p> <p>SAXS cannot distinguish pores from particles and therefore cannot be used for powders consisting of porous particles. This method assumes that particles are isotropic and spherically shaped, and thus has limited applicability to powders containing particles whose morphology is far from spherical e.g. non-spherical nano-objects such as carbon nanotubes. In addition, due to the need for a concentrated sample, an interference effect between particles may arise.</p>	<p>Particles in powder and suspension form</p> <p>Size range: 1-300 nm</p>	<p>Average particle size for a sample, estimated by mathematical adaption of a diffractogram.</p>
<p>X-ray diffraction (XRD) (BS EN 13925-1, BS EN 13925-2 and BS EN 13925-3)</p> <p>XRD estimates the average particle size by mathematical adaptation of a simulated diffractogram to real measurement. Enables crystallinity to be quantified with high statistical</p>	<p>Single crystal or polycrystalline materials</p> <p>Crystallite size range: ~1-100 nm</p>	<p>Average particle size for a sample, estimated by mathematical</p>

<p>relevance, and avoids the need for representative sampling.</p> <p>Crystal structures of existing phases and equipment- and sample-specific parameters must be known. It is important to note that particle size does not equal crystallite size. Other factors can also influence the peak width, such as microstrain, lattice defects and temperature factors. Larger crystalline samples (>1mg) are required for analysis.</p>		<p>adaption of a diffractogram.</p>
<p>Dynamic Light Scattering (DLS)/Photon Correlation Spectroscopy (PCS) (ISO/22412:2008; ISO/13321:1996; ASTM E2490 – 09)</p> <p>Enables rapid and simple estimation of an average particle size and measurement of the broadness of the size distribution of sub micrometre-sized particles or droplets dispersed in liquids. For nanoparticles in suspension, DLS/PCS is one of the most commonly employed techniques providing in situ characterisation of size and size distribution and is often applied with zeta potential measurements to provide an indication of the particle suspension stability with respect to time and medium. Only a small quantity of sample is needed, and in the particle size range < 100 nm, no refractive indices are necessary. DLS/PCS is of particular benefit to toxicity assessment as it measures size in solutions that more accurately resemble the exposure conditions. An extension of this technique for high concentration opaque suspensions is Photon Cross Correlation Spectroscopy (PCCS), which provides particle size and stability of nanoparticle suspensions.</p> <p>However, extensive sample dilution is necessary. This method is of limited use when particles are difficult to maintain in a dispersed state or when particles of > 2 µm in size are present. This method is temperature sensitive and only enables low resolution. Optical parameters must be known for data analysis, and this method is not suitable for particles with different optical properties.</p> <p>It is noted that Dynamic Light Scattering (DLS) does not provide a full particle size distribution. DLS measures fluctuations in the intensity of scattered light caused by Brownian motion, from which the hydrodynamic diameter is calculated, enabling estimation of the particle size distribution. Thus, even though DLS does not measure particle size distribution directly, this method provides a good background for the estimation of the full particle size distribution. The method also provides a number (the 'polydispersity index') indicating the polydispersity of the particle population. There are several software routines that facilitate the calculation of a particle size distribution from DLS data, but the adequacy and the comparability of these routines needs to be further evaluated (Lövestam et al., 2010).</p>	<p>Particles or droplets dispersed in liquids</p> <p>Size range: 1 - 1000 nm</p>	<p>Size distribution based on mass/number. Average particle size and polydispersity index (dimensionless; measure of broadness of the size distribution).</p>

4.1.115 It is recommended that the text under the current Table R.7.1-30 (R.7a, pg. 147) is amended to the following:

4.1.116 Using the methods listed in Tables R.7.1-30 to R.7.1-33, the following information should be presented (as appropriate):

- Sample preparation methods and analysis methods used
- Lot number, sample number
- Suspending medium, temperature, pH
- Concentration (relevant to particles or fibres)
- Representative image(s) from microscopy
- Particle size distribution histogram from the applied measurement technique
- Average particle size(s) for resolvable peaks in the distribution, as mass, number and surface area per unit volume as appropriate
- Expected % change of reported values in the future (e.g. variations between production batches)
- Reference all Standards (e.g. ISO) and reference materials used.

4.1.117 It is recommended that the following paragraph is inserted after the bullet point list in the text under the current Table R.7.1-30 (R.7a, pg. 147):

4.1.118 Rules for the graphical representation of particle size analysis data in histograms, density distributions and cumulative distributions are specified in ISO 9276-1:1998. It also establishes a standard nomenclature to be followed to obtain the distributions mentioned above from the measured data. In a graphical representation of particle size analysis data, the independent variable, i.e. the physical property chosen to characterise the size of the particles, is plotted on the abscissa (x-axis). The dependent variable, which characterises the measure and type of quantity (e.g. number, mass) is plotted on the ordinate (y-axis). ISO 9276-2:2001 provides the relevant equations for the calculation of average particle sizes or average particle diameters and moments from a given particle size distribution. It is assumed that the size distribution is available as a histogram. It is nevertheless also possible to apply the same mathematical treatment if the particle size distribution is represented

by an analytical function. It is furthermore assumed in ISO 9276-2:2001 that the particle size of a particle of any other shape may also be represented by the diameter of an equivalent sphere, e.g. a sphere having the same volume as the particle concerned.

4.1.119 It is recommended that the paragraph starting “It is advantageous to have accurate information...” in the text under the current Table R.7.1-30 (R.7a, pg. 147) is updated to the following:

4.1.120 It is advantageous to have accurate information about the propensity of materials to produce particulate aerosol (including the *dustiness* of the material). No single method of dustiness testing is likely to represent and reproduce the various types of processing and handling used in industry. The measurement of dustiness depends on the test apparatus used, the properties of the dust and various environmental variables. The measurand of dustiness is the ratio of the inhalable dust produced by the dustiness test procedure, in milligrams, to the test mass of material used for the test, in kilograms. There are a number of methods for measuring the dustiness of bulk (non-nanoscale) materials, based on the biologically relevant aerosol fractions defined in EN 481. Two methods (the rotating drum method and the continuous drop method) are detailed in EN 15051 “Workplace atmospheres – Measurement of the dustiness of bulk materials – Requirements and reference test methods” (CEN, 2006).

The two methods in EN 15051, however, provide quite different results. A recent comparison of dustiness results for a range of minerals based on the two methods revealed a difference in classification for the respirable fraction for 50% of the tested materials. Considering the inhalable fraction, classification was different for 60% of the tested materials. There was no trend in the data. Consequently, a recommendation has been given within CEN to revise the standard. It is recommended to take the above information into account if results derived from these methods are intended to be used for classification and labelling purposes. However, an order of relative dustiness could be achieved by applying the same method to a range of materials.

The particle size distribution of a dust cloud may be different from the powder source. Studies on dust generation by free falling powders have demonstrated that the manner in which the powder is handled may be as important as the dust generating capacity of the material, in terms of the resulting exposure (e.g. Heitbrink et al., 1992). Falling height has an important influence on dust generation and release for more than one reason. The higher the impact, the more dissemination of dust there is. Moreover, the greater the falling height, the greater flow of entrained air, which favours dust dissemination. This shows the importance of process design and adequate work practices.

There have been many interesting studies on material flow which demonstrate that the influence of the various factors is not so obvious. For example, it is sometimes erroneously assumed that a powdered material with a larger proportion of coarse particles offers less dust hazard; however, a higher proportion of coarse particles in the material may actually increase dustiness due to a *decrease in the cohesion of the material as the proportion of coarse particles increases* (Upton et al., 1990), and also due to the agitation of the fine particles as there are more collisions with large particles. The higher the impact between particles, the more dissemination of dust there is.

The aerosolisation/sampling methods in Table R.7.1-31 are used in the determination of the distribution of respirable particles and (to a lesser extent) the distribution of inhalable particles. These methods generate aerosol test atmospheres and require coupled particle detection instrumentation.

The particle detection methods in Table R.7.1-33 can be used to characterise the distribution of aerosolised particles. These methods are preferred since they measure particles in the air and as such the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD), but are subject to limitations. All particle size instrumentation have ranges of particle size limited by the principle of operation. This is almost two decades. Therefore more than one type of instrument is often used with overlapping size ranges. Often depending on the material, these size distributions may not match exactly, because different measuring principles deliver different equivalent diameters. Moreover, the lower sizes of 1nm to 3 nm cannot be accurately measured in

aerosol measurement instrumentation because of diffusion losses in tubes or at the inlet of the instruments. Depending on the number based particle size distribution the particle number concentration will be determined too low and particle counters with different valid lower size limit will give different particle number concentrations. Aerosolisation of substances for particle size distribution characterisation also results in a degree of artificiality if the engineering set-up introduces an upper limit on the aerosol size as a result of the operational conditions (e.g. flow rate and exit orifice). The upper size limit can be predicted using Stoke's equation. Other methods that measure inhalable fractions only or that give no detailed distributions are detailed in Table R.7.1-32.

- 4.1.121 It is recommended that the last sentence of the current first paragraph on pg. 148 (R.7a) stating "These methods are only applied if light microscopic examination indicates likelihood that fibres are present." is removed.
- 4.1.122 It is recommended that the title of **Table R.7.1-31** (R.7a, pg. 149) is modified to "Methods to generate/sample airborne dispersed or nebulised particles".
- 4.1.123 It is recommended that cascade impaction, rotating drum method and continuous drop method are retained in Table R.7.1-31 and light scattering/diffraction is moved to a new table R.7.1-33 entitled "Methods of measuring airborne dispersed or nebulised particles".

4.1.124 It is recommended that Table R.7.1-31 (R.7a, pg. 149) is moved to directly below Table R.7.1-30 and updated as follows:

Method and details	Material and size range	Data type
<p>Cascade impaction</p> <p>Cascade impactors can be used to obtain the size distribution of an aerosol (or a dust cloud). Air samples are drawn through a device which consists of several stages on which particles are deposited on glass or glass fibre. Particles will impact on a certain stage depending on their size. The cut off size can be calculated from the jet velocities at each stage by weighing each stage before and after sampling and the MMAD derived from these calculations.</p> <p>A well established technique to measure the distribution of particles of respirable or inhalable size. However, cascade impaction may fail to describe the dimension of high aspect ratio nanoparticles when they no longer follow aerodynamic rules (Ma-Hock et al., 2007). Conventional cascade impactors will have size selective stages limited to the capture of particles greater than ~250 nm. This is a sampling method and also requires aerosolisation.</p> <p>ISO/TR 27628:2007 provides an informative description.</p>	<p>Particles in an aerosol</p> <p>Size range: 0.1-20 µm and 0.5-80 µm</p>	<p>MMAD can be determined via an appropriate coupled analytical technique.</p>
<p>Electrical Low Pressure Impactor (ELPI)</p> <p>ELPI is a type of cascade impactor that combines inertial collection with electrical particle detection to provide near-real-time aerosol size distributions for particles larger than 7 nm in diameter. Aerosol particles are charged in a unipolar ion charger before being sampled by a cascade impactor. The upper size limit of the instrument is 10 µm, but in practice reliable data can be obtained only up to about 2.5 µm due to significant losses at larger particle sizes. Collected aerosol particles are available for offline analysis, but this is also a limitation as it does not provide a direct measurement. It does however enable a range of off-line analytical methods to be used with samples, including electron microscopy and chemical speciation. ELPI has useful application in relation to exposure estimation.</p> <p>Data from the lowest stage have relatively large uncertainty due to losses and uncertainties of the true size channel width.</p> <p>ISO/TR 27628:2007 provides an informative description.</p>	<p>Particles in an aerosol</p> <p>Size range: 7 nm – 10 µm</p>	<p>MMAD can be determined via an appropriate coupled analytical technique.</p>

<p>Rotating drum method (EN 15051)</p> <p>This method is based on size selective sampling of an airborne dust cloud produced by the repeated lifting and dropping of a material in a rotating drum. Air drawn through the drum passes through a specially designed outlet and a 3-stage fractionating system consisting of two porous polyurethane foams and a membrane filter. The mass of dust collected on each collection stage is determined gravimetrically to give a direct measure of the biologically relevant size fractions. This method simulates a wide range of material handling processes in industry and determines the biologically relevant size functions of a material in the airborne state. Full size distributions can be obtained by analysing the contents on the dust collection stages.</p> <p>This method is suitable to determine the distribution of particles of respirable or inhalable size. Rotating drum dustiness tests are usually performed as three replicate tests and need quite large amounts of test material, typically 300–600 g. It has been highlighted that such large amounts of test material may not be practical if very toxic and/or costly materials are to be tested and there is a need for test systems that can be operated under controlled atmospheric environments using much smaller amounts of material (Schneider & Jensen, 2008).</p>	<p>Dry powders/granulates/friable products</p> <p>Size range: 0.5-10,000 µm</p>	<p>MMAD can be determined via an appropriate coupled analytical technique.</p>
<p>Continuous drop method (EN 15051)</p> <p>This method is based on the size selective sampling of an airborne dust cloud produced by the continuous single dropping of material in a slow vertical air current. The dust released by dropping material is conducted by the airflow to a sampling section where it is separated into the inhalable and respirable fractions.</p> <p>This method is suitable to determine the distribution of particles of respirable or inhalable size. The continuous single-drop method requires a total amount of 500 g for the required five single-test runs. It has been highlighted that such large amounts of test material may not be practical if very toxic and/or costly materials are to be tested and there is a need for test systems that can be operated under controlled atmospheric environments using much smaller amounts of material (Schneider & Jensen, 2008).</p>	<p>Dry powders/granulates/friable products</p> <p>Size range: 0.5-10,000 µm</p>	<p>MMAD can be determined via an appropriate coupled analytical technique.</p>

4.1.125 It is recommended that a new **Table R.7.1-33** entitled “Methods of measuring airborne dispersed or nebulised particles” is inserted directly after Table R.7.1-32 (R.7a, pg.150), with content as follows (N.B. This has implications for the numbering of Tables in the rest of R.7.1.)

Method and details	Material and size range	Data type
<p>Scanning Mobility Particle Sizer (SMPS) (ISO 15900:2009; ISO 10808:2010; ISO 28439:2011)</p> <p>SMPS operates by charging particles and fractionating them based on their mobility when passing between electrodes. This method combines a Differential Mobility Analyser (DMA) and an Optical Particle Counter (OPC). SMPS detects and counts nanoparticles, and enables measurement of the particle size distribution and count median diameter of nano aerosols, up to 10^9 particles /cm³. This method also allows evaluation of nanoparticle surface area, mass dose, composition and dispersion to support effective analysis of inhalation toxicity testing results. SMPS also has useful application in relation to exposure estimation.</p> <p>Measurement with SMPS is the only currently available method that meets all of the following requirements in the size range below 100 nm: i) measurement of particle size distribution during particle exposures in a continuous manner with time resolution appropriate to check stability of particle size distribution and concentration; ii) measurement range of particle sizes and concentrations covers those of the nanoparticle aerosols exposed to the test system during the toxicity test; iii) particle size and concentration measurements are sufficiently accurate for nanoparticle toxicity testing and can be validated by ways such as calibration against appropriate reference standards; iv) resolution of particle sizing is sufficiently accurate to allow conversion from number-weighted distribution to surface area-weighted or volume-weighted distribution.</p> <p>However, SMPS is relatively slow and requires a scanning approach to measure different size intervals in series. This method is restricted to ambient temperatures below 35 °C (due to evaporation of butanol in the CPC) and requires aerosolisation of the sample. SMPS cannot distinguish between agglomerates and primary particles. For non-spherical particles (e.g. HARN), estimation of diameter and mass concentration by SMPS can result in significant error. Assembling data of measurements from SPMS and OPC to provide a whole picture of particle size distribution is not appropriate, due to the different principles employed by the two methods (Ma-Hock et al., 2007). It is important to know the stability of the source, since rapid changes of the size distribution, particle concentration, or both, can affect measurement of the size distribution. This is relevant to consider for nanomaterials, which have a high tendency to agglomerate in the</p>	<p>Particles in an aerosol</p> <p>Size range: ~3 – 800 nm</p>	<p>Size distribution based on number counted (number count per size interval). From the distribution, MMAD can be calculated, with knowledge of the density of the particles.</p>

atmosphere.		
<p>Fast Mobility Particle Sizer (FMPS)</p> <p>FMPS enables determination of the size distribution of sub-micrometer aerosol particles, up to 10^7 particles / cm^3 (depending on particle size). Measurements can be made with a time resolution of one second or less, enabling visualisation of particle size distributions in real time. However, FMPS is typically less sensitive than the SMPS at low particle concentrations.</p>	<p>Particles in an aerosol</p> <p>Size range: ~5 - 560 nm</p>	<p>Size distribution based on number counted (number count per size interval). From the distribution, MMAD can be calculated, with knowledge of the density of the particles.</p>
<p>Diffusion batteries</p> <p>The operation of diffusion batteries is based on the Brownian motion of the aerosol particles. Depositional losses through diffusion are a function of particle diameter. By measuring diffusion-based deposition rates through systems with varying geometries, it is possible to determine particle size distribution. The deposition systems are usually placed together in series to form a diffusion battery. The diffusion battery can be designed for determination of particle sizes as low as 2 nm depending upon instrument setup. This method has useful application in relation to exposure estimation.</p> <p>The primary property measured is the diffusion coefficient of the particles and this has to be converted to particle diameter. The instrument needs to be operated with a particle counter (typically a continuous flow Condensation Particle Counter) in order to determine the number concentration before and after each diffusion stage. Inversion of the raw data to real size distribution is complex and the solutions of the equations do not give unambiguous results in the case of polydisperse aerosol size distributions.</p> <p>ISO/TR 27628:2007 provides an informative description of this method.</p>	<p>Particles in an aerosol</p> <p>Size range: 0.005 – 0.1 μm</p>	<p>Particle number in intervals according to diffusion diameter, from which the median diffusion diameter can be determined with knowledge of the density of the particles.</p>
<p>Optical Particle Counter (OPC)</p> <p>OPC is a widely used method for detecting and counting aerosolised particles, and operates across a wide temperature range (0 – 120 °C). Enables agglomerates/aggregates of primary particles to be measured and counted. OPC has useful application in relation to exposure estimation.</p>	<p>Particles in an aerosol</p> <p>Size range: 0.3 – 17 μm</p>	<p>Particle number concentration</p>

<p>However, OPC is insensitive to particles smaller than approximately 100-300 nm in diameter and provides insufficient coverage of potential primary particle. Assembling data of measurements from SPMS and OPC to provide a whole picture of particle size distribution is not appropriate, due to the different principles employed by the two methods (Ma-Hock et al., 2007).</p> <p>ISO/TR 27628:2007 provides an informative description of this method.</p>		
<p>Laser scattering/diffraction</p> <p>In general, the scattering of the incident light gives distinct pattern which are measured by a detector. This technique is particle property dependent – i.e. material has unique scattering and diffraction properties which are also particle size dependent. It is important to calibrate the instrument with similar material (of the same size range as the material to be measured). Laser scattering techniques are suitable for geometric particles, viz spheres, cubes and monocrystals. Particle size will be established optically. The MMAD can be calculated by means of a calculation correction.</p> <p>The method is suitable to determine the distribution of particles of respirable and inhalable size. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 1:3 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.</p>	<p>Particles of all kind</p> <p>Size range: 0.06-100 µm</p>	<p>Particle size/size distribution*, from which mass median diameter can be calculated, with knowledge of the density of the particles.</p>
<p>Light scattering aerosol spectrometer (LSAS)</p> <p>LSAS is a type of light scattering instrument, applicable for measuring the size, number concentration and number/size distribution of particles suspended in a gas. LSAS can be used for the determination of the particle size distribution and particle number concentration at relatively high concentrations of up to 10^{11} particles/m³.</p> <p>The large measurement range of LSAS may result in high uncertainty in nanoscale measurements. Measurements may be dependent on the reflectivity of particles. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 1:3 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.</p>	<p>Particles in an aerosol</p> <p>Size range: 0.06 - 45 µm</p>	<p>Particle size/size distribution*, from which mass median diameter can be calculated, with knowledge of the density of the particles.</p>

- 4.1.126 It is recommended that the bullet point list in the text under the current Table R.7.1-31 (R.7a, pg. 150) is removed, as this has been combined with the updates suggested earlier which is relevant to all tables of methods.
- 4.1.127 It is recommended that Table R.7.1-32 “Methods that measure inhalable fractions only or that give no detailed distributions” (R.7a, pg. 151) is moved to directly below Table R.7.1-31.
- 4.1.128 It is recommended that the sub-section on “Reference substances” directly after the current Table R.7.1-32 (R.7a, pg. 151) is removed, as this is now sufficiently covered in R7.1.1.3 sub-section on “Experimental data” (R.7a, pg. 27) following earlier proposed updates.
- 4.1.129 **R.7.1.14.2 Available information on granulometry**
- 4.1.130 **Published data on granulometry**
- 4.1.131 It is recommended that current text in R.7.1.14.2 sub-section “Published data on granulometry” (R.7a, pg. 152) is updated to the following:
- 4.1.132 Particle size measurements have been published in the peer-reviewed literature. No electronic databases that are specific to particle size data could be found at the time of publication.
- 4.1.133 **R.7.1.14.3 Evaluation of available information on granulometry**
- 4.1.134 It is recommended that the first sentence of the first paragraph of R.7.1.14.3 Evaluation of available information on granulometry (R.7a, pg. 152) is updated to the following:
- 4.1.135 The particle size distribution characterisation is carried out on the material under investigation and, where appropriate, on the airborne dust.
- 4.1.136 It is recommended that the second sentence of the first paragraph of R.7.1.14.3 Evaluation of available information on granulometry (R.7a, pg. 152) is updated to the following:
- 4.1.137 It is important to note that the original particle size distribution is highly dependent on the industrial processing methods used and care should be

taken to ensure that the measurement and assessment activity considers any changes to the particle size distribution by subsequent environmental or human transformations.

4.1.138 The asterisk within the fourth sentence starting “Great care should be taken on*...” should be removed as it does not link to any information.

4.1.139 The first sentence of the second paragraph of R.7.1.14.3 Evaluation of available information on granulometry (R.7a, pg. 152) is recommended to be updated to:

4.1.140 Methods which determine the particle size distribution and MMAD need the generation of representative test atmospheres using suitable generation equipment and correct sampling techniques.

4.1.141 **Experimental data on granulometry**

4.1.142 No change required.

4.1.143 **Non-experimental data on granulometry**

4.1.144 No change required.

4.1.145 **Remaining uncertainty on granulometry**

4.1.146 The last sentence of the first paragraph of R.7.1.14.3 Evaluation of available information on granulometry sub-section “Remaining uncertainty on granulometry” (R.7a, pg. 152) is recommended to be updated to:

4.1.147 The effect of impurities should be considered when measuring fibre length and particle size distributions.

4.1.148 It is recommended that the following paragraph is inserted at the end of the current R.7.1.14.3 Evaluation of available information on granulometry sub-section “Remaining uncertainty on granulometry” (R.7a, pg. 152):

4.1.149 Aerosolisation of substances for particle size distribution characterisation also results in a degree of artificiality if the engineering set-up introduces an upper limit on the aerosol size as a result of the operational conditions (e.g. flow rate and exit orifice). The upper size limit can be predicted using Stoke’s equation.

