JRC TECHNICAL REPORTS

Evaluation of the availability and applicability of computational approaches in the safety assessment of nanomaterials

Final report of the
Nanocomput project

Andrew Worth, Karin Aschberger, David Asturiol Bofill, Joseph Bessems, Kirsten Gerloff, Rabea Graepel, Elisabeth Joossens, Lara Lamon, Taina Palosaari, Andrea Richarz

2017

How to cite this report: Worth, A., Aschberger, K., Asturiol Bofill, D., Bessems, J., Gerloff, K., Graepel, R., Joossens, E., Lamon, L., Palosaari, T. and Richarz, A., *Evaluation of the availability and applicability of computational approaches in the safety assessment of nanomaterials,* EUR 28617 EN, Publications Office of the European Union, Luxembourg, 2017, ISBN 978-92-79-68708-2, doi:10.2760/248139, JRC106386.

All images © European Union 2017, except:

Page 11, ECHA, Figure 1.1, 2009. Source: European Chemicals Agency (ECHA), Helsinki
Page 252, ECHA, Figure 4.2, 2016. Source: European Chemicals Agency (ECHA), Helsinki
Page 278, Eric Wieser, Figure 4.6, 2010. Source: Wikimedia Commons [CC BY-SA 3.0]

Executive Summary

This is the final report of the Nanocomput project, the main aims of which were to review the current status of computational methods that are potentially useful for predicting the properties of engineered nanomaterials, and to assess their applicability in order to provide advice on the use of these approaches for the purposes of the REACH regulation. Since computational methods cover a broad range of models and tools, emphasis was placed on Quantitative Structure-Property Relationship (QSPR) and Quantitative Structure-Activity Relationship (QSAR) models, and their potential role in predicting NM properties. In addition, the status of a diverse array of compartment-based mathematical models was assessed. These models comprised toxicokinetic (TK), toxicodynamic (TD), *in vitro* and *in vivo* dosimetry, and environmental fate models. Finally, based on systematic reviews of the scientific literature, as well as the outputs of the EU-funded research projects, recommendations for further research and development were also made.

The Nanocomput project was carried out by the European Commission's Joint Research Centre (JRC) for the Directorate-General (DG) for Internal Market, Industry, Entrepreneurship and SMEs (DG GROW) under the terms of an Administrative Arrangement between JRC and DG GROW. The project lasted 39 months, from January 2014 to March 2017, and was supported by a steering group with representatives from DG GROW, DG Environment and the European Chemicals Agency (ECHA).

Background information

The first part of this report (Chapters 0-2) provides background information.

Chapter 0 provides the terms of reference of Nanocomput, and is intended to orient the reader, linking the project objectives to different chapters in this report.

Chapter 1 provides the scientific background and regulatory context to the rest of the report. It identifies the properties that drive the toxicity and fate of NMs, gives an overview of standard test methods for measuring physicochemical properties and toxicity, and explains the different kinds of alternative (non-animal) approaches that are being developed for regulatory purposes. In addition, an overview of the EU regulatory framework for NMs (REACH and other pieces of legislation) is provided.

Chapter 2 presents an overview of software tools (including models and databases) that are available for predicting the toxicity and fate of NMs. The emphasis is on the different kinds of (mathematical) modelling approaches being used. Practical considerations and opportunities for developing computational models are also discussed. The chapter also includes experience in the grouping of NMs for the purpose of read-across, and proposals for NM categorisation schemes.

Assessment of the availability and applicability of computational methods for NMs

Chapter 3 provides a systematic review of the model landscape, based on a detailed and systematic survey of the scientific literature. This includes an analysis of current status of QSPR and QSAR models. A quantitative structure-property relationship (QSPR) is a mathematical model that uses key descriptors (chemical features or physicochemical properties) to make predictions of other physicochemical properties, whereas a QSAR is a

similar type of model except that the descriptors are used to make predictions of a biological activity (such as a toxicological endpoint). In this report, QSPRs and QSARs are treated in the same way, since they both employ statistical learning methods to identify useful descriptors and/or establish the form of the correlative model between descriptors and predicted property/activity.

The development of QSPRs and QSARs for NMs is still in its infancy, and been a challenge for a number of reasons. Traditionally, QSPR and QSAR modelling has applied to substances in solution, typically undissociated molecules, rather than particles. At present, relatively few theoretical descriptors are available for particles, although experimental descriptors may be useful (provided they can be measured reliably). A further complication is that particles do not typically form a homogeneous collection of species – they may undergo aggregation/agglomeration processes, adsorb and desorb macromolecules present in the surrounding medium, and may (partially) dissolve as well, leading to a distribution of masses/sizes/shapes (i.e. polydispersity). Rather than modelling a single species, it may therefore be necessary to model a distribution / mixture of species, which is increasingly difficult the more the material deviates from monodispersity. Furthermore, as with most 'classical' chemicals, *the mode of toxicological action is often unknown*, *making it difficult to identify, a priori, the most relevant and predictive descriptors.* Finally, in the case of QSARs, a lack of reliable biological data has also hindered model development.

In spite of these challenges, the development of QSPRs and QSARs for NMs has been a growing area of research. The analysis of QSPR/QSAR landscape identifies the properties and endpoints that are most often predicted, the availability of datasets for modelling, the descriptors (properties) that are most often used as predictors, as well as the statistical techniques most often applied. A detailed review of the literature identified 44 QSPRs (with solubility being the most frequently modelled endpoint) and 78 QSARs (with *in vitro* cytotoxicity endpoints being the most frequently modelled). It is concluded that while many of the QSPRs may be relevant for filling data gaps under REACH, very few QSARs directly predict a REACH endpoint. Some QSARs predict generic biological "endpoints", for example based on the integration of readouts from multiple *in vitro* methods. Thus, in general, QSARs are more likely to be useful for prioritising chemicals of concern, and for supporting read-across arguments, rather than for directly filling data gaps.

The QSAR Model Reporting Format (QMRF), as a tool for documenting and reporting QSPR/QSARs, was found to be useful, but requires a few additional fields to capture relevant particle properties.

Chapter 3 also includes an analysis of the current status of compartment-based models, including TK and TD models, *in vitro* and *in vivo* dosimetry models, and environmental fate models.

TK models simulate the time-dependent concentration of particles (including NMs) in one or more biological compartments of an organism. These include physiologically based kinetic (PBK) models that are based on physiologically relevant compartments and processes, as well as classical toxicokinetic (CTK) models that simulate key ADME properties by aggregating compartments into simpler model structures. In the context of regulatory risk assessment, these models could be used to reduce uncertainties in extrapolating toxicity data (e.g. acute-to-chronic, within and between species, route-to-route), and therefore

modify the assessment factors applied in the determination of Derived No Effect Levels (DNELs). The literature review revealed the availability of 19 TK models, including 13 PBK models and 6 CTK models. These models are applicable to a total of 15 different NMs including metals, metal oxides, polymeric and carbon-based nanomaterials, with metal NMs being the most commonly modelled materials (10 out of 19 models). The PBK models are of varying complexity (from 3 to 20 compartments), with blood, liver, spleen, kidneys and the reticuloendothelial system (RES) being the most represented compartments among the different models. Since NMs are captured and retained by the RES these cells (monocytes in the blood, reticular cells in the spleen, and Kupffer cells in the liver), these models could provide a means of predicting the potential for bioaccumulation, which is a property of concern.

TD models simulate the intensity and time-course of substance-induced effects on a biological system (e.g. prediction of the inflammatory response of macrophages under exposure to NMs). While these models are still very much in their infancy, they can be used to provide mechanistic insights and to support the experimental design of toxicological studies. The literature review revealed the availability of just four such models.

Dosimetry models simulate the local concentration or dose of particles (including NMs) in a defined *in vitro* or *in vivo* system. These include respiratory tract dosimetry models, which simulate the fate of inhaled NMs in the respiratory tract based on the physical and physiological factors that influence the deposition, clearance, and retention of inhaled particles. These models can be used to extrapolate toxicologically effective doses from animal studies to humans. The literature review revealed the availability of 7 respiratory tract models.

Dosimetry models also include *in vitro* dosimetry models that simulate the fate of particle (including NMs) within *in vitro* test systems, based on kinetic processes such as diffusion, sedimentation and advection. By simulating the fraction of administered particles that deposit on cells as a function of time, these models can be used to support *in vitro* test design and the interpretation of toxicologically relevant *in vitro* effects for a given applied concentration. The literature review revealed the availability of 5 *in vitro* dosimetry models.

Environmental fate models, developed to predict environmental concentrations in the environment or consumer exposure via the environment, were categorised into two types of models – material flow (MF) models and process-based environmental fate models. MF models typically track the materials from production to use and further to end-of-life stages and identify at each stage how much of a material is released into a technical (e.g. waste water treament plant) or environmental (e.g. air, soil, water) compartment. Process-based fate models determine the transport (e.g. advection and deposition) and fate (partitioning between compartments) of a material in an environmental system by modelling physicochemical processes, such as aggregation and sedimentation. Based on a systematic literature review, a total of 27 publications for MF models and 54 publications for process-based fate models (out of 100) were characterised.

The MF models were found to differ widely in terms of the NMs modelled, the types of NM-containing product categories considered (e.g. cosmetics, biocides, paints, textiles), the number of compartments and life cycle stages, scale (e.g. local, national) and modelling approach (static, dynamic, deterministic, stochastic). The MF models are applicable to a limited range of NMs, these being metals (mostly Ag), metal oxides (mostly TiO_2, ZnO and

CeO) and carbon-based NMs (mostly MWCNT). On the whole, MF models are based on the mass of NMs and transfer / release factors between compartments. The physicochemical properties of NMs, their environmental transformations (e.g. oxidation, reduction, aggregation, agglomeration) and detailed transfer and fate processes (e.g. sedimentation, resuspension, partitioning) are not generally taken into account. In predicting environmental concentrations of a NM, background levels are not generally taken into account. Major sources of uncertainty in these models relate to the amounts of a NM produced and allocated to different product categories, and the quantification of use patterns.

Process-based environmental fate models were also found to be a diverse range of models, but could be broadly categorized into two groups depending on whether they describe fate and transport in aquatic (including multimedia box models) or soil media. These models vary considerably in terms of temporal and spatial scales, but tend to take physicochemical properties, transformations, and other fate and transport processes (e.g. agglomeration, aggregation, sedimentation, dissolution) into account. They also differ in terms of the modeling approach (static *vs* dynamic), and the level of mechanistic detail considered. For example, some models apply colloidal chemistry kinetic equations to describe particle aggregation and sedimentation. Aquatic media models are applied to a restricted number of NMs, mostly metals and metal oxides. Models describing the fate and transport of NMs in soil media can be considered a special class of models in the sense that they attempt to account for the transport and retention of colloids in porous media. Many of these models are based on colloidal filtration theory (CFT), which include advection-diffusion equations as well as a sink term for (reversible or irreversible) colloid-collector interactions. Again, a major source of uncertainty in these models concerns the input parameters (mass loadings) which are sometimes derived from the outputs of MF models.

In terms of applicability under REACH, MF models provide approximate estimates of releases to major environmental compartments, which could be used in low-tier risk assessments. In contrast, process-based models are not generally intended for reliable PEC estimation, but rather to take into account the mechanistic processes underlying transport and fate, and to evaluate the influence of natural variability in the environmental fate and transport of NMs in aquatic and soil environments.

In addition, a few models were identified that predict the bioaccumulation of NMs in aquatic species (*Daphnia magna* and *Chlamydomonas reinhardtii*). These are generally single compartment models based on uptake and depuration (elimination) processes, and could be used to identify concerns for toxicity to environmental species.

The detailed results of the literature review supporting Chapter 3 are provided as supplementary materials (Excel-based inventories of models and their characteristics using standardised reporting templates; S1 for QSAR and QSPR models, and S2 for PBK, dosimetry and environmental fate models). In order to systematically capture the characteristics of QSPR and QSAR models, a modified version of the QMRF was developed. In contrast to QSPR and QSAR models, there is no international (e.g. OECD) standard for reporting kinetic, dynamic, dosimetry and fate models. Therefore, in order to systematically capture the characteristics of these types of models, a reporting template had to be developed from scratch.

Chapter 4 reports the results and conclusions of two case studies on grouping and read-across, which were carried out to explore the practicalities of reading across toxicological

properties between different physical forms (analogues) of the same substance. The two case studies focus on the genotoxicity of TiO$_2$ and carbon nanotubes (CNT), respectively. The aim was to explore the practical process of grouping and read-across between analogues, the applicability of the ECHA Read Across Assessment Framework (RAAF), with a view to sharing the lessons learned about the overall process. Thus, the conclusions obtained for specific substances should not be regarded as recommendations for regulatory action.

In the TiO$_2$ genotoxicity case study, the result of *in vitro* comet assay was selected as the endpoint to be predicted for two targetTiO$_2$ NMs based on a set of 6 source NMs. This endpoint was selected for practical reasons, being the most data rich endpoint based on extensive literature search. The source TiO$_2$ NMs had sizes from 5-93 nm, crystal types of anatase and rutile, some were coated and others uncoated, and were mostly spherical. More than 150 physicochemical properties for each of these NMs were gathered and used to inform the read-across.

The grouping hypothesis was based on the observation that coated nano TiO$_2$ produced negative results in the *in vitro* comet assay while the uncoated forms were positive. The mechanism through which the coating prevents genotoxicity of TiO$_2$ is not well understood and the literature points to a possible combination of effects. The conduction band of TiO$_2$ falls in the cellular redox region and therefore shows that TiO$_2$ has the potential to damage DNA and other cellular components. *Some studies show that PEG-coated nano-TiO$_2$ is negative in the comet assay, while uncoated forms are positive*. This can be explained by two different phenomena. The first is that the coating can act as a "bumper" preventing the physical contact between the NM and cellular target (e.g DNA) that is needed to cause genotoxicity; the second is the fact that coated NMs show better dispersibility, lower cytotoxicity and sedimentation rates than uncoated NMs. Some studies have shown that dispersions of nano-TiO$_2$ with agglomerates of more than 200nm were positive in the comet assay, while the smaller agglomerates were negative. It is difficult to identify a single mechanism of action for the genotoxicity of some nano-TiO$_2$ and probably multiple mechanisms are contributing to the toxicity. Both of the identified mechanisms have the consequence that coating prevents the genotoxicity of nano-TiO$_2$ as determined by the *in vitro* comet assay.

The grouping hypothesis was supported by the use of chemoinformatic techniques such as hierarchical clustering, principal components analysis (PCA), and random forest (RF). Hierarchical clustering showed two clear clusters of NMs: the coated ones and the rest. The PCA indicated that the main differences between NMs are due to particle size, crystal type, and the presence of coating. All the other properties were shown to be less relevant. Finally, the RF methodology showed that the most important properties for predicting the genotoxicity of nano-TiO2 are all related to the presence or absence of coating.

In the CNT case study, physicochemical, *in vitro* genotoxicity and *in vivo* genotoxicity data were compiled for 19 MWCNT and 2 reference materials (carbon black and asbestos). The physicochemical data were not reported consistently in the source publications which made it difficult to compare analogues in terms of their physicochemical properties. The genotoxicity data were selected by applying a number of data quality considerations. While a range of genotoxicity endpoints were available, the case study focused on five endpoints which were the most data rich: *in vitro* gene mutation in mammalian cells, *in vitro* DNA

damage (Comet assay), *in vivo* DNA damage (Comet assay), *in vitro* chromosome aberration (micronucleus assay), and *in vivo* chromosomal aberrations (micronucleus assay). Based on a weight of evidence across all reliable studies, the 19 MWCNT were all considered non-genotoxic. It was therefore hypothesised that this conclusion may be extrapolated to other MWCNTs within the assessed ranges of size (length and width), surface coating, and content of oxidising impurities. Target substances were those analogues having a data gap for at least one of the five genotoxicity study types.