4.1.150 R.7.1.14.4 Conclusions on granulometry

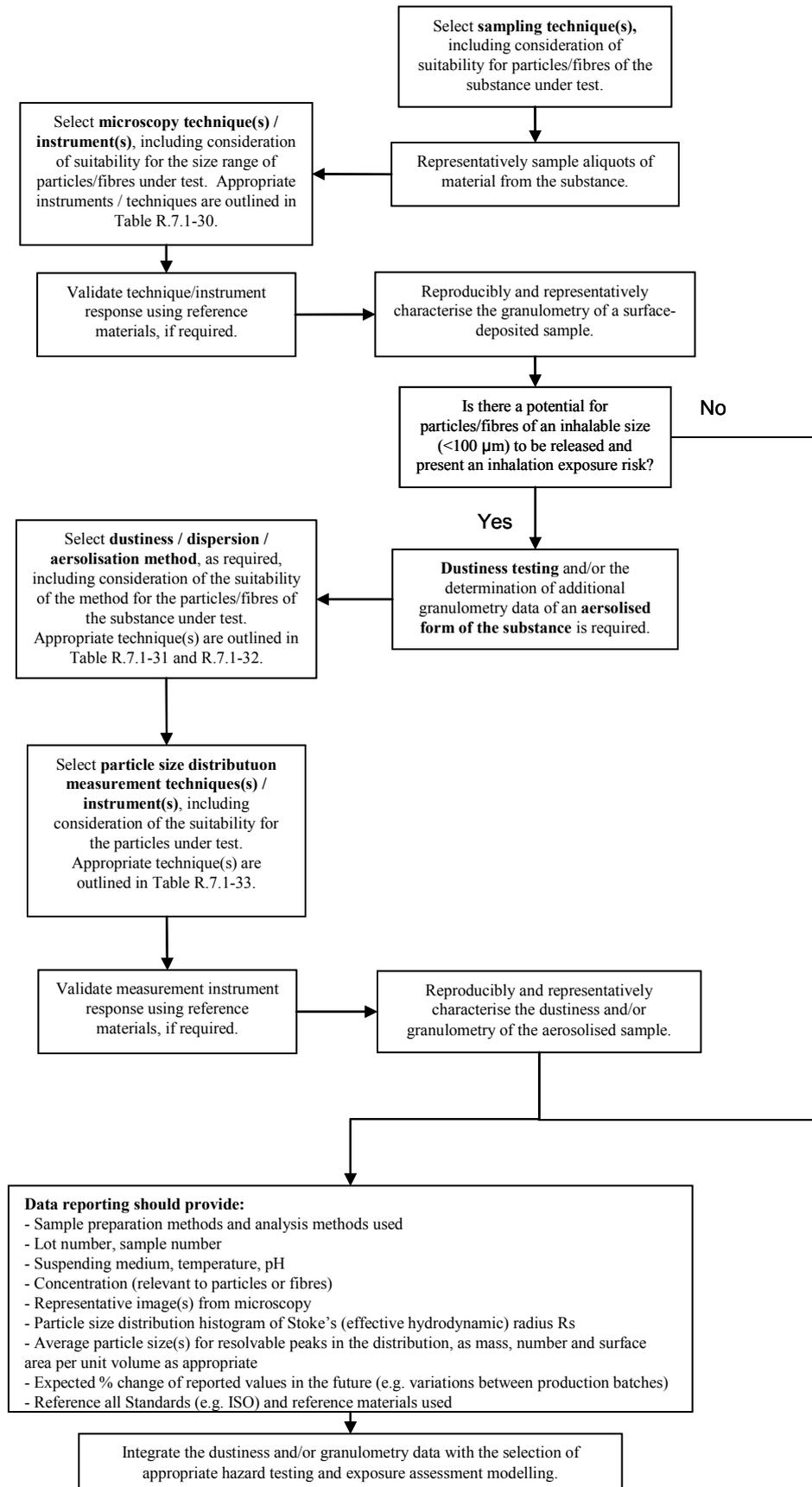
4.1.151 It is recommended that the current text in section R.7.1.14.4 Conclusions on granulometry (R.7a, pg. 153) is updated to the following:

4.1.152 The potential release of particles into the workplace or environment is an important consideration in the design and operation of many industrial processes and safe handling of substances. Release of particles may present a safety hazard and could cause adverse health effects to humans and affect the environment. It is therefore important to obtain data about the propensity of substances to be released as particles or fibres, allowing risks to be evaluated, controlled and minimised. Measurement of the release of particles from powdered substances has similarities to the conventional measurement of the dustiness of a powder, but with significant differences in the methods and instrumentations suited to different particle size ranges.

In addition, the particle size distribution is needed to inform the decision regarding which route of administration is most appropriate for the acute toxicity and 28-day base set animal studies. A number of methods are provided for determining the particle size fractions which are then used to assess the possible health effects resulting from inhalation of airborne particles in the workplace. A number of methods covering different ranges of particle sizes are available though none of them is applicable to the entire size range. Multiple techniques should be used where possible in order to formulate a complete understanding of the particle properties, and the optimum set of required techniques should be selected based on the specific substance and form under investigation.

4.1.153 R.7.1.14.5 Integrated testing strategy (ITS) for granulometry**4.1.154 Figure R.7.1-7 Integrated Testing Strategy for Granulometry**

4.1.155 It is recommended that the current Figure R.7.1-7 Integrated testing strategy for granulometry (R.7a, pg. 145) is deleted and replaced with the following Figure, which has been developed to be consistent with the recommendations for updated Guidance text for Granulometry:



4.1.156 **Examples and case studies on granulometry**

4.1.157 With regard to the examples and case studies on granulometry in the current guidance (R.7.1.14.5), a more appropriate description of the issues and typical data from granulometry should be developed, which will be dependant upon the accepted composition of the granulometry Information Requirement (i.e. whether shape and surface area are sub-ordinate to granulometry or included as additional relevant specific intrinsic properties). At this time it is recommended that current text is removed.

4.1.158 Suitable text for inclusion in this section can be developed for nanomaterials from the detailed work undertaken in the RIP-oN2 project. However, this needs to be accompanied by other text on non-nano materials before recommending update to guidance, which is out with the remit of the RIP-oN2 project.

4.1.159 **R.7.1.14.6 References on granulometry**

4.1.160 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.1.14.6 (R.7a, pg. 154):

4.1.161 ASTM E2490 – 09. “Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS)”, ASTM.

4.1.162 BS EN 13925-1:2003. “Non-destructive testing. X-ray diffraction from polycrystalline and amorphous materials. General principles”, BSI, WEE/46.

4.1.163 BS EN 13925-2:2003. “Non-destructive testing. X-ray diffraction from polycrystalline and amorphous materials. Procedures”, BSI, WEE/46.

4.1.164 BS EN 13925-3:2005. “Non-destructive testing. X-ray diffraction from polycrystalline and amorphous materials. Instruments”, BSI, WEE/46.

4.1.165 ISO 9276-1:1998. “Representation of results of particle size analysis -- Part 1: Graphical representation”, ISO TC 24/ SC 4.

4.1.166 ISO 9276-2:2001. “Representation of results of particle size analysis -- Part 2: Calculation of average particle sizes/diameters and moments from particle size distributions”, ISO TC 24/ SC 4..

- 4.1.167 ISO 10808:2010. "Nanotechnologies -- Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing", ISO TC 229.
- 4.1.168 ISO/TR 12885:2008. "Nanotechnologies -- Health and safety practices in occupational settings relevant to nanotechnologies"
- 4.1.169 ISO 13318-1:2001. "Determination of particle size distribution by centrifugal liquid sedimentation methods -- Part 1: General principles and guidelines" ISO TC 24/SC 4
- 4.1.170 ISO 13318-2:2007. "Determination of particle size distribution by centrifugal liquid sedimentation methods -- Part 2: Photocentrifuge method", ISO TC 24/SC 4
- 4.1.171 ISO 13318-3:2004. "Determination of particle size distribution by centrifugal liquid sedimentation methods -- Part 3: Centrifugal X-ray method", ISO TC 24/SC 4
- 4.1.172 ISO 13321:1996. "Particle size analysis -- Photon correlation spectroscopy", ISO TC 24/ SC 4.
- 4.1.173 ISO 13322-1:2004. "Particle size analysis -- Image analysis methods -- Part 1: Static image analysis methods", ISO TC 24/ SC 4.
- 4.1.174 ISO 13322-2:2006. "Particle size analysis -- Image analysis methods -- Part 2: Dynamic image analysis methods" ISO TC 24/ SC 4.
- 4.1.175 ISO TS 13762:2001. "Particle size analysis -- Small angle X-ray scattering method", ISO TC 24/ SC 4.
- 4.1.176 ISO 15900:2009. "Determination of particle size distribution -- Differential electrical mobility analysis for aerosol particles", ISO TC 24/SC 4.
- 4.1.177 ISO 20998-1:2006. "Measurement and characterization of particles by acoustic methods -- Part 1: Concepts and procedures in ultrasonic attenuation spectroscopy" ISO TC 24/ SC4.
- 4.1.178 ISO 22412:2008. "Particle size analysis -- Dynamic light scattering (DLS)", ISO TC 24/ SC4.

- 4.1.179 ISO/TR 27628:2007 "Workplace atmospheres – Ultrafine, nanoparticle and non-structured aerosols – Inhalation exposure characterization and assessment", ISO TD 146/SC 2.
- 4.1.180 ISO 28439:2011. "Workplace atmospheres -- Characterization of ultrafine aerosols/nanoaerosols -- Determination of the size distribution and number concentration using differential electrical mobility analysing systems", ISO TC 146/SC 2.
- 4.1.181 Lövestam, G., Rauscher, H., Roebben, G., Sokull Kluttgen, B., Gibson, N., Putaud, J.-P. & Stamm, H. 2010, "Considerations on a definition of nanomaterial for regulatory purposes", JRC Reference Reports, Joint Research Centre, European Commission.
- 4.1.182 Ma-Hock, L., Gamer, A., Landsiedel, R., Leibold, E., Frechen, T., Sens, B., Huber, G., van Ravenzwaay, B. 2007, "Generation and Characterization of Test Atmospheres with Nanomaterials," Inhalation Toxicology, 19(10), pp. 833-848.
- 4.1.183 OECD. 2009, "Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)21, Paris.
- 4.1.184 Schneider, T. & Jensen, K.A. 2008, "Combined single-drop and rotating drum dustiness test of fine to nanosize powders using a small drum", Annals of Occupational Hygiene, vol. 52, no. 1, pp. 23-34.
- 4.1.185 World Health Organization (WHO). (1997). Determination of airborne fibre number concentrations. A recommended method, by phase-contrast optical microscopy (membrane filter method). Geneva.
- 4.1.186 Subject to acceptance of the recommended guidance changes above, the following references would need to be removed from R.7.1.14.6 (R.7a, pg. 154):
- 4.1.187 Brandrup, J. and Immergut, E.H. (1989) eds "Polymer Handbook", 3rd edition, Wiley.
- 4.1.188 Community Bureau of Reference (1979) "Certification Report on Particles of Defined Particle Size", Brussels

- 4.1.189 Hazard Prevention and Control in the Work Environment: Airborne Dust, WHO/SDE/OEH/99.14 Chapter 1 – Dust: Definitions and Concepts
- 4.1.190 HSE (1996) The Notification of New Substances (NONS): Guidance Document on methods for measuring Particle Size Distribution

4.1.191 **Guidance update for “GRANULOMETRY” recommended if the scope of the term “Granulometry” and the corresponding information requirement IS considered to include the recommended additional relevant specific intrinsic properties “Shape” and “Surface Area”**

4.1.192 **Part B: Hazard Assessment**

4.1.193 **B.6.1.4 Other physico-chemical properties**

4.1.194 It is recommended that the following paragraph on Granulometry is added to the end of section B.6.1.4. (PART B – HAZARD ASSESSMENT, pg. 21), following the paragraph ending “...are preferred over other determinations of K_{ow} ”:

4.1.195 **Granulometry**, the characterisation of particle-related properties which can include size distribution, shape and surface area, amongst other properties, is an important parameter to consider particularly from a hazard assessment perspective. Determination of the particle size fractions are used to assess the possible health effects resulting from inhalation of airborne particles in the workplace. The inhalable size range of particles is important in determining not only if the situation poses an inhalation problem, but also where in the respiratory tract the particles may deposit. Therefore, the particle size distribution can be used as an argument when considering inhalation testing. A number of methods covering different ranges of particle sizes are available, although none of them is applicable to the entire size range. The particle size distribution is needed in order to decide which route of administration is most appropriate for the acute toxicity and 28-day base set animal studies. Particle size is also a factor in environmental exposure assessment. Shape is an important parameter in the characterisation of particles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape. Specific surface area is an important parameter in the characterisation of nanomaterials, with emerging evidence of quantitative value as an additional dose metric / descriptor for hazard assessment. The specific surface area will dictate the surface charge in cases where nanomaterials are surface functionalised, with direct consequences on nanomaterial interaction (i.e., agglomeration) with

other naturally occurring particulate, route of exposure as a function of surface ligand-biological interface and mechanisms of toxicity.

4.1.196 **R.7.1.1.1 Information requirements on physico-chemical properties**

4.1.197 **Table R.7.1-1**

4.1.198 It is recommended that the phrase “Granulometry (particle size distribution)” in Table R.7.1-1 (R.7a, pg. 18) is clarified to the word “Granulometry”, consistent with the Regulation and Guidance.

4.1.199 **R.7.1.1.4 General testing strategy for physico-chemical properties**

4.1.200 **Tier 3**

4.1.201 It is recommended that the term “Particle Size Distribution” under R.7.1.1.4 “Tier 3” (R.7a, pg. 36) should be amended to state “Granulometry”, consistent with the Regulation and Guidance.

4.1.202 **Figure R.7.1-1 Tiered testing scheme on physico-chemical testing**

4.1.203 It is recommended that the “particle size distribution” box in Tier 3 of Figure R.7.1-1 (R.7a, pg. 37) is updated to state “Granulometry”, consistent with the Regulation and Guidance.

4.1.204 **R.7.1.1.6 Overall consistency of the physico-chemical profile**

4.1.205 **Table R.7.1-5 Summary of use of physico-chemical properties**

4.1.206 It is recommended that the Granulometry row in Table R.7.1-5 (R.7a, pg. 44) is updated as follows:

Test	Impact on other physico-chemical test	Impact on toxicology	Impact on ecotoxicology	Impact on Risk assessment
Granulometry		<p>Computation of inhalable, thoracic and respirable fractions as a function of size of particles.</p> <p>Knowledge of high aspect ratio particles and specific surface area may inform interpretation of some toxicity test results.</p> <p>Particle shape is an important parameter in the characterisation of nanoparticles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media.</p>		

4.1.207 **R.7.1.14 GRANULOMETRY**

4.1.208 The suggested new guidance chapters for shape and surface area, provided in Section 4.2 of this document, are organised according to the standard chapter structure of R.7a. It is therefore proposed that, if the Information Requirement of Granulometry subsumes shape and surface area, the updated R.7.1.14 Granulometry chapter should become an amalgamation of: i) the proposed updated guidance on Granulometry (which would now equate to particle size distribution guidance), ii) suggested guidance text for shape iii) suggested guidance text for surface area, sub-divided by sub-headings for each property under the standard section headings of the chapter.

4.1.209 An existing example of this approach, for reference, is the current guidance chapter R.7.1.10 FLAMMABILITY (R.7a, pg. 121) which incorporates guidance for: i)

pyrophoricity, ii) flammability on contact with water, and iii) flammability, structured simply by including sub-headings for each property under the standard section headings of the chapter.

4.1.210 In the interests of avoiding the repetition of large amounts of text within this report, a merged granulometry chapter of these three sets of proposed text has not been produced here.

4.1.211 **Definition of granulometry**

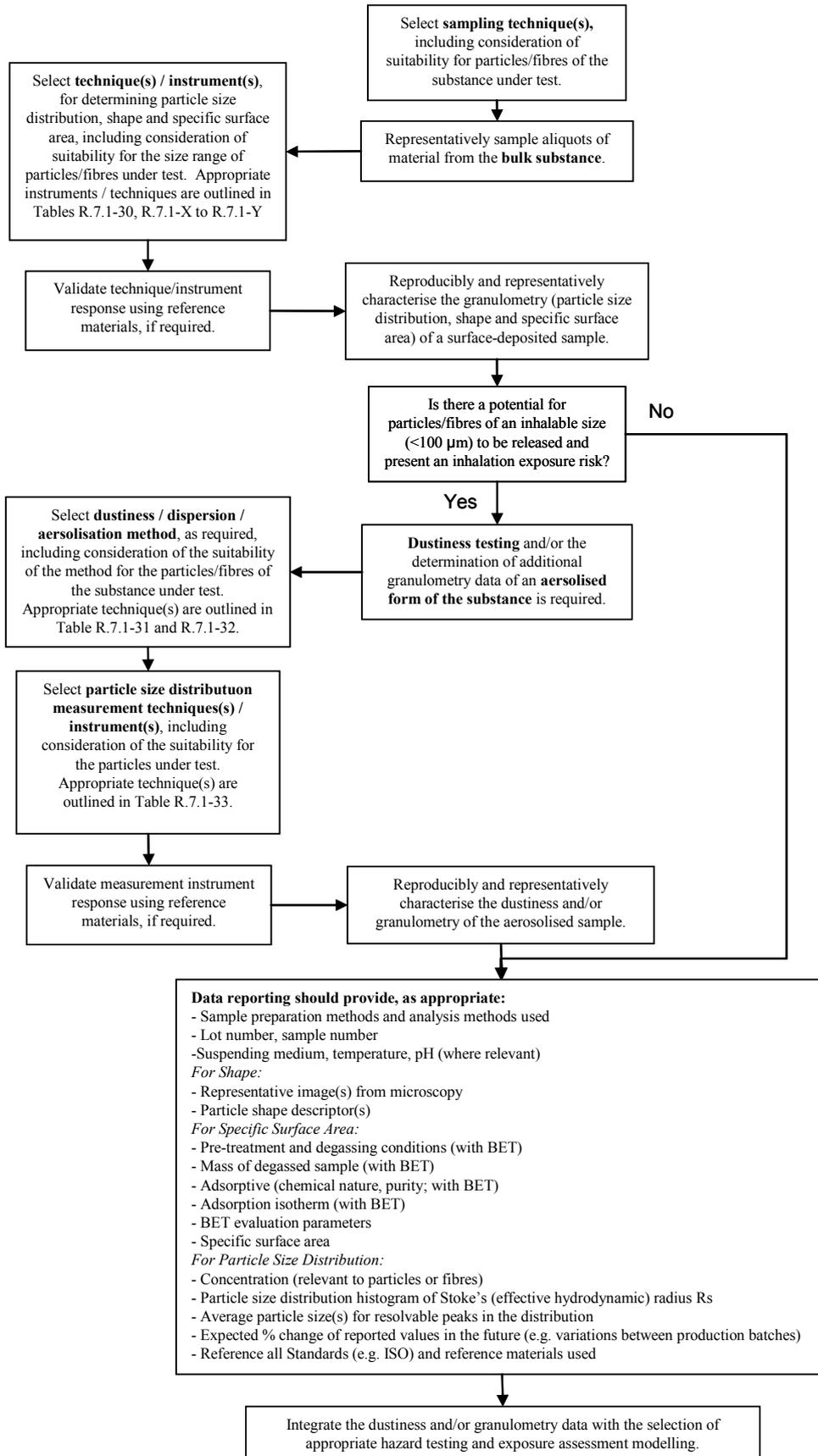
4.1.212 The only additional amended text that would be needed using this approach is an updated definition of Granulometry, encompassing all three properties. It is recommended that the following definition of Granulometry should be used in this case (R.7a, pg. 143):

4.1.213 Particles exist as populations with a range of sizes, shapes, morphologies and compositions, which may be altered by their surrounding environment and the action of forces. Granulometry can be defined as the characterisation of particle-related properties which can include size distribution, shape and surface area, amongst other properties.

4.1.214 Definitions of particle size distribution, shape and surface area, as provided earlier in this document, should then be included under property-specific sub-headings below this.

4.1.215 **R.7.1.14.5 Integrated testing strategy for granulometry**

4.1.216 In the case of R.7.1.14.5 (R.7a, pg. 153), it is proposed that the previously recommended updated integrated testing strategies for particle size distribution, shape and surface area could be combined as follows to provide an overall ITS for Granulometry (if the Information Requirement of Granulometry subsumes shape and surface area):



4.1.217 OTHER ISSUES

4.1.218 Integrated testing strategies

4.1.219 It is recommended that, during their overall review of the guidance, ECHA harmonises what is stated in Annex VII-X with what is stated in the general and specific ITS sections of Guidance R.7a.

4.1.220 **R.7.1.15.1 Information requirements on adsorption/desorption**

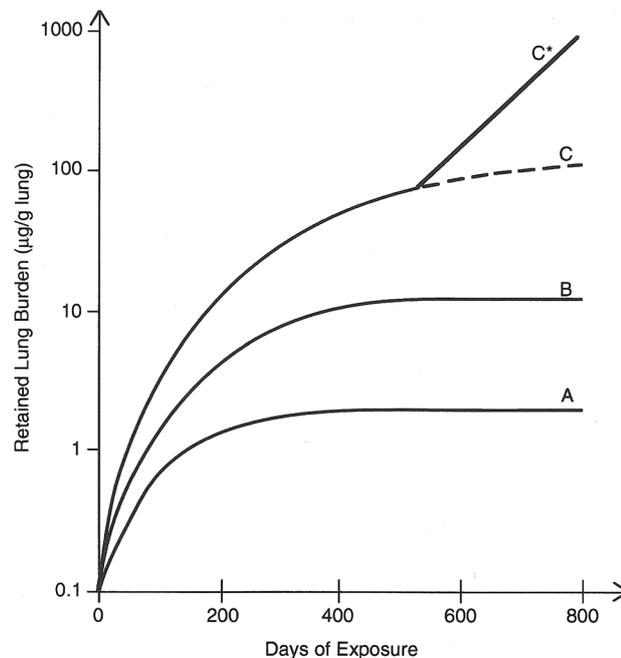
4.1.221 The following text is recommended to be added as a sixth paragraph in R.7.1.15.1 (R.7a, pg. 157), following the paragraph ending "...higher tiered testing data accordingly":

4.1.222 With regard to nanomaterials the distribution coefficient K_d has to be based on actual testing using one of the methods for the measurement of adsorption outlined in Table 7.1-33 since estimations of K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}) have no or questionable merit when it comes to nanomaterials.

4.1.223 TOXICOLOGICAL INFORMATION REQUIREMENTS

- 4.1.224 The current R.7A guidance document does not provide for a general introduction to the endpoints or issues, as it does for physico-chemical properties in Section R.7.1. It is proposed that development of a new introductory section is considered, facilitating the inclusion of the cross-cutting toxicological aspects, such as those proposed below from the considerations for nanomaterials. This should also acknowledge the importance of characterisation and sample preparation, which have been recommended for inclusion in the introduction of R.7a.
- 4.1.225 Within RNC/RIP-oN2/B1/2/FINAL, RNC/RIP-oN2/B2/2/FINAL and RNC/RIP-oN2/B3/2/FINAL several aspects of guidance have been identified that, whilst not requiring a specific amendment, could benefit from the insertion of an advisory note. Specifically this was identified in situations such as the consideration of lung overload phenomena, assay interference and the suitability of bacterial mutation assays for assessing particles. Due to their applicability to several endpoints, advisory notes on lung overload and assay interference should be included in the Introduction to R.7 or in a new toxicological specific introduction prior to the commencement of Section R.7.2. which marks the beginning of the toxicology section of Guidance R.7a with *Skin and Eye Irritation/Corrosion and Respiratory Irritation*.
- 4.1.226 These advisory notes do not propose a protocol but aim to provide useful advice and references to relevant resources. These advisory notes are outlined below with a special emphasis on nanomaterial examples and could need to be complemented with notes related to other materials.
- 4.1.227 **Advisory note on the consideration of rat lung overload within inhalation toxicity assessment**
- 4.1.228 The term 'lung overload' or 'particle overload' as it is also known, is a phenomenon associated with exposure to poorly soluble, low toxicity (PSLT) particles and occurs when a threshold dose of particles is achieved within the lung. During chronic exposure to PSLT particles, the lung burden of particles increases until a steady state or equilibrium is achieved between deposition and clearance of particles (Miller 2000) as shown by the A, B and C traces in Figure R.7-Z. Below the lung overload threshold, particles are cleared via

normal mechanisms at a normal clearance rate, generating little or no appreciable response.

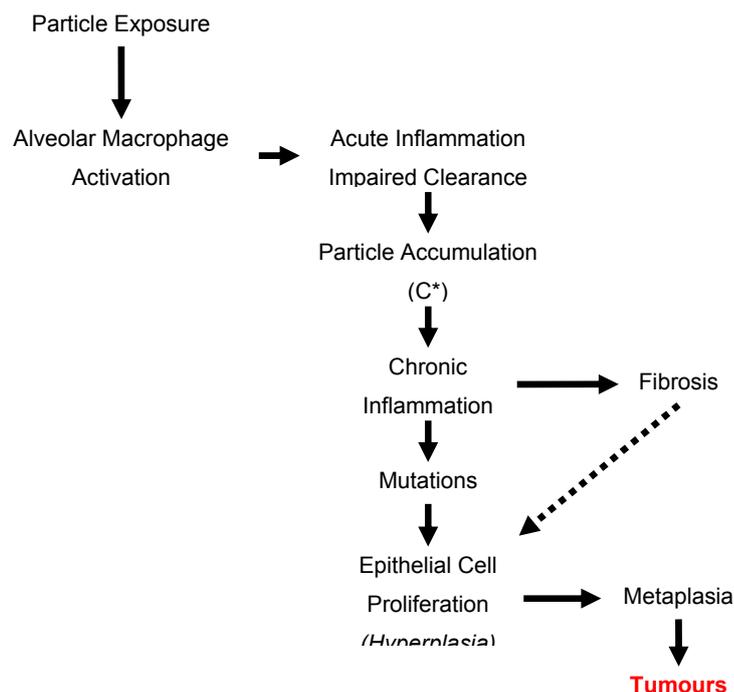


4.1.229 **Figure R.7-Z:** Schematic representation of the relationship between retained lung burden and length of exposure leading to the phenomenon of lung overload. Curves A, B, and C are associated with progressively increasing exposure concentrations. If the exposure level is sufficiently high and the length of exposure sufficiently long, alveolar macrophage-mediated clearance of particle can be overwhelmed. When this occurs, retained lung burden increases linearly with further exposure (curve C*). Reproduced from Miller (2000).

4.1.230 However, once threshold has been reached, the clearance mechanisms of the lung become overloaded which is typified by a progressive reduction of particle clearance from the deep lung, reflecting a breakdown in alveolar macrophage (AM)-mediated dust removal due to the loss of AM mobility. This is shown in the C* trace of Figure R.7-Z1 whereby at the point of threshold,

particle retention occurs exponentially rather than an equilibrium being established (as demonstrated by the dashed line).

4.1.231 The result of this rapid net increase in particle accumulation is lung inflammation, cessation of alveolar-mediated clearance and an increase in accumulation of particle laden macrophages within the lung alveoli. The continued build up of particles leads to a higher rate of transfer to lymph nodes and accumulation of particles in the lung interstitia. Persistent inhalatory exposure leads to chronic inflammation which in turn is likely to lead to fibrosis, alveolar cell proliferation (hyperplasia), the conversion of cells to cell types not normal associated with the specific lung location (metaplasia). The final result maybe local tumour formation (neoplasia) as shown in Figure R.7-Z2 (Mauderly 1996; Miller 2000; Oberdorster 1996). This occurs only at high particle inhalatory exposure and is known as the overload phenomenon.



4.1.232 **Figure R.7-2:** Suggested pathogenic sequence of effects of chronically inhaled particles in rats. Adapted from Oberdörster (1996).

4.1.233 The driving force behind this cascade of effects is thought to be the particle load rather than an intrinsic property of the particles themselves. The situation

of overload is most commonly associated with repeated inhalation exposure to particles but it can also occur after single or repeated instillation of particles into the lung (due to high deposition fraction as result of direct instillation) or possible as a result of a single massive inhalation exposure (Mauderly 1996). As such since this phenomenon occurs at high level of inhalatory exposure, it is often argued that the observed effects are a product of the experimental condition and not necessarily a true reflection on the intrinsic toxic potential of the particles to cause inflammation, fibrosis and cancer. Indeed this also raises the question of particular sensitivity to lung overload between different species (e.g. between different experimental species or between an experimental species such as rats and humans). In a comparative study assessing the long-term pulmonary response rats mice and hamsters to inhalation of pigmentary grade titanium dioxide, the authors found species differences. Lung burden was shown to be lower in hamsters at concentrations which caused overload in rats and mice. Also the inflammatory and pathological responses were less severe in mice than rats and diminished with time irrespective of the similar lung burdens (Bermudez et al. 2002).

4.1.234 It should however be noted that this is only the case for PSLT particles. Exposure to highly reactive or toxic particles may cause inflammation, fibrosis and cancer at non-overload conditions due to intrinsic properties of the particles themselves. Inflammation, fibrosis and cancer in rats arising from high exposure to PSLT particles could be a result of the exposure conditions (overload) rather than a result of an intrinsic particle property.

4.1.235 The question of which dose metric best describes the association between deposited dose in the lung, overload conditions and subsequent pathogenic effects is particularly pertinent. There have been several suggested metrics with the first being particle volume as suggested by Morrow et al. (1988). Morrow hypothesised that overload begins when 6 percent of the macrophage volume is filled with particles and total cessation of AM-mediated clearance occurs when 60 percent of the macrophage volume is filled. Such a driver of lung overload has also been more recently suggested for carbon nanotubes (Pauluhn 2010). However, two further metrics have been discussed as important in driving lung overload. The first is surface area and there are several studies which suggest that, as metric, particle surface area correlates

well with induced pathogenic events (Elder et al 2005; Borm et al. (2004)). In a study by Tran et al. (2000), data from a series of chronic inhalation experiments on rats with two poorly soluble dusts, titanium dioxide and barium sulphate was analysed. The results indicated that when lung burden was expressed as particle surface area, there was a clear relationship with the level of inflammation and translocation to the lymph nodes. Most usefully, the authors suggested that based on the shape of the statistical relationship for lung response to particles, indicated the presence of a threshold at approximately 200-300 cm² of lung burden. In relation to surface area as a driving metric, due to their known high level of surface area, the potential for overload effects may be increased with those nanomaterials which exhibit a high biologically accessible surface area.

- 4.1.236 The third suggested metric is that of mass. Whilst some studies indicate mass as a less sensitive indicator of lung overload (Warheit et al. 1997) there is a study showing an improved relationship between the mass of three forms of PSLT particles, and the generation of inflammation due to lung overload.
- 4.1.237 The generation of overload conditions may be seen as a point of weakness within a study design and hinder accurate risk assessment due to the suggested differences in species susceptibility introducing further uncertainty. Indeed in a retrospective analysis by Valberg et al. (2009) they analysed studies considering the lifetime tumour occurrence in rats after repeated dose short term intratracheal instillation of 19 different PSLT particles. Including other draw backs within the studies (such as the lack of low-dose studies) the authors pointed towards significant issues with study design that resulted in lung overload in the test subjects. They argued that the response of rats to PSLT particle lung overload is stereotyped and unique to that species and pointed towards human exposure to demonstrate this. Specifically workers historically exposed to potentially lung-overloading burdens of inhaled dust (e.g., coal workers, underground miners using diesel equipment) do not exhibit an established lung-cancer excess despite the potential for lung overload. As such in rats, when the lung-overload threshold is exceeded, rats develop lung tumours from ongoing inflammation as opposed to particle-specific toxicity, whilst humans do not Valberg et al. (2009).

- 4.1.238 Based on this evidence the authors suggested that the reported results for PSLT particles were not a reliable basis for predicting human lung cancer risk. Such a criticism could be placed on all studies of PSLT particles to which, due to dosing regimes (e.g. intratracheal bolus) or exposure levels may generate overload conditions.
- 4.1.239 The interpretation of data obtained after high doses of PSLT particles should be approached with caution and appropriate discussion should be given to the mechanistic driver behind any pathogenic effects detected. The reason for this is to establish the relevance to human and if alteration of the default assessment factors is warranted or appropriate in the derivation of exposure limits.
- 4.1.240 For further information, review articles covering this subject include Miller (2000) which provides an excellent in depth discussion of particle deposition, clearance and lung overload; Borm et al. (2004) which discusses the importance of overload in the context of risk assessment.
- 4.1.241 Subject to acceptance of the recommended guidance changes above, the following references would need to be included:
- 4.1.242 Bermudez E, Mangnum JB, Asgharian B, Wong BA, Reverdy EE, Hexp PM, Warheit DB, Everitt JI. (2002) "Long-term pulmonary response of three laboratory rodents to subchronic inhalation of pigmentary titanium dioxide particles". *Toxicol Sci*; 70:86 –97.
- 4.1.243 Borm PJ, Schins RP, Albrecht C. (2004) "inhaled particles and lung cancer, part B: Paradigms and risk assessment". *Int J Cancer*. May 20;110(1):3-14.
- 4.1.244 Donaldson K. "Nonneoplastic lung responses induced in experimental animals by exposure to poorly soluble nonfibrous particles." *Inhal Toxicol*. 2000 Jan-Feb;12(1-2):121-39.
- 4.1.245 Elder A, Gelein R, Finkelstein JN, Driscoll KE, Harkema J, Oberdörster G (2005). " Effects of subchronically inhaled carbon black in three species. I. Retention kinetics, lung inflammation, and histopathology" *Toxicol Sci*. Dec;88(2):614-29.

- 4.1.246 Mauderly JL. "Usefulness of animal models for predicting human responses to long-term inhalation of particles." *Chest*. 1996 Mar;109(3 Suppl):65S-68S.
- 4.1.247 Miller F. J. (2000) "Dosimetry of particles in laboratory animals and humans in relationship to issues surrounding lung overload and human health risk assessment: A critical review." *Inhal Toxicol*; 12:19-57
- 4.1.248 Morrow PE. "Possible mechanisms to explain dust overloading of the lungs." *Fundam Appl Toxicol*. 1988 Apr;10(3):369-84.
- 4.1.249 Mossman BT. "Mechanisms of action of poorly soluble particulates in overload-related lung pathology." *Inhal Toxicol*. 2000 Jan-Feb;12(1-2):141-8.
- 4.1.250 Oberdorster G., (1996) "Significance of particle parameters in the evaluation of exposure-dose-response relationships of inhaled particles". *Inhal Toxicol*; 8(suppl):73-89
- 4.1.251 Pauluhn J. "Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures." *Toxicol Sci*. 2010 Jan;113(1):226-42. Epub 2009 Oct 12.
- 4.1.252 Tran CL, Buchanan D, Cullen RT, Searl A, Jones AD, Donaldson K. "Inhalation of poorly soluble particles. II. Influence Of particle surface area on inflammation and clearance." *Inhal Toxicol*. 2000 Dec;12(12):1113-26.
- 4.1.253 Warheit DB, Hansen JF, Yuen IS, Kelly DP, Snajdr SI, Hartsky MA. (1997) "Inhalation of high concentrations of low toxicity dusts in rats results in impaired pulmonary clearance mechanisms and persistent inflammation." *Toxicol Appl Pharmacol*;145(1):10-22.
- 4.1.254 Valberg PA, Bruch J, McCunney RJ. (2009) "Are rat results from intratracheal instillation of 19 granular dusts a reliable basis for predicting cancer risk?" *Regul Toxicol Pharmacol*.;54(1):72-83.

- 4.1.255 **Advisory note on the consideration of assay inhibition/ enhancement (interference)**
- 4.1.256 Various nanomaterials have been on occasion found to interfere with several commonly used assays utilised to determine their cellular or toxic effects. For example, some nanomaterials may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanoparticles may bind to assay components including the substrates (such as CNT with the reagent in MTT assays; Belyanskaya *et al.* 2007) or the biomarker being measured, (such as LDH and cytokine proteins, see for example Davoren *et al.* 2007).
- 4.1.257 A summarised list of potential sources of interferences with commonly used assays has been developed by Kroll *et al.* (2009) and reproduced within Table 7.2.X.

4.1.258

Table 7.2.X: Nanoparticle interference with cytotoxicity assays (reproduced from Kroll et al., 2009)

Cytotoxicity assay	Detection principle	NP interference	Altered readout	Particle/Reference
Cell viability				
MTT	Colorimetric detection of mitochondrial activity	Adsorption of substrate	Reduced indication of cell viability	Carbon nanoparticles
LDH	Colorimetric detection of LDH release	Inhibition of LDH	Reduced indication of necrosis	Trace metal-containing nanoparticles
Annexin V/ Propidium iodide	Fluorimetric detection of phosphatidylserine exposure (apoptosis marker) Propidium iodide-staining of DNA (necrosis marker)	Ca ²⁺ -depletion Dye adsorption	Reduced indication of apoptosis Reduced indication of necrosis	 Carbon nanoparticles
Neutral red	Colorimetric detection of intact lysosomes	Dye adsorption	Reduced indication of cell viability	Carbon nanoparticles
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of apoptosis	Trace metal-containing nanoparticles especially Zn ²⁺
Stress response				
DCF	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles
Inflammatory response				
ELISA	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles Metal oxide nanoparticles

4.1.259

It should be noted that this list is not exhaustive and proper testing should be performed where possible as a matter of course to check for inhibition or enhancement of test results.

4.1.260

Within certain standard methodologies such as ISO/FDS 29701 (Nanotechnologies - endotoxin tests on nanomaterial samples for in vitro systems), the method requires the use of sample 'spikes' (addition of a known sample control to the test sample) to test for inhibition or enhancement of the spiked control. This is calculated by assessing the returned value against the

expected value which should be a cumulative value of the spike and sample. Any alteration to this may indicate inhibition (return of a value less than expected) or enhancement (return of a value greater than expected) of the assay. The use of sample spikes is encouraged as it allows a simple yet effective method of investigating potential assay interference and would give greater confidence in derived results. This is especially important due to the uncertainty that surrounds the effect of nanomaterials on the performance of routinely used assays.

- 4.1.261 The use of such methods to investigate possible inhibition or enhancement of results should be carried out wherever possible irrespective of standard method requirement; however this may not always be possible. In many of the studies reported it is not possible to ascertain whether the assays were adequately controlled to assess for interference. Thus, as a general precaution, it is advisable to use more than one assay to assess the endpoint or effect in question, as advised by Landsiedel et al. (2009) for establishing genotoxicity. The potential for inhibition or enhancement of the test result may impact on numerous test methods. In certain cases, the potential for assay interference has been identified for some nanomaterials, for example carbon nanotubes are suggested to interfere with the MTT assay (Wörle-Knirsch et al. 2006) and as such may cause issues with tests such as OECD TG 431/EU B.40 Human Skin Model tests (EPISKIN™, EpiDerm™) due to their use of the MTT assay. However knowledge on nanomaterial assay interference is incomplete and so precautions to ensure the validity of an assay, such as the mentioned use of control spikes could be used.
- 4.1.262 Due to the potential for interference resulting in misleading results in numerous assays, utmost care should be taken in testing for such interference to validate obtained results.
- 4.1.263 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.2:
- 4.1.264 Belyanskaya L., Manser P., Spohn P., Bruinink A., Wick P. 2007, "The reliability and limits of the MTT reduction assay for carbon nanotubes – cell interaction," Carbon, vol. 45, pp. 2643–2648.

- 4.1.265 Davoren, M., Herzog, E., Casey, A., Cottineau, B., Chambers, G., Byrne, H.J. and Lyng, F.M. 2007, "In vitro toxicity evaluation of single-walled carbon nanotubes on human A549 lung cells", *Toxicol In Vitro*, vol. 21, no. 3, pp. 438-448.
- 4.1.266 Kroll, A., Pillukat, M.H., Hahn, D., Schneckeburger, J. 2009, "Current in vitro methods in nanoparticle risk assessment: Limitations and challenges," *European Journal of Pharmaceutics and Biopharmaceutics*, 72(2), 370-377.
- 4.1.267 Landsiedel, R., Schulz, M., Kapp, MD., Oesch, F. 2009, "Genotoxicity Investigations on Nanomaterials: Methods, Preparation and Characterization Test Material, Potential Artifacts and Limitations - Many Questions, Some Answers," *Mutation Research*, 681(2-3), 241-58.
- 4.1.268 The following update to Guidance should be considered for insertion into Section R.7.7 under the 'In vitro data' heading on page 380 prior to table R.7.7-2:
- 4.1.269 **Advisory note on the consideration of bacterial assay interference**
- 4.1.270 Assessment of substances with regard to genotoxicity is generally based on a combination of tests to assess effects on three major end points of genetic damage associated with human disease: gene mutation, clastogenicity and aneuploidy.
- 4.1.271 One such test, the bacterial reverse mutation (Ames) test (OECD TG 471/EU B.12/13: Bacterial reverse mutation test (in vitro)), uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (Ames et al., 1975; Maron et al., 1983; Gatehouse et al., 1994). The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid (histidine). The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain (OECD TG471, 1997). A positive test indicates that the test substance might act as a mutagen, or hold carcinogenic potential (as cancer is often linked to DNA damage).

- 4.1.272 Generally, the major drawback of the Ames test is that it is difficult to translate prokaryotic data for eukaryotic genotoxicity testing, and the test is known to generate false positive results (Khandoudi et al., 2009). Indeed, it is now clear from the results of international collaborative studies and the large databases that are currently available for the assays evaluated, that no single assay can detect all genotoxic substances (Eastmond et al., 2009).
- 4.1.273 In relation to nanomaterials, a recent review of the applicability of genotoxicity tests to NM questioned whether the Ames test was accurately representative of NM genotoxicity (Landsiedel et al., 2009). The Landsiedel study reported that of those studies reviewed, results were predominantly negative (5/6 studies). The group speculated that it is likely that some NMs are not able to cross the bacterial wall, whilst others kill the test organism as they are bactericidal.
- 4.1.274 Based on this evidence, it is advisable that any data harvested from such bacterial mutation tests should be followed up with other assays after the initial screening, perhaps via implementation of a battery of standardised genotoxicity testing methods covering an as wide as possible variety of potential genotoxic mechanisms.
- 4.1.275 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.7:
- 4.1.276 Ames, B.N., McCann, J. and Yamasaki, E. 1975, "Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Maron, D.M. and Ames, B.N. (1983). Revised Methods for the Salmonella Mutagenicity Test. Mutation Res., 113, 173-215.
- 4.1.277 Eastmond DA, Hartwig A, Anderson D, Anwar WA, Cimino MC, Dobrev I, Douglas GR, Nohmi T, Phillips DH, Vickers C (2009) "Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme." Mutagenesis. 2009 Jul;24(4):341-9

- 4.1.278 Gatehouse, D., Haworth, S., Cebula, T., Gocke, E., Kier, L., Matsushima, T., Melcion, C., Nohmi, T., Venitt, S. and Zeiger, E. (1994). Recommendations for the Performance of Bacterial Mutation Assays. *Mutation Res.*, 312, 217-233.
- 4.1.279 Khandoudi, N., Porte, P., Chtourou, S., Nessler, F., Marzin, D., and Le Curieux, F., "The presence of arginine may be a source of false positive results in the Ames test," *Mutation Research*, vol. 679, no. 1-2, pp. 65–71, 2009
- 4.1.280 Landsiedel, R., Schulz, M., Kapp, MD., Oesch, F. 2009, "Genotoxicity Investigations on Nanomaterials: Methods, Preparation and Characterization Test Material, Potential Artifacts and Limitations - Many Questions, Some Answers," *Mutation Research*, 681(2-3), 241-58.
- 4.1.281 **UPDATE TO GUIDANCE ON EXISTING INFORMATION REQUIREMENTS**
- 4.1.282 ***R.7.3 Skin and eye irritation/ corrosion***
- 4.1.283 *Skin and eye irritation: Non-testing Data*
- 4.1.284 R.7.2.3.1 Non-human data on irritation/ corrosion
- 4.1.285 We propose the insertion of the following statement to inform users of Guidance of the current ambiguity surrounding the use of non-testing data for nanomaterials
- 4.1.286 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis only.
- 4.1.287 The statement would be applicable for insertion as well into the following sections of R.7.2:

- R.7.2.3.1 Non-human data on irritation/ corrosion - Page 203 appended to paragraph 3
- R.7.2.4.1 Non-human data on irritation/ corrosion - Page 215 appended to paragraph 4
- Also appendix R7.2.2-2 (p241 under the contents of Appendix 7.2-2 list), R7.2.2-3 (p245 under the contents of Appendix 7.2-3 list)

4.1.288 Within Figure (Table) R.7.2-2 section 5a/b (skin irritation/corrosion) and Figure R.7.2-3 section 5, the following amendments to the ITS table's footnotes should be made:

4.1.289 Page 227 R.7.2-2 section 5a/b footnote 'f'.

4.1.290 Conclusion on no classification can be made if the *in silico* model has been shown, in a scientifically justified manner, to predict adequately the absence of the classified effect and also fulfils the requirements of Annex XI. For nanomaterials the use of *in silico* models has yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.291 Page 232 R.7.2-3 section 5 footnote 'e'.

4.1.292 Conclusion on no classification can be made if the *in silico* model has been shown to predict adequately the absence of the classified effect and also fulfils the requirements of Annex XI. For nanomaterials the use of *in silico* models has yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.293 **R.7.3 Skin and respiratory sensitisation**

4.1.294 *Skin and respiratory sensitisation: Non-testing Data*

4.1.295 As previously mentioned, the use of *in silico* approaches such as (Q)SAR, groupings and read across are not yet sufficiently developed for nanomaterials and as such the following insertion should be made:

4.1.296 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.297 This insertion would be made into the following sections:

- R.7.3.3.1 Non-human data for skin sensitisation – Page 259 appended to paragraph 7
- R7.3.4.1 Non-human data on skin sensitisation - Page 267 appended to paragraph 1
- R7.3.5.1 Non-human data on respiratory sensitisation - Page 271 appended to paragraph 1

4.1.298 A footnote should be added and linked to the first step of the ITS (box 1) so that the step reads:

4.1.299 Gather and evaluate existing information (human-, animal-, *in vitro*-, (Q)SAR^a, read across^a and category^a data) on skin sensitisation according to Annex VI, step 1.

4.1.300 And a new footnote read:

4.1.301 ^a For nanomaterials the use of *in silico* models has yet to be established or accepted. Therefore the use of non-testing approaches for

nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.302 **R7.4: Acute Toxicity**

4.1.303 *Acute Toxicity: Non-testing Data*

4.1.304 It is suggested that the following insertion should be made into acute toxicity Guidance R.7.4 in the use of non-testing approaches:

4.1.305 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.306 This insertion would be made into the following sections:

- R.7.4.3.1 Non-human data on acute toxicity – Page 291 appended to paragraph 3
- R.7.4.4.1 Non-human data on acute toxicity – Page 297 appended to paragraph 1
- R.7.4.5.1 Conclusions on suitability for Classification and Labelling – Page 301 appended to paragraph 2

4.1.307 The following change is recommended for stage 4 of the ITS (Generation of new data/proposal for testing strategy), 3rd paragraph page 306:

4.1.308 The route of exposure to be used for acute toxicity evaluation depends on the nature of the substance (e.g. gas or not, molecular weight, log K_{ow} , solid with inhalable particle size (e.g. nanomaterials)) and should reflect the most likely route of human exposure.

4.1.309 In addition, Figure R.7.4-1 (ITS for acute toxicity endpoint) should be amended such that the question “is the substance gaseous” is substituted with “is the substance inhalable”.

4.1.310 ***R7.5 - Repeated Dose Toxicity***

4.1.311 *Repeat Dose Toxicity: Non-testing Data*

4.1.312 It is also suggested that the following insertion should be made into repeated dose toxicity Guidance R.7.5 in the use of non-testing approaches:

4.1.313 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.314 This insertion would be made into the following sections:

- R.7.5.3.1 Non-human data on repeated dose toxicity– Page 314 inserted between paragraphs 4 and 5
- R.7.5.4.1 Non-human data on repeated dose toxicity– Page 320 appended to paragraph 6

4.1.315 For the ITS, the following paragraph is suggested for inclusion into R.7.5.6.2 page 335:

4.1.316 As mentioned within section R.7.5.3.1a, the use of (Q)SAR approaches in addressing data gaps is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis. In the use of inhalation exposure as a route to test for repeated dose toxicity of poorly soluble low toxicity (nano)particles, the issues surrounding lung

overload should be considered. (Further discussion of lung overload can be found in the Lung Overload Advisory Note).

4.1.317 The ITS states that at the 10 t/y or more tonnage threshold “the use of a combined repeated dose toxicity study with the reproductive/developmental toxicity screening test (OECD TG 422) is recommended if an initial assessment of repeated dose toxicity and reproductive toxicity is required”. However, according to OECD WPMN (reference) modifications to this guideline are needed before it can be used for the inhalation route instead of the oral route. Therefore the above sentence in R.7.5.6.3 should be altered to read:

4.1.318 The use of a combined repeated dose toxicity study with the reproductive/developmental toxicity screening test (OECD TG 422) is recommended if an initial assessment of repeated dose toxicity and reproductive toxicity is required. However in the case of inhalation as a route of exposure, as may be the most likely route for (nano)particle exposure, further modification of OECD TG 422 may be required with full justification.

4.1.319 ***R.7.6 Reproductive and developmental toxicity***

4.1.320 It is suggested that the following insertion should be made into reproductive toxicity Guidance R.7.6 in the use of non-testing approaches:

4.1.321 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.322 This insertion would be made into the following sections:

- R.7.6.4.1 Non-human data on reproductive toxicity– Page 355 inserted between paragraphs 4 and 5

4.1.323 For the ITS, the following text should be inserted into the Stage 2 paragraph under Section R.7.6.6.2:

4.1.324 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.325 **R.7.7 Mutagenicity and Carcinogenicity**

4.1.326 It is suggested that the following insertion should be made into reproductive toxicity Guidance R.7.6 in the use of non-testing approaches:

4.1.327 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.