Since all analogues were treated as non-genotoxic, supervised learning could not be applied to identify properties that may distinguish between genotoxic and non-genotoxic nanoforms. However, unsupervised learning (hierarchical clustering and principal component analysis [PCA]) could be applied to explore structural similarities between the nanoforms. Hierarchical clustering showed that clusters were mainly driven by the size (length and diameter) and surface area of the MWCNT, but not by the presence of impurities or type of functionalization. PCA revealed that the analogues could be clustered according to length, surface area, ROS generation, and combustion elemental analysis (CEA) of H and N. Again, *the type of functionalization did not play a role in clustering*. These results suggest that genotoxicity does not seem to be (solely) responsible for the initiation of carcinogenicity following MWCNT inhalation. To the extent that *in vivo* studies reveal the potential for carcinogenicity following MWCNT exposure, this is likely to be due to the persistence of the fibres, resulting in persistent release of reactive oxygen species and inflammation. Accordingly, the presence of genotoxicity *in vivo* may be a secondary effect, resulting from ROS generation and inflammation. There is evidence that the persistence of CNTs in biological compartments is related to their morphological (size and shape) and mechanical (rigidity) characteristics (as with larger fibres).

The two case studies show how data from different sources can be gathered, interpreted and combined, and how the ECHA guidance on the grouping and read-across can be applied at a practical level. The case studies also illustrate how chemoinformatic techniques could be applied to support a grouping hypothesis. A detailed analysis of different physicochemical properties like zeta potential, polydispersibility index and particle size distribution measured in different media and with different sonication treatments is provided in the annexes.

The two read-across case studies were also analysed in terms of their underlying uncertainties with a view to evaluating the applicability of ECHA's Read-Across Assessment Framework (RAAF) for NMs. The RAAF was developed as a structured approach to promote consistency in the evaluation of read-across arguments. Out of the six RAAF scenarios defined by ECHA, scenario 6 best reflects the NM case studies. Both the TiO_2 and MWCNT case studies are based on category approaches (reading-across from a group of substances to a target), different compounds (in this case nanoforms) having the same type of effect, and no variations in effect (e.g. Comet assay result either positive or negative). It was found that the RAAF is useful and applicable for evaluating read-across of NM properties, although some of the considerations ("assessment elements") need to be interpreted more broadly to capture nan-specific issues. In particular, the following uncertainties need to be considered for NMs: a) the *high variability of measurements* used in the physicochemical characterisation of NMs (e.g. zeta potential); b) the fact that *similarity cannot be based on chemical (e.g. molecular) structure alone*, as for conventional chemicals, but should also consider physical form and relevant physicochemical properties; c) experimental *artefacts affecting the interpretation of in vivo and in vitro toxicity studies* (in particular those relating

to kinetics, e.g. sedimentation of particles onto cellular monolayers); d) *transformation processes* (e.g. metal ion speciation, dissolution, agglomeration/ aggregation) which may also vary over time; and e) *effects of coatings*, which may be intentionally added, or adsorbed from the environment (coronas).

The detailed results supporting Chapter 4 are provided as supplementary materials (Excel-based datasets for the grouping and read-across case studies).

Recommendations for further research and development

Based on the content of previous chapters, and a detailed review of unpublished deliverables requested from EU-funded research projects (Framework Programmes, Horizon 2020), **Chapter 5** summarises the scientific and technical state of the art, and makes recommendations for further research and development, with a view to increasing the availability and uptake of computational methods. The systematic review of the recent EU project research into nanosafety assessment showed that considerable progress has been made towards addressing the challenges of modelling nanomaterials. However there is still a fragmentation in the scientific results and a lack of coordination, and the lack of public access to the results and tools is preventing their uptake and use in regulatory decision making.

Overall conclusions and recommendations

Chapter 6 presents the overall conclusions from the Nanocomput project, including lessons learned in conducting literature reviews and research-based case studies on grouping and read-across. A number of recommendations are also offered with a view to overcoming current shortcomings in our knowledge of NM behaviour, and in the availability of tools (such as databases and predictive models) and practical guidance to use such tools in the regulatory assessment of NMs. The overall conclusions and recommendations are structured according to 6 main themes which are related to: 1) the inherent scientific uncertainties in our understanding of NM behaviour, 2) data quality and availability; 3) the availability of predictive models (the "model landscape"); 4) the practicality of applying the REACH guidance on grouping and read-across; 5) the utility of the ECHA Read-Across Assessment Framework (RAAF) in evaluating and documenting uncertainties in the read-across of NM properties; and 6) the need for infrastructures and a one-stop hub to support the application of predictive models in nanosafety assessment.

Acknowledgements

Disclaimer

The report presents the findings and conclusions of the authors, but does not represent an official view of the JRC, DG GROW, or other European Commission services.

Abbreviations

CNT	Carbon nanotube
DG ENV	Directorate-General for Environment (EC)
DG GROW	Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs (EC)
EC	European Commission
ECHA	European Chemicals Agency
ISO	International Organization for Standardization
JRC	Joint Research Centre (EC)
MWCNT	Multi-walled carbon nanotube
NM	Nanomaterial
OECD	Organisation for Economic Cooperation and Development
PCA	Principal Components Analysis
QSAR	Quantitative Structure-Activity Relationship
QSPR	Quantitative Structure-Property Relationship
RAAF	Read-across Assessment Framework (ECHA)
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive Oxygen Species
SCCS	Scientific Committee on Consumer Safety (EC)
TD	Toxicodynamic
TK	Toxicokinetic
TiO_2	Titanium dioxide (titania)
WPMN	Working Party on Manufactured Nanomaterials (OECD)

Table of Contents

0   Terms of Reference and report overview

The Nanocomput project was carried out by the European Commission's Joint Research Centre (JRC) for the Directorate-General (DG) for Internal Market, Industry, Entrepreneurship and SMEs (DG GROW) under the terms of an Administrative Arrangement (No 33269) between JRC and DG GROW. Some of the work, aimed at compiling inventories of computational models, was carried out under the terms of a JRC subcontract with Leitat Technological Centre (Spain).

The project lasted 39 months, from January 2014 to March 2017, and was supported by a steering group with representatives from DG GROW, DG Environment and the European Chemicals Agency (ECHA).

The main aims of Nanocomput were to review the current status of computational methods that are potentially useful for predicting the properties of engineered nanomaterials, and to assess their applicability in order to provide advice on the use of these approaches for the purposes of the REACH regulation. The technical terms of reference in Administrative Arrangement defined four main tasks:

Task 1: Current status of computational methods for estimating the intrinsic properties and toxicological effects of manufactured nanomaterials (NMs);

Task 2: Assessment of the applicability of available models/approaches in meeting the data requirements of REACH;

Task 3: Recommendations on the use of computational methods;

Task 4: Recommendations for further research and development activities.

A brief explanation of these tasks, and associated subtasks, with links to parts of this report is given in the following Table:

Table 0.1. Relationship between Nanocomput tasks (defined in the project terms of reference) and parts of this report

	Tasks	Chapter	Appendices and supplementary materials[1]
T1	1.1 Review on grouping, QSAR, QSPR and TK models applied to NMs	The conceptual basis of the different modelling approaches is explained in **Chapter 1**, whereas practical considerations that need to be considered when developing such models is discussed in **Chapter 2**. **Chapter 2** also includes a list of relevant NM databases, and describes efforts at developing database ontologies. Approaches for grouping NMs for various purposes (including read-across) are also reviewed in **Chapter 2**. This includes research proposals as well as experience gained in regulatory assessments. An extensive and systematic review of the modelling literature is summarised in **Chapter 3**. Information on (as yet unpublished) models under development in EU funded research projects is summarised in **Chapter 5**.	Appendices IV and V gives a list of EU (IV) and other (V) funded research projects having a stated aim to develop computational models or grouping approaches.
	1.2 Compile model inventories	Templates for the structured reporting of QSPR/QSAR and PBK/fate/dosimetry models were developed, and used to systematically capture information on model characteristics from the scientific literature. The QPSR/QSAR and PBK/fate/dosimetry model templates are described in **Chapter 3.**	The model inventories for QSPR/QSAR and TK/TD/dosimetry/fate models are provided as supplementary materials S1 and S2 (Excel workbooks). The individual QSPR/QSAR model descriptions in document format are also provided as supplementary material

Tasks		Chapter	Appendices and supplementary materials[1]
			S3.
	1.3 QMRF evaluation	The suitability of the QMRF for NM-relevant models is evaluated in **Chapter 3**.	Appendix VI outlines a proposal for extending the QMRF to capture NM-relevant properties.
T2	2.1 Practicality and applicability of the different identified approaches according to REACH	The status of REACH information requirements and guidance documentation is reviewed in **Chapter 1**. Existing grouping proposals are reviewed in **Chapter 2**, including comments on their practicality and applicability for the purposes of REACH. Based on the results of a detailed literature review, **Chapter 3** provides an assessment of the practicality and applicability of QSPR/QSAR models as well as TK/TD/dosimetry/fate models.	Appendix III gives an overview of the Standard Information Requirements (REACH Annexes VI-X) Appendix VII illustrates the reporting format developed to systematically information on TK/TD/dosimetry/fate models
	2.2 Evaluate computational methods and models for the purposes of grouping NMs	Building on the conclusions of **Chapters 2 and 3** (Task 2.1), the practicality and applicability of grouping approaches, including the use of supervised and unsupervised machine learning techniques, is explored through two read-across case studies (nano TiO2 and MWCNTs) in **Chapter 4**.	The details of the computational machine learning techniques (clustering and PCA) for the nano TiO2 case study are provided in Appendix X.
T3	3.1 Grouping NMs according to similarity	In Chapter 4, practical recommendations on how to group NMs considering compositional, physicochemical and in vitro descriptors, in accordance with draft ECHA guidance, are provided. These recommendations are based on the experience gained in carrying out two read-across case studies (nano TiO2 and MWCNTs).	

	Tasks	Chapter	Appendices and supplementary materials[1]
	3.2 QSAR and read across in integrated assessment approaches	Background information on integrated assessment approaches, such as Integrated Testing Strategies (ITS) and Weight of Evidence (WoE) is provided in **Chapter 1**. The use of WoE as an integral part of the grouping and read-across approach is illustrated in **Chapter 4**. Research gaps, in terms of the need for new QSPRs and QSARs, are identified in Chapter 5.	The dataset for the MWCNT case study is given in Supplementary Material S4.
	3.3 Use of models and approaches to predict NM physicochemical properties	Existing models and approaches to predict NM physicochemical properties are described in **Chapter 3**. Ongoing research developments are reported in **Chapter 5**.	
T4	4.1 R&D on approaches assessed under Task 2 4.2 Public dissemination and update of the models inventories developed under Task 1	The challenges in developing and applying predictive approaches for NMs are described in **Chapter 1**. Based on the literature reviews (Chapter 3), the lessons learned in the case studies (Chapter 4), and review of EU project deliverables, recommendations for further research and development activities are made in **Chapter 5**. A proposal for public dissemination and future updating of the model inventories is provided in **Chapter 5**. Overall conclusions are presented in **Chapter 6.**	Appendices IV and V give a list of EU funded (IV) and other (V) research projects having a stated aim to develop computational models or grouping approaches.

1) Supplementary materials S1-S4 are available upon request

1 Background information

1.1 Introduction

Manufactured nanomaterials (NMs) are being increasingly included in a variety of goods and products, because of their novel physical and chemical characteristics (European Commission, 2012). There are concerns, however, that the very same characteristics may also lead to environmental and human health risks.

In the EU nanomaterials have been defined by some legal pieces. Legally binding definitions are included in the Cosmetic Products Regulation (EC) 1223/2009, the Biocidal Products Regulation (EU) 528/2012/EC and Regulation (EU) 1169/2011 on Provisions of Food Information to the Consumers (FIC Regulation). In addition in 2011 the EC adopted a Recommendation (2011/696/EU) on the definition of the term 'nanomaterial' with the goal to promote consistency in the interpretation of the term nanomaterial for legislative and policy purposes in the EU (EC, 2011a). This definition is broadly applicable across different regulatory sectors, however not legally binding. The EC Definition applies to all particulate NMs regardless of their origin, i.e. natural, incidental or manufactured. It refers to a size range of 1 – 100 nm and established also a threshold of 50% or more particles <100 nm in the number size distribution which in specific cases can be lowered to 1% (Appendix II). This size range has been proposed in several definitions, including ISO/TS 12805:2011 by the International Organization for Standardization (ISO).

The NM definition in the Biocidal Products Regulation is based on the EC Definition, while the definitions for cosmetic products and food were implemented previously and contain some relevant differences. Although comprising a comparable size range (<100 nm) the main difference is the restriction to intentionally manufactures (or produced) materials, which is further restricted for cosmetic products to insoluble or biopersistent material. This size range has been proposed in several definitions, including ISO/TS 12805:2011 by the international Organization for Standardization (ISO).

The revision of the EC definition and an alignments of the definitions of NM in cosmetic products and food with the EC definition are currently are under discussion (JRC internal information; Rauscher et al., 2014; Roebben et al., 2014).

To evaluate and manage the environmental and health impacts of chemicals, risk assessment (RA) is considered a pertinent approach that can be adapted to assess the potential risks caused by NMs. RA is a process by which scientific and regulatory principles are applied in a systematic approach to address qualitatively and/or quantitatively the likelihood that humans or environmental species may be harmed due to potential exposure to chemicals.

Regulation 1907/2006 concerning the Registration, Authorisation and Restriction of Chemicals (REACH) (EU, 2007; European Parliament and Council, 2006a), requests registrants to demonstrate the safe use of chemicals including NMs. REACH is intended to ensure chemical safety while promoting innovation and competitiveness as well as reducing the use of *in vivo* testing through the use of non-animal alternatives such as *in silico*, *in vitro* methods and *in chemico* methods, read across and weight of evidence.

This chapter provides background information on the information requirements for NMs with special focus on REACH, as well as the scientific and technical basis for understanding their behaviour (fate and effects). The REACH regulation (section 1.2) and risk assessment

approach (section 1.3) are briefly introduced, and an overview is given (section 1.4) of key physicochemical (PC) properties that are relevant for characterising and understanding the behaviour (fate and biological effects) of NMs. This chapter also touches on the scientific basis of NM behaviour (section 1.5), since this understanding is important in the development and application of standard (section 1.6) and alternative approaches to animal testing (section 1.7).

1.2 Information requirements for risk assessment - legal provisions and guidance

In the EU, substance and sector-specific pieces of legislation provide a binding framework to ensure the safety of substances and products on the market (manufactured or imported). NMs are implicitly covered or explicitly addressed (e.g. cosmetics and biocidal products), depending on the applications and its legislative framework. The 'substance' definition of REACH applies to chemicals irrespective of size, shape and physical state, and thus its provisions apply also for NMs, even if there are currently no provisions that explicitly refer to NMs (EC, 2008; European Parliament and Council, 2006a).

1.2.1 Chemical substances under REACH

According to the (second) Regulatory Review on Nanomaterials (EC, 2012c), REACH sets the best possible framework for the risk management of NMs when they occur as substances or mixtures. More specific requirements may be introduced through the possible revision of some of the REACH Annexes. In addition further ECHA guidance for REACH registrants is needed.

The provisions of REACH contain extensive obligations for manufacturers to generate and assess data on chemicals (including PC properties, manufacture, uses and hazardous properties) and to demonstrate that risks can be adequately controlled during their use. All chemicals manufactured or imported in quantities higher than 1 t/y have to be registered with basic information requirements (for overview, see Appendix III). In addition, for all chemicals manufactured or imported in quantities higher than 10 t/y, a chemical safety assessment (CSA) has to be performed and documented in the chemical safety report (CSR).

Under REACH, different forms of one substance (e.g. solids, suspensions, powders, nanomaterials, etc.) are considered within a single registration of a substance (EC, 2012c). However, the registrant must ensure the safety of all included forms and provide adequate information to address the different forms in the registration. This means that more than one endpoint study for different forms may be required, or different forms within one registration may have a different hazard classification.

The REACH approach to hazard assessment and risk characterisation, with its built-in flexibility, makes it suitable for NMs (EC, 2012c). Based on RIPoN (REACH Implementation Project on Nanomaterials) Reports (Hankin et al., 2011), ECHA has published specific guidance for NMs (ECHA, 2012b, 2012c, 2012d). In addition, ECHA has set up a Nanomaterials Working group to give advice on scientific and technical issues in relation to NM under REACH.

The information collected or generated under REACH is used e.g. for priority setting, classification and labelling, chemical safety assessment and PBT/vPvB[1] assessment. It needs to be adequate for both classification & labelling and for chemical safety assessment if the latter is required (triggered by classification or PBT assessment). REACH Article 10 defines the information requirements to be submitted for registration and Annex I outlines the general provisions for assessing substances and preparing chemical safety reports. Annexes VII to X more specifically detail the standard information requirements. Information on intrinsic properties is mainly dependent on the tonnage at which the substance is brought on the market (see overview in Appendix III). Column 2 of each of these Annexes (VII to X) defines the specific rules for adaptation of the standard information requirements as defined in column 1, i.e. when a specific step (test) does not need to be conducted, or further studies may be considered in case of positive results. Guidance notes on fulfilling the requirements of Annexes VII to X are presented in REACH Annex VI. This Annex contains also the information requirements which are independent from the tonnage, i.e. general registration information, identification of the substance, manufacture and use, classification and labelling, and guidance on the safe use. REACH Annex XI defines the 'General rules for adaptation of the standard testing regime set out in Annexes VII to X'. The basis for the use of alternative approaches under these rules are laid down by Article 13 and 25.

Article 25(1) states that *testing on vertebrate animals shall be undertaken only as a last resort*. According to Article 13(1):

> *"information on intrinsic properties of substances may be generated by means other than tests, provided that the conditions set out in Annex XI are met. In particular for human toxicity, information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods, for example, in vitro methods or qualitative or quantitative structure-activity relationship models or from information from structurally related substances (grouping or read-across)."*

As described in Annex XI, testing can be waived or replaced by other information and methods when: i) testing is not scientifically necessary; ii) testing is technically not possible, or iii) Substance-tailored exposure–driven testing (i.e. exposure-based waiving) is appropriate. The types of test that can be waived or replaced by alternative tests are dependent on the tonnage and the requirements of the respective Annex (see Appendix III). Guidance is provided in Chapter R.5: 'Adaptation of information requirements' of the ECHA 'Guidance on information requirements and chemical safety assessment' (ECHA 2011).

REACH Annex XI also explains when alternative approaches such as (Q)SAR, grouping and read-across, *in vitro* methods and weight of evidence can be used instead of testing to indicate the presence or absence of a certain dangerous property (see section 1.7).

Results obtained from valid qualitative or quantitative structure relationship models — (Q)SARs — may be used to indicate the presence or absence of a certain dangerous property, if: i) the scientific validity of the (Q)SAR model has been established, ii) the substance falls within the applicability domain of the (Q)SAR model, iii) the results are adequate for the purpose of classification and labelling and/or risk assessment and iv) the

---

[1] Persistent, Bioaccumulative and Toxic (PBT), very Persistent and very Bioaccumulative (vPvB)

adequate and reliable documentation of the applied method is provided (REACH Annex XI, 1.3).

Substances whose physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern as a result of structural similarity may be considered as a group, or 'category' of substances and PC, human health effects and environmental effects or environmental fate may be predicted from data for reference substance(s) within the group by interpolation to other substances in the group (read across approach) (REACH Annex XI, 1.5) (see also section 1.7.4)

Guidance on how to assess (Q)SARs if conditions are met and on the technically and scientifically justified methodology for the grouping of substances is provided in 'Chapter R.6: QSARs and grouping of chemicals' of the 'ECHA Guidance on information requirements and chemical safety assessment' (ECHA 2008).

The current minimum standard information requirements as described in the REACH Annexes may not address all NM-specific PC properties and characteristics; especially the physicochemical properties may not be sufficient to adequately characterise the nanomaterial and discriminate it from other forms. However, it is already possible to document relevant information in IUCLID (International Uniform Chemical Database), which is the electronic tool used for submissions of REACH dossiers to ECHA. IUCLID included in September 2013 the 13 OECD Harmonised Templates (OHTs) which cover NM-specific endpoints (http://www.oecd.org/ehs/templates/).

The impact assessment on the possible amendment of the REACH Annexes to more specifically address NMs has been finalised and the potential elements were presented as 'Informal Considerations' (non-paper) at the May 2014, March 2016 and March 2017 meetings of the 'Subgroup of the REACH and CLP Competent Authorities on Nanomaterials' (CASG Nano). The proposed amendments include for example more detailed information on substance identity and highlight the existence of nanoforms of the same substance. The proposal also includes a definition of nanoform which is in accordance with the European Commission Recommendation of 18 October 2011 (EC, 2011a) on the definition of nanomaterial.

1.2.2   Cosmetic products

Certain ingredients of cosmetics require an authorisation based on a scientific risk assessment and an inclusion in Annexes IV (colorants), V (preservatives) and VI (UV filters) to Regulation (EC) No 1223/2009 (European Parliament & Council, 2009). Some of these substances may be particles at the nanoscale. The Cosmetic Products Regulation provides a definition of nanomaterial (insoluble/partially soluble or biopersistent and intentionally manufactured) as well as mechanisms for notification, labelling and safety evaluation of cosmetic products containing nanomaterials. It also imposed bans on testing finished products and ingredients on animals as well as on marketing of such products and ingredients (from March 2013).

The EU Scientific Committee for Consumer Safety (SCCS) released a guidance on the 'Safety Assessment of Nanomaterials in Cosmetics' (SCCS, 2012) where it is recommended that the nanospecific physicochemical properties are considered in the safety assessment. It highlights the need for special considerations in relation to the safety of NMs, in view of the

possible distinct properties, interactions, and/or effects that may differ from conventional forms of the same materials. It covers and gives recommendations addressing the main elements of risk assessment, i.e. material (physicochemical) characterisation, exposure assessment (e.g. likelihood and extent of translocation of NMs across biological barriers), and toxicological evaluation (e.g. consideration of nano-related aspects such as particulate form or possible interaction with biological entities). The guidance presents an overview of available methods of toxicological evaluation of nanomaterials for each endpoint and concludes that at present in view of current limitations an approach using *in vitro* assays only is too premature to be applied for risk assessment. Concerning read-across, the SCCS considers that in the absence of a sufficient knowledge base on nanomaterial properties, behaviour, and effects, a category approach to risk assessment is currently not feasible for NMs, and risk assessment of each nanomaterial needs to be carried out on a case-by-case basis. The SCCS is, however, open to consider the application of read-across and mathematical models that enable a category approach to conduct risk assessment of NMs in the future, when new knowledge will increase the understanding of the key parameters that drive the properties, biological interactions and toxicological effects of NMs (SCCS, 2012).

Further guidance on NMs data submission is provided in the SCCS 'Notes of guidance for the testing of cosmetic ingredients and their safety evaluation' it is stressed again that specific properties should be taken into consideration in safety assessment (SCCS, 2015) and in the Memorandum on 'Relevance, Adequacy and Quality of Data in Safety Dossiers on Nanomaterials' (SCCS, 2014), SCCS highlights the importance of submitting data that are relevant, adequate and of good quality in support of risk assessment and also highlights that when NMs are submitted under the same dossier, the possibility to read-across should be justified.

1.2.3 Biocidal products

The risk assessment and authorisation of NMs in biocidal products is addressed by the Biocidal Products Regulation (EU) No 528/2012 (European Parliament and Council, 2012). It states that for the approval of NMs as active substances and for subsequent product authorisation, the test methods applied to the NMs shall be accompanied by an explanation addressing their scientific appropriateness, taking into consideration the specific characteristics of each NM. The information requirements for active substances are outlined in Annex II of the Regulation, whereas those for the biocidal product are given in Annex III. The general rules for the adaptation of the data requirements for biocides are presented in Annex IV of Biocidal Products Regulation (EU) No 528/2012. These are in accordance with Annex XI of REACH regulation. Specific guidance for nanomaterials is in preparation.

1.2.4 Plant protection products

The Plant Protection Products Regulation (EC) 1107/2009 (European Parliament and Council, 2009) does not make any explicit reference to NMs. The data requirements for active substances and plant protection products are set out in Annexes II and III to Directive 91/414/EEC (Union & Council of the European Union, 1991). Guidance on how to assess the risk of NMs in plant protection products is provided by the EFSA Guidance on the Risk Assessment of the Application of Nanoscience and Nanotechnologies in the Food and Feed Chain (EFSA, 2011).

1.2.5 Food production

The use of nanotechnology and nanomaterials in food production is currently covered by EC Regulation No 258/97 concerning 'novel foods' and 'novel food ingredients' (European Parliament and Council, 1997) which addresses food not consumed to any significant degree in the EU prior to May 1997. The proposal for the revised regulation (European Commission, 2013) addresses NM and nanotechnology more explicitly by covering *'foods modified by new production processes such as nanotechnology and nanoscience and food or vitamins, minerals and other substances containing or consisting of 'engineered nanomaterials'*. A pre-market approval (safety assessment and authorisation) is required for novel food as well as for food additives.

In 2011 EFSA issued a 'Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain' (EFSA, 2011) providing a practical approach for assessing potential risks arising from applications of nanoscience and nanotechnologies in food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides. This guidance is now being updated.

This document provides guidance to applicants for data generation on the PC characterisation and on testing approaches to identify and characterise hazards arising from the properties of NMs. It describes 6 exposure scenarios, depending on the possible transformations of the NM before and after ingestion of the food/feed and for each of these scenarios the type of data needed to conduct a risk assessment is specified. When no exposure to NM is verified for example by data indicating no migration[2] from food contact materials or by demonstration of complete degradation/dissolution with no possible absorption of NM, the information requirements can be reduced. Direct use of NM in food or feed which are transformed to non-nanoforms in a food/feed matrix and before ingestion can be treated as and follow the guidance for non-nanoforms. For such NMs only local effects in the gastrointestinal tract and possible absorption before transformation should be considered. A comparison should indicate whether the nanoform has increased, less or similar hazard as compared to the non-nanoform.

1.3   Risk assessment

The three major steps in a risk assessment (or to prepare a CSA under REACH) include (a) hazard assessment, (b) exposure assessment and (c) risk characterisation (REACH Annex I ; ECHA, 2009; European Parliament and Council, 2006a).

The hazard assessment has the objective to identify the hazards of the substance, assess their potential effects on human health and the environment, and determine, where possible, the no-effect levels (threshold levels). If a substance meets the criteria for classification as dangerous, or is identified as PBT or vPvB, it will undergo the exposure assessment and risk characterisation.

---

[2] The guidance document does not include details on analytical or mathematical methods for detecting migration of NMs to food and feed; the analytical approach should be well justified by the applicant.

The exposure assessment serves to measure or estimate the dose or concentration of the substance to which humans and the environment are, or may be, exposed in specific exposure scenarios which cover all identified uses and life stages of the substance. An exposure assessment is required only for hazardous substances in REACH.

For the risk characterisation, Derived No Effect Levels (DNELs) extrapolated from the *in vivo* no-effect levels are compared with the levels of exposure to assess whether the risk is controlled. When a no-effect level cannot be quantitatively determined, a qualitative or semi-quantitative approach is used.
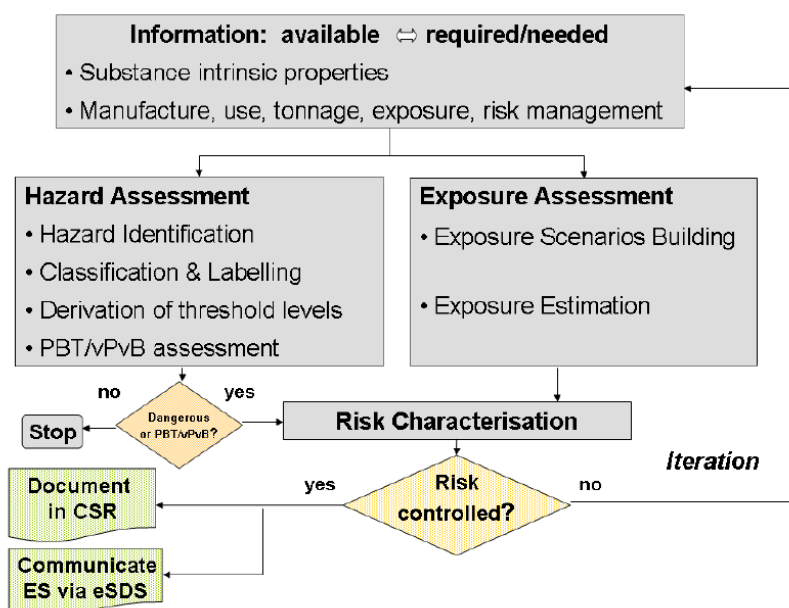
Figure 1.1 Overview of the Chemical Safety Assessment process under REACH (ECHA, 2009)**.**

Although the necessity of an exposure assessment and risk characterisation is dependent on the results of the hazard assessment, exposure information may play an important role in influencing it. In situations where human or environmental exposure is absent or so low that additional effects information is not requested to improve the risk management, exposure-based waiving of hazard information can be considered (Annex XI of REACH, section 3 on substance tailored exposure-driven testing, European Parliament and Council 2006). Reducing/minimising exposure is therefore an important risk management option in the development and production of NMs and NM-containing products, especially when there are still scientific uncertainties in the hazard assessment.

In contrast to exposure-based waiving, additional (targeted) testing can be triggered if the risk characterization indicates a need to investigate further the effects on humans or the environment for certain exposure situations. The exposure assessment also informs the hazard assessment in terms of which routes of exposure (e.g. inhalation) and which concentrations are the most relevant to test. Exposure-based adaptations are an integral part of the ECHA Guidance on Information Requirements and Chemical Safety Assessment, Chapter R5: Adaptation of Information requirements (ECHA, 2011). According to this guidance, exposure based adaptations (EBA) can be considered when they can be justified

based on no release, strictly controlled conditions, absence of exposure, or no significant exposure. In practice, exposure based adaptations of the standard hazard testing regime under REACH require reliable information on use and exposure, which is often not available for NMs.

The applicability of current risk assessment methodologies for NMs has been assessed by the EC scientific committees including the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), the Scientific Committee on Consumer Safety (SCCS), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA). SCENIHR (SCENIHR, 2009) concluded that *'risk assessment methodologies for the evaluation of potential risks of substances and conventional materials to man and the environment are widely used and are generally applicable to nanomaterials. However, specific aspects related to nanomaterials still require further development. As there is not yet a generally applicable paradigm for nanomaterial hazard identification, a case-by-case approach for the risk assessment of nanomaterials is still warranted.*' Similar conclusions were made by the scientific committee of EFSA for NM applications in food and feed (EFSA Scientific Committee, 2009).

The Scientific Committees as well as OECD have identified that for NMs further development of some standardised and validated methods are required, for example for sample preparation and characterisation, exposure data and models and some endpoints in toxicological test guidelines (section 1.6). Harmonisation and standardisation of measurement and test methods in support or risk assessment of NM is being promoted through the OECD and by a Commission Mandate to the European Standards Organisation (EC, 2012c).