4.1.328 This insertion would be made into the following sections:

- R.7.7.3.1 Non-human data on mutagenicity – Page 379 appended to paragraph 2
- R.7.7.4.1 Non-human data on mutagenicity – Page 383 appended to paragraph 6
- R.7.7.10.1 Non-human data on carcinogenicity – Page 407 appended to paragraph 1
- R.7.7.11.1 Non-human data on carcinogenicity – Page 412 appended to paragraph 5

4.1.329 For the ITS, inclusion of the following paragraph in R.7.7.6.2 as a new second paragraph is suggested:

- 4.1.330 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.
- 4.1.331 A new first paragraph for Section R.7.7.6.3 *standard information requirement at annex VII* is recommended:
- 4.1.332 Solid particles, including some nanomaterials, may not penetrate the cell wall of bacteria and as such this assay may not allow a robust evaluation of (nano)particle mutagenicity as discussed in the bacterial mutagenicity advisory note [TO BE INSERTED IN R.7.7 UNDER THE 'IN VITRO DATA' HEADING ON PAGE 380]. Therefore, bacterial mutation assay should not be used as a single test for (nano)particle mutagenicity, but instead used in conjunction with a range of mammalian cell gene mutation tests to reduce the potential for confounded results due to interference with a test method.
- 4.1.333 For carcinogenicity, the inclusion of the following statement is recommended at the end of the first paragraph of the section R.7.7.13.2 *Preliminary considerations*.
- 4.1.334 The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.
- 4.1.335 Within Figure R.7.7-2 a footnote should be attached to all occurrences of 'non-testing data', specifically section 'Annex VII-IX' top left hand box and section 'Annex X' top box, stating:

- 4.1.336 ^a For nanomaterials the use of *in silico* models has yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis.
- 4.1.337 **R.712 Guidance on Toxicokinetics**
- 4.1.338 Page 149, R.7.12.2.1, First paragraph, 7th line. The sentence currently states that:
- 4.1.339 “However, the physico-chemical characteristics of the substance will change if the substance undergoes metabolic transformation and the physico-chemical characteristics of the parent substance may not provide any clues as to the identity, distribution, retention and elimination of its metabolites.”
- 4.1.340 Within biological fluids, there is the potential that other processes, as well as metabolism may alter the substance and as such the following amendment has been suggested:
- 4.1.341 “However, the physico-chemical characteristics of the substance will change if the substance undergoes metabolic transformation or other physical or chemical modification in the test system, and the physico-chemical characteristics of the parent substance may not provide any clues as to the identity, distribution, retention and elimination of its metabolites.”
- 4.1.342 Page 194, Appendix R.7.12-2, Sub heading ‘gastro intestinal absorption models’,
- 4.1.343 The first sentence states that:
- 4.1.344 “In order to be absorbed from the GI tract, substances have to be present in solution in the GI fluids, and from there have to cross the GI wall to reach the lymph or the venous portal blood.”
- 4.1.345 Transport of nanoparticles across the gastrointestinal mucosa can occur via several different pathways (summarised by Powell et al. 2010) and has been demonstrated from for some insoluble particles such as latex beads (Hussian and Florence 1998), TiO₂ (Wang et al. 2007) and gold nanoparticles (Schleh et al. 2011) have been shown to be absorbed from the GI tract into the systemic circulation. As such, this suggests that particles do not need to be in suspension but instead can translocate from a suspension of insoluble particles. As particle may translocate which are is

suspension as a dispersion and not actually as a solution, an appendment to the sentence to the following has been suggested:

4.1.346 “In order to be absorbed from the GI tract, substances have to be present in solution in the GI fluids, and from there have to cross the GI wall to reach the lymph or the venous portal blood. In the case of particulates, the possibility for small sized particles in the nanometer size range to translocate across the GI wall in particulate form should be considered.”

4.1.347 **ECOTOXICOLOGICAL INFORMATION REQUIREMENTS**

4.1.348 **R.7.8.4.1 Data on aquatic pelagic toxicity**

4.1.349 For nanomaterials, some novel endpoints and observations in fish and invertebrate test may provide useful information (e.g. for supporting PNEC-derivation and/or understanding the possible mode of action) on the material tested. At the moment, corresponding methods are well-established in the scientific literature (see Smith et al. 2007, Bouskill et al. 2006) but are not internationally validated for these endpoints. For fish tests these additional observations can include fish ventilation rate, gill pathologies, mucus secretion and brain pathology, animal behaviour (Smith et al. 2007, Federici et al. 2007) and activity levels of relevant antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST) (Oberdörster 2004, Wang et al. 2008, Zhu et al. 2008, Klaper et al. 2009, Kim et al. 2010, Wong et al. 2010). For invertebrates like Daphnia these additional observations can include heart rate, hopping frequency and appendage movement cycle frequency (Lovern and Klaper, 2007). As with existing endpoints, the NOEC, LOEC or EC50-values (e.g. concentration that causes e.g. 50% increase in ventilation rate in fish or heart rate in daphnia) could be estimated and used in PNEC derivations if these EC50-values are the most sensitive endpoints and considered relevant in the regulatory risk assessment framework.

4.1.350 The following additional text for guidance is proposed for R.7.8.4.1:

4.1.351 “For nanomaterials, the provision of data on the following parameters (as part of an ensemble of data on additional relevant endpoints considered by the registrant to be of value) is recommended: fish ventilation rate, gill pathologies, mucus secretion and

brain pathology, animal behaviour, and activity levels of relevant antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST). “Nevertheless, as a thorough understanding of the relevance and impact on the ecosystem and population of such parameters in view of the regulatory risk assessment is missing, their relevance for overall ecotoxicity assessment remain supportive until further research would indicate otherwise.

4.1.352 The following text is recommended to be added as a new fifth paragraph in R.7.8.4.1 (R.7b, pg. 26), following the paragraph ending "... before reviewing a test report.":

4.1.353 “NANOMATERIALS: With regards nanomaterials and aquatic pelagic toxicity, the recommendations set out in the OECD Guidance Manual for testing (OECD, 2009) and Preliminary Guidance Notes on Sample Preparation and Dosimetry for nanomaterials (OECD, 2010) need to be taken into consideration, especially in regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, characterization of samples (prepared from stock dispersions) prior to administration/testing and possibly during and at least at the end of the test.”

4.1.354 **R.7.8.6 References on aquatic pelagic toxicity**

4.1.355 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.8.6:

4.1.356 Kim, K.T., Klaine, S.J., Cho, J., Kim, S.H. & Kim, S.D. 2010, "Oxidative stress responses of *Daphnia magna* exposed to TiO₂ nanoparticles according to size fraction", *The Science of the total environment*, .

4.1.357 Klaper, R., Crago, J., Barr, J., Arndt, D., Setyowati, K. & Chen, J. 2009, "Toxicity biomarker expression in daphnids exposed to manufactured nanoparticles: Changes in toxicity with functionalization", *Environmental Pollution*, vol. 157, no. 4, pp. 1152-1156.

4.1.358 Lovern SB, Strickler JR, Klaper R., 2007, "Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C₆₀, and C₆₀H_xC₇₀H_x)", *Environ Sci Technol.*, vol. 4, no. 12, pp. 4465-70.

- 4.1.359 OECD. 2009, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme. First revision", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV, Paris.
- 4.1.360 OECD. 2010, "Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2010)25, Paris.
- 4.1.361 Oberdorster, E. 2004, "Manufactured nanomaterials (Fullerenes, C-60) induce oxidative stress in the brain of juvenile largemouth bass", *Environ.Health Perspect.* vol. 112, no. 10, pp. 1058-1062.
- 4.1.362 Smith, C.J., Shaw, B.J. and Handy, R.D. 2007, "Toxicity of single-walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects", *Aquatic Toxicology*, vol. 82, no. 2, pp. 94-109.
- 4.1.363 Templeton, R.C., Ferguson, P.L., Washburn, K.M., Scrivens, W.A. and Chandler, G.T. 2006, "Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod", *Environ.Sci.Technol.*, vol. 40, no. 23, pp. 7387-7393.
- 4.1.364 Wang, J., Liu, Y., Jiao, F., Lao, F., Li, W., Gu, Y., Li, Y., Ge, C., Zhou, G., Li, B., Zhao, Y., Chai, Z. and Chen, C. 2008, "Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO₂ nanoparticles", *Toxicol*, vol. 254, no. 1-2, pp. 82-90.
- 4.1.365 Wong, S. W. Y. Leung, P.T. Y., Djurišić, A. B. Leung, K.M.Y. 2010. Toxicities of nano zinc oxide to five marine organisms: influences of aggregate size and ion solubility. *Anal Bioanal Chem* (2010) 396:609–618
- 4.1.366 Zhu, X.S., Zhu, L., Lang, Y.P. and Chen, Y.S. 2008, "Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1979-1985.
- 4.1.367 **R.7.8.9.1 Laboratory data on toxicity to sediment organisms**
- 4.1.368 The following text is recommended to be added as a new third paragraph in R.7.8.9.1 (R.7b, pg. 124), following the paragraph ending "...categories is given in Section R.6.2.":
- 4.1.369 "Estimates based on results from "equilibrium partitioning methods" are limited to distribution of a substance in molecular form. However, substances may also be distributed in the environment as particles (caused by abrasion/weathering of anthropogenic materials) and hence extrapolation based on partitioning may not be relevant. In such a case the partitioning method may underestimate exposure of soil and sediment environments and

overestimate the exposure of water. If the particle size is small also air distribution may occur. There are no estimation methods available for particle distribution so this has to be dealt with on a case-by-case basis. With regard to nanomaterials, the recommendations set out in the OECD Guidance Manual for testing (OECD, 2009) and Preliminary Guidance Notes on Sample Preparation and Dosimetry for nanomaterials (OECD, 2010) need to be taken into consideration, especially in regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, characterization of samples (prepared from stock dispersions) prior to administration/testing and possibly during and at least at the end of the test.

4.1.370 **R.7.8.10.1 Laboratory data on toxicity to sediment organisms**

4.1.371 The following text is recommended to be added as a new fifth paragraph in R.7.8.10.1 (R.7b, pg. 127), following the paragraph ending "...categories is given in Section R.6.2.":

4.1.372 "Estimates based on "partitioning" are limited to distribution of a substance in molecular form. However, substances may also be distributed in the environment as particles (caused by abrasion/weathering of anthropogenic materials) and hence extrapolation based on partitioning may not be relevant. In such a case the partitioning method may underestimate exposure of soil and sediment environments and overestimate the exposure of water. If the particle size is small also air distribution may occur, at least in the local perspective. There are no estimation methods available for particle distribution so this has to be dealt with on a case-by-case basis. With regard to nanomaterials, the recommendations set out in the OECD Guidance Manual for testing (OECD, 2009) and Preliminary Guidance Notes on Sample Preparation and Dosimetry for nanomaterials (OECD, 2010) need to be taken into consideration, especially in regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, characterization of samples (prepared from stock dispersions) prior to administration/testing and possibly during and at least at the end of the test."

4.1.373 R.7.8.13 References on toxicity sediment organisms

4.1.374 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.8.13:

4.1.375 OECD. 2009, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme. First revision", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV, Paris.

4.1.376 OECD. 2010, "Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2010)25, Paris.

4.1.377 R.7.9.3 Information on degradation/biodegradation and its sources

4.1.378 The following text is recommended to be added as a third paragraph in R.7.9.3 (R.7b, pg. 163), following the paragraph ending "...higher tiered testing data accordingly":

4.1.379 With regard to nanomaterials it should be noted that the OECD biodegradability test methods have been developed and validated principally for assessment of organic compounds whereas many nanomaterials are principally inorganic and even carbon-based nanomaterials arguably tend to be of an inorganic nature. However, surface coating and functionalizations might be organic and consist of biodegradable materials. Methods measuring carbon dioxide production or oxygen uptake are applicable, but they require large amounts of test material. If several conclusive aerobic degradation tests indicate very low or negligible degradation, then other aerobic degradation tests will most likely also be negative and it may be useless to proceed with additional tests. It may be better to decide to skip the more elaborate test, and conclude that the substance is not biodegradable (OECD, 2010).

4.1.380 R.7.9.7 References on biodegradation

4.1.381 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.9.7:

4.1.382 OECD. 2010, "Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2010)25, Paris.

4.1.383 **R. 7.10.3.2 Non-testing data aquatic bioaccumulation**

4.1.384 The following text is recommended to be added as a final paragraph in R.7.10.3.2 (R.7c, pg. 16), following the paragraph ending "... data on the chemical of interest":

4.1.385 With regard to nanomaterials, it is not possible to make log Kow or solubility estimations since nanomaterials are dispersed and not in solution. Measured BCF values are required and stability and changes in e.g. aggregation and agglomerate size are of vital importance to consider. There is furthermore a need to emphasise that for nanomaterials that undergo dissolution like Ag0 it is very important to get information, if possible, on the form of the substance present in the animal tissue. The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for the environment must be scientifically justified.

4.1.386 **R.7.11.3.1 – Laboratory data a) Non-testing data**

4.1.387 The following text is recommended to be added as a final paragraph in R.7.11.3.1 a) Non-testing data (R.7c, pg. 111), following the paragraph ending "... as in the ITS in Section R.7.11.6.":

4.1.388 Estimates based on "partitioning" are limited to distribution of a substance in molecular form. However, substances may also be distributed in the environment as particles (caused by abrasion/weathering of anthropogenic materials) and hence extrapolation based on partitioning may not be relevant. In such a case the partitioning method may underestimate exposure of soil and sediment environments and overestimate the exposure of water. If the particle size is small also air distribution may occur. There are no estimation methods available for particle distribution so this has to be dealt with on a

case-by-case basis. animal tissue. The use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for the environment must be scientifically justified.

4.1.389 **R.7.11.3.1 – Laboratory data b) testing data**

4.1.390 The following text is recommended to be added as a final paragraph in R.7.11.3.1 (R.7c, pg. 111), following the paragraph ending "...reliability, adequacy, relevance and completeness":

4.1.391 With regard to nanomaterials, the recommendations set out in the OECD Guidance Manual for testing (OECD, 2009) and Preliminary Guidance Notes on Sample Preparation and Dosimetry for nanomaterials (OECD, 2010) need to be taken into consideration, especially in regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, characterization of samples (prepared from stock dispersions) prior to administration/testing and possibly during and at least at the end of the test.

4.1.392 **R.7.11.7 REFERENCES**

4.1.393 Subject to acceptance of the recommended guidance changes above, the following references would need to be included into R.7.11.7

4.1.394 OECD. 2009, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme. First revision", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV, Paris.

4.1.395 OECD. 2010, "Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2010)25, Paris.

4.2 NEW CHAPTERS FOR ENDPOINT SPECIFIC GUIDANCE

4.2.1 In the event that the regulatory interpretation of the term Granulometry and consequently the associated information requirement does not include “shape” and “surface area”, it is recommended that two new chapters (entitled Shape and Surface Area) are included into R.7a. Proposed text has been drafted below. In this draft of the chapter contents, figure and table legends have been given generic numbers (R.7.1-X for all proposed figures and tables in the Shape chapter and R.7.1-Y for Surface Area). It is evident from the position in the chapter and description in the text which tables/figures are being referred to. Permission would be required from ISO to reproduce the suggested images.

4.2.2 R.7.1.19 SHAPE

4.2.3 Solid particulates/granulates with identical composition can have a variety of well- or ill-defined shapes, including spheres, rods, tubes, fibres and plates, which may have different physical, chemical, and biological properties. Shapes are determined by the way in which the entities are bound together and particles will assume the shape that minimises free energy and is kinetically achievable under given environmental conditions. Particle shape is an important parameter in the characterisation of some nanoparticles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Knowledge of high aspect ratio particles may inform interpretation of some toxicity test results.

4.2.4 Definition of shape

4.2.5 Shape is a qualitative or, at best, semi-quantitative geometrical description or dimension-less term(s) of the extremities of the particle or collections of particles, their agglomerates or aggregates, that make up the material under investigation (adapted from OECD, 2009).

4.2.6 Particles may have readily definable shapes such as spheres, rods, or defined crystal morphologies. More often, particle shape is much more variable and ‘shape factors’ such as sphericity, circularity, aspect ratio, convexity and fractal dimension are needed to characterise shape.

- 4.2.7 ISO 9276-6:2008 specifies rules and nomenclature for the description and quantitative representation of particle shape and morphology. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape.
- 4.2.8 *Macrodescription* is a description of the overall form of a particle in terms of the geometrical proportions of the particle. In general, simple geometrical descriptors calculated from the size measurements made on the particle silhouette are used. Low-order Fourier descriptors can also be regarded as macrodescriptors.
- 4.2.9 *Mesodescription* provides information about details of the particle shape and/or surface structure that are in a size range not much smaller than the particle proportions, like Barrett's roundness and concavity (Barrett, 1980).
- 4.2.10 The following mesodescriptors can be defined:
- a) morphological mathematical descriptors, computing robustness and largest concavity index;
 - b) a concavity tree, providing general insight into the organisation of concavities and their complexity;
 - c) angularity descriptors, determining those parts of the boundary that are active in the abrasion process:
 - i. an angularity factor, selecting the apices on corners which are coincident with the convex hull because it is these points that will make contact with the surface of another particles,
 - ii. a quadratic spike parameter, taking into account those spikes that are outside a circle, of area equal to that of the particle, centred over the particle centroid,
 - iii. slip chording, generating information on the number of cutting edges and their sharpness in the facet signature waveform;
 - d) fractal dimension, providing data on the overall structural complexity by consideration of a larger measurement step;

- e) Fourier descriptors, of higher order than macrodescriptors, specifying the smaller-scale components of morphology;
- f) bending energy, measuring the overall complexity of contour lines.

4.2.11 *Microdescription* determines the roughness of shape boundaries using two of the descriptors mentioned above:

- fractal dimension, measured using a measurement step smaller than that used for structural description;
- higher-order Fourier descriptors/coefficients for surface-textural analysis.

4.2.12 **R.7.1.19.1 Information requirements on shape**

4.2.13 The study does not need to be conducted if the substance is marketed or used in a non-solid or non-granular form. Shape determination requires information on water solubility. Fibre length and diameter distribution require information on the fibrous nature of the product and on stability of the fibrous shape under electron microscope conditions.

4.2.14 The summary should include a microscopy image of the particle and a qualitative or semi-quantitative geometrical description of the extremities of the particle and/or collections of particles, agglomerates or aggregates that make up the material under investigation. Size-independent macro-, micro- and meso-shape descriptors (examples are ratios of extensions in different directions; unit [meter/meter] such as aspect ratio or fractal dimension are available (ISO 9276-6:2008) and should be used wherever possible. A combination of terms and/or measurands may be needed to describe shape; this is essential to circumvent the challenges already foreseeable where materials are capable of concurrently exhibiting multiple shapes in a sample which may present different hazard potentials. Information should also be included on the temperature at which measurements were made, purity of the sample used, physical state, method used and reference substance used (if any).

4.2.15 The level of inspection used in a method is a very practical criterion for the classification of the method, since many methods provide shape information at different size levels. Another convenient way of classifying methods is to

differentiate between those which derive shape descriptors from particle images and those which derive shape descriptors from physical properties:

a) Calculation of geometrical descriptor/shape factors:

Geometrical shape factors are ratios between two different geometrical properties, such properties usually being some measure of the proportions of the image of the whole particle or some measure of the proportions of an ideal geometrical body that envelops, or forms an envelope around, the particle. These results are macroshape descriptors similar to an aspect ratio.

b) Calculation of dynamic shape factors from physical equivalent diameters:

These shape factors are similar to geometrical shape factors except that at least one physical property is considered in the comparison. Usually, the results are expressed as the ratio of equivalent diameters, e.g. Stokes sedimentation velocity to volume-equivalent diameter x_{Stokes}/x_V .

c) Morphological analysis

Morphological analysis descriptors give mean values of particle shape that are not much smaller than the proportions of the whole particle. A typical example is concavity analysis.

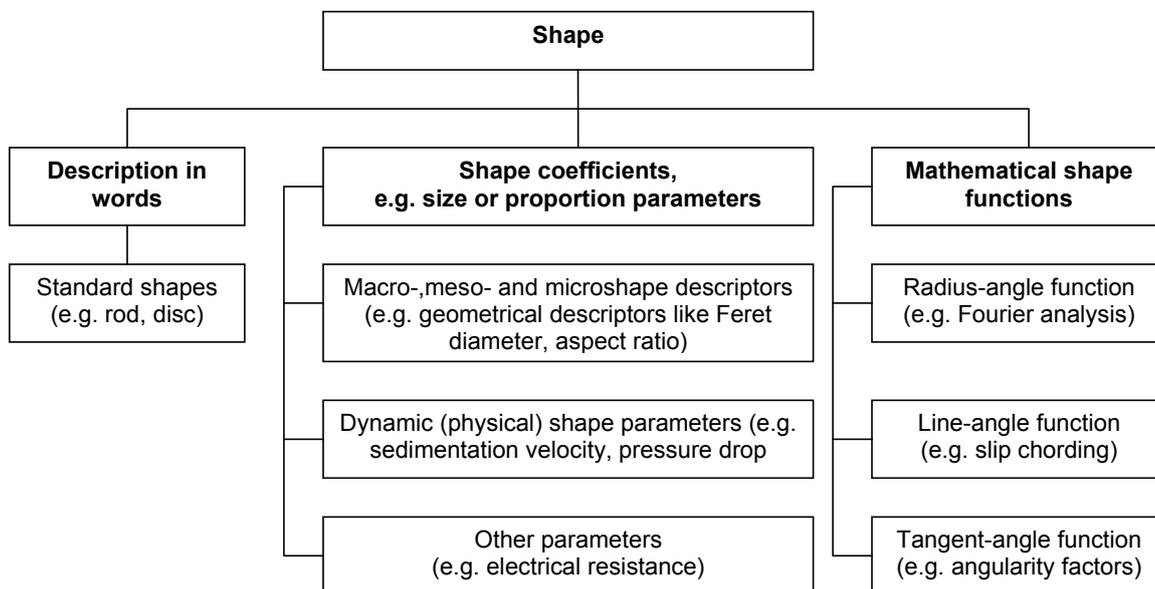
d) Analysis of the contour line (shape boundary):

Multiple operations on the grey-level pixel image of a particle can produce a set of shape descriptors which can be correlated with the topology or surface texture of the particle.

e) Analysis of the physical spectra:

Multiple operations on, or the mathematical treatment of, the physical spectra of a single particle can extract the shape of information as a set of descriptors. Such a procedure has been described for shape analysis by azimuthal light scattering and diffraction spectroscopy.

4.2.16 **Figure R.7.1-X Classification of some methods for shape description (adapted from ISO 9276-6:2008)**



4.2.17 In the context of hazard assessment of nanomaterials, there are three forms in which properties should be characterised: “as produced”, “as dosed / as exposed”, and at the point(s) of interaction within the organism (which are sometimes collectively referred to as “as tested”, but this and the equally un-specific term *in situ* require some further description of the context). “As dosed / as exposed” should reflect as much as possible the state of the substance to which humans and /or environment are exposed. The latter (at the point of interaction with the organism) is the most challenging measurement, because invasive techniques usually cannot be used without compromising the integrity of the organism and possibly invalidating the test, but acknowledged to be of more interest to advancing mechanistic toxicology rather than to regulatory toxicology. Although potentially confounded by issues of artefacts, insufficient statistical reliability, and difficulties in measurement and interpretation, an indirect way of assessing this form is through post-exposure evaluation, examining the shape distribution (i.e. a description of the proportion of particles with particular shapes in a sample) of particles in cells, tissues, organs or the environmental compartment after exposure.

- 4.2.18 **R.7.1.19.2 Available information on shape**
- 4.2.19 **Testing data on shape**
- 4.2.20 The characterisation of particle properties requires very careful sampling and sample splitting practices to be followed. ISO 14488:2007 specifies methods for obtaining a test sub-sample from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative of the whole sample with a defined confidence level. Further information is available in the Sample Preparation section of the Introduction to R.7.
- 4.2.21 A number of different methods for the qualitative or semi-quantitative description of particle shape and morphology are available (Table R.7.1-X). The shape of particles is usually determined by electron microscopy (e.g. TEM, SEM), which includes many qualitative and semi-quantitative techniques to investigate the morphology (size and shape) and also the aggregation state.
- 4.2.22 The choice of an appropriate shape description method depends on the measurement technique available and the particle system under examination (in particular its size range). Methods based on mathematical operations on contour lines (e.g. fractal dimension analysis or Fourier analysis) require a relatively high resolution of particle images. This may be obtained by using a scanning electron or light microscope. Apart from such factors, the results of shape analysis may also be significantly affected by sample preparation (e.g. by the sample size and its representativeness of the whole sample) by particle orientation in 2D-analysis.

4.2.23 **Table R.7.1-X Methods for the qualitative or semi-quantitative description of particle shape and morphology**

Method details	Material and size range	Data type
<p>Transmission Electron Microscopy (TEM)</p> <p>TEM can be used for samples collected from the air or prepared in suspension on a TEM grid, including those from separation and sampling instruments. Powder preparation is very easy and fast for this method. Enables qualitative assessment of size and shape of particles, and differentiation between agglomerates and primary particles. TEM has a very high local resolution (nm) and is capable of imaging lattice planes and individual rows of atoms with resolution better than 0.2 nm. Additions to TEM can provide further information e.g. Scanning Transmission Electron Microscopy (STEM), High-Resolution TEM (HRTEM) or in-situ measurements using Environmental TEM, which offers the potential for dispersed samples to be characterised.</p> <p>However, TEM is a highly work-intensive method and requires manual preparation of samples. Dispersions need to be diluted (to ca. 1%) or prepared into work-intensive cryo-sections. Drying samples under vacuum for analysis may alter the size and shape of the particles being characterised. An extremely small area of the sample is analysed, which might not be representative enough. The comparatively small share of evaluated particles (ca. 1,000) results in limited statistical precision. Only a two-dimensional projection of particles is visible and can be evaluated; and the interpretation of pictures is difficult. Picture analysis is impossible if agglomeration is significant. Contours of particles may not be clearly resolved in some samples. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects.</p> <p>Further informative information on this method is available in ISO/TR 27628:2007. ISO 13322-1:2004 and ISO 13322-2:2006 provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.</p>	<p>Particles in solid, powder and suspension form.</p> <p>Size range: < 0.1 – 10 µm. Particularly suitable for the particle size range of 1 - 500 nm.</p>	<p>Image, providing opportunity to determine macro-, meso- and micro-descriptors of shape</p>

<p>Scanning Electron Microscopy (SEM)</p> <p>SEM can be used for samples collected from the air or prepared in suspension on a SEM grid, including those from separation and sampling instruments. Sample preparation is easier than for TEM, and only a small quantity of sample needed. Testing possible with undiluted dispersions and emulsions. SEM enables non-destructive testing of samples, and provides an image of the sample structure with very precise determination of size and shape at high local resolution. This method can be used in-situ as Environmental SEM.</p> <p>A representative sample of the material must be used. Where samples are not electrically conducting, plasma sputter-coating the surface-adhered particles with a layer of a conducting material is often required. This process may modify the sample being characterised. Only a small section of the sample is pictured and imaging is limited to surface features. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects.</p> <p>Further informative information on this method is available in ISO/TR 27628:2007. ISO 13322-1:2004 and ISO 13322-2:2006 provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.</p>	<p>Particles in solid, powder and suspension form.</p> <p>Size range: < 0.01 – 10 µm.</p> <p>Particularly suitable for the particle size range of 10 nm – µm</p>	<p>Image, providing opportunity to determine macro-, meso- and micro-descriptors of shape</p>
<p>Scanning Probe Microscopy (SPM)</p> <p>SPM includes both atomic force microscopy and scanning tunnelling microscopy (STM), which are all based, with some minor modifications, on a scanning probe (called the tip), which is moved across a substrate where particles have been deposited. SPM techniques allow individual nanoparticles and aggregates to be profiled in three dimensions from which shape can be studied. This is an advantage over SEM and TEM, which can measure only two dimensions. Air samples or liquid dispersions can be assessed, including those from separation and sampling instruments. SPM images give directly the three-dimensional morphology of complex samples such as carbon nanotubes, and can resolve simultaneously both their atomic structure and the electronic density. SPM enables rapid sample analysis under ambient conditions, and requires minimal sample</p>	<p>Particles in air or dispersed in a liquid</p> <p>Size range: 1nm – 8 µm</p>	<p>Image, providing opportunity to determine macro-, meso- and micro-descriptors of shape</p>

<p>preparation.</p> <p>For analysis, the sample must disperse onto and adhere to a substrate. The roughness of the substrate must be less than the size of the particles being measured to avoid a lack of clarity regarding image interpretation. Although SPM can resolve horizontal and vertical details to fractions of a nanometre, it is unable to deal with large changes in vertical profile occurring over a few nanometres.</p> <p>ISO TR/27628:2007 provides an informative description of this method.</p>		
<p>Optical microscopic examination</p> <p>It is preferable to prepare samples directly in order not to influence shape and size of the particles.</p> <p>This method provides images for the characterisation of the shape and distribution of samples of respirable and inhalable particles and does not refer to airborne dust or dispersed or nebulised particles.</p> <p>Optical microscopy can be used to examine likelihood of fibres present by comparing similarities to known fibrous or fibre releasing substances or other data. Extreme care required during sample preparation to avoid fibre breaking and clumping. Care should also be taken to avoid contamination by airborne fibres. Samples might be prepared by (a) producing suspensions in water by gentle hand agitation or vortex mixing or (b) transfer of dry material onto copper tape either directly or by spraying of the dry fibres by use of atomiser or pipette. Length and diameter distributions should be measured independently at least twice and at least 70 fibres counted. No two values in a given histogram interval should differ by > 50% or 3 fibres, whichever is larger. The presence of long thin fibres would indicate a need for further, more precise measurements.</p>	<p>Particles of all kinds, including fibres.</p> <p>Size range: 0.2–5000 μm.</p> <p>Fibre diameters as small as 0.2 μm and as large as 100 μm and lengths as small as 5 μm and as large as 300 μm.</p>	<p>Image, providing opportunity to determine macro-, meso- and micro-descriptors of shape</p>

4.2.24 Using the methods listed in Table R.7.1-X, the following information should be presented:

- Sample preparation methods and analysis methods used
- Lot number, sample number

- Suspending medium, temperature, pH
- Representative image(s) from microscopy
- Shape descriptor(s)
- Reference all Standards (e.g. ISO) used and reference materials used

4.2.25 **Published data on shape**

4.2.26 No electronic databases that are specific to particle shape data could be found at the time of publication. Software used with commercial instruments characterising shape by image analysis often contain libraries of reference shapes to categorise the particles under test.

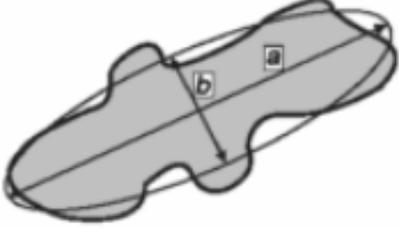
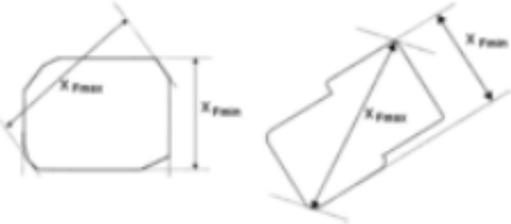
4.2.27 **R.7.1.19.3 Evaluation of available information on shape**

4.2.28 **Experimental data on shape**

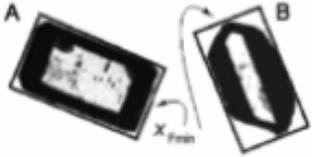
4.2.29 Shape is very often not a specific physico-chemical property of a substance. The original shape is highly dependent on the industrial processing methods used and can also be affected by subsequent environmental or human transformations. In that respect any published data on shape will only be pertinent to that particular sample or process.

4.2.30 Macroshape descriptors represent the geometrical proportions of particles. Most of them are ratios of descriptors of different geometrical properties. Geometrical (Table R.7.1-X) and proportion (Table R.7.1-X) descriptors of macroshape, mesoshape descriptors (Table R.7.1-X), combination of shape descriptors (Table R.7.1-X) and roughness descriptors (which represent microshape properties) (Table R.7.1-X) are available (ISO 9276-6:2008). Fractal dimensions are necessary to distinguish between mesoshape (concavity) and microshape (descriptors).

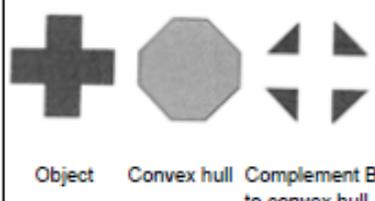
4.2.31 Table R.7.1-X Geometric macroshape descriptors (reproduced from ISO 9276-6:2008)

<p>Legendre ellipse of inertia</p> 	<p>An ellipse with its centre at the particle's centroid and with the same geometrical moments, up to the second order, as the original particle area</p> <p>The major and minor axes are given by x_{Lmax} and x_{Lmin}, respectively</p> <p>Robust measurement</p> <p>For equations, see Clause A.2</p>
<p>Feret diameters x_{Fmax}, x_{Fmin}</p> 	<p>Distances between parallel tangents</p> <p>Maximum diameter x_{Fmax} corresponds to the "length" of the particle</p> <p>Minimum diameter x_{Fmin} corresponds to the "breadth" of the particle</p>
<p>Length x_{LF}</p>	<p>Feret diameter perpendicular to the minimum Feret diameter</p>
<p>Geodesic length x_{LG}, thickness x_E</p> 	<p>Better approximations for very long and concave particles, such as fibres</p> <p>Robust method of determining x_{LG} as an approximation for geodesic length and x_E, using the following equations for an area- and perimeter-equivalent rectangle:</p> $A = x_E \cdot x_{LG} \quad P = 2(x_E + x_{LG})$

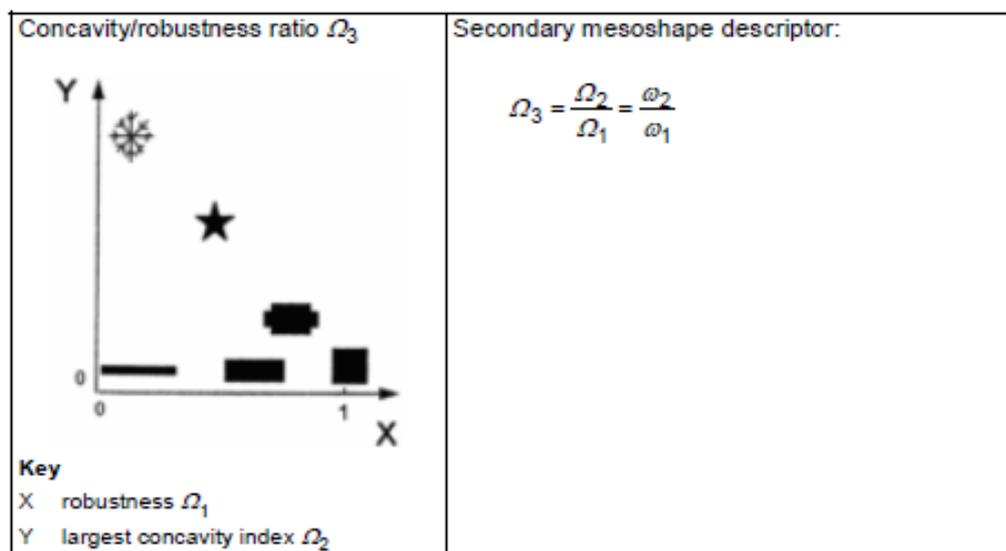
4.2.32 Table R.7.1-X Proportion macroshape descriptors (reproduced from ISO 9276-6:2008)

Ellipse ratio	<p>Ellipse ratio = x_{Lmin} / x_{Lmax}</p> <p>where x_{Lmin} and x_{Lmax} are the lengths of the axes of the Legendre ellipse</p> <p>(Also used: elliptical shape factor)</p> <p>More robust parameter than aspect ratio</p>
Aspect ratio	<p>For not very elongated particles:</p> <p>Aspect ratio = x_{Fmin} / x_{Fmax}</p>
Elongation	<p>For very elongated particles, such as fibres:</p> <p>Elongation = x_E / x_{LG}</p> <p>(Also used: eccentricity)</p>
Straightness	<p>For very elongated particles (reciprocal of curl):</p> <p>Straightness = x_{Fmax} / x_{LG}</p>
Irregularity (modification ratio)	<p>Relationship between the diameter of the maximum inscribed circle d_{imax} and that of the minimum circumscribed circle d_{cmin}:</p> <p>Irregularity = d_{imax} / d_{cmin}</p> <p>(Also used: modification ratio)</p>
	
Compactness	<p>Degree to which the particle (or its projection area) is similar to a circle, considering the overall form of the particle:</p> <p>Compactness = $\frac{\sqrt{(4A/\pi)}}{x_{Fmax}}$</p> <p>Roundness Rn is also used, but is less robust:</p> <p>$Rn = 4A / \pi x_{Fmax}^2$</p>
Extent	<p>Extent = $\frac{A}{x_{Fmax} \cdot x_{Fmin}}$</p> <p>(Also used: bulkiness)</p>
	<p>Ratio of the Feret box area to the projected area:</p> <p>Box ratio = A / A_{box}</p> <p>$A_{box} = x_{Fmin} \cdot x_{LF}$</p> <p>Very sensitive to orientation</p>

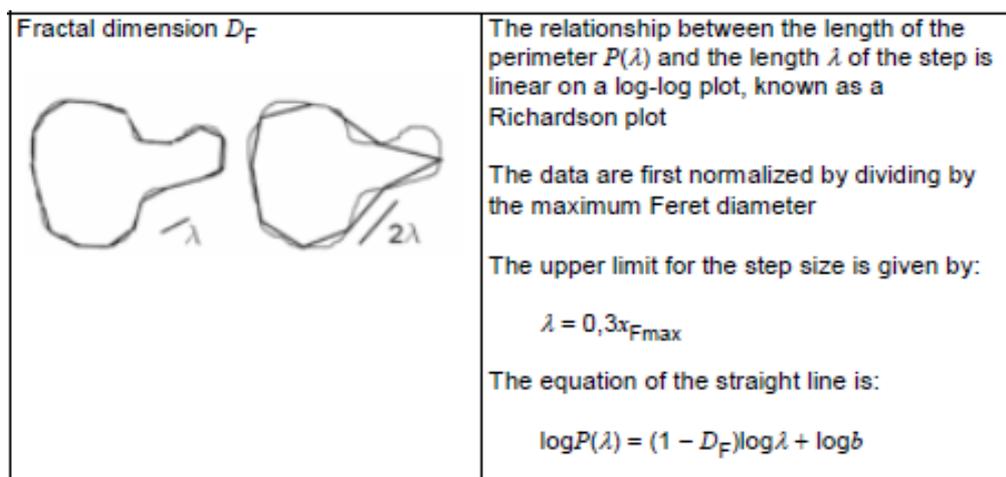
4.2.33 Table R.7.1-X Mesoshape descriptors (reproduced from ISO 9276-6:2008)

Wadell's sphericity Ψ'	$\Psi' = (x_V/x_S)^2 = \pi \cdot x_V^2/S$
Circularity C	<p>Degree to which the particle (or its projection area) is similar to a circle, considering the smoothness of the perimeter:</p> $C = \sqrt{\frac{4\pi A}{P^2}} = \frac{x_A}{x_P}$ <p>(Term under square root sign is called the form factor, FF)</p>
Solidity	<p>Measure of the overall concavity of a particle:</p> $\text{Solidity} = A/A_C$ <p>where A_C is the area of the convex hull (envelope) bounding the particle</p> <p>Global surface concavity index (CI) and concavity are also used:</p> $\text{CI} = \frac{A_C - A}{A} \quad \text{Concavity} = \frac{A_C - A}{A_C}$
Convexity	$\text{Convexity} = P_C/P$ <p>where P_C is the length of the perimeter of the convex hull (envelope) bounding the particle</p>
Average concavity	$\Psi_{FP} = \frac{\bar{x}_F}{x_P}$ <p>where the angle-average Feret diameter \bar{x}_F is given by:</p> $\bar{x}_F = \frac{1}{\pi} \int_0^\pi x_F(\alpha) d\alpha$
<p>Particle robustness Ω_1</p> 	$\Omega_1 = \frac{2\omega_1}{\sqrt{A}}$ <p>where ω_1 is the number of erosions necessary to make the silhouette disappear completely</p>
<p>Largest concavity index Ω_2</p> 	$\Omega_2 = \frac{2\omega_2}{\sqrt{A}}$ <p>where ω_2 is the number of erosions necessary to make the residual of the silhouette, set with respect to the convex hull of area A_C, disappear completely</p>

4.2.34 **Table R.7.1-X Combination of shape descriptors (reproduced from ISO 9276-6:2008)**



4.2.35 **Table R.7.1-X Roughness descriptor (reproduced from ISO 9276-6:2008)**



4.2.36 **Non-Experimental data on shape**

4.2.37 At present, there are no QSPR/QSAR tools available for accurately predicting particle shape. Therefore the property will need to be experimentally determined.

4.2.38 **Remaining uncertainty on shape**

4.2.39 It is useful to distinguish between aggregates and agglomerates. While an aggregate may be considered to be permanent in most situations, agglomerates may break up under certain circumstances. As small particles often form

agglomerates, sample pre-treatment (e.g. the addition of dispersing agents, agitation or low-level ultrasonic treatment) may be required before the shape can be determined. However, great care must be taken to avoid changing the shape or size of the particle during sample preparation and the influence of any dispersant on testing results.

4.2.40 A combination of terms and/or measurands may be needed to describe shape; this is essential to circumvent the challenges already foreseeable where materials are capable of concurrently exhibiting multiple shapes in a sample which may present different hazard potentials.

4.2.41 Problems associated with image analysis are manifold and errors can be introduced in the generation of shape descriptors. These errors can exist at many levels, but most of them are fundamentally different from those observed in the more traditional techniques used for the characterisation of dispersed matter. Such shape descriptor errors are usually introduced by the protocols necessary to perform calculations on any given image (ISO 13322-1:2004, Annex D). The common sources of errors which occur when performing image analysis and in the comparison of image analysis protocols include image resolution, binarization and algorithms for calculating shape descriptors (ISO 9276-6:2006).

4.2.42 **R.7.1.19.4 Conclusions on shape**

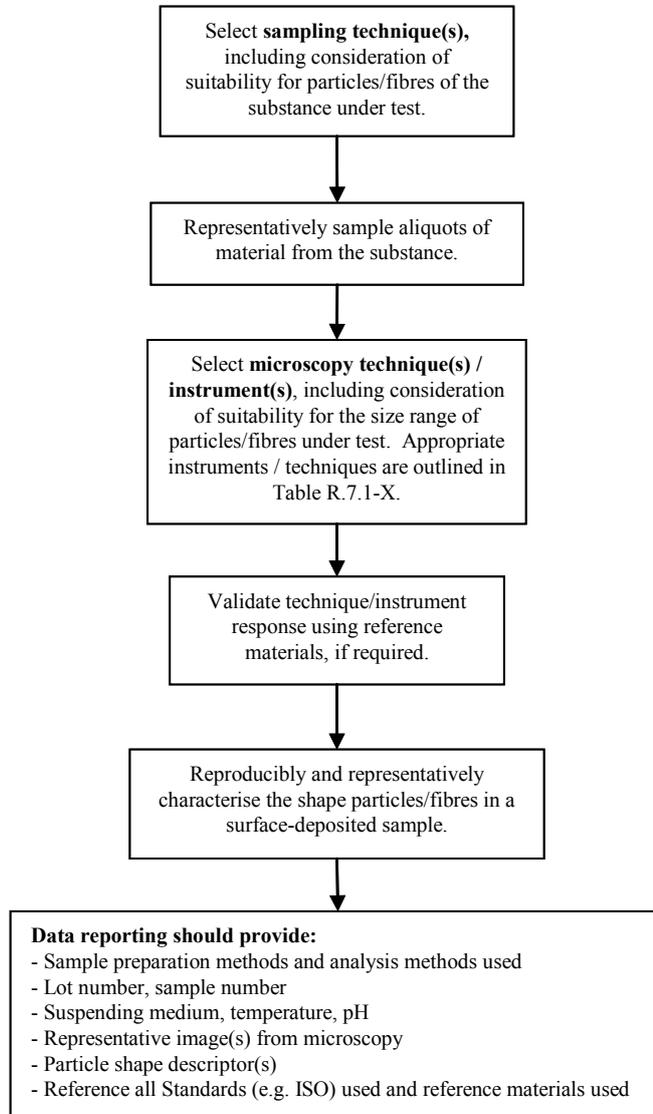
4.2.43 Shape is an important parameter in the characterisation of particles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape. The shape of particles is usually determined by electron microscopy (e.g. TEM, SEM), which includes many qualitative and semi-quantitative techniques to investigate the morphology (size and shape) and also the aggregation state.

4.2.44 **Concluding on C&L and Chemical Safety Assessment**

4.2.45 Shape is not used as a classification and labelling criterion. However, it can be used in the chemical safety assessment in considering risks associated with the substance.

4.2.46 R7.1.19.5 Integrated testing strategy (ITS) for shape

4.2.47 The following schematic diagram (Figure R.7.1-X) presents an integrated testing strategy for shape.



4.2.48 R7.1.19.6 References on shape

4.2.49 Barrett, P.J. 1980, "The shape of rock particles, a critical review", *Sedimentology*, 27, pp. 291-303

4.2.50 ISO 9276-6:2008 "Representation of results of particle size analysis. Part 6: Descriptive and quantitative representation of particle shape and morphology". ISO/TC 24.

- 4.2.51 ISO 13322-1:2004. "Particle size analysis -- Image analysis methods -- Part 1: Static image analysis methods", ISO TC 24/ SC 4.
- 4.2.52 ISO 13322-2:2006. "Particle size analysis -- Image analysis methods -- Part 2: Dynamic image analysis methods" ISO TC 24/ SC 4.
- 4.2.53 ISO 27628:2007 "Workplace atmospheres – Ultrafine, nanoparticle and non-structured aerosols – Inhalation exposure characterization and assessment", ISO TC 146/SC 2.
- 4.2.54 OECD. 2009, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme. First revision", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV, Paris.

4.2.55 R.7.1.20 SURFACE AREA

- 4.2.56 For particle-based substances, the surface plays an important role in influencing the physical and chemical interactions. As chemical reactions take place at surfaces, a sample of material with a high specific surface area to volume ratio can be expected to have a higher reactivity than a sample of the same material with a low specific surface area to volume ratio.
- 4.2.57 Surface area is an important parameter in the characterisation of nanoparticles, with emerging evidence of quantitative value as a dose metric or descriptor for hazard assessment. The total surface area should not be confused with the specific surface area where smaller particles have a larger specific surface area independent of whether they are present as primary, agglomerated or aggregated particles (SCENIHR, 2009). For nanoscale materials, the reduction in size is accompanied by an inherent increase in the surface-to-volume ratio.
- 4.2.58 The specific surface area will dictate the surface charge in cases where nanomaterials are surface functionalised. This in turn has direct consequences on (a) nanomaterial interaction (i.e., agglomeration) with other naturally occurring particulate matter (i.e., contaminant vectors); (b) route of exposure as a function of surface ligand-biological interface (i.e., bioaccumulation pathway, bioavailability); and (c) mechanisms of toxicity (e.g., dose response curves normalized for surface area may indicate different results compared to results presented on a per mass basis)" (OECD, 2009).
- 4.2.59 The volume specific surface area (VSSA) is determined from the entire particulate powder material including the whole size range distribution, with all external and/or internal surfaces. It characterises the entire particulate surface area per volume of a solid and/or powder material. The VSSA can be used to distinguish dry solid nanostructured material from non-nanostructured material based on its integral material surface area per material volume (SCENIHR, 2010; Kreyling et al., 2010).
- 4.2.60 The toxicity of some nanoparticles has been demonstrated in a number of studies to be related to their small size and therefore high surface area (e.g. Duffin et al., 2002, Duffin et al., 2007, Stoeger et al., 2006; Oberdörster et al., 2005). In addition, it has been observed in several nanotoxicity studies that effects correlate with surface area (e.g. Brown et al., 2001; Donaldson et al., 1998; Oberdorster et al., 1992; Tran

et al., 2000) to a greater extent than mass as a dose metric. Other studies have demonstrated that the mass or volume may be a better descriptor in some cases. No scientific consensus has been reached at this stage regarding whether a single metric will be appropriate or possible given the complexity of different toxicological profiles and physico-chemical characteristics.

4.2.61 Definitions of surface area

4.2.62 Surface area is defined as the area of the exposed surface of a single particle, or more general, the area of the exposed surface of a certain amount of a material (OECD, 2009).