Several (regulatory) risk assessments on NM-containing products have been completed and various products in different sectors have been authorised (Second regulatory review 2012: 20 medicines, 3 food contact materials, 4 cosmetic products) (EC, 2012c). The SCCS (and its predecessor committees) has performed a number of risk assessments on nanomaterials used as cosmetic ingredients (Table 1.1)

Table 1.1. SCCS risk assessments on nanomaterials used as cosmetics

Nanomaterial	Document[1]
Titanium dioxide as UV-Filter, as pigment	SCCNFP/0005/98
Zinc oxide in suncream(	SCCP/1147/07
TiO2	SCCS/1516/13 + SCCS/1539/14
ZnO	SCCS/1489/12 + SCCS/1518/13
ETH 50 (nano and non-nano)	SCCS/1429/11
MBBT	SCCS/1460/11
Silica	SCCS/1545/15
Carbon Black	SCCS/1515/13
Hydroxyapatite	SCCS/1566/15
Additional coatings for TiO2 (nano form) as UV filter in dermally applied cosmetic products	SCCS/1580/16
Titanium Dioxide (nano form) as UV-Filter in sprays –	SCCS/1583/17 (in finalization)

[1] Documents available at: https://ec.europa.eu/health/scientific_committees/consumer_safety/opinions_es

1.4 Properties that drive NM behaviour (fate and toxicity)

Table 1.1 provides a list of properties that have been identified as relevant for understanding fate and effects of NMs. These properties have been identified from the discussions of the OECD Working Party on Manufactured Nanomaterials (WPMN) (OECD, 2014g), Member States for REACH implementation, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2009), the Scientific Committee on Consumer Safety (SSCS, 2012) as well as the scientific literature. Information requirements for NMs under REACH is at the moment not yet finalised: inclusion of relevant properties in REACH submissions is under discussion; such properties include crystalline phase, dustiness, crystallite size, zeta potential, pore density, radical formation potential, surface chemistry and functionalization, specific surface area, dispersability, (photo)catalytic activity, particle size distribution, agglomeration/aggregation states (OECD, 2010, 2014g).

Properties in Table 1.2 have been organised following the scheme proposed by Sellers et al. (2015) into chemical identity, particle characteristics, fundamental transport and behaviour, reactivity/activity, which is consistent with the properties suggested in ECHA's voluntary Practical Guide on grouping NMs for read-across (ECHA, 2017b). In the Sellers proposal, the chemical identity group comprises a NM including impurities and surface coating, and information on crystal structure. The particle characteristics group may include shape, size related properties and porosity. The transport and behaviour group covers properties that influence the movement of a NM in the environment or within the organism, and may depend on the NM or on the environment, like water solubility, dispersibility, zeta potential. The reactivity/activity group comprises properties that are exclusively related to the

reactivity of the NM as a whole, like radical formation potential, catalytic activity, protein binding properties, hydrolysis, dissociation constant.

There is a distinction to be made between intrinsic and extrinsic properties. Intrinsic properties are characteristics of the material itself that do not account for interactions with other components (OECD, 2010a). Extrinsic properties are characteristics resulting from interactions occurring at the interface (i.e. boundary) and the surrounding medium (which may be an environmental or biological matrix or medium in an experimental test system). This distinction is important in the development of *in silico* models for PC properties. With regard to this distinction, physicochemical properties listed in Table 1.2 are to be considered as intrinsic properties that aim at defining the nature of the pristine NMs. Any property that may be modified by the interaction of the NM with the environment can be considered as an extrinsic property, and may be then defined according to the environment (e.g. behaviour properties may be considered also extrinsic properties).

Table 1.2. Physicochemical properties and their relevance in (eco)toxicological assessment. The table shows the list of parameters that can be submitted following the voluntary ECHA Practical Guide, and those that are under discussion by the OECD (WPMN). Physicochemical properties that are considered relevant in the literature are also added to the table.

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
Chemical identity	Chemical composition	This refers to the chemical identity and atomic arrangement of the nanomaterial; it shall consider also impurities and additives [purity is expressed as the percentage of the intended NM present].	The type of elements involved in the core as well as the form or valence of those elements are relevant in toxicology.	Definition is applicable to NMs (ECHA, 2014a; OECD, 2010).
	Crystal type[3]	The specific arrangement of a chemical group (crystallite) in the three dimensional space. In some cases more crystal types of the same NM may observed: in this case the relevant information is the fraction of the different crystalline forms that are present (OECD, 2010).	This is a recognized indicator of the biological impact of NMs (Aitken et al., 2011; Landsiedel et al., 2010).	Definition is under discussion (OECD, 2010).
	Crystallite size	Size of the crystal or grain.	Important for ensuring comparability between tests and for interpreting data from the tests.	Definition is under discussion (OECD, 2010).
	Surface chemistry	Chemical nature, including composition, of the outermost layers of the nano-object.	It allows the recognition of the various modifications of the surfaces of nanomaterials that will lead to numerous potential interactions and will play a key role in determining: i) fate in natural aqueous systems; ii) colloidal stability and iii) exposure (OECD, 2010, 2014g).	Definition is under discussion (OECD, 2010).

---

[3] Crystalline phase in OECD (2010)

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
	Surface functionalization, coating	Functionalization is mainly related to the introduction at a surface of chemical groups which are intended to subsequently react with other species (Rivolo, 2012). Surface modification, functionalization and coating are considered in RIP-oN 1 (European Commission, EC, & IHCP JRC, 2011) as synonymous with surface treatment.	Surface treatment is considered to affect environmental fate and health safety of NMs (OECD, 2014g).	Definition is under discussion (OECD, 2010).
Particles characteristics	Basic morphology	NMs with the same composition may have different shape (i.e. spheres, rods, tubes, fibres and plates), which may be related to different physical, chemical, and biological properties. Shape is defined by OECD (2010) as the (semi-)qualitative geometrical description or dimensionless term of the extremities of the particle or collections of particles, their agglomerates or aggregates, of the material under investigation. Recognised shape descriptors are sphericity, circularity, aspect ratio, convexity and fractal dimension. ISO (2012d) defines three levels of detail in describing the shape, described by the geometric form of the external boundary of the particle and refer to convexity, perimeter, circularity at different scale levels (macro-, meso-, and micro-shape descriptors, u.m. m/m)[4].	The elongation (or aspect) ratio (together with rigidity) is an important determinant of the potential health effects of fibrous materials, including NMs.	Considered as shape, definition is under discussion (ECHA, 2012b; OECD, 2010).

---

[4] Macrodescriptors are defined from size measurements made on the particle silhouette; mesodescriptors are morphological mathematical descriptors, computing robustness and largest concavity index, a concavity tree, providing general insight into the organization of concavities and their complexity and

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
	Particle size	The physical dimension of the smallest discrete form of a substance under specified measurement conditions. If a group of particles are of differing sizes they may be described by a particle size distribution (OECD, 2010).	Once internalised, particle size may also affect the distribution within the body, and the toxicity at both the point of entry and distally. Size distribution is not a static parameter; it may also change during the course of (environmental) toxicity testing (as well as during the life cycle of the material) due to e.g. partial dissolution, interaction with test media or preferential absorption of smaller particles (ECHA, 2017b).	
	Pore density	Pore density is defined measure of the void (i.e. 'empty') spaces in a material, and is a fraction of the volume of voids over the total volume [g/cm^3].	Pore density has relevance in the fate of NMs as it influence their kinetics (i.e. sedimentation rate and size distribution as suspended material) in different media (J. Meesters, Koelmans, Quik, Hendriks, & van de Meent, 2014; Antonia Praetorius, Scheringer, & Hungerbühler, 2012).	Definition is under discussion (OECD, 2010).

angularity descriptors, fractal dimension, Fourier descriptors, bending energy; microdescriptors determines the roughness of shape boundaries using fractal dimension and higher-order Fourier descriptors/coefficients for surface and textural analysis (ISO, 2012d).

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
	Specific surface area (SSA)	SSA is the surface area of the particle SA [m^2] per unit mass m [g] SSA=SA/m [m^2/g] (Wolfgang G. Kreyling, Semmler-Behnke, & Chaudhry, 2010). It is an intensive property and hence allows expressing the information related to the surface area in a comparable way, independent on the quantity of the available material. The area of the exposed surface of a single particle plays an important role in influencing the physical and chemical interactions of the particle in the media. SCENIHR (SCENIHR, 2009) and ECHA (2012b) consider also the concept of volume specific surface area (VSSA) [m^2/cm^3] $$VSSA = \frac{SA}{V} = SSA \cdot \rho \qquad (1)$$ Where ρ is material density and V [cm3] is the volume of the material. VSSA is proposed as a measurement that can be used to distinguish dry solid nanostructured material from non-nanostructured material at VSSA≥60 m^2/cm^3 (Wolfgang G. Kreyling et al., 2010).	Chemical reactions take place at surfaces, hence high SSA represents high reactivity. SSA is relevant for a number of parameters for (eco)toxicological and risk assessment. It will dictate the surface charge density in cases where nanomaterials are surface functionalised. This in turn has direct consequences on (a) nanomaterial interactions (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 normalised for surface area may indicate different results compared to results presented on a per mass basis).	Definition is under discussion (ECHA, 2012b; OECD, 2010).
Fundamental transport/behaviour	Dispersibility	Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase). 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	It may influence the ability of the NMs to reach and enter the cell (OECD, 2010).	Definition is under discussion (OECD, 2010).

18

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
		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 (Hankin et al., 2011).		
	Water solubility	Water Solubility/Dispersibility refers to the mass proportion of a given sample of nanomaterial which is held in water solution or as a colloidal suspension in water as a function of time or where the sample of nanomaterial loses its particulate character as it changes from a particle form to a molecular form (OECD, 2010). [u.m.: µg/L; mg/L; g/L; g/cm³; kg/m³; ppb; vol%]	If a nanomaterial is soluble, it is likely to be presented to the *in vitro/in vivo* test system in a molecular or ionic form and can be expected to elicit the same response as more usual chemical forms of the material (with different dissolution rate compared to the bulk substance). If the nanomaterial under investigation is insoluble in biological or environmental media then it will be presented to the test system in particulate form and might elicit a different response from that expected based on the chemical composition.	Already required in REACH (ECHA, 2012b; European Parliament and Council, 2006a).
	Dissolution rate	According to experimental evidence, the rate of dissolution of soluble materials increases with decreasing particle size and differs in different media; dissolution rate is relevant as a process	Dissolution rate is considered in several studies as a measure for NMs biodurability; it is then related to NMs (bio)persistence (K. A. Jensen,	Not required in REACH.

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
		when dealing with NMs behaviour (Utembe, Potgieter, Stefaniak, & Gulumian, 2015).	Kembouche, & Nielsen, 2013; Utembe et al., 2015)	
	Hydrophobicity/ Hydrophilicity	Hydrophobicity refers to the 'water-avoiding' behaviour of a nonpolar molecule or group that has low affinity for water molecules. Hydrophilicity refers to the 'water-liking' behaviour of a polar molecule or group that has high affinity for water molecules	Influences protein binding to NM (Aggarwal, Hall, McLeland, Dobrovolskaia, & McNeil, 2009; Landsiedel et al., 2012), and characterizes inhibitory activity towards various enzymes (Gallegos, Burello, & Worth, 2009). Hydrophilicity is one of the most relevant descriptors in uptake models (V Chandana Epa et al., 2012).	Not required in REACH.
	Dustiness	This is the propensity of a material to generate airborne dust during its handling. The measure of interest is the degree to which a given nanomaterial can remain in the air column before settling. Interactions of NMs with other common airborne particulate matter should be studied.	It provides a basis for estimating the potential for inhalation exposure (Lidén, 2006).	Definition is under discussion (OECD, 2010).
	Zeta potential (surface charge)	Defined as the charge at the particles interfaces. It is a repulsive inter-particle force, because a colloidal, charged system is a stable system (Fermin & Riley, 2010): therefore, ionization enhances the particles stability. In fact, a charged surface attracts counter-ions in its vicinity. Therefore, when two particles approach each other, their diffuse layers will overlap and the resultant repulsive force may outweigh the attractive Van der Waals attraction, rendering the	Zeta potential can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds	Definition is under discussion (OECD, 2010).

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
		suspension stable [mV]. Van der Waals forces are expressed by the Hamaker constant, which is related only to the properties of the interacting bodies and the medium and consists of the summation of intermolecular interactions (Hamaker, 1937). This constant could be useful in determining the degree of agglomeration and sorption (Sellers et al., 2015).	repulsion and the dispersion will break and flocculate. In nanotoxicology, zeta potential (surface charge) plays a key role in determining the (1) degree of colloidal interaction which is itself a function of the pH and ionic strength of the bulk solution; and (2) bioavailability when considering mass transport through charged membranes.	
	Steric hindrance	Repulsion short-range effects rising from surface characteristics (e.g. presence of polymers) (Elimelech, Gregory, Jia, & Williams, 1995b).	It influences agglomeration (SCENIHR, 2010) and protein binding (Landsiedel et al., 2012) of NMs. It is considered relevant as a property driving toxicity of NMs (Tomasz Puzyn et al., 2011a).	Not required in REACH.
Reactivity	Protein binding	Mechanism of chemical interaction with proteins. It can be covalent (irreversible; common with electrophilic toxicants such as nonionic and cationic electrophiles and radical cations) or non-covalent (apolar interactions or the formation of hydrogen and ionic bonds and is typically involved in the interaction of toxicants with targets such as membrane receptors, intracellular receptors, ion channels, and some enzymes) (Casarett & Doull, 2008).	Protein binding results in increased stability of NMs (Vandebriel & De Jong, 2012) and affects biodistribution of NMs throughout the body (Aggarwal et al., 2009).	Not required in REACH.
	Hydrolysis	Cleavage of chemical bonds by the addition of water.	Uptake is a modulated by hydrolysis, and hence may influence exposure to a	Already required in REACH

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
	Catalytic activity	This is the ability of some NMs to speed up certain reaction as a catalyst [mol/(g*s); mol/(g*h)].	It causes ionization events leading to free radical production (WHO, 2012).	Definition is under discussion (OECD, 2010).
	Photocatalytic activity	This is the ability of some NMs to speed up a certain photoreactions as a catalyst, eventually in combination with light (sunlight, ultraviolet light) [mol/(g*s); mol/(g*h)].	It is recognized as a relevant PC property in toxicity testing (SCENIHR, 2010). Photocatalytic activity may drive oxidative stress under UV light (Ken Donaldson et al., 2013).	Definition is under discussion (OECD, 2010).
	Conduction band (valence band, band gap)	Energy required to free an electron from its bond to an atom [eV].	Substances with conduction band in the range of cellular membrane redox potential (-4.12 to -4.84 eV) are possibly causing oxidative stress (Burello & Worth, 2011a; Haiyuan Zhang et al., 2012a).	Not required in REACH and not considered in OECD recommendations.
	Electrophilicity and Nucleophilicity (acidity and basicity)	Electrophilicity is the attraction to electrons; electrophiles tend to accept electrons. Nucleophilicity is the tendency to donate an electron pair to an electrophile in a reaction.	Electrophilicity/Nucleophilicity is related to reactivity of NMs (Burello, 2013; Albert Poater, Gallegos Saliner, Solà, Cavallo, & Worth, 2010)	Not considered in REACH and not considered in OECD recommendations.
	Radical formation potential	This is the ability of a substance to produce reactive oxygen species (ROS), including for instance superoxide radical O2·, hydrogen peroxide H2O2, and hydroxyl radical OH·.	These ROS compounds exert severe cellular damage such as oxidations of DNA, proteins or lipids that may cause cell death (Rivolo et al., 2012).	Definition is under discussion (OECD, 2010).
	Redox potential	Measure of the tendency of a chemical species to acquire electrons and thereby be reduced [mV].	Oxidizing substances may interact with cells thus altering their redox balance and causing oxidative stress (Burello &	Already required in REACH (European

Category	Term	Definition [u.m.]	Relevance in (eco)toxicological assessment	REACH requirement or OECD recommendation
			Worth, 2013). This is related to the conduction band.	Parliament and Council, 2006a).
	Dissociation constant	It is the reversible reaction of a substance resulting into two or more chemical species, which may be ionic; the process may be represented as RX$\leftrightarrow$R+X. he dissociation constant K is expressed as the ratio of concentrations of the species on either side of the equation at equilibrium: $$K = \frac{[R][X]}{[RX]}$$ Where the cation R+ is hydrogen, the substance can be considered an acid, and so this constant becomes an acid dissociation constant (Ka) (ECHA, 2014a).	It is related to protein binding (Vilaseca, Dawson, & Franzese, 2013; S.-T. Yang, Liu, Wang, & Cao, 2013) and may be important for interpreting the agglomeration of NMs (Bruinink, Wang, & Wick, 2015).	Already required in REACH (European Parliament and Council, 2006a).