4.2.63 Surface area as an extensive quantity depends on the amount of the material, and therefore a better comparable characteristic is the ratio of the surface area to the mass of a certain amount of a material. This is the so called specific surface area which is an intensive quantity and thus independent of the amount of the material. The volume specific surface area (VSSA) of a material is an ensemble measurement, only valid for the entire material as analysed; if a fraction/subset of the material (e.g. fractionated by size) is analysed, this subset will have a different VSSA which may be above or below the VSSA of the initial entire material.

4.2.64 Specific surface area = surface area of a material divided by its mass [SI unit: m^2/kg].

4.2.65 Volume specific surface area = density multiplied by the mass specific surface area [SI unit: m^2/cm^3].

4.2.66 R.7.1.20.1 Information requirements on surface area

4.2.67 The study does not need to be conducted if the substance is marketed or used in a non-solid or non-granular form. Specific surface area requires information on water insolubility. Fibre length and diameter distributions require information on the fibrous nature of the product and on stability of the fibrous shape under electron microscope conditions.

4.2.68 The summary should include a determination of the specific surface area [m^2/kg] and (where appropriate) the calculated volume specific surface area [m^2/cm^3] of the material under investigation, the temperature and conditions at which

measurements were made, purity of the sample used, physical state, method used and reference substance used (if any).

4.2.69 **R.7.1.20.2 Available information on surface area**

4.2.70 **Testing data on surface area**

4.2.71 The characterisation of particle properties requires very careful sampling and sample splitting practices to be followed. ISO 14488:2007 specifies methods for obtaining a test sample from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative of the whole sample with a defined confidence level. Further is available in the Sample Preparation section of the Introduction to R.7.

4.2.72 By far the most common technique for measurement of the surface area of particles is by gas absorption measurements using Brunauer, Emmet and Teller (BET) adsorption isotherm theory (Table R.7.1-Y) (Brunauer et al., 1938). This is a high vacuum method and requires a clean, dry sample of the nanomaterial. Nitrogen is the most common adsorbate, although many other gases such as argon, carbon dioxide, or krypton are also used. The BET technique involves measuring the amount of adsorbate released on vaporisation. The BET surface represents the surface area that is freely accessible to gases. The primary particle diameter (assumed as equivalent sphere diameter) is subsequently calculated from already available specific surface area and density of particles. Although this method provides measurement of two parameters simultaneously, i.e. size as well as surface area, the drawback of this procedure is in the assumption of a mono-dispersed spherical system which reports only an average size and does not provide the size distribution or a surface area distribution.

4.2.73 Emerging techniques for measuring particle surface area of nanoparticles in dispersion are being commercialised but are not yet standardised, such as the NMR analysis system for specific surface area determination of nano dispersions. This technique is based on the fact that liquid in contact with or “bound” to the surface of a particle behaves differently from that of the “free” liquid. Bound liquid molecules undergo restricted motion while free liquid can move unrestricted. The NMR relaxation time of liquid “bound” to the particle surface is much shorter than that of “free” liquid, the difference can be several orders of magnitude. In most situations

there is a rapid exchange between liquid molecules on the surface and in the rest of the fluid, and an average relaxation time can be measured; this is then a direct measure of the amount of available particle surface area.

4.2.74 Table R.7.1-Y Brunauer, Emmet and Teller (BET) for determination of surface area

Method and details	Material	Data type
<p>BET method (ISO 9277:2010; ISO 18757:2005)</p> <p>Enables determination of the total specific external and internal surface area of by measuring the amount of physically absorbed gas. Commonly applied to determine the surface area of nanomaterials. Allows an assessment of the agglomeration state of powders.</p> <p>Method assumes a mono-dispersed spherical system and provides a measurement of the surface area of a dry particle, which is not necessarily representative of the surface area of the particle when dispersed in the exposure medium. In order to ensure proper working conditions and correct data evaluation, the apparatus performance should be monitored periodically using a surface-area reference material. The BET method cannot reliably be applied to solids which absorb the measuring gas.</p> <p>ISO 9277:2010 is applicable to adsorption isotherms of type II [disperse, nonporous or macroporous solids] and type IV [mesoporous solids, pore diameter between 2-50 nm]. ISO 18757:2005 is applicable for determination of the total specific external and internal surface area of disperse or porous [pore diameter > 2 nm] fine ceramic materials.</p>	Disperse or porous solids (e.g. powders)	Specific surface area (m ² /kg)

4.2.75 Using the BET method, the following information should be presented:

- sample preparation methods and analysis methods used
- lot number, sample number
- pre-treatment and degassing conditions, e.g. degassing in a vacuum or in inert gas flow, temperature and duration of degassing;
- mass of degassed sample;
- adsorptive (chemical nature, purity);
- adsorption isotherm (n_a , plotted against relative pressure, p/p_0), measurement temperature;

- evaluation parameters: multipoint or single-point determination, BET plot or range of linearity, monolayer amount, BET parameter C , molecular cross-sectional area used;
- specific surface area;
- references for all Standards (e.g. ISO) and reference materials used.

4.2.76 **Published data on surface area**

4.2.77 No electronic databases that are specific to particle surface area data could be found at the time of publication.

4.2.78 **R.7.1.20.3 Evaluation of available information on surface area**

4.2.79 **Experimental data on surface area**

4.2.80 Surface area is not a specific physico-chemical property of a substance. Any published data on surface will only be pertinent to that particular sample or process.

4.2.81 **Non-Experimental data on surface area**

4.2.82 At present, there are no QSPR/QSAR tools available for accurately predicting the surface area of nanomaterials. Therefore the property will need to be experimentally determined.

4.2.83 **Remaining uncertainty on surface area**

4.2.84 In many cases specific surface area measurements are derived quantities that depend on the nature of the probe molecule. (OECD, 2010). In the case of porous materials, it is often useful to distinguish between external and internal surface. The external surface is usually regarded as the envelope surrounding the discrete particles or agglomerates, but is difficult to define precisely because solid surfaces are rarely smooth on an atomic scale. The external surface include all the prominences and also the surface of those cracks which are wider than they are deep; the internal surface comprises the walls of all cracks, pores and cavities which are deeper than they are wide and which are accessible to a test gas (the adsorptive). In practice, the demarcation depends on the methods of assessment and the nature of the pore size distribution; hence accessibility of pores depends on the size and shape of gas molecules, the area of, and the volume enclosed by, the

internal surface as determined by gas adsorption will depend on the adsorptive molecules (molecular sieve effect).

4.2.85 Not all particulate materials are amenable to a meaningful VSSA determination, for example where the specific surface area of substances with complex structural assemblies where the internal components are intrinsically not measurable.

4.2.86 **R.7.1.20.4 Conclusions on surface area**

4.2.87 For particle-based substances, the surface plays an important role in influencing the physical and chemical interactions. Surface area is an important parameter in the characterisation of nanoparticles in particular, with emerging evidence of quantitative value as a dose metric / descriptor for hazard assessment. The surface area will dictate the surface charge in cases where nanomaterials are surface functionalised, with direct consequences on nanomaterial interaction (i.e., agglomeration) with other naturally occurring particulate, route of exposure as a function of surface ligand-biological interface and mechanisms of toxicity (OECD, 2009). By far the most common technique for measurement of the surface area of particles is by gas absorption measurements using Brunauer, Emmet and Teller (BET) adsorption isotherm theory.

4.2.88 **Concluding on C&L and Chemical Safety Assessment**

4.2.89 Surface area is not used as a classification and labelling criterion. However, it can be used in the chemical safety assessment in considering risks associated with the substance.

4.2.90 **R.7.1.20.5 Integrated testing strategy (ITS) for surface area**

4.2.91 The tiered approach to testing (Section R.7.1.14) combined with the choice of an appropriate test method and implemented in conjunction with the ITS for granulometry (R.7.1.14.4) represents an integrated testing strategy for specific surface area.

4.2.92 **R.7.1.20.6 References on surface area**

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4.3 RESEARCH & DEVELOPMENT REQUIREMENTS

4.3.1 The following section outlines proposals for further R&D in relation to enhancing the evidence base of relevance/applicability of the information requirements, test methods or testing strategies for nanomaterials in the context of REACH. These recommendations do not preclude the determination and consideration of data where it can be feasibly gathered. It is not within the remit of the RIP-oN2 project to develop a *strategy* for addressing the research & development needs. Where it is identified that an important R&D need exists by other organisations (e.g. by OECD) or on the basis of the evidence and guidance reviewed, by default a high priority is assigned to specific properties, endpoints and/or methods. All others are considered to be of lower priority for further research and development and are indicated using an asterisk.

4.3.2 In relation to the recommendations for guidance which introduce additional information requirements (i.e. shape, surface area), we recommend that emerging standards and guidance documents, and implementations from the OECD Sponsorship Programme be considered.

4.3.3 PHYSICO-CHEMICAL PROPERTIES

4.3.4 EXISTING INFORMATION REQUIREMENTS

4.3.5 R.7.1.4 Relative density

4.3.6 Further research is required to assess the applicability of OECD TG 109 to nanomaterials, and modify if necessary (OECD, 2009).

4.3.7 R.7.1.6 Surface tension

4.3.8 Further research is required to assess the applicability of OECD TG 115 for nanomaterials, and modify if necessary (OECD, 2009).

4.3.9 R.7.1.7 Water solubility

4.3.10 On the basis of SCENIHR's 2009 opinion and OECD's conclusion in ENV/JM/MONO(2009)21, further work is required to determine the applicability of TG 105 and whether the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does

not completely dissolve. Further research required to resolve/overcome issues with agglomeration of nanomaterials in water solubility tests (RNC/RIP-oN2/B1/2/FINAL, 4.9-16).

- 4.3.11 ISO/AWI TR 13014 may contain information of relevance to consider in relation to the solubility and dispersibility of nanomaterials and it is recommended that this standard be reviewed when it reaches Final Draft International Standard (FDIS) status ((RNC/RIP-oN2/B3/2/FINAL, 6.1.10) for possible future citation in updated REACH Guidance.
- 4.3.12 **R.7.1.8 Partition coefficient N-octanol/water**
- 4.3.13 Further research is required to assess the applicability of OECD TG 107, 117 and 123 for nanomaterials, and modify if necessary (OECD, 2009).
- 4.3.14 **R.7.1.10 Flammability**
- 4.3.15 Further research required into applicability of current flammability test methods for nanopowders (RNC/RIP-oN2/B4/2/FINAL, table 4.9), building on initial work by NANOSAFE 2 (RNC/RIP-oN2/B3/2/FINAL, 3.1.75-85). This should include the validation of methods and the development of Standard Operating Procedures (SOP).
- 4.3.16 **R.7.1.11 Explosive properties**
- 4.3.17 Further research needed into applicability of current explosivity test methods for nanopowders (RNC/RIP-oN2/B4/2/FINAL, table 4.10), building on initial work by NANOSAFE 2 (RNC/RIP-oN2/B3/2/FINAL, 3.1.75-85). This should include the validation of methods and the development of Standard Operating Procedures (SOP).
- 4.3.18 **R.7.1.14 Granulometry**
- 4.3.19 The definition and scope of the term “Granulometry” under REACH is required to be developed and agreed (RNC/RIP-oN2/B2/2/FINAL, 5.1.4). This is a priority requirement.
- 4.3.20 OECD TG 110 is required to be updated and extended to cover nanoparticles, including acknowledgement of other standardised equipment (e.g. Centrifugal

Sedimentation) (RNC/RIP-oN2/B4/2/FINAL, table 4.13 Method B). Method A of OECD TG 110 (sedimentation, or centrifugation) is not applicable to nanomaterials (OECD, 2009), as it is useful only in the range $2 \mu\text{m} < R_s < 100 \mu\text{m}$. However, alternative standardised equipment (e.g. centrifugal sedimentation) can be used in accordance with this method. Method B of OECD TG 110 (electron microscopy) requires a necessary but minor deviation in the data reporting for nanomaterials (i.e. particles/fibres of less than 5 microns in length and less than 100 nm in diameter).

4.3.21 The following draft standards are recommended to be reviewed when they reach FDIS status, with a view to assessing their relevance for incorporation into the REACH Guidance on Granulometry (RNC/RIP-oN2/B3/2/FINAL, 6.1.46-47):

- ISO/AWI TS 10797, which describes the characterisation of SWCNT using transmission electron microscopy (currently at Committee Draft stage);
- ISO/AWI TS 10798, which describes the characterisation of SWCNT using scanning electron microscopy (SEM) and energy dispersive X-ray spectrometry analysis (currently at Committee Draft stage)
- ISO/CD 12025, which aims to provide a general framework for determining nano-object release from powdered engineered nanoparticles into the gaseous surroundings by means of analysis of the generated aerosols particles (currently at Committee Draft Stage).

4.3.22 **R.7.1.15 Adsorption / Desorption**

4.3.23 Further work may be required to establish the relevance of this property for nanomaterials. A consensus opinion on this matter could not be reached within the Project Consortium.

4.3.24 **R.7.1.17 Dissociation constant**

4.3.25 Further work is required to assess the applicability of OECD TG 112 for nanomaterials, and modify it, should this be necessary (OECD, 2009).

4.3.26 **ADDITIONAL RELEVANT SPECIFIC INTRINSIC PROPERTIES**

4.3.27 **Shape**

4.3.28 ISO/AWI TS 10797, which describes the characterisation of SWCNT using transmission electron microscopy, (currently at Committee Draft stage) is recommended to be reviewed when it reaches FDIS status, with a view to assessing its relevance for incorporation into REACH Guidance on shape (RNC/RIP-oN2/B3/2/FINAL, 6.1.62).

4.3.29 **Specific Surface Area**

4.3.30 Further work required to develop suitable reference materials for nanomaterials, to be used in methods such as BET (RNC/RIP-oN2/B4/2/FINAL, table 4.18; RNC/RIP-oN2/B3/2/FINAL 4.1.233, 6.1.67). This should include the validation of material and method combinations and the development/adoption of a Standard Operating Procedures (SOP). Should the new work item under ISO/TC 229 on "Generic requirements for reference materials for development of methods for characteristic testing, performance testing and safety testing of nano-particle and nano-fibre powders" be accepted, this work may produce important outputs of relevance to the characterisation of nanomaterials.

4.3.31 The validity of calculating surface area based on particle size measurements (obtained via methods such as SMPS, SAXS etc.) requires further investigation for nanomaterials (RNC/RIP-oN2/B4/2/FINAL, table 4.18 SMPS/FMPS) and the development/adoption of a Standard Operating Procedure (SOP).

4.3.32 **Porosity**

4.3.33 Further research into the relevance of the property of porosity is required, in terms of both its influence on the (eco)toxicological effects of nanomaterials and the applicability of available methods for nanomaterials (RNC/RIP-oN2/B2/2/FINAL, 5.2.19; RNC/RIP-oN2/B4/2/FINAL table 4.19). This will likely emerge as part of the OECD Sponsorship Programme, as porosity is included on the list of properties to be investigated. The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.34 Surface energy

4.3.35 Further research is needed in order to investigate the influence of surface energy on (eco)toxicological effects and to develop robust methods for the determination of the surface energy of nanomaterials. (RNC/RIP-oN2/B4/2/FINAL, table 4.20). The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.36 Surface chemistry

4.3.37 Further research is required into the relationship between surface chemistry and (eco)toxicological effects, and the utility of subsequent data for hazard assessment in a regulatory context. This will likely emerge from the OECD Sponsorship Programme, as this property is one of those under consideration. The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.38 It is recommended that ISO/DTR 14187 (which looks specifically at the surface chemical analysis of nanostructured materials), ISO/NP TS 10812 (which outlines the use of Raman Spectroscopy for the characterisation of SWCNT) and ISO/AWI TR 13014 (which aims to provide guidance on physico-chemical characterisation for manufactured nano-objects for toxicological testing), should be reviewed when they reach FDIS stage.

4.3.39 Surface acidity

4.3.40 Further research is required into surface complexation models and methods to study the surface acidity of nanomaterials (OECD, 2010), as well as the relationship between surface acidity and (eco)toxicological effects of nanomaterials (RNC/RIP-oN2/B4/2/FINAL, table 4.21). The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.41 Surface charge

4.3.42 Further research into the relationship between surface charge (zeta potential) and (eco)toxicological effects of nanomaterials is required (RNC/RIP-oN2/B3/2/FINAL, 3.1.253-257, 4.1.120-128, 5.1.37-40; RNC/RIP-oN2/B4/2/FINAL, table 4.22). The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.43 ISO/TC 24 is currently in the process of developing two standards on zeta potential determination, ISO/DIS 13099-1 and ISO/DIS 13099-2. Although these standards are not specifically for the characterisation of nanomaterials, it is recommended they be reviewed once published (RNC/RIP-oN2/B3/2/FINAL, 6.1.77) for possible future citation in updated REACH Guidance.

4.3.44 Redox potential

4.3.45 Research into the applicability of standard electrochemical methods, as well as alternative methods, for determining the redox potential of nanomaterials is required (RNC/RIP-oN2/B3/2/FINAL, 4.1.176-178; RNC/RIP-oN2/B4/2/FINAL, table 4.23). The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.46 Cell-free ROS/RNS production capacity

4.3.47 Further research required into the relationship between ROS/RNS generating capacity and (eco)toxicological effects of nanomaterials, as well as the development of standard measurement methods for nanomaterials (RNC/RIP-oN2/B4/2/FINAL, table 4.24). The R&D requirement includes basic research to establish the relevance of the property and applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.48 GENERAL CONSIDERATIONS

4.3.49 As part of the PARTICLE_RISK project, Zuin et al. (2010) developed an evidence-based approach for undertaking a preliminary ranking of nanoparticles in terms of their hazard potential. This involves assigning a hazard rating of high, moderate or

low to a specific nanoparticle across a range of “indicators” (physico-chemical or toxicological endpoints), according to a ranking table. The weight-of-evidence approach developed in PARTICLE_RISK has the potential to inform the development of thresholds for hazard testing based on nanomaterial type in the context of REACH. However, the current ranking values were based on measurement data which differed in their uncertainty and reliability, and further research data is required to enable a robust set of criteria/thresholds for hazard assessment within the physico-chemical property information requirements to be developed.

4.3.50 A number of key general points have been noted from the review of the scientific literature (RNC/RIP-oN2/B3/2/FINAL, section 4.1) for further consideration in the context of research and development requirements to further establish or validate emerging outputs. These include:

- It has been suggested that adequate particle characterisation should be performed in three distinct phases, primary, secondary, and tertiary, where: primary characterisation is performed on particles as-synthesised or as-received in its dry native state; secondary characterisation is performed on particles in the wet phase as a solution or suspension in aqueous media; tertiary characterisations are performed on particles following interactions with cells under *in vivo* or *in vitro* conditions (Sayes and Warheit, 2009). Characterisation after administration is particularly advantageous where the possibility of physico-chemical changes in the material before and after administration exists. Hence, there is need to establish the limits of when characterisation of nanomaterials as-produced or as-supplied (which is the most direct and currently realistic approach to obtaining physico-chemical information about the material being studied) can represent the properties of the material when in contact with the environment in which it is being observed, for example in air or physiological environments of *in vivo* or *in vitro* assays;
- A wider set of reference materials and procedural standards (including for sampling and measurement) are urgently required to overcome the problem of inconsistent data (Lead and Wilkinson, 2006; Borm et al., 2006). Some progress has been made recently regarding reference materials for

characterisation, but standardised nanoparticles are not yet widely available and researchers have to rely on commercially available, often not well-characterised nanoparticles (Linsinger et al., 2011).

- It is highly recommended that NanoImpactNet (NIN) protocols are reviewed when published with a view to assessing their relevance for incorporation into the REACH Guidance (RNC/RIP-oN2/B3/2/FINAL, 3.1.192-194). Protocols submitted to NIN thus far relate to exposure systems, particle preparation, particle characterisation, oxidative stress, cytotoxicity and exposure assessment.
- There is a need to develop (and include in the REACH Guidance, when available) standardised and validated methodologies for sampling nanomaterials and accompanying SOPs.
- There is a need to develop clear guidance on *in situ* characterisation and testing. This requires basic research to establish the applicability of methods, the validation of methods and the development of Standard Operating Procedures (SOP).

4.3.51 There are also a number of generic standards under development by ISO which, although they may not provide information of relevance to a specific REACH Information Requirement or additional relevant specific intrinsic property, are important to consider in the future (RNC/RIP-oN3/B3/2/FINAL, 6.1.99-6.1.105).

4.3.52 ISO/DTR 13121, which describes a process for evaluating, addressing, making decisions about, and communicating the potential risks of developing and using engineered nanoscale material (currently at FDIS stage). While this technical report does not address specific methods for characterising the physico-chemical properties of nanomaterials, it does provide guidance for establishing a physico-chemical profile of the nanomaterial as part of an overall risk evaluation process and is recommended to be reviewed once published.

4.3.53 ISO/DTR 13121 (currently at Committee Draft stage) is recommended to be reviewed once published as it highlights the importance of characterising the physico-chemical properties of a nanomaterial over its entire life cycle, and suggests that any anticipated changes in relevant physical and chemical properties

across the lifecycle of the material should be noted. For these reasons, the document suggests that it may be necessary to characterise the material at multiple points, unless there is good reason to expect that the material will remain unchanged. It provides the recommendation that the properties of the nanomaterial should be compared to those of the corresponding bulk (non-nanoscale) materials, where appropriate, to determine the nature and extent to which the properties are different. This information will be useful to further assess the possibility of read-across of data from the bulk form to the nano-form. Currently, ISO/DTR 13121 lists the following properties as being relevant to characterise in relation to the risk assessment of nanomaterials: chemical composition (including surface coating), molecular and crystal structure, physical form and shape (at room temperature and pressure), particle size, size distribution and surface area, particle density, solubility (in water and biologically relevant fluids), dispersibility, bulk density, agglomeration state, porosity, surface charge and surface reactivity.

4.3.54 There are also two further draft standards under development by ISO, which are recommended to be reviewed once published, as they are expected to provide valuable information in relation to the physico-chemical characterisation of nanomaterials, namely ISO/AWI TS 11931-1 and ISO/AWI TS 11937-1 which describe characteristics and measurement methods for nano-calcium carbonate and nano-titanium dioxide, respectively.

4.3.55 In addition, ISO/TC 229 has a new work item on "Generic requirements for reference materials for development of methods for characteristic testing, performance testing and safety testing of nano-particle and nano-fibre powders" currently out for ballot. This work will undoubtedly produce important outputs of relevance to the characterisation of nanomaterials, but it is unlikely that this will be available for some time.

4.3.56 **TOXICOLOGICAL ENDPOINTS & TESTING**

4.3.57 The following section outlines proposals for further R&D in relation to toxicological information requirements, test methods or testing strategies for nanomaterials, based on the outcomes of the gap analysis (RNC/RIP-oN2/B4/2/FINAL).

4.3.58 R.6 QSARS AND GROUPING OF CHEMICALS

4.3.59 The advice provided on the “scientific basis for the categorisation of nanomaterials and application of *in silico* methods, read-across and category approaches for deriving hazard information for nanomaterials from the information on bulk substances or from comparison between nanomaterials” concluded that a fully prescribed category approach is not yet possible for nanomaterials. As such, the approach presented is a higher level overview and suggests where such groupings could potentially be applied. However further development and validation of both testing methods as well as the category approach itself is required before a true category approach can be taken.

4.3.60 The use of QSAR/read across/grouping is not recommended unless scientifically justified and validated by experimental values in the same range that applies to the characteristics of the (nano)material.

4.3.61 It is recommended that the advice provided be considered for further development of a possible new sub-section on nanomaterials under R.6.2.5 Guidance on specific types of categories.

4.3.62 NON-TESTING *IN SILICO* APPROACHES

4.3.63 The potential value of the development of validated *in silico* or non-testing approaches for nanomaterials, as with all substances under REACH, cannot be over stated and as such this could be considered a high, short term to medium term priority. However as discussed in sections 4.2 and 4.3 of this document, such approaches are not yet sufficiently developed for nanomaterials. The use of non-testing data, where justified and acceptable is beneficial in several ways not least due to the reduction of testing resources required for registration improving the speed in which substances can be registered and associated cost.

4.3.64 Where *in silico* approaches are based on extensive data sets, especially those consisting of human epidemiological data for analogous materials, these may allow a clearer insight into the potential long term effects of materials in a ‘real world’ situation that may not be as accurately gleaned from standard test methods using animal models. This again may be particularly pertinent in the case of complex disease state for which extrapolation between animal models and humans maybe

difficult such as for carcinogenicity. As such further research development and validation of such approaches should be considered one of the highest priorities to ascertain their applicability to nanomaterials. Such R&D should focus on the following areas of non-testing approaches:

- 4.3.65 Basis for grouping or categorisation of nanomaterials based upon physico-chemical characteristics could allow the broad identification of hazardous properties which would inform of the need for further testing and potential endpoints to be tested. A preliminary scheme is suggested based upon the current scientific evidence behind drivers of (nano)particle toxicology and has been discussed. Thorough characterization of NM and efficient sharing of this data along with hazard information are important conditions for the rapid development in this area.
- 4.3.66 The development of a greater understanding and implementation of (Q)SAR approaches would be enormously beneficial to allow rapid screening of materials based upon structural/ chemical attributes with defined toxicological importance.
- 4.3.67 Further R&D into read-across is also required for validation of non-testing approaches. However the nature of the read-across requires consideration. By this, we mean read-across between differing forms of particle, both within the nano-dimension (nanoparticle to nanoparticle read-across) or between a bulk (non-nano dimension) material and a nanomaterial of the same composition (bulk to nano read-across). The benefit of this is that testing data that has already been obtained for a bulk compound may be usable and applicable for nanomaterials and as such reduce or remove the need for further testing (reducing animal testing).
- 4.3.68 **CONSIDERATIONS FOR STUDY DESIGN**
- 4.3.69 Investigations undertaken and reported to date have highlighted a number of key issues or gaps in existing testing strategies which may influence the outcome of studies, and thus should be observed closely in the consideration of nanomaterials within the context of REACH.

- 4.3.70 In relation to the manner in which experiments are set up, factors such as the exposure method, dose selected, species used, cell type under investigation and in the case of photo-reactive nanomaterials such as some metal oxides, light conditions (Warheit *et al.* 2005) all have the potential to impact on the toxicity of nanoparticles, indicating that the experimental set up is very influential.
- 4.3.71 A summary and discussion of those issues considered to be of importance in this respect is provided below.
- 4.3.72 **Dispersion**
- 4.3.73 As discussed at various points within the RNC/RIP-oN2/2/B3/FINAL report, it is clear that dispersion impacts upon the potential toxicity of nanomaterials in animals or cells. A multitude of dispersing processes have been utilised by investigators to improve the dispersion including solvents (such as acetone), surfactants (such as pluronic), proteins (albumin and serum) or mechanical processes (such as centrifugation, or sonication). At this time it is not possible to conclude which techniques are most appropriate, but in designing experimental protocol for consideration of nanomaterials in the context of REACH, it is essential that such techniques should try to mimic realistic exposure scenarios, routes and avoid interference by dispersants.
- 4.3.74 **Selection of Dose**
- 4.3.75 Many investigations reported have used very high doses, raising the question of whether the toxic effects observed are likely to derive from dose used or the material. In particular, at high doses, the aggregation of CNT is promoted, and so the toxicity that transpires *in vivo* is potentially a result of the blockage of airways and blood vessels, rather than to a specific toxic effect.
- 4.3.76 In order to inform selection of relevant nanomaterial exposure concentrations to be used within *in vitro* and *in vivo* experiments, information regarding the human exposure levels is required as a reference. However, at the current time this is severely lacking and thus exposure assessment is of key importance to developing sound dose selection for future studies
- 4.3.77 In relation to dosing regime, many investigations of nanomaterial toxicity have used a single dose administered to animals or cells. Within occupational or consumer

settings, it is more likely that normal nanomaterial exposures (non-accidental) will occur over a period of time, depending on their application.

4.3.78 **Selection of exposure route and duration**

4.3.79 Testing methods for initial *in vivo* toxicity assessment normally use the oral route of exposure. However for the testing of nanomaterials, this may not be the ideal first candidate. As a general observation, repeated dose studies with lower doses over a long time period and use of a route of exposure appropriate to the end use of the nanomaterial in question, are likely to be of greater relevance in consideration of the potential risk of nanomaterials within occupational or consumer settings, than extremely short exposures at high doses. In addition, the use of chronic studies will also allow for the more relevant identification of the potential carcinogenic consequences of nanomaterial exposure.

4.3.80 **Interaction of nanoparticles with biological molecules**

4.3.81 Attention should be directed to consideration of the role that interaction of nanoparticles with biological molecules plays in altering their behaviour within biological systems. It is known that on entering the body, particles immediately become coated in biological molecules, including proteins. It is hypothesized that this coating can influence particle behaviour and toxicity, with different particles having different capacities to bind different molecules. Furthermore, there is a possibility that the particles can alter the protein structure and function (and thus behaviour), which again may contribute to toxicity. Further research is required to generate a greater understanding of this complex area, and it is advised that consideration should be given in future experimentation to the role that such interactions may play in toxicity.

4.3.82 **Use of physico-chemical data to inform experimental design**

4.3.83 It was been highlighted both within the toxicology and physico-chemical sections of RNC/RIP-oN2/B3/2/FINAL that the physico-chemical characteristics of particles used (size, crystallinity, functionalisation, contamination etc.) can impact on their toxicity. Thus, as these factors are able to influence the findings obtained, a thorough physico-chemical characterisation should be undertaken and used as

justification of the relevancy of the experimental approach used in future toxicological investigations undertaken.

4.3.84 In addition, a specific consideration in relation to metal nanomaterials is determination of whether any toxicity observed derives from their small size, is mediated through the release of ions from particles, or perhaps a combination of both. Elucidation of this is essential for the hazard characterisation of metal nanoparticles within the context of REACH

4.3.85 **Target Organ Toxicity considerations**

4.3.86 At the current time, there is a paucity of data relating to the systemic transfer of particles following exposure via the lungs, skin and gut, and this should be a focus of future experiments. The benefit of this data is to identify target organs which particles may preferentially transfer to and/or accumulate in. Such organs would therefore be identified for specific system/ organ toxicity studies (Appendix R.7.5-1). Studies have focused on dermal and pulmonary toxicity of particles and demonstrated the transfer of test nanoparticles in small amounts (~1-8%). However there is an absence of data on the consequences of exposure to the gastrointestinal tract, and within damaged/diseased skin. Once systemically available, target organs currently identified include the liver, kidney, cardiovascular system and brain. The effect of particle interaction with these organs requires further elucidation to establish if the small percentage of transfer is toxicologically significant and if repeat exposure leads to retention of particles within such organs/ systems resulting in a build up of dose.

4.3.87 Due to the sensitivity of the system, the transfer of nanoparticles to the reproductive system, in particular the foetus should be considered a very high short term priority.

4.3.88 Another interesting attribute which requires further investigation is which physico-chemical parameters influence particle transfer rates and retention? In the study by Semmler-Behnke et al (2008) they demonstrated the importance of particle surface charge in particle translocation by demonstrating an increase in translocation to 8% of the instilled dose into the lungs.

4.3.89 Adoption of standardised 'controls' to assist assessment of toxicity

4.3.90 The use of both nanoparticulate and non-particulate controls (such as carbon black or asbestos within CNT studies, or zymosan within inflammation studies) has been reported on numerous occasions. The choice of controls can, to some extent be driven by the hypothesis being tested (and links to determination of the physico-chemical characteristics responsible for toxicity). The use of such benchmark controls (those for which extensive background information is available) provides a useful indication of the relative toxicity of the nanomaterial under investigation versus other particles or reagents of known toxicity (e.g. Warheit *et al.* 2004, Shvedova *et al.* 2005). In addition, the use of comparative testing between nano and non-nano forms of a substance should be encouraged as this would provide high value, within study comparisons of different size forms which would help inform the feasibility and process of bulk to nano read-across. Thus, comparative testing and use of benchmarking controls could be encouraged for the hazard identification and characterisation of nanomaterials within the context of REACH. However this needs to be considered in relation to the need for extra control groups, thus requiring extra animals within testing regimes which may outweigh the potential benefits. As such the most appropriate use of benchmark controls could be within in vitro testing, including their use in the development of in vitro methods. This would provide a link and point of comparison between previous in vivo results using the benchmark material and the in vitro results using the benchmark material and the new test substance. In relation to comparative testing, R&D which specifically addresses the issue of bulk and nano-form comparison should be undertaken to elucidate grounds for read across.

4.3.91 Interference of nanomaterials with toxicity assays

4.3.92 Nanomaterials have been on occasion found to interfere with several commonly used assays utilised to determine their cellular or toxic effects. For example, some nanoparticles may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanoparticles may bind to assay components including the substrates (such as CNT with the reagent in MTT assays; Belyanskaya *et al.* 2007) or the biomarker being measured, (such as LDH and cytokine proteins, see for example Davoren *et al.* 2007).

4.3.93 As the potential for inhibition or enhancement of the test result may impact on numerous test methods, some of which are known or suspected as outlined in RNC/RIP-oN2/2/B4/FINAL report Section 5 (e.g. OECD TG 431/EU B.40 Human Skin Model tests (EPISKIN™, EpiDerm™), and some may not be currently known. As such further R&D to ascertain potential grounds of inhibition/enhancement is required within this area so that test methods can be amended appropriately or recommendations made against their use within guidance in situations of confirmed reliability problems with nanomaterials. As a R&D requirement, a greater understanding of inhibition/enhancement could be considered a short term goal of high priority.

4.3.94 **ENDPOINT SPECIFIC R&D PRIORITIES**

4.3.95 As echoed in RNC/RIP-oN2/B1/2/FINAL paragraph 5.15, in the utilisation of inhalation exposure to (nano)particles and other substances it would be prudent to consider the utilisation of Bronchoalveolar Lavage (BAL) data as a standard practice in inhalation studies and possibly, a requirement. The justification of the usefulness of BAL in improving sensitivity of inhalation study results is given in the following paragraphs and the guidance alterations are outlined in the relevant sections of the following Guidance endpoint sections.

4.3.96 BAL is a methodology developed to sample the cells of the airspaces of the lungs, the airways and alveoli, which allow monitoring of pathological processes that are occurring there. The normal population of the BAL are the cells that normally move around on the surface of the airspaces ingesting microbes and dust and turning over the fluids that line the airspaces. This normal population is predominantly alveolar macrophages with a small population, normally less than 10% of lymphocytes and 1% polymorphonuclear leukocytes (PMN) in humans (Balbi et al., 2007). This pattern of the normal cell population is much the same in rats and humans. Any damage or disease process that is ongoing in the lungs is reflected in a change in the cell population in the BAL, as immuno-inflammatory cells are recruited into the lungs and this cellular exudate appears in the air spaces. In general the more severe the damage or injury, the greater the cellular exudate and the greater is the change in the inflammatory cell population in the BAL and the deviation from normalcy in the BAL cell population. Thus in the most severe lung diseases in humans, acute respiratory distress syndrome (ARDS) or pneumonia for

example, the BAL is overwhelmed with neutrophils the defining cell of acute inflammation. In asthma or other immunopathology of the lung, eosinophils and lymphocytes can become prominent revealing the unique underlying role of the immune system. Along with cells the BAL fluid also contains a sample of the proteins and other biomolecules that are present in the airspaces and lining fluid. Thus extra information on the levels of exudated protein, cytokines, products of oxidative stress, cytoplasmatic enzymes indicative of membranolysis etc. can also be obtained, which illuminate the underlying cellular pathology.

4.3.97 The use of BAL in particle toxicology (Henderson, 2006) is predicated on the understanding that BAL provides a quantitative index of the extent of lung damage and subsequent inflammation that arises in response to pulmonary deposition of dust. BAL is considered to be much more sensitive to early effects of particle-induced injury, detecting the injury before an overt pathological change can be seen in histological sections of rat lungs or where changes are minimal making them impossible to quantify in sections. Practice and a huge number of published studies has shown that the lung injury caused by inhalation of a damaging dust follows a well defined course of developing inflammation that can be tracked in the BAL as increasing numbers of cells, the proportion of PMN and levels of inflammatory markers . In general the inflammation evident in the BAL profile undergoes a decline when the exposure ceases, although the most toxic dusts cause inflammation that persists after exposure ceases. Exposure to low toxicity dusts causes only a very slight change in the population in the BAL, typically increased numbers of macrophages and the accumulation of particle-laden macrophages in the airspaces. This is a normal 'physiological' response and if the exposure to such a low toxicity dust is moderate then even long term exposure shows little other change in the BAL and little pathological change. If the exposure is to a more intrinsically toxic dust there is a more rapid change in the BAL with accumulation of PMN in the BAL even at low lung burdens of such a dust and accompanying pathological changes such as fibrosis in the long term.

4.3.98 **R7.3: Skin and Respiratory sensitisation**

4.3.99 There is currently no information requirement for respiratory sensitisation under REACH but the issue of respiratory sensitisation and associated health effects such as occupational asthma is substantial. Some nanomaterials have been shown to

produce allergic type inflammation in the lung (Cho et al. 2010) raising the question if certain forms of nanomaterials may be allergenic. The requirement to evaluate respiratory sensitisation is hampered by the availability of acceptable and validated test methods. Indeed current R.7a Guidance in relation to respiratory sensitisation states the “No *in vitro* tests specific for respiratory sensitisation are available yet, owing to the complexity of the mechanisms of the sensitisation process.”. Guidance also reports that whilst several *in vivo* test methods have been published, these are not yet validated or internationally accepted. Owing to the importance of potential respiratory sensitisation to human health, it would prudent to further develop assays for the detection of respiratory sensitisers so that respiratory sensitisation may become a future information requirement. This is important not just for nanomaterials, but also other substances.

4.3.100 In addition the cytokine profiling of BAL obtained from exposed animals to detect a Th2 (allergic type) cytokine profile may allow the elucidation of respiratory sensitisation and shows again the usefulness of BAL. This would however require further R&D to ascertain its predictiveness for clinical manifestation of allergic disease.

4.3.101 **R7.4: Acute Toxicity**

4.3.102 Acute toxicity as an endpoint is used for both classification and labelling purposes as well as for a chemical safety assessment for acute exposure. This is also reflected in the recent OECD GD39 document which states that “acute inhalation toxicity data are used to satisfy hazard classification and labelling requirements, to estimate the toxicity of mixtures and to assess human health and environmental risks”. The use of simple lethality is a blunt measure of negative effects and is unsuitable for the derivation of accurate human exposure limits. Indeed, as mentioned within the R.8 guidance for the derivation of human derived no effect levels (DNEL), where no NOAEL is apparent, e.g. use of LD50 or LC50 data, then a much higher assessment factor should be applied. The limitation of acute toxicity measurements is to such a blunt endpoint is not necessary and guidance states that in relation to CSA, information on acute toxicity is not normally limited to availability of LD50 or LC50 value. Specifically it suggests clinical signs of toxicity, local irritant effects time of onset and reversibility of the toxic effects. These are

attributes that could not be evaluated using lethality and body weight would be of only limited use.

4.3.103 The following include alterations to the current standard test methods to improve sensitivity and allow for nano-relevant routes of exposure. As such these would require a change to the guidance suggested test method, but not to the Guidance document itself.

4.3.104 The use of gross necropsy is an insensitive method of establishing pathogenic effects as it would only reveal effects manifesting in surface changes visible to the naked eye. Therefore using only gross pathology would not identify potentially serious pathogenic effects such as large scale cell death, internal organ haemorrhaging (not visible that the organ surface) and test substance/ inflammatory cells accumulation within an organ. As a result we would propose that the following test methods be updated to include a more extensive evaluation of the pathogenic effects via the use of extended pathology / histology (ENVJM MONO 2009/21) of the internal organs, over and above the currently required gross pathology:

- Acute toxicity: Oral - OECD TG 420 (EU B.1 bis) (Acute oral toxicity – Fixed dose procedure)
- Acute toxicity: Oral - OECD TG 423 (EU B.1 tris) (Acute oral toxicity – Acute toxic class method)
- Acute toxicity: Oral - OECD TG 425 (Acute oral toxicity – Up-and-down procedure)

4.3.105 As discussed in paragraphs 5.2.18 to 5.2.20, the use of BAL aides in the toxicological evaluation of (nano)particles as well as other substances by providing a quantitative index of the extent of lung damage and subsequent inflammation that arises in response to pulmonary exposure top attest substance or (nano)particle. . Such a quantitative index of sub-lethal effects would allow the derivation of no effect levels which can be more easily and confidently used within a CSA for hazard assessment and the derivation of acute human exposure limits. BAL is considered to be much more sensitive to early effects of particle-induced injury, detecting the injury before an overt pathological change can be seen in histological sections of rat lungs or where changes are minimal making them impossible to quantify in sections. It is

not suggested that BAL is performed on live animals as this is associated with welfare issues and within the toxicological literature is more commonly performed on euthanized animals at the cessation of the experiment/ timepoint to detect pathogenic effects (e.g. Pauluhn 2010). The use of BAL is considered an enhancement of current test procedures and does not necessarily constitute the use of extra groups of animals. Indeed rather than the superficial analysis of body weight and/ or gross necropsy, the dead animal would also be subject to BAL and analysis of the BAL fluid so that more information may be gleaned from the animal thereby maximising its use within an experiment. The use of such non-lethal endpoints such as lung inflammation detected by BAL is also extolled within the recent OECD GD39 document concerning acute toxicity which states that “the non-lethal endpoints at the lower end of the concentration response curve might be just as useful as lethal endpoints”. The benefits of the use of BAL to improve the usefulness of experimental data and maximise the use of experimental animals very apparent. BAL is also commonly used within those inhalation studies using nanomaterials reported within the peer review literature including for acute toxicity (Ellinger-Ziegelbauer, 2009).

4.3.106 Because of the relative benefits of BAL, and as recommended use within the RNC/RIP-oN2/B1/2/FINAL paragraph 5.15. we propose the incorporation of BAL as standard into the following acute toxicity test methods:

- OECD TG 403 (EU B.2) (Acute inhalation toxicity)
- Draft OECD TG 433 (“Acute Inhalation Toxicity, Fixed Dose Procedure”)
- Draft OECD TG 436 (“Acute Inhalation Toxicity, Acute Toxic Class Method”)

4.3.107 **R7.5 - Repeated Dose Toxicity**

4.3.108 We propose the incorporation of BAL as standard into the following acute toxicity test methods:

- Repeated Dose: Inhalation - OECD TG 412 / EU B.8 Subacute Inhalation Toxicity: 28-Day Study

- Repeated Dose: Inhalation - OECD TG 413/EU B.29 Subchronic Inhalation Toxicity: 90-day Study
- Repeated Dose: Inhalation - OECD TG 422: Combined repeated dose toxicity / reproductive screening study

4.3.109 Inhalation is thought to be the most likely route of nanoparticle exposure, however methodology for the use of inhalation exposure for this method appears to be lacking. We recommend following the advice of the OECD WPMN as stated in RNC/RIP-oN2/B1/2/FINAL that the following methodology should be amended to include an appropriate OECD standard inhalation exposure protocol in combination with the reproductive & developmental toxicity screening endpoint.

- Neurotoxicity - OECD TG 424 (rodents)

4.3.110 **Reproductive Toxicity** (relevance to R.7.6 reproductive and developmental toxicity, and R.7.5 repeated dose toxicity)

4.3.111 We recommend following the advice of the OECD WPMN as stated in RNC/RIP-oN2/B1/2/FINAL that the following methodology should be amended to include an appropriate OECD standard inhalation exposure protocol in combination with the reproductive & developmental toxicity screening endpoint.

- Reproductive and developmental toxicity - OECD TG 422: Combined repeated dose toxicity / reproductive screening study

4.3.112 In relation to reproductive effects of nanomaterials, there exist three *in vitro* tests which, following further research and development to investigate their suitability for use as assays for nanomaterials, might together offer a route by which to reduce and refine the use of animals within reproductive toxicity testing. These are:

- the embryonic stem cell test for embryotoxicity (EST),
- the micromass embryotoxicity assay, and
- the whole rat embryo embryotoxicity assay.

4.3.113 The EST uses two cell lines (mouse embryonic stem cells (ES) and mouse 3T3 fibroblast cells) and three endpoints (inhibition of differentiation of the ES cells,

inhibition of ES and 3T3 cell viability, and inhibition of ES and 3T3 cell proliferation) to classify the embryotoxic potential of test chemicals.

- 4.3.114 The micromass test evaluates effects of a test substance on the differentiation and growth of micromass cultures of rat limb bud. Limb bud cultures provide a model which represents various developmental processes in cartilage histogenesis such as cell to cell communication, cell proliferation and cell differentiation.
- 4.3.115 The whole rat embryo culture uses isolated and cultured early post-implantation rat embryos to study embryotoxic effects of chemicals on cultured embryos. Comparison of controls and chemical exposed cultures over a 48-hour period allows assessment of delays or malformation during the development of certain organ systems.
- 4.3.116 All three assays have undergone and passed ECVAM validation (ECVAM, 2002). For the embryonic stem cell test (EST), the accuracy of correlation between *in vitro* and *in vivo* data was 78%. For the micromass embryotoxicity assay, *in vitro* to *in vivo* correlation was 70%; and for the whole rat embryo embryotoxicity assay, correlation was 80%. Following conduction of formal validation studies by ECVAM, all three were deemed to be “scientifically validated tests which were ready to be considered for regulatory purposes” by ECVAM’s Scientific Advisory Committee (ECVAM, 2002).
- 4.3.117 There exists limited evidence of research into the applicability of these tests to nanomaterials to date. However, with relation to the EST, paragraph 3.2.70 of the RNC/RIP-oN2/B3/2/FINAL report states that Park et al. (2009) suggested that *in vitro* embryonic stem cell differentiation test may be a valuable tool to test embryotoxicity of nanomaterials.
- 4.3.118 Thus, it is considered that following further research and development to investigate the applicability of the assays to testing for nanotoxicology, the three tests may offer a route by which to reduce and refine the use of animals within reproductive toxicity testing.
- 4.3.119 **Inflammation & cytotoxicity** (with relevance to R.7.2 Skin and eye irritation/corrosion and respiratory irritation, R.7.3 Skin and respiratory sensitisation, R.7.4 acute toxicity, and R.7.5 repeated dose toxicity)

- 4.3.120 The use of assays to detect markers of inflammation, fibrosis and cytotoxicity provide valuable tools to improve current test methods to detect the effects of nanoparticle exposure. This has value in both improving the sensitivity by detecting sub-clinical changes and generating mechanistic information which can inform the potential for disease outcomes which would further improve risk assessment. Current test methods are focused towards clinical pathology with a modest range of clinical chemistry markers, few of which inform as to the level or type of inflammation or sub-clinical fibrosis and as such could be seen as a relatively insensitive method of establishing pathogenic effects of nanoparticles. The use of markers of cytotoxicity, (such as lactate dehydrogenase release (LDH)), inflammation (cytokines and chemokines) and fibrosis have been long used in the research community as a sensitive way of establishing effects, have also been extensively used in nanotoxicological research and are frequently discussed throughout the RNC/RIP-oN2/B3/2/FINAL report and within large scale EU funded studies (e.g. NanoKem, paragraph 3.2.133).
- 4.3.121 An example is the use of cytokine and chemokine measurements in samples obtained from testing subjects either from primary target organs (e.g. the lung via BAL fluid) or from the circulation. A cytokine/chemokine profile provides information both as to the intensity and type of inflammatory reaction generated by exposure to a test substance which is very relevant for nanomaterials (hence inclusion herein) and other substances. For example, profiling can indicate the polarisation towards a TH1 (cell-mediated) pro-inflammatory response or TH2 (humoral immunity) type response which can indicate allergy/ sensitisation.
- 4.3.122 Therefore the use of such an array type approach to inform as to what is occurring at the cellular level can actually inform of the scale and type of a response. This may mean potentially greater information can be obtained from a single experiment which could result in the need for fewer test protocols (and hence animals used) through the merging of experimental approaches. For example a repeated dose inhalation study may also inform as to the generation of respiratory sensitization. This is an example of maximising the information gleaned from a single animal leading to the reduction and refinement of animal testing.
- 4.3.123 The use of such assays as cytokine/ chemokine measurements, cytotoxicity and markers of pro-fibrogenic effects can be used both with in vitro and in vivo analysis

and provide a useful point of comparison between such systems. The dual use at this stage of R&D would go some way to developing a comparative knowledge base that may allow a reduction or replacement of animal testing, and is an approach taken within the NanoCare project discussed in RNC/RIP-oN2/B3/2/FINAL report paragraph 3.2.30. As such when considering alterations to guidance and identification of areas requiring further development for testing nanomaterials, it seems prudent to put in place steps (via further R&D) towards the ultimate goal of a reduction in animal testing for both nanomaterials and other substances as the range of in vitro alternatives to in vivo testing is current deficient in guidance for all substances.