1.4.1 Theories underlying environmental and biological fate

The OECD published 118 guidelines for the testing of chemicals, and the WPMN evaluated which of these is applicable to NMs (OECD, 2009b). This review concluded that 4 out of 22 test guidelines for physical chemical properties are applicable to NM. 16 guidelines might be applicable under some circumstances, or to some classes of NM. Two guidelines are not applicable to NM or, if applicable, provide no useful information.

Standard methods are needed in order to fulfil the information requirements listed in Table 1.3. Measurement methods and protocols for providing information listed in the previous table are not yet identified, although several projects are developing measurement protocols (see Appendixes IV and V). In particular, NanoReg developed several SOPs on particle size distribution (Mast & De Temmerman, 2016; Gottardo et al, 2017).

The OECD WPMN is also aiming at providing test guidance for the determination of PC properties for NMs to support the submission of information on substances within IUCLID.

Table 1.3 lists the methods for relevant PC and environmental fate properties according to the OECD WPMN discussions (OECD, 2014b, 2014g). Characteristics that are not reported in the OECD guidelines are not listed here. When an OECD guidance is available for the identified PC property, its applicability as addressed in OECD (2009b) is reported in a dedicated column. When OECD guideline is not available, other relevant guidelines are reported (OECD, 2009b, 2014g).

Table 1.3. List of the existing methods supporting the measurement of NM properties

PC property	OECD guideline[5]	Applicability for NMs testing	ISO standards	Other available methods
Dispersion/ agglomeration/ aggregation	None available (OECD, 2014g)	-	Centrifugal liquid sedimentation, dynamic light scattering, small- angle x-ray scattering; single-particle ICPMS, particle tracking analysis and field flow fractionation; TEM and x-ray diffraction (ISO, 2013a)	-

---

[5] More about OECD's Test Guidelines Programme, see http://www.oecd.org/env/testguidelines.

PC property	OECD guideline[5]	Applicability for NMs testing	ISO standards	Other available methods
Water solubility	105	Existing guidance needs to be revised (OECD, 2014g)	-	Solubility of particles of size below 4 mm are considered in CEN (2002)
Zeta potential	None available (OECD, 2014g)	-	Electrophoretic and electro-acoustic methods are available (ISO, 2012c, 2013b, 2102)	-
Composition of nanomaterial	None available (OECD, 2009b)	-	ISO documents are also available (ISO, 2011a, 2011b, 2011c, 2012a, 2012b); One is specific for NMs (ISO, 2011d)	Bulk: Elemental Analysis, ICPMS, EDX, EELS, dynamic-SIMS, 3D – Atom Probe (OECD, 2009b).
Particle size distribution – dry and in relevant media	110	Might be applicable to NMs under certain circumstances (OECD, 2009b)	Many techniques are available from ISO, many of them were recently revised (ISO, 1996, 2004, 2007a, 2007b, 2007c, 2009b, 2010b)	On TEM analyses (ASTM, 2009a, 2009b, 2010)
Basic morphology	None available (OECD, 2009b)	-	SEM and TEM (ISO, 2011a, 2012a, 2012b) Specific guidelines for carbon nanotubes (ISO, 2012a)	-
Crystallite type	-	-	-	Crystalline phase guidance is available from Japanese Industrial Standards (JIS, 2007)

PC property	OECD guideline[5]	Applicability for NMs testing	ISO standards	Other available methods
Crystallite size	-	-	Grain size is determined via SEM and TEM (ISO, 2004, 2012b); a guideline is available for aerosols (ISO, 2009a)	Powder XRD, HR-TEM, Raman spectroscopy (JIS, 2005; OECD, 2009b)
Surface chemistry	None available (OECD, 2014g)	-	Available guidelines for single walled carbon nanotubes are given by ISO (ISO, 2010a, 2011b); plus other guideline on other methods (ISO, 2002, 2006b, 2010c)	Fourier-transform infrared spectroscopy, time-of-flight secondary-ion mass spectrometry, matrix-assisted laser desorption time-of-flight mass spectrometry, auger electron spectroscopy, XPS, electron energy loss spectroscopy, UV-vis, molecular spectroscopy; ICP-MS, ICP-AAS, LC-MS, GC-MS, TGA (OECD, 2014g) Other guidance (Stefaniak et al., 2005)

PC property	OECD guideline[5]	Applicability for NMs testing	ISO standards	Other available methods
Specific surface area (SSA)	None available (OECD, 2014g)	-	BET (ISO, 2010b)	Brunauer Emmett Teller (BET) method and transmission electron microscopy (TEM) methods (ISO, 2003; OECD, 2009b, 2014g)[6]; small-angle X-ray scattering (SAXS) Form metal powders (American Society for Testing and Materials, 2010)
Photocatalytic activity	None available (OECD, 2014g)	-	-	DCFH – fluorescence based analysis for reactive oxygen species(possible nanoparticle interference with the assay). Colorimetric methods (possible nanoparticle interference with the assay). Electron spin resonance. Quartz crystal microbalance. Gas-phase techniques such as gas-chromatography
Hydrolysis	111	Might be applicable to NMs under certain circumstances (OECD 2009a)	-	-

---

[6] ISO methods are not defined specifically for NMs.

PC property	OECD guideline[5]	Applicability for NMs testing	ISO standards	Other available methods
Dustiness	-	-	-	Relevant guideline on airborne dust (not specific to NMs) (ASTM, 1990; CEN, 2006)
Pore density	None available (OECD, 2014g)	-	BET (OECD, 2014g); methods are available from ISO but these are not specific to NMs (ISO, 2005, 2006a, 2007d)	-
Octanol-water partition coefficient, where relevant	107, 117, 123	Non suitable for NMs (exception: fullerene)		Other kinetics shall be considered for NMs; methods are not sufficiently developed to become standardised (OECD, 2014g)
Hydrophobicity	116	Applicable to NMs (OECD, 2009b)	-	Hyrophobicity characterisation method developed by JRC (Desmet et al, 2017)

1.5 Understanding the fate and toxicity of NMs

Exposure of humans and the environment to NMs can occur throughout their life cycle, from their production, manufacture (incorporation into products), use and disposal. The different phases in life cycle may affect NM properties and hence their interactions with environmental and biological media (Gottschalk et al.)

Once humans are exposed to NMs, the particles can travel throughout the body and deposit in target organs, where they may penetrate cell membranes and even enter organelles such as mitochondria. They can therefore affect biology at different levels (Günter Oberdörster, Oberdörster, & Oberdörster, 2005), which may result in injurious responses.

This section is aimed at: 1) introducing well-established theories that have been applied in investigating the fate of NMs in environmental and biological systems; 2) summarising the available information on the kinetics of NMs; and 3) reporting evidence on their toxicodynamics.

1.5.1 Theories underlying environmental and biological fate

The fate of NMs in environmental media and biological systems is partly determined by particle-particle and particle-surface[7] interactions leading to agglomeration/aggregation processes that may result in colloidal instability and gravitational sedimentation. These processes are relevant in both aqueous and gaseous compartments.

Agglomeration and aggregation kinetics in fluid media

The behaviour of NMs in fluids is dependent on particle-specific properties, the chemistry of the surrounding medium (e.g. pH, ionic strength, ionic composition, presence of proteins or colloids) and hydrodynamic conditions (Ilinskaya & Dobrovolskaia, 2013a, 2013b; Petosa, Jaisi, Quevedo, Elimelech, Tufenkji, et al., 2010).

The transport of NMs in fluids follows Brownian diffusion, a random and chaotic motion of particles resulting in collisions between the particles and fluid molecules surrounding them. The trajectory of a given particle is of self-similar nature, meaning that any magnified portion of the trajectory would look qualitatively similar to the original one (Elimelech, Gregory, Jia, & Williams, 1995a).

Depending on the environmental conditions and on intrinsic PC properties, NMs can agglomerate or aggregate in fluid media (see Table 0.1 for definitions). Agglomerates can de-agglomerate in presence of stabilizing agents such as proteins (Rivolo et al., 2012). Changes in particle size resulting from agglomeration or aggregation may influence the interactions of NMs with natural colloids (e.g. humic and fulvic acids) and macromolecules (e.g. proteins, peptides) (Klaine et al., 2008), as well as their reactivity and toxicity. Although the processes of agglomeration and aggregation are relevant in predicting the fate of NMs, there are no theoretical models capable of distinguishing between the two processes. Ongoing research is focusing on identifying which environmental parameters influence particle coalescence (Yanjie Li et al., 2015; Joris T K Quik et al., 2012) and on the parameters that can be used to model and predict the fate of NMs (Cornelis, Pang, Doolette, Kirby, & McLaughlin, 2013; J. T. K. Quik, Velzeboer, Wouterse, Koelmans, & van de Meent, 2014). In these models, aggregate formation is assumed to be irreversible, and aggregation and agglomeration processes are not distinguished (Elimelech, Gregory, Jia, & Williams, 1995c; Pippa, Dokoumetzidis, Demetzos, & Macheras, 2013).

In the following paragraphs the main theories considered in modelling aggregation/agglomeration processes are presented.

DLVO theory

The classical DLVO (Derjaguin, Landau, Verwey and Overbeek) theory of colloidal stability has been proposed to address the kinetics of agglomeration processes. DVLO theory combines the opposing effects of the van der Waals attractive force and the electrostatic repulsive force due to the so called 'double layer' of counterions, i.e. zeta potential for NMs. The repulsive force depends on the double layer potential and thickness, the particle radius

---

[7] A surface is conceptualized by an infinite flat plate and is relevant in studying the deposition of NMs (S. Lin & Wiesner, 2012). In some instances a natural colloid interacting with a NM is assumed as a surface because of the big difference in dimension (J. Meesters et al., 2014).

and the dielectric constant of the medium. The potential energy curve as a function of the separation distance between particles has an energy barrier and two minima, as shown in Figure 1.2.
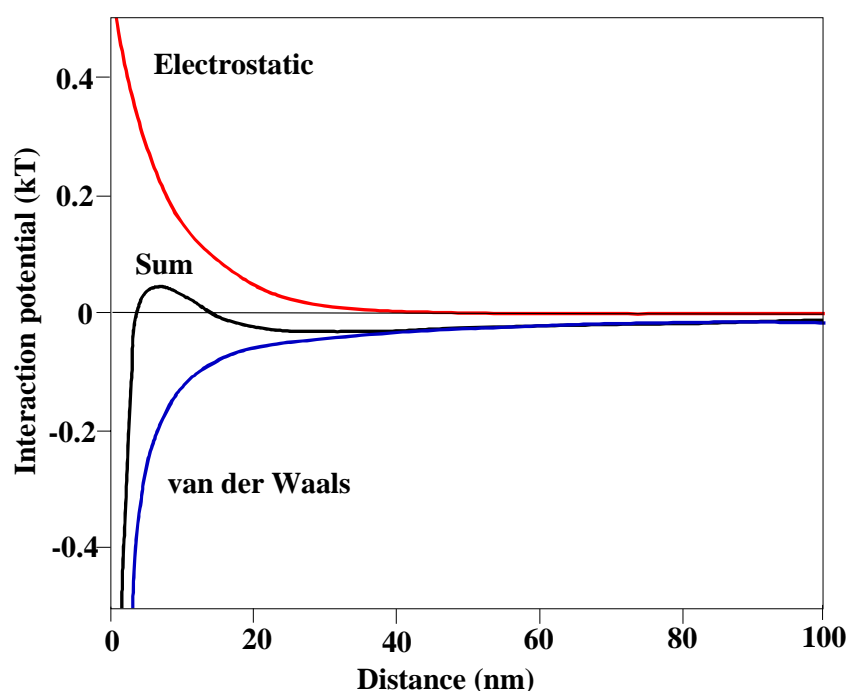
Figure 1.2. Potential energy curve for the interaction between colloidal particles. The interaction depends on a balance between attractive van der Waals forces and repulsive electrostatic forces.

General rules can be used to determine colloidal stability. For example, the thicker the double layer around the particles, the greater the repulsion between them. Repulsive forces between particles decrease when the pH of the medium is close to the isoelectric point (IEP) value of the particle. An increased concentration of an inert electrolyte causes the electrical double layer potential to decrease, and the same effect is observed when an organic, water miscible, solvent, is present, which causes the dielectric constant of the medium to decrease. Repulsive forces between particles can be caused by adsorption of polymers, proteins, non-ionic detergents and polyelectrolytes. The intentional coating of particles with polymers causes steric stabilization, although partial coverage can lead to agglomeration.

Van der Waals forces are expressed by the Hamaker constant, which is related only to the properties of the interacting bodies and the medium and consists of the summation of intermolecular interactions (Hamaker, 1937). In aqueous environments, when particles approach each other (coalescence) or a surface (deposition), the overlap of the diffuse electric double layers results in electrostatic double layer interactions.

Non-DLVO interactions that are relevant for the agglomeration/aggregation and deposition of NMs in an aqueous environment include steric interactions, magnetic forces (for iron-based NMs), and hydration forces (Petosa, Jaisi, Quevedo, Elimelech, Tufenkji, et al., 2010). Expressions describing the extent of steric forces have been derived for particles with adsorbed layers of polymers or surfactants that might lead to steric repulsion. Magnetic materials exhibit dipole behaviour that overcomes the particle-particle interactions. Finally, particles that carry hydrophilic material such as proteins at their surfaces may attract water molecules and hence have a hydrated surface. Such particles will exert a repulsive

interaction thus influencing NMs stability (Healy, Homola, James, & Hunter, 1978). Parameters for calculation of non-DLVO interactions are not available and this is the reason why this theory is not extensively applied (Haiyuan Zhang et al., 2012a).

DLVO is taken into consideration in environmental fate models in absence of measurements for calculating the attachment efficiency α (J. Meesters et al., 2014). According to the DLVO theory, the total interaction energy between suspended particles can be evaluated as the sum of the attractive van der Waals and the repulsive electrical double-layer energies. The aggregation efficiency α is defined as (Equation 2):

$$\alpha \approx k_{Debye} 2r_i e^{\left(-\frac{V_{max(i,j)}}{k_b T}\right)} \tag{2}$$

Where $V_{max(i,j)}$ is the total interaction energy between particles i and j; k_b is the Boltzmann constant; T is the temperature; k_{Debye} is a parameter related to the length of the double layer (Pashley & Karaman, 2005), r_i is the radius of the particle i. Other authors have proposed different calculations of α, for instance by taking into consideration the relevance of both interaction energy and Brownian diffusion in the transport of NMs (Wen Zhang, Crittenden, Li, & Chen, 2012; Wen Zhang, Rittmann, & Chen, 2011). The DLVO theory is also taken into consideration in biological environments in predicting protein binding to NMs (Vilanova, Franzese, & Barnabei, 2014), where it is applied in simulating NM-protein interactions[8].

Smoluchowski-Friedlander theory

Smoluchowski (1917) and Friedlander (1977) calculated the kinetics of spherical dispersed particles in fluids, and this theory is sometimes considered to represent the kinetics involved in aggregation and coagulation processes such as flocculation and Ostwald ripening. Simplified versions of the Smoluchowski equation are considered for instance in the calculation of aggregation constants (Jiang, Oberdörster, & Biswas, 2008; J. T. K. Quik et al., 2014), as a theory at the base of model development (Arvidsson, Molander, Sandén, & Hasellöv, 2011).

This approach considers three types of kinetic process: Brownian diffusion (perikinetic aggregation, due only to particle diffusion, equation 3), fluid motion (orthokinetic aggregation, that happens in presence of flow, equation 4), and differential settling (equation 5) (Elimelech et al., 1995c):

$$k_{ij} = \frac{2kT}{3\mu} \frac{(a_i + a_j)^2}{a_i a_j} \tag{3}$$

$$k_{ij} = \frac{4}{3} G(a_i + a_j)^3 \tag{4}$$

$$k_{ij} = \left(\frac{2\pi g}{9\mu}\right)(\rho_s - \rho)(a_i - a_j)(a_i + a_j)^3 \tag{5}$$

Where k_{ij} are the different collision rate constants of particles (or aggregates) i and j, k is the Boltzmann constant, T is the absolute temperature, a_i, a_j are the particle radii, μ is the viscosity of the fluid, G is the shear rate, g is the acceleration due to gravity, ρ is the density of the fluid and ρ_s is the density of the particle. The diffusion coefficient of a spherical particle undergoing Brownian motion is expressed by the Einstein Stokes equation (Equation 6), from which Smoluchowski derived the definition for the perikinetic rate constant (Equation 3):

$$D_i = \frac{kT}{6\pi a_i \mu} \tag{6}$$

Assuming each collision is effective in forming an aggregate, it is possible to write the rate of change of concentration of aggregates as equation 7:

$$\frac{dn_k}{dt} = \frac{1}{2} \cdot \sum_{\substack{i=1 \\ i+j=k}}^{i=k-1} k_{i,j} n_i n_j - n_k \sum_{k=1}^{k=\infty} k_{i,k} n_i \tag{7}$$

Where the first term on the right-hand side represents the rate of formation of k-fold[9] aggregates by collision of any pair of aggregates, i and j, such that i+j=k. The second term accounts for the loss of k-fold aggregates by aggregation with any other particles or aggregates. The terms k_{ij} and k_{ik} are the appropriate rate constants (equations 3 to 5), n_i and n_j are number concentrations of different aggregates - n_i particles of size i, n_j particles of size j.

These equations have been combined in several applications aimed at studying the behaviour of NMs in environmental media (Arvidsson, Molander, Sandén, et al., 2011; Buffle, Wilkinson, Stoll, Filella, & Zhang, 1998; Petosa, Jaisi, Quevedo, Elimelech, Tufenkji, et al., 2010; J. T. K. Quik et al., 2014) and in human exposure (EC, 2012b). For instance, Arvidsson et al. apply Equation 7 in defining an environmental fate kinetic model where the number of particles of k-fold aggregates varies in time as a function of aggregation efficiencies[10] $\alpha_{i,j}$ (see definition in Table 1.3), adding a term for sedimentation and one term for advection in the system. In the field of human exposure this equation was applied in the ENPRA EU FP7 project for the estimation of coagulation kinetics of particles in the aerosol, where particles of any size can coagulate to form aggregates (EC, 2012b).

Considering the low environmental concentrations of NMs and the corresponding lower probability of interaction of two NMs in real fluids, scientists in the field identified heteroagglomeration/aggregation rather than homoagglomeration as a relevant process to

---

[9] Equations are defined assuming an initial dispersion of identical particles that after a period during which aggregation occurs, contains aggregates of various sizes and different concentrations: n_i particles of size i, etc. where n_i refers to the number concentrations of different aggregates and 'size' implies the number of primary particles comprising the aggregate; we can speak of 'i-fold' and 'j-fold' aggregates (Elimelech et al., 1995c)

[10] Arvidsson et al. (2011) refers to $\alpha_{i,j}$ as *collision efficiency*; since it is defined as irreversible collision, it corresponds to aggregation efficieny (Elimelech et al., 1995c)

be considered when addressing the environmental fate and transport of NMs (J. Meesters et al., 2014), but the inherent challenges in the prediction are still high as there is no clear measurement technique nor recognised protocol for the measurement of attachment efficiencies that is so far only possible though the application of semi-empirical models (Wen Zhang et al., 2012).

Following these approaches, and in order to express the kinetics of interactions of different nature, different authors defined homo- and hetero- attachment efficiencies α_{homo} and α_{hetero} (definitions in Table 1.4) (Arvidsson, Molander, Sandén, et al., 2011; J. Meesters et al., 2014; Antonia Praetorius, Scheringer, & Hungerbühler, 2012).

Table 1.4. Relevant parameters taken into account by multimedia modelling (MMM) approaches. The table reports definitions of the identified parameters that are used as input values in MMMs.

Parameter relevant for environmental fate models	Definition
Collision (or attachment, or aggregation) efficiency, also referred to as 'sticking probability' or 'stickiness coefficient', α	It is defined as the fraction of collisions that are effective in agglomeration in Elimelech et al. (Elimelech et al., 1995c). It is recalled by Praetorius et al. (Antonia Praetorius, Scheringer, & Hungerbühler, 2012) and by Quik et al. (J. T. K. Quik et al., 2014) as attachment efficiency. If there is strong repulsion between particles then practically no collision gives an aggregate and α=0. When there is no significant net repulsion or when there is an attraction between particles, then the collision efficiency can approach unity. Zhang et al. (Wen Zhang et al., 2012) reports that aggregation kinetics of various ENMs have been extensively studied using attachment efficiency (α), commonly determined by normalizing the hydrodynamic size growth rate in initial aggregation curves to the growth rate under the favourable (or fast) aggregation condition in which the ionic strength is equal to or greater than the critical coagulation concentration.
Homo- and Hetero- agglomeration / aggregation rate constants (also defined as coagulation or flocculation rate constants according to Elimelech et al. 1995b).	It is the rate constant governing agglomeration/aggregation processes in kinetic models (Arvidsson, Molander, Sandén, et al., 2011). The homo-agglomeration rate constant is referred to as the interactions between NMs of the same type and is defined as $k_{hom\text{-}agg}=\alpha_{hom}\,k_{colli,j}$ where α_{hom} is attachment efficiency for homo-agglomeration and $k_{colli,j}$ is the collision rate constant. Hetero-agglomeration/aggregation is the interaction of natural colloids or of suspended particulate matter with ENMs. Praetorius et al. (Antonia Praetorius, Scheringer, & Hungerbühler, 2012) defines it as $k_{het\text{-}agg}=\alpha_{het}\cdot k_{colli,j}$ where α_{het} is the attachment (or collision) efficiency for hetero-agglomeration.
Collision rate constant for particles of type i and j	It depends on factors such as particle size and transport mechanism. It is usual to assume that the collision rate is independent of colloid interactions and depends only on particle transport (see equations 3-5).

Brownian motion applies also in the gas medium and diffusion is considered the main mechanism for collision of NM particulates in aerosols. Reflecting this, some authors have defined the polydisperse coagulation coefficient as a function of particle size and diffusivity in air (J. Meesters et al., 2014). Polydisperse particles are particles of different sizes (i.e. particles and their agglomerates or aggregates).

Agglomeration kinetics are also taken into account in the kinetics of NMs in *in vitro* systems, where the process is considered to be affected by NM concentration, surface chemistry and zeta potential (Teeguarden, Hinderliter, Orr, Thrall, & Pounds, 2007), where the concepts of gravitational settling and diffusion are also taken into account.

Fractal approaches

The Einstein Stokes equation (6) is also applied in the analytical technique of Dynamic Light Scattering (DLS) assuming well-dispersed primary spherical particles (Landsiedel et al., 2010). When agglomerates or aggregates are formed, their loose packing can be accounted for by considering the aggregate/agglomerate fractal dimension (Wendel Wohlleben, 2012).

The term 'fractal' comes from the Latin adjective *fractus*, that means 'to break', intended as creating irregular fragments (Mandelbrot, 1982). Fractal geometry is an extension of conventional Euclidean geometry that allows size-dependent measures to change in a non-integer or fractional way when the scale changes. This characteristic can be described by assigning a fractional number – a fractal dimension – to the dimension of the object. The relationship between the mass M of an agglomerate/aggregate and its size L can be expressed in terms of a fractal dimension:

$$M \sim L^{d_f} \tag{8}$$

Where M is the mass of the particles L is a linear measure of size[11] and d_f is the mass fractal dimension (Elimelech et al., 1995c). Aggregates and agglomerates are considered fractal objects with fractal dimension $d_f < 3$. When equation 8 is valid at different scales, then aggregates have a self-similar structure, independent of the scale of observation.

Fractal approaches have been applied so far in a series of studies that have demonstrated that scale invariance is a common characteristic of biological systems, ranging from tissues to cultured cells, nucleus and chromatin (Metze, 2013; Moreno et al., 2011). In colloidal chemistry, fractal distribution is assumed in diffusion-limited agglomeration leading to fractal morphology characterised by a $d_f \approx 2.1$ (M. Y. Lin et al., 1990). Being fractal, the structure of the colloidal aggregates can be studied in more detail in terms of the relationship of the cluster structure and its aggregation kinetics.

Fractal dimension d_f is mentioned in the ECHA guidance on information requirements and chemical safety assessment regulatory requirements (Appendix R7-1) in relation to the definition of NM shape (ECHA, 2012b). It is also considered as a structural descriptor in QSAR applications (T. Le, Epa, Burden, & Winkler, 2012) as well as environmental fate models (Arvidsson, Molander, Sandén, et al., 2011; Haoyang Haven Liu & Cohen, 2014b; Antonia

---

[11] L is a measure of length of a particle or aggregate/agglomerate. It may be the gyration radius of the aggregate or the largest diameter of an irregular aggregate.

Praetorius, Scheringer, & Hungerbühler, 2012) where it is used as a rough estimate of the density of the aggregates .

1.5.2 Human kinetics

Pre-absorption processes

Human biokinetics/toxicokinetics/pharmacokinetics (all synonymous but from now on named toxicokinetics or TK) is the science that studies the concentration-time course of a chemical substance in blood, tissues and excreta. It is dependent on the rates and extents of absorption, distribution, metabolism and excretion (ADME). Distribution, metabolism and excretion together are sometimes called 'disposition' where metabolism and excretion may be lumped to the term 'elimination'.

TK for classical substances starts with absorption. Absorption is usually defined as the passage across an outer lining (membrane) such as the gastrointestinal (GI) tract epithelium, the skin or the epithelium covering the airways. For soluble chemicals, the rate of absorption is not considered to be limited by the dissolution process. Thus absorption is considered to occur directly from the mucus layer (produced by mucous membranes) in which the substance is dissolved, from the GI fluid, or from a very thin aqueous layer covering the skin.

For NMs, the situation may be different. Some NMs are relatively soluble and once dissolved, the resulting ions behave like the soluble chemicals. Other NMs are poorly soluble but may still undergo various kinds of transformation (e.g. change in speciation and/or attachment of organic molecules (Schultz et al., 2014) h taken from a paper on aquatic and terrestrial organisms in the environment (C. Schultz et al., 2014). This may occur in humans following airborne exposure that results in deposition in the aqueous lung lining phase, oral exposure that might result in release from the food matrix in the aqueous contents of the GI tract or dermal exposure that might result in migration a consumer product or direct deposition (following airborne exposure or skin contact with contaminated surfaces in occupational settings) towards the thin aqueous layer on the epidermis.

The above implies that 'pre-absorption' processes can be influential on the systemic bioavailability of NMs (the amount that becomes available in the systemic circulation relative to the total exposure). The low or absence of solubility implies that there is 'time' for other processes: to occur; for example, local clearance (i.e. removal) may occur at the same time and leave less NM for absorption. In other words, unlike soluble chemicals, NMs can be cleared to a varying extent already before absorption.

Inhalation exposure. Considering the inhalation pathway, it is recognised that particles smaller than 10 μm can enter the lung. Particle deposition in the lung depends on particle size, density, and hygroscopicity (ability of a substance to attract and hold water molecules from the surrounding environment), and is influenced by the local anatomy and airflow as reviewed recently by Braakhuis et al. (Braakhuis, Park, Gosens, De Jong, & Cassee, 2014). They report that NMs with diameters in the range 10-100 nm enter preferentially the alveolar areas. For particles in the mentioned diameter range, the deposition of NMs is mainly governed by diffusion of the NMs in the inhaled air (Brownian motion) and the density is less relevant; whereas for particles (or agglomerates) larger than 100 nm diffusion is less likely but the density more and more is determining the deposition. Probably, shape

and the extent of absorption contribute as well. Once deposited in the lung, (partially) soluble NMs dissolve (partially) in the lining fluid (mucus layer) of the epithelium where inert NMs might form non-dissolved but colloidal suspensions. Local clearance from the airways occurs as macrophages transport non-dissolved NMs (single and agglomerated but still relatively small NMs) by mucociliary transport up to the laryngopharynx (W. Yang, Peters, & Williams, 2008). This can be followed by swallowing after which absorption in the GIT may occur via the so-called 'mucociliary escalator'. This is the transport by macrophages from the lower lung compartments 'upwards' to the bronchioles where it can be followed by coughing and swallowing. As an example, for agglomerates of 7 and 20 nm gold nanoparticles, macrophage mediated escalation followed by fecal excretion is the major pathway of clearing the inhaled NPs from the lungs in rat (Balasubramanian et al., 2013).

Local clearance may also result from the solubility of the NM, as suggested by Konduru et al. (2014) in a study on the biokinetics and effects of barium sulfate nanoparticles following inhalation and instillation in rats. Pulmonary exposure to instilled BaSO4 NPs caused dose-dependent lung injury and inflammation. Four-week and 13-week inhalation exposures to a high concentration (50 mg/m3) of BaSO4 NPs elicited minimal pulmonary response and no systemic effects. Instilled and inhaled BaSO4 NPs were cleared quickly yet resulted in higher tissue retention than when ingested. Injected BaSO4 NPs localized in the reticuloendothelial organs and redistributed to the bone over time. BaSO4 NP exhibited lower toxicity and biopersistence in the lungs compared to other poorly soluble NPs such as CeO2 and TiO2. Based on these observatiosn, particle dissolution was regarded as a likely mechanism.

Oral exposure. NMs can reach the GI tract via intake of food or water, or accidental ingestion, or following inhalation and clearance by the mucociliary escalator. Once ingested, NMs face different environments while passing through the GI tract. An important factor is the pH value as it affects the net surface charge of the NM (Burello, 2013). In the stomach, it ranges from 1.5-2.0 in the fasting state and might rise up to 7.0 after ingestion of a meal. Upon increasing motility of the stomach, the contents are transported to the small intestine, where pancreatic bicarbonate secretion leads to neutralisation of the chyme to a pH ranging from 6.4 to 7.5, depending on the intestinal section. The surface area of the small intestine is amplified immensely towards the more distal part, due to the presence of crypts (the crypts of Lieberkühn) and projections (microvilli), thereby facilitating nutrient absorption (altogether, the intestine features a surface area of 200-300 m^2). In addition to the wide pH range a NM has to face while passing the GI tract, a series of proteins is secreted, such as mucins, lactoferrin, albumin and other factors which are capable of mineral chelation. These proteins have the potential to influence the absorption of a NM by affecting corona formation, solubility, agglomeration and aggregation.

Dermal exposure. Personal care products such as sun screens may contain nano-TiO$_2$ or nano-ZnO, due to their efficiency in filtering UV light and their transparency (Nohynek & Dufour, 2012).

Absorption
NMs may enter the body via different routes, including inhalation, ingestion and penetration of the skin).

Upon inhalation exposure. Soluble NMs that dissolve in the lining fluid of the lung epithelium can be transferred to the blood and distributed to the whole body (Günter

Oberdörster et al., 2005). Solubility (rate and extent of dissolution) depends on chemical composition, size, coating, stability and on the biological environment (Braakhuis et al., 2014). In the respiratory tract, less soluble NMs may be absorbed via cell-mediated active translocation from the site of deposition through the lung epithelium to interstitial sites. From there NMs may be directed to the local lymph nodes and as lymph nodes are drained by blood they may ultimately reach the systemic blood circulation. Uptake from the site of deposition into systemic blood may also happen directly by crossing the lung barrier in the alveoli (Borm et al., 2006). Once less soluble NMs are deposited in the alveoli, there is a competition between alveolar macrophages and lung endocytes for cellular uptake. Cellular uptake by alveolar macrophages may result in local clearance via the mucociliary transport before absorption to the systemic circulation can occur (as mentioned above). Once taken up by endocytes, cell-mediated transport across the epithelial membrane results in absorption.

Upon oral exposure. The level of NM uptake within the GI tract appears to be relatively low, albeit dependent on properties such as size, surface structure, chemical composition and charge (Landsiedel et al., 2012). Various uptake routes are relevant for various size distributions: endocytosis through 'regular' epithelial cells, uptake by M-cells (phagocytising enterocytes in the Peyer's Patches) and possibly also persorption (via gaps at the villous tip when enterocytes are lost to the gut lumen) and paracellular uptake (Powell, Faria, Thomas-McKay, & Pele, 2010; Stern & McNeil, 2008). Interspecies differences occur, for example because there are more M-cells in rodents compared to humans (Hagens, Oomen, de Jong, Cassee, & Sips, 2007).

Upon dermal exposure. The number of studies on skin penetration of NMs is limited. When Zn was applied topically as labelled ZnO-nano (Osmond-McLeod et al., 2014), most of healthy and intact skin surface area seemed impermeable to the NM. Only the hair follicles and the openings of the sweat glands be available for some particle penetration. Also, the effect of damaged skin needs further investigation (EASAC & JRC, 2011). Furthermore, most studies seem to be performed under static conditions where flexing of the skin during exposure might enhance penetration (Günter Oberdörster et al., 2005).

The SCCS has evaluated the dermal penetration potential of several NM ingredients in cosmetics, including TiO2 (SCCS/1516/13 and SCCS/1539/14), ZnO (SCCS/1489/12 and SCCS/1518/13) and Carbon Black (SCCS/1515/13). In its Opinion on TiO2 (nano form)" the SCCS concluded: "*From the limited relevant information provided in the submission, and the information from open literature, the SCCS considers that TiO2 nanomaterials in a sunscreen formulation are unlikely to lead to: systemic exposure to nanoparticles through human skin to reach viable cells of the epidermis, dermis, or other organs.*" (SCCS/1539/14). However, the SCCS also state that: "*Although there is no conclusive evidence at present to indicate penetration of TiO2 nanoparticles through the skin to viable cells of the epidermis, a number of studies have shown that they can penetrate into the outer layers of the stratum corneum, and can also enter hair follicles and sweat glands.*" For ZnO NPs the SCCS stated that there is no indication for dermal penetration of ZnO NPs based on the available scientific literature. However, a very small proportion of Zn ions that are released from the ZnO NPs may be available for systemic exposure after dermal application (SCCS/1489/12). The SCCS also found an "overall lack of dermal absorption of carbon black nanoparticles" (SCCS/1515/13).

A study funded by the Danish EPA used *in vitro* (EpiDerm™) and *in vivo* (mouse, human skin graft) models to investigate whether the size or surface coating of TiO2 NPs and ZnO NPs has

an effect on the dermal penetration/absorption of the nanoparticles (DK EPA, 2015). Based on their results, it was concluded that "dermal penetration of TiO2 and ZnO NPs did not occur at or above the limit of detection of the used experimental methods. Should absorption of TiO2 and ZnO nanoparticles occur at levels below the detection limit of the assays used herein, the systemic dose would be very small (far lower than the doses used in the studies discussed above) and so highly unlikely to cause systemic toxicity based on toxicological evidence in rodents."

Distribution

Once systemically available (following absorption), poorly soluble NMs typically distribute via the systemic circulation or within phagocytising cells via the lymph drainage to various parts of the body. Distribution via blood is followed by rapid uptake by organs and tissues containing significant phagocytising capacity. Thus distribution is mainly to the liver and spleen although distribution to heart, kidneys and immune-modulating organs has been reported as well (Landsiedel et al., 2012; Günter Oberdörster et al., 2005; van Kesteren et al., 2014). General characteristics such as rate of dissolution, surface treatment, chemical composition, shape, agglomeration, aggregation all may have an impact on distribution (where and how much). In whatever tissue (including blood), NMs can bind with different affinities to biological targets such as peptides, proteins, lipids and DNA to form a corona (Iseult Lynch, Feitshans, & Kendall, 2015). NMs are reported to be able to distribute to the central nervous system via sensory neurons in the nose (Oberdörster et al., 2005). A recent paper argues that nanomaterials have the right size and shape for interacting with transport proteins such as apolipoproteins, offering them access to all cells via the low-density lipoprotein receptor (Iseult Lynch et al., 2015).

Metabolism / Dissolution / Transformation / Bio-Nano interaction

Metabolism, defined as the enzyme-mediated conversion from one chemical species to another (a metabolite), is probably not relevant for NMs, not least because they are generally too large to fit into the active site of the well-known biotransformation enzymes. However, some abiotic oxidation may occur at the outer surface of NMs. Two other transformation processes are more relevant for NMs: dissolution into ions, and corona formation through bio-nano interactions (Iseult Lynch et al., 2015). These processes will depend on the physicochemical characteristics of the NM as well as those of the environment (e.g. pH, the presence of salts and proteins).

Once the NM is exposed to a protein rich environment, a protein corona is formed. This is the biomolecule coating that forms around nanoparticles upon contact with biological molecules. It is formed because of the high surface reactivity of the NMs resulting in adsorption of various molecules (mainly proteins). Desorption also occurs at different time scales depending on the binding energy of the nanoparticle-protein interaction. Characteristic binding forces are van der Waals interactions, hydrogen bonds, hydrophobic interactions, electrostatic interactions and π - π stacking (Wolfram et al., 2014). There is evidence that the corona is stratified into two layers, one of which is 'soft' (loosely associated macromolecules, characterised by short exchange times) and the other 'hard' (tightly bound, characterised by long exchange times) (Monopoli et al., 2011; Monopoli, Aberg, Salvati, & Dawson, 2012).

The corona is continuously exchanging with the proteins in the environment and its composition varies in time and depending on the environmental conditions (Cedervall et al., 2007). The corona is stable for a longer time than the typical time scale of cellular uptake, thus acting as cell 'mediator' in the interaction of the NM with cell receptors (F. Wang et al., 2013). Wang also found that after entering the cell, the nanoparticle and the corona reach the lysosomes where peptides are degraded by lysosomal enzymes. Subsequently, membrane damage may occur following release of the lysosomal content into the cytosol and subsequent cell death due to apoptosis.

Qualitatively, the composition of the protein corona on a given NM, at a given time, depends on the concentration and type of the proteins in the surrounding medium (e.g. plasma) and their affinity for the NM surface. It is generally assumed that proteins with high concentrations in plasma and high association rates will initially cover the surface of the NMs. Over time, however, these proteins will dissociate and will be replaced by proteins of lower concentration, slower exchange rates but higher affinities (Vroman & Adams, 1969). Therefore, as NMs distribute from the blood to various locations or between different cell compartments, the evolution of the protein corona will change the surface properties of the NMs, changing also their toxicological profiles (Burello, 2013; Iseult Lynch et al., 2015).

The plasma membrane is a highly selective and effective barrier that limits the entry and exit of large macromolecular substances and materials. Neverthless, NMs are capable of enntering cells, either through one of the several endocytic pathways, or by passive penetration of the plasma membrane. Folloing endocytosis, NMS are enclosed within the early endocytic vesicles and are thus not directly carried into the cytosol. In contrast, the nanomaterials internalized via membrane penetration enter the cytoplasm directly. The term 'endocytosis' can be broadly divided into pinocytosis ("cell drinking") and phagocytosis ("cell eating"). Pinocytosis is commonly involved in the internalization of fluids and molecules by small vesicles, whereas phagocytosis is the process by which the cells such as monocytes/macrophages, neutrophils and dendritic cells engulf large particulate matter and are form intracellular phagosomes. The mechanisms of cellular uptake by endocytosis have been reviewed elsewhere (Salatin and Khosroushahi, 2017).

Excretion

Excretion via the usual routes (biliary, urinary, via mammal glands, via saliva) is generally unknown. The few studies available, however, suggest that excretion is very slow. The longer the half-life, the longer (and more expensive) the study needed for a reliable assessment of the rate of excretion. This is however highly relevant information, since the longer the half-life, the higher the risk for bioaccumulation (Savolainen et al., 2013).

Generally, the largest proportion of ingested NMs in food appears to be excreted directly via the feces, the excretion rate is usually above 90% (Hillery, Jan, & Florence, 1994; Landsiedel et al., 2012; van Kesteren et al., 2014). Only upon intravenous administration, limited biliary secretion into the faeces was found for polystyrene NMs (Landsiedel et al., 2012).

For very small quantum dots (up to approximately 20 nm which is dependent on the coating) as well as for carbon nanotubes, urinary excretion seems relevant (Landsiedel et al., 2012).

Elimination (sum of solubilisation and excretion)

Once absorbed and phagocytised, insoluble NMs are eliminated (cleared) from the body to a minor extent. For many NMs, the whole-body elimination half-lives are significantly longer than the absorption half-lives, which means that the rate of appearance in the body is larger than the rate of disappearance. Persistency is an early indication of accumulation upon repeated exposure. As far as the authors are aware, however, no studies have been reported that investigate the time-course of tissue concentrations of NMs upon repeated exposure. What has been investigated is the time-course after a single exposure or after the last exposure in recovery studies. This has been recognised as a data gap and need for further investigation in the EU (Savolainen et al., 2013). The results of a recent preliminary risk assessment based on computer modelling of human intake and clearance of nano silica showed that the concentration of Si in the liver is expected to keep on increasing for about 2 years (more than 500 days) before a steady-state is reached between uptake and elimination (van Kesteren et al., 2014).

1.5.3 Toxicodynamics

Once a NM reaches an organ or a cell following exposure, it may exert different toxicological effects (Alarifi et al., 2013; E.-J. Park et al., 2008; Vandebriel & De Jong, 2012).

NMs have been implicated in a range of cellular interactions that may result in cytotoxicity and other cellular responses (Lai, 2012). These include: 1) interaction with the plasma membrane that physically disrupts membrane processes (ion transport, signal transduction) and may lead to cell death; 2) biochemical interaction with membrane receptors, leading to activation of signal transduction pathways; 3) uptake into the cell via endocytosis with subsequent effects on intracellular macromolecules and organelles; 4) interaction with mitochondria leading to alterations in metabolism, ROS production and interference with the anti-oxidant defence (E.-J. Park et al., 2008; X.-R. Xia, Monteiro-Riviere, & Riviere, 2010); 5) DNA binding and damage, leading to the arrest of cell cycle division and protein synthesis; 6) interaction with the cytoskeleton, halting vesicular trafficking and causing mechanical instability and cell death; and 7) interaction with proteins, lipids, and other biomolecules leading to different types of corona and biological effects. Depending on the nanomaterial, oxidative stress results in inflammation, DNA adduct formation and apoptosis (Cheng, Jiang, Wang, Chen, & Liu, 2013; Ken Donaldson & Poland, 2012).

The reticuloendothelial system (RES) primarily consists of monocytes and macrophages which are accumulated in lymph nodes, spleen and liver. Once NMs reach the liver, they may induce systemic DNA damage and mutagenesis because of uptake and subsequent induction of systemic inflammation. Macrophages in the RES of the liver and the spleen are known to take up particles bound with serum proteins, which can trigger autoimmune responses (A. Nel, Xia, Mädler, & Li, 2006). Once NMs reach liver and spleen, they have the potential to induce acute toxicity associated with histological changes in terms of focal infiltration with phagocytic and inflammatory cells, loss of neurons, microgliosis, astrogliosis and apoptosis (Knudsen et al., 2014).

Once taken up by macrophages NMs can interfere with their physiological function. Autophagy is an active uptake mechanism during which a portion of the cytoplasm or old and damaged organelles is engulfed by double or multi membrane structures (autophagosomes). These organelles later fuse with lysosomes, so that their contents can be

digested by lysosomal enzymes. Autophagy can be activated by cells as a pro-survival mechanism in response to cellular stress, and to sustain energy production via alternative routes, but it can also be involved in a cell death program, depending on the stimuli and the cell type (F. Wang et al., 2013). This protective mechanism could be disturbed by NM uptake and may lead to the enhancement of NM-induced toxicity. Moreover, the NM may directly interact with the lysosomal membrane of the macrophage, inducing oxidative stress, cell death and inflammation (Ho, Wu, Chein, Chen, & Cheng, 2011; W G Kreyling, 1992; Wolfgang Koch, 2008).

As a consequence of inflammation, the NLRP-3 inflammasome is activated, a process that has been described for both crystalline and amorphous SiO_2, but also for TiO_2 (Hornung et al., 2008; Tschopp & Schroder, 2010; Winter et al., 2011). Particles of low solubility and toxicity (LSLTP) may cause inflammation in proportion to their specific surface area and their zeta potential (Duffin, Tran, Brown, Stone, & Donaldson, 2007; Tran, Buchanan, Cullen, Searl, & Jones, 2000), and their adverse effects have been demonstrated to be associated with their intrinsic inflammatory potency (Bakand, Hayes, & Dechsakulthorn, 2012; Günter Oberdörster et al., 2005). As it has been shown that these LSLTP induce rat lung overload at a certain threshold level, Donaldson and co-workers considered dosimetry, rather than particle number or size, as the driving factor for inflammatory responses *in vivo* (Donaldson et al., 2008). In fact, the determined threshold value for TiO_2 and $BaSO_4$ was found to be $1 \text{ cm}^2/\text{cm}^2$ of the proximal alveolar region (PAR, site of high particle retention in the lung), as calculated using previously published results (Tran et al., 2000), below which the inflammatory response would not be triggered. This was found true not only for nanoparticles, but also for their micron-sized counterparts. Equal results could be found in *in vitro* studies using A549 lung epithelial type II cells and IL-8 as the relevant readout for pro-inflammatory effects (K Donaldson et al., 2008). In a later publication, Donaldson and Poland underpinned the comparability of mechanisms of toxicity for nano- and micron-sized particles and state that the dose is the main trigger for adverse effects (Donaldson and Poland, 2013). Taking into account above considerations, surface area was recommended as the most appropriate metric in particle toxicity testing.

Inhaled particles are known to play an important role not only in fibrosis or cancer development, but also in chronic obstructive pulmonary disease (COPD) and asthma (Borm et al., 2006; Geiser et al., 2014). An important player in the relationship between inflammation and carcinogenesis is the formation of ROS during inflammatory phagocyte respiratory burst. Also macrophages are known to play a significant role in inflammatory processes induced by NMs in the lungs: they are a key player in the uptake and removal of inhaled NMs (Ken Donaldson et al., 2013). Vandebriel and De Jong (2012) report that the toxic effect of ZnO NMs is due to its solubility resulting in increased intracellular concentration of Zn^{2+}. A correlation exists between particle size, surface charge and pulmonary inflammation, as indicated by neutrophil influx and the induction of proinflammatory mediators: the smaller the particles, the greater the inflammatory response(Ken Donaldson et al., 2013; Mura et al., 2011).

Chronic inflammation in the liver may occur when biopersistent NMs cannot be removed, and inflammatory cells are continuously recruited. *In vivo* experiments with injected (intraperitoneal or intravenously) TiO_2 have reported increases in general markers for liver damage (such as ALT or AST) (Duan et al., 2010; Huiting Liu et al., 2009), inflammation (increase in pro-inflammatory cytokines and/or infiltration of inflammatory cells) (Cui et al.,

2011; Ma et al., 2009), oxidative stress (Huiting Liu et al., 2010; Soliman, Attia, Hussein, Mohamed, & Ismail, 2013), apoptosis , necrosis as well as fibrosis (Alarifi et al., 2013; J. Chen, Dong, Zhao, & Tang, 2009). Upon treatment via oral gavage, similar findings were reported: apoptosis, necrosis or inflammation (Cui et al., 2011), as well as general liver damage after treatment with higher concentrations of TiO_2 (starting from 125 mg/kg BW, as detected via above-mentioned markers) (Duan et al., 2010) were found usually above subacute exposure (60 and 30 days, respectively). Also, specific DNA damage has been reported after oral gavage (Sycheva et al., 2011) and administration via the drinking water (Trouiller, Reliene, Westbrook, Solaimani, & Schiestl, 2009), respectively. It must be noted however that the reported effects were only found at the highest concentration groups (200 mg/kg BW and 500 mg/kg BW, respectively). ZnO and SiO_2 NMs are also reported to induce liver damage and inflammation. Vandebriel and De Jong (2012) report that intravenous instillation and oral administration of ZnO nanoparticles result in accumulation to the liver (but also to the spleen, lungs and kidney); oral exposure of nanoparticulate SiO_2 also leads to accumulation to the liver (van der Zande et al., 2014). Intravenous administration of SiO_2 nanoparticles cause DNA damage in the liver (Downs et al., 2012). When lower doses induce an adverse effect, such as influx of inflammatory cells, a recovery to normal levels is sometimes reported (Ali Kermanizadeh, 2013).

In the blood, NMs can mechanically obstruct the vasculature which leads to congestion in multiple vital organs and subsequent organ failure. For example, there are reports of nanoparticle-induced coagulopathies (i.e. coagulation disorders caused by perturbation of the blood coagulation system), dependent on the size and charge of the NM, which determines if a NM has pro- or anti-coagulant properties. A consequence can be disseminated intravascular coagulation (DIC), which can be either acute or chronic and, if untreated, may lead to multiple organ failure and death (Ilinskaya & Dobrovolskaia, 2013a, 2013b).

Moreover, NMs such as TiO_2 have been found to cause an imbalance in haemostasis and thus a disturbance of the immune system. For example, *in vivo* studies in mice reported decreased proliferation of T and B lymphocytes as well as natural killer cells has been (Duan et al., 2010); exposure was occurring through intragastric administration. A disturbance of the cell cycle can result from interactions of TiO_2 with proteins, as reported in *in vitro* studies on human lung epithelial cells (Prasad et al., 2013).

Thus, NMs can cause adverse effects through their interactions with cells in target organs and with proteins in the blood and tissue fluids. An increasing number of studies are investigating these effects and their relationships with the PC properties of NMs.

1.6   Standard Test Guideline methods for toxicity testing

The OECD provides test guidelines (TGs) which contain of internationally harmonised test methods for Mutual Acceptance of Data, used by government, industry and independent and certified laboratories. Originally, they were developed to determine the safety of chemicals and chemical preparations. Their use for safety assessment of NMs has been evaluated and published in the OECD's 'Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials' (OECD, 2009a). A number of toxicity endpoints are listed as well as the respective test guidelines and their possible implementation for nanotoxicity testing.

More recently, results from expert workshop recommendations have been published, such as on genotoxicity (OECD, 2014d) or inhalation toxicity testing (OECD, 2011). Inhalation toxicity testing has been further discussed by an expert group (OECD, 2012b). This group has considered issues such as the NM size range and the analysis of the broncho alveolar lavage (BAL) fluids. The links between the toxicity test results and these parameters are considered essential, as is knowledge on toxicokinetics, to determine for example lung burden and biokinetics. The TG 417 on toxicokinetics was discussed for its applicability for NM toxicity testing, as discussed at the OECD expert meeting on the Toxicokinetics of Manufactured Nanomaterials in 2014 where challenges for its application to NMs toxicological assessment were identified (OECD, 2016d). A guidance document on a tiered testing approach for preliminary assessment of NMs hazard using *in vitro* methods is foreseen to be available in 2017. Table 1.5 lists the discussed endpoints, the respective OECD test guideline(s) and their current applicability status.

Furthermore, the OECD has published a living document on NM sample preparation and dosimetry, with the latest update being made available in 2012 (OECD, 2012a). The issue on the right dosimetry is discussed equally to previous publications by Donaldson and colleagues (K Donaldson et al., 2008; Ken Donaldson & Poland, 2013), stating that *the mass metric appears not always to be the most appropriate or relevant one. Indeed for some nanomaterials the results may be better expressed as a function of surface area or particle number because particle size and specific area may play a main role in determining the toxicity of nanomaterials*. However, it is also acknowledged that this is -currently- often still a challenging task. Other issues raised in this document are on storage and stability of NM or the appropriate test media, making the point that it is key to know and report the exact composition of the prepared sample. Furthermore, the document points out the fundamental importance of proper PC characterisation of the materials and lists the relevant properties.

Table 1.5. Endpoints, the discussed method, the respective OECD test guideline(s) and their current applicability status. This status is under regular review. *In vitro* test methods are in bold.

Endpoint	Method	OECD Test Guideline	Applicability for nanotoxicity testing
Acute toxicity	oral exposure	420, 423, 425	Appropriate for initial investigation (OECD, 2009a)
Repeated dose toxicity (28 and 90 days) in rodents	oral exposure, to detect neurotoxic and immunotoxic effects, effects on the reproductive and endocrine system	407, 409	Applicable; enhancement of methods to detect NM-specific effects (e.g. cardiovascular effects) needs to be considered (OECD, 2009a)
	inhalation	412, 413	Not specifically intended for the testing of NMs (OECD, 2009a)
Skin and Eye irritation	*in vivo* application	404, 405	Applicable (OECD, 2009a)
Skin corrosion	**Transcutaneous Electrical Resistance Test (TER), Reconstructed Human Epidermis (Rhe) Test Method, *In vitro* Membrane Barrier Test Method**	430, 431, 435	Applicable; however some assays (e.g. MTT assay or other assays using metabolically converted vital dyes) may not be appropriate (OECD, 2009a)
Skin sensitization	local lymph node assay	429	Most appropriate method, permits estimation of the potency of the sensitization reaction (OECD, 2009a)
Genotoxicity	**Bacterial reverse mutation assay**	471	Not applicable (OECD, 2014d)
	***In vitro* mammalian cell gene mutation assay**	473, 476	Applicable (OECD, 2014d)
	***In vitro* micronucleus assay**	487	Applicable after modification (OECD, 2014d)
Reproductive and developmental toxicity	oral exposure	421, 422, 414, 415, 416	Applicable for oral exposure (inhalation exposure would require additional considerations) (OECD, 2009a)
Toxicokinetics	oral exposure, Intravenous (IV) administration, dermal or inhalation exposure (as applicable)	417	Not applicable (OECD, 2009a)

In additional to these TGs, several different working groups are currently dealing with the fate of NMs in aquatic media as discussed in a rolling work plan. Here, new TGs/ guidance documents for determining different PC properties, on their ecotoxicological and environmental effects (OECD, 2014c) or aquatic and algal toxicity are being discussed or developed further.

1.7 Alternative methods

The risk assessment of chemicals has traditionally been based on toxicity studies on animals, mainly rodents like rats and guinea pigs, which have served as surrogates for humans. With a growing concern about animal welfare and technological advances, new possibilities to determine the toxic properties of substances that do not require the use of animals are increasingly available. Alternative (non-animal) methods can be used as exploratory (e.g. to better understand mechanisms of action) or predictive tools (to extrapolate observations to the whole organism level).

In vitro experiments provide an important source of information on possible (toxic) effects of chemicals at the cellular level, however for many endpoints, they still don't represent a full replacement for *in vivo* tests, with the exception of some validated and accepted methods (for example for eye irritation/corrosion) that can be found in the designated database DB-ALM: http://ecvam-dbalm.jrc.ec.europa.eu/.

Another alternative to *in vivo* testing is the use of computer-based techniques commonly referred to as *in silico* tools. When little is known about the activity of substances, *in silico* tools are usually used to cluster and group those that are 'similar', i.e. have common structural features, metabolites, or PC properties and are thus expected to have similar biological activity (ECHA, 2008; OECD, 2012c, 2012d). This is the basis of the read-across approach in which the unknown toxic effects of a chemical of interest are deduced from the known effects of its analogues.

Differently from read-across, quantitative structure-activity relationship (QSAR) models are *in silico* tools that correlate specific activities (toxicity endpoint) of a series of substances with structural features. QSARs can thus be used to predict the activities of substances from their own properties, without the need to know the activities of analogues.

Physiologically based toxicokinetic (PBTK) models are another set of tools that at least in theory have *in vivo* predictive potential based on an understanding of anatomy and physiology, and on the application of mathematical modelling to data derived from *in silico* and *in vitro* models. In this case, predictions are not of a toxicological endpoint, but of the kinetics of absorption, distribution, metabolism and excretion resulting in an overall time-course of a chemical in blood plasma or organs.

Given their reductionist nature and uncertainties in their applicability, alternative methods do not usually represent stand-alone alternatives to classical test methods. However, it is expected that approaches based on the integrated use of multiple alternative methods, commonly called Integrated Testing Strategies (ITS) or Integrated Approaches to Testing and Assessment (IATA), will help to minimise or avoid animal testing.

1.7.1 Adverse Outcome Pathways (AOPs)

An Adverse Outcome Pathway (AOP) is a conceptual construction that portrays existing knowledge concerning the link between a molecular initiating event (MIE) and an adverse outcome (AO), by capturing the sequential chain of causally-linked events at different levels of biological organisation (Ankley et al., 2010). AOPs provide a means of systematically organising mechanistic knowledge on the biological and toxicological effects of chemicals, they can guide the further development of alternative methods, facilitate the integration and use of newer 'non-standard' data such as HTS and 'omics, and support the identification of knowledge gaps to inform intelligent testing strategies.

An AOP programme was launched by the OECD in 2012 (http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm). Specific rules are applied to AOP development. For example, one AOP always consists of a single MIE and a single AO, but can have multiple key events (KEs) which are causally linked by the so-called key event relationships (KERs). This leads to a simplified and 'linear' representation of a mechanism of toxicity. The AO can be defined at various levels: in humans, an AO can relate to the whole population, but also to individual organ damage, for example liver fibrosis or skin sensitisation; in environmental science, the AO relates to the death or reproductive impairment of an individual (e.g. a fish) which ultimately has consequences for the whole population. To promote the harmonised development of AOPs, an AOP-wiki was launched in September 2014 (https://aopkb.org/).

So far, AOPs have been developed on the basis of chemical-induced MIEs (Gerloff et al., 2017). However, due to the unique properties of NMs, their mechanisms of toxicity might not be directly comparable to those of chemicals in solution. In particular, NM-triggered MIEs could be different, and the toxicokinetics of NMs plays a major role in determining this (for example, solubility might vary depending on the biological environment of toxicological action).

1.7.2 In silico methods

In silico methods comprise a diverse range of techniques that solely use a computer to obtain an outcome. They are used to extract patterns and knowledge from large amounts of data (i.e. perform data mining) and to use this knowledge to build models (i.e. machine learning) that are able to predict the outcome of an experimental test thereby avoiding the need to carry it out. The terms 'data mining' and 'machine learning' are often confused as they employ the same methods but with different goals.

Supervised and unsupervised methods

Data mining methods may be categorized as either supervised or unsupervised. In unsupervised methods, no target variable is identified as such. They can be used during the early stages of an investigation to detect patterns, find features that can be useful for categorization, and gain insight into the nature or structure of the data. In contrast, supervised methods are mostly applied to reproduce and/or predict patterns found in the data.

The most common unsupervised data mining method is clustering, which is the grouping of objects into meaningful categories. Given a representation of N objects, a clustering exercise will assign the N objects to k clusters with respect to a similarity measure. There are various

clustering algorithms. Here we briefly describe two of the most common ones: hierarchical clustering and k-means clustering.

Hierarchical clustering assigns each object (e.g. NM) to a cluster. Initially, N objects are assigned to N clusters, each containing just one object. The distances (similarities) between the clusters are therefore the same as the distances (similarities) between the items they contain. In a second step, the most similar pair of clusters is identified and these are merged into a parent cluster so that there is one cluster less. The distances between this new cluster and all the other clusters are recalculated in the third step. Then, steps 2 and 3 are repeated until the whole dataset has been merged into a single cluster.

An example of hierarchical clustering is shown in Figure 1.3. In the ENPRA project (www.enpra.eu), various NMs (ZnO, TiO2, Ag, and CNTs) were tested with different coatings, crystalline structures and sizes. The NMs were clustered with respect to their lowest observed effect concentrations obtained for a series of cytotoxicity, genotoxicity, inflammation, and oxidative stress tests (Asturiol & Worth 2012 unpublised results).
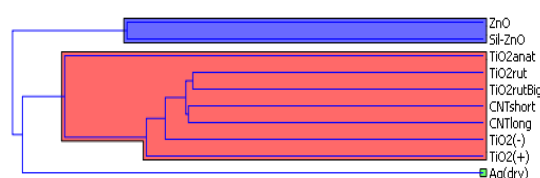
Figure 1.3. Hierarchical clustering of a set of NMs based on their toxicological profiles

The way in which the distance between the clusters is calculated (single-linkage, complete-linkage, average-linkage, etc.) determines the hierarchy of the cluster. If a pre-determined number of clusters is desired, the dendrogram is cut across the appropriate number of branches/links.

K-means clustering is an iterative algorithm that groups data into a pre-set number of clusters (K) which is defined by the user. In a first step, the algorithm randomly guesses the centre locations of the clusters. In a second step, each data point is assigned to its closest centre. In a third step, each cluster finds the centroid of the points that they are assigned to, i.e. the clusters find their centre. Steps 2 and 3 are then repeated until convergence is achieved, i.e. there is no variation in the position of the centroids.

Supervised learning consists of finding a model/rule that relates the data associated with objects (e.g. NMs descriptors or properties) to a pre-defined class (e.g. carcinogen/non-carcinogen, sensitiser/non-sensitiser, etc.). Datasets of objects with known classes are defined as *training sets* and are used to train the model. The trained model will be applied to a *test set* of compounds for its validation. If the training sets are sufficiently diverse (general), the model will be applicable to a wide variety of compounds (large applicability domain). Otherwise, its applicability domain will be specific to a small family of compounds (small applicability domain). For example, given a database that consists of a list of chemicals with their structural features (e.g. presence of heteroatoms, presence of rings, number of conjugated bonds, etc.) and their toxic activity (e.g. carcinogen or non-carcinogen), a supervised machine learning algorithm will find a function or pattern that differentiates carcinogens from non-carcinogens. The function or model could be as simple as this:

compounds containing aromatic nitro groups and with MW<1000Da are indicators of carcinogens (Benigni & Bossa, 2006). Given the database of carcinogens and non-carcinogens, the model 'learns