- 4.3.124 In addition when conducting testing, including comparative testing between in vitro and in vivo as well as between materials (e.g. bulk and nano) there should be a consideration of appropriate metric. The consideration of metric should be based on the driving effect of toxicity (the biologically effective dose) and may be different depending on the chemistry, shape and form of the material in question. Ideally several metrics could be considered and measured to allow generated data to be expressed in different ways to ascertain which is the most appropriate.
- 4.3.125 Those assays which provide useful points of comparison or improve the sensitivity of current clinical-chemistry performed during testing are highlighted as priority areas for the short/medium term. Those assays which inform more towards the mechanistic basis of understanding the pathogenic effects are seen as a lower priority (*) and could be considered as long-term research targets. The reasoning and approach discussed herein are equally applicable to the subsequent test assay sections
- Pro-Inflammatory effects *in vitro/ vivo* – Cytokine, chemokine and their receptor expression and transcription e.g. IL-8, GRO, TNF- α , IL-1 β
 - Pro-fibrogenic effects *in vitro/ vivo* – Pro-fibrotic mediators e.g. TGF- β , IL-6, IL-10, EGF etc
 - Pro-fibrogenic effects *in vivo* – Histopathological examination e.g. using Collagen staining (e.g. Sirius Red, Trichrome)

- *Pro-Inflammatory effects in vitro - Cell signalling and Transcriptional activation e.g. AP1, NFkB, STAT, NRF2, MAP Kinase
- *Pro-fibrogenic effects – Collagen production, type and biochemical modifications e.g. Hydroxyproline, pro-collagen peptides

4.3.126 *Cytotoxicity*

- Cytotoxicity - Propidium Iodide
- Apoptosis - Annexin V
- Cytotoxicity – LDH release
- *Cytotoxicity – Neutral Red uptake
- *Cytotoxicity - Trypan Blue

4.3.127 **Oxidative stress** (of mechanistic value to R.7.2 Skin and eye irritation/ corrosion and respiratory irritation, R.7.4 acute toxicity, R.7.5 repeated dose toxicity as well as R.7.7 mutagenicity and carcinogenicity)

4.3.128 The generation of oxidative stress can impact on numerous systems within the body and can arise through several mechanisms. These can include direct particle/ chemical production of oxidants, an inhibition of normal cellular process leading to an increase in intracellular oxidants/ decrease in intracellular antioxidants or through the stimulation of oxidant production by inflammatory cells though the generation of inflammation leading to a pro-oxidant environment (Schins 2002). The result of oxidative stress can be the activation of oxidant sensitive pro-inflammatory transcription factors such as nuclear factor kappa B (Rahman and MacNee 2000) leading to inflammation and direct genetic damage potentially leading to mutation and/or carcinogenesis. The use of assays both in vitro and in vivo can further improve the sensitivity of current biomarkers of adverse effects and point further inform as to other pathological effects (e.g. high levels of oxidative stress and antioxidant depletion could point towards an increased need for evaluation of mutagenicity and carcinogenicity). As the use of oxidative stress markers provides a more mechanistic understanding of the effects caused by nanoparticle exposure and is considered a driver of pathogenic effects, these could be considered as a

lower priority of the longer term which may inform hazard evaluation but may not currently be of overt use to risk assessment.

- *Oxidative Stress: Antioxidant capacity - Glutathione Depletion (intra- and extra-cellular)
- *Oxidative Stress: Redox sensitive dyes e.g. DCFH, DHE etc.
- *Oxidative Stress: Antioxidant capacity - vitamin C depletion
- *Oxidative Stress: Antioxidant capacity - TEAC (trolox equivalent anti-oxidant capacity)
- *Oxidative Stress: Antioxidant capacity - ORAC (oxygen radical anti-oxidant capacity)
- *Oxidative Stress: Antioxidant capacity – FRAP (Ferric/Reducing Antioxidant Power)
- *Oxidative Stress: Antioxidant capacity – Mitochondrial dysfunction
- *Oxidative Stress: Antioxidant capacity - Antioxidant gene/protein expression and transcription factors (HO-1 expression etc.)

4.3.129 As expressed before, there should be a consideration of appropriate metric when developing and utilising test of oxidative stress. The consideration of metric should be based on the driving effect of toxicity (the biologically effective dose) and may be different depending on the chemistry, shape and form of the material in question. Ideally several metrics could be considered and measured to allow generated data to be expressed in different ways to ascertain which is the most appropriate.

4.3.130 **Genotoxicity**

4.3.131 The establishment of the genotoxic effects of particles is critical in understanding the long term implication of particle exposure. However, how these assays relate to actually presentation of carcinogenic effects (e.g. tumour formation) is still required and should be a focus of R&D in the short term. If found to be adequately predictive the development of standardised methods would be considered a further priority. The result would be an improvement in the current approach to generating

mutagenicity/ carcinogenicity data for which robust data is currently difficult and costly to obtain. In addition, the consideration of alternative metrics to mass should be made and ideally several metrics could be considered and measured to allow generated data to be expressed in different ways to ascertain which is the most appropriate.

4.3.132 As such the following assays, commonly used within the research community are suggested for further R&D:

- Mutagenicity: Oxidative adducts of DNA e.g. 8-OH-dG (Khalil et al. 2011)
- Mutagenicity: Lipid adducts e.g. N-1,N2 malondialdehyde-2'-deoxyguanosine (M1dG) (Blair 2008)
- *DNA Repair reporter assays: e.g. GreenScreen assay (Benton et al. 2008)

4.3.133 **Particle translocation**

4.3.134 At the current time, there is a paucity of data relating to the systemic transfer of particles following exposure via the lungs, skin and gut, and this should be a focus of future experiments. The benefit of this data is to identify target organs which particles may preferentially transfer to and/or accumulate in. Such organs would therefore be identified for specific system/ organ toxicity studies (Appendix R.7.5-1). Studies have focused on dermal and pulmonary toxicity of particles and demonstrated in some cases the transfer of test nanoparticles in small amounts (~1-8%). However there is an absence of data on the consequences of exposure to the gastrointestinal tract, and within damaged/diseased skin. Once systemically available, target organs currently identified include the liver, kidney, cardiovascular system and brain. The effect of particle interaction with these organs requires further elucidation to establish if the small percentage of transfer is toxicologically significant and if repeated exposure leads to retention of particles within such organs/ systems resulting in a build up of dose.

4.3.135 Due to the sensitivity of the system, the transfer of nanoparticles to the reproductive system, in particular the foetus should be considered a very high short term priority.

4.3.136 Another interesting attribute which requires further investigation is which physico-chemical parameters influence particle transfer rates and retention? In the study by

Semmler-Behnke et al (2008) they demonstrated the importance of particle surface charge in particle translocation by demonstrating an increase in translocation to 8% of the instilled dose into the lungs.

- 4.3.137 In order to address the issue of nanoparticle toxicokinetics, there is the need to develop improved methods to detect particles so as to establish the likelihood and rate of particle transfer across a barrier and tissue accumulation. In particular improvements both in the sensitivity of test methods as well as ways in which to detect particles is needed especially for those which do not lend themselves to easy identification via detection of metal ions, radiolabels etc. Also as outlined in the RIVM nanosilver case study (Pronk et al. 2009), there are also issues with identifying if the detected substance is in particulate form, ionic form or both.
- 4.3.138 **Cardiovascular toxicity**
- 4.3.139 In terms of toxicological information, RIVM (2009) proposes the following base set information requirements for nanomaterials: toxicokinetic testing; repeated dose toxicity testing for the inhalation route (or other routes, depending on the anticipated exposure routes), preferably with inclusion of additional parameters to the standard repeated dose toxicity study, such as cardiovascular and/or inflammatory parameters.
- 4.3.140 One endpoint specifically identified as an additional endpoint, albeit subordinate to the repeat dose toxicity Information Requirement, is cardiovascular toxicity.
- 4.3.141 An overview of the rationale for acknowledging this endpoint in updated guidance is provided.
- 4.3.142 Within the repeated dose toxicity information requirement (R.7.5) there are guidance and requirements for different levels of histopathology and haematological analysis with differing exposure periods. However, only analysis of the gross pathology of the heart is performed and specifically addresses the cardiovascular system, As such it would not detect such cardiovascular disease as atherosclerosis, indeed atherogenic effects are not demonstrated in standard laboratory strain rodents and are usually only shown in transgenic apolipoprotein E knockout mice which, in relation to the presence of atherosclerotic plaques more accurately reflect the normal human condition. Within appendix (1) to R.7.5 there is some information

as to the testing strategy for specific system/ organ toxicity. However this does not specifically specify the cardiovascular system as a target. Instead the guidance states “*Specific investigation (or further investigation) of any organ/system toxicity (e.g. immune, endocrine or nervous system) may sometimes be necessary and should be addressed on a case- by-case basis.*”

- 4.3.143 However there exists robust information to suggest that the cardiovascular system is a particular target of concern for (nano)particles. Indeed much of this evidence stems from robust epidemiological studies and other experimental studies, highlighting the link between air pollution (e.g. PM₁₀, PM_{2.5}) and adverse cardiovascular effects. Among the various components of air pollution, particulate matter (PM) has been reported as the main driver of harmful effects on health. Increases in the air concentration of particles with an aerodynamic diameter of less than 10 µm (PM₁₀) are clearly related to more adverse cardiovascular effects (Mills et al. 2009). Notably, epidemiological studies reported the relationship between increased mortality from cardiac disease and particulate pollution, even at a very low mass concentration (Pope et al. 1992, Schwartz 1994). Inhalation studies have demonstrated that nanoparticles are inhaled and retained in the lungs (Ferin et al. 1992). This led to the development of the “ultrafine hypothesis” which highlighted the role of nanoparticles in the health effects of air pollution.
- 4.3.144 The hypotheses relating to cardiovascular effects of PM in general, and thought to be particularly relevant to nanoparticles, have been summarised by Donaldson et al. (2005) and are shown in the following:
- 4.3.145 1) Particle-induced lung inflammation affects the endothelium, thrombotic potential, fibrinolytic balance and atheromatous plaque activity in ways that favour plaque rupture and thrombosis;
- 4.3.146 2) Particles enter the interstitium and/or cause inflammation which affects the autonomic nerve endings that regulate the heart rhythm leading to dysrhythmia;
- 4.3.147 3) Particles translocate to the blood and have direct effects on the endothelium, plaques and thrombogenic mechanism.

- 4.3.148 Thus it is suggested that (nano)particles may generate cardiovascular effects either via systemic release of non-particle mediators generated as a result of (nano)particle exposure, interaction with the autonomic nervous system, or with direct interaction with components of the cardiovascular system itself due to particle transfer in the blood stream.
- 4.3.149 In various models nanoparticles are shown to be highly potent in these three areas of effect i.e. certain nanoparticles can be very potent at causing inflammation, some interstitialise readily and also it has been demonstrated that some can gain access to the blood (although in small amounts but the level of translocation required to cause an effect has not been established). For these reasons combustion derived nanoparticles, the principal nanoparticle in ambient air, are implicated in the cardiovascular effects of PM in these studies.
- 4.3.150 Whilst further epidemiological studies are required in order to validate this theory, there is an increasing weight of evidence to support this hypothesis but research is required to ascertain if nanoparticles are indeed to the crucial driver behind PM cardiovascular effects. It has been demonstrated in humans that a good correlation exists between an increase in PM_{2.5} and increased cardiovascular mortality and morbidity (Miller et al. 2007). Indeed, it has also been suggested that particle number is a better metric to correlate with the risk of heart attack (Seaton et al. 2010). Moreover, numerous studies are strengthening this proposal by studying the potential mechanisms by which nanoparticles could affect the cardiovascular system.
- 4.3.151 Applying such a theory of cardiovascular toxicity to nanomaterials, *in vivo* studies have suggested that exposure to CNT could be associated with vascular damage & pro-thrombic responses (Radomski et al. 2005). Wang et al, (2007) identified markers of cardiac damage following oral administration of TiO₂ nanoparticles.
- 4.3.152 A number of *in vitro* investigations have also suggested that nanoparticles have the propensity to stimulate cardiovascular disease. For example, Radomski et al. (2005) showed the CNT were capable of promoting platelet aggregation *in vitro*, and Helfenstein et al. (2008) demonstrated that SWCNT and TiO₂ were capable of promoting an inflammatory response in cardiac cells. Metal oxides have also been shown to detrimentally affect endothelial function, negatively impact on cardiomyocyte function (Courtois et al, 2008), disturb normal cardiac

electrophysiology, enhance, and promote an inflammatory response in blood vessels (Oesterling et al, 2008).

- 4.3.153 In animal models such as apolipoprotein E knockout mice, it was suggested that the ultrafine component of PM_{2.5} had more pro-atherogenic effects than its fine component compared to control, promoting plaque growth and destabilization (Araujo JA et al. 2008). Disruption of atherosclerotic plaque leads to thrombus formation (atherothrombosis) and the risk of acute ischaemic events such myocardial infarction. Atherothrombosis is the main cause of cardiovascular death. It has been suggested that nanomaterials could have pro-thrombotic properties via various mechanisms such as increased expression of tissue factor and accumulation of fibrin and platelets on the endothelium creating a pro-thrombotic environment. Moreover, it has been suggested that exposure to PM could alter the vasomotor properties of the vascular system (for review see Mills et al. 2009).
- 4.3.154 Thus, to ascertain whether the cardiovascular system may be a specific target for toxicity further R&D is required. As outlined above, several studies have been conducted as to the cardiovascular effects of nanoparticles administered by indirect routes (e.g. via inhalatory or oral routes) but a large number of studies are conducted using direct intravenous routes of exposure. Such a route of exposure is of little value in the REACH regulatory context for risk assessment. However it may offer some mechanistic information as to the effect of blood borne nanoparticles, although the dose of particles in the blood arising from IV injection compared to the one via translocation is likely to be several orders of magnitude higher.
- 4.3.155 Due to the prominent link between particle exposure in the form of air pollution (particularly from combustion generated nanoparticles) and adverse cardiovascular effects, it seems pertinent to ascertain if nanoparticles can cause or exacerbate cardiovascular effects. Further research into establishing and understanding the contribution that particle exposure has to cardiovascular disease, including if such effects are threshold or non threshold in nature, is seen as a priority which should be addressed within the short/ medium term. In addition, consideration to the driving metric should be made so that test and exposure data can be expressed in the most appropriate and useful way. In order to protect human health, appropriate test methods that are sufficiently sensitive and predictive need to be established and developed within a frame that ensures that they are standardised and

internationally recognised. Whilst tests systems are in development for research purposes and mechanistic evaluation, these are not yet suitable for regulation. The development of suitable regulatory test methods could be judged as a medium term priority.

4.3.156 ECOTOXICOLOGICAL ENDPOINTS & TESTING

4.3.157 The importance of sample preparation and characterisation in conducting ecotoxicological tests and the potential impact this may have on the reliability of test data is universally acknowledged within the project consortium. In doing so, we have proposed amendments to the guidance document at the relevant junctures directing registrants to the relevant section on sample preparation for consideration of a range of issues such as dispersibility/ solubility, aggregation/agglomeration state, characterisation of actual test concentration during exposure etc. The impact of such issues is considered further more generally followed by more specific issues. It should be noted that whilst the consortium does agree that a consideration must be made of the issues surrounding sample preparation and measurement, there is not consensus on the effect this may have on the suitability of the tests list subsequently.

4.3.158 GENERAL CONSIDERATIONS

4.3.159 Aquatic testing

4.3.160 Water solubility is an essential parameter in ecotoxicological testing and data should be available prior to any aquatic effects testing. Failure to do so could result in testing above the solubility limit leading to misinterpretation of the results. Poorly soluble substances are defined by OECD (2000) as substances with a limit of solubility <100 mg/L although technical problems are more likely to occur at <1mg/L. Based on the summary of difficult substance testing issues provided in the guidance (i.e. Table 7.8-3) the following issues are relevant in regard to nanomaterials and need to be explored further:

1. Even if nanomaterials are put into suspension, it may be difficult to maintain and verify concentrations due to problems in developing an analytical method – such methods should be developed and be put in place to allow analysis;
2. Many nanomaterials are likely to be lost from the water column and hence results expressed in terms of nominal concentration might exceed the true concentration of the substance in the test medium – a better understanding

of nanomaterials aggregation/agglomeration, dispersibility and sedimentation is needed and methods to verify this need to be developed;

3. Many nanomaterials have low water-solubility and physical effects (e.g. entrapment) may occur if the test concentration is significantly above water solubility which again influences the ecotoxicity of the nanomaterials tested – a better understanding of the mechanisms behind entrapment and physical effect is needed as well as methods to verify such effects need to be developed; and
4. Nanomaterials can be highly hydrophobic and insufficient test duration might lead to non-steady state conditions, and effectiveness of aquatic tests needs to be explored further and validated.

4.3.161 These issues are important and should be addressed within ecotoxicology studies by verifying exposure concentration over time which may require the further development and implementation of methods and analysis to allow.

4.3.162 **Soil testing**

4.3.163 In regard to soil ecotoxicity studies there is a fundamental need for an analytical method capable of verifying the actual exposure concentration in the soil and over time. There is also a need to develop an analytical method to verify nanomaterial concentrations, aggregations/agglomeration behaviour and stability of nanomaterials in soil. The applicability of soil tests for nanomaterials needs to be explored further and validated.

4.3.164 **ENDPOINT SPECIFIC R&D PRIORITIES**

4.3.165 In relation to the general issues above, the view of part of the project consortium was that further R&D should be conducted in relation to the following tests to validate them for nanomaterials. Furthermore, it was also concluded that there is a need for the development of an analytical methods to verify nanomaterial concentrations, aggregations/agglomeration behaviour and stability of nanomaterials in a range of environments including aquatic and soils.

4.3.166 *R.7.8.4.1 Data on aquatic pelagic toxicity*

- Growth inhibition study aquatic plants (algae preferred)

- Short-term toxicity testing on invertebrates (preferred species Daphnia)
- Long-term toxicity testing on invertebrates (preferred species Daphnia), (unless already provided as part of Annex VII requirements)
- Short-term toxicity testing on fish
- Long-term toxicity testing on fish, (unless already provided as part of Annex VIII requirements)
- Fish early-life stage (FELS) toxicity test
- Fish short-term toxicity test on embryo and sac-fry stages
- Fish, juvenile growth test

4.3.167 *R.7.8.9.1 Laboratory data on toxicity to sediment organisms*

- Long-term toxicity to sediment organisms

4.3.168 *R.7.8.15 Information requirements for toxicity to STP microorganisms*

- Activated sludge respiration inhibition testing

4.3.169 *R.7.9.2.1 Annex VII (Registration tonnage >1 t/y -<10 t/y)*

- Ready biodegradability

4.3.170 *R.9.2.2 Annex VIII (Registration tonnage ≥ 10 t/y)*

- Hydrolysis as a function of pH

4.3.171 *R.7.10.3.1 Laboratory data on aquatic bioaccumulation*

- Bioaccumulation in aquatic species, preferably fish

4.3.172 It should be noted that OECD 305 is currently under revision in order to include dietary route of exposure and hence it might be worthwhile to consider the dietary exposure route against water exposure.

4.3.173 *R.7.10.16.1 Laboratory data on avian toxicity*

- Long-term or reproductive toxicity to birds

4.3.174 *R.7.11.3.1 Laboratory data*

- Effects on soil micro-organisms
- Soil short-term toxicity to invertebrates
- Long-term toxicity testing on soil invertebrates, unless already provided as part of Annex IX requirements
- Short-term toxicity to plants
- Long-term toxicity testing on plants, unless already provided as part of Annex IX requirements

4.3.175 This however is not universally acknowledged within the consortium as the request for updates of existing OECD testing guidance is not completely inline with the preliminary conclusion by OECD WPMN. Their conclusions state that many of the OECD testing guidance in their current wording is applicable with the reinforced statement that attention needs to be given to measuring, dosing, delivery and tracking of the substance in the testing system.

4.3.176 The importance of issues surrounding sample preparation, characterisation and dosimetry are agreed upon within the consortium. In line with the conclusions of OECD WPMN (ENV/JM/MONO(2009)21, it is recognised that there is a need for a guidance document(s) for sample preparation and dosimetry which the OECD WPMN feel should be independent from the existing OECD guidance documents perhaps rather than update each guidance document separately.

4.3.177 **Additional Relevant Specific Intrinsic Properties**

4.3.178 There exists debate within the project consortium as to the usability and applicability of a range of potential additional endpoints and biological markers of toxicity identified within the RNC/RIP-oN2/B2/2/FINAL (paragraphs 5.4.1 to 5.4.7) and RNC/RIP-oN2/B3/2/FINAL (paragraphs 4.3.7 to 4.3.13) reports. These additional endpoints/ markers areas follows:

4.3.179 R.7.8.4.1 Data on aquatic pelagic toxicity

- Fish ventilation rate
- Fish gill pathologies
- Fish Mucus secretion
- Fish brain pathology
- Animal behaviour
- Oxidative stress fish (CAT, SOD, GPX, GST)
- *Daphnia heart rate
- *Daphnia hopping frequency
- *Number of cycles per minute of daphnia in appendage movement
- *Trojan horse effect of nanomaterials

4.3.180 In support of these proposed endpoints it was noted in the review of the literature that a number of new significant effects for nanomaterials have been observed in e.g. fish (see Smith et al. (2007) and Federici et al. (2007) reviewed in RNC/RIP-ON2/B3/2/FINAL section 4.3.7-4.3.13 and Stone 2009). On the basis of these findings, candidate endpoints considered include ventilation rate, gill pathologies, mucus secretion, brain and gill Zn and Cu, Na+K+-ATPase activity, behavioural changes, and changes in the brain. These may provide additional information requirements not currently in Technical Guidance Documents and aid the assessment of a nanomaterial's ecotoxicity. Although standardised guidance documents are not available from the OECD or other international standardisation bodies, methods for establishing these endpoints have been available in the literature for some time (see Smith et al. 2007, Bouskill et al. 2006).

4.3.181 A large number of studies on C60, TiO₂, ZnO, and CdSe quantum dots in crustaceans and fish have reported observing increased and/or decreased activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST) (Oberdörster

2004, Zhu et al. 2008, Wang et al. 2008, Klaper et al. 2009, Wong et al. 2010), which are associated with the defence system to oxidative stress (Klaper et al. 2009, Kim et al. 2010). Collectively, this suggests that the use of such biochemical biomarkers for oxidative stress may provide useful data for predicting the ecotoxicity of nanomaterials, although generic applicability across all species has yet to be established. Although standardised guidance documents are not available from the OECD or other international standardisation bodies, methods for establishing these endpoints have again been available in the literature for some time.

- 4.3.182 In relation to the 'Trojan horse effect' of nanomaterials, there are indications that nanomaterials increase both the toxicity and the bioaccumulation potential of known environmental pollutants in comparison with "bulk" materials and with suspected important differences between nano and non-nano and with insufficient evidence basis for guidance to be provided (RNC/RIP-ON2/B3/2/FINAL (paras 4.3.51 to 4.3.57, 4.3.96 to 4.3.98). Reported effects as well as the development of test methods need to be explored further and validated as a mid-term priority. Development of an analytical method to verify nanomaterial concentrations, aggregations/agglomeration behaviour and stability of nanomaterials in water is furthermore needed as well as exploration of the feasibility of using this endpoint in regulatory guidance.
- 4.3.183 The opposing position regarding the use of these endpoints/ biomarkers is based within the conclusions of the RNC/RIP-ON2/B1/2/FINAL report (para 6.42) which concluded that it is currently premature due to significant lack of scientific justification for the relevance of these endpoints, to include them in a regulatory context. Therefore, further research is needed to develop reliable methods and understanding of these endpoints but not only for nanomaterials but also for substances in general. It is also important to remember that according to the current assessment of the adverse effects on the environment. Individuals are not studied but populations. Some of these endpoints impose a paradigm shift in this respect which demand a significant increase in knowledge before such can be included in guidance for industry in how to fulfil legal obligations. However, the relevance of these parameters for regulatory purposes is questionable nor should it be raised as a nano-specific issue. This is an animal test for research purposes and is inconsequential for regulatory purposes.

4.3.184 Despite the opposing opinions in relation to these identified new endpoints and biomarkers, it is noted by the project consortium as a whole that whilst there is evidence of the use of these endpoints within the literature, before definitive conclusions can be proposed, further R&D is required. Specifically the applicability of these endpoints for nanomaterials needs to be explored further and validated as well as exploration of the feasibility of using this endpoint in regulatory guidance. In addition, further R&D is required for the development of international standards that could support regulatory guidance and in addition there is a need to develop analytical methods to verify nanomaterial concentrations, aggregations/agglomeration behaviour and stability of nanomaterials in water.

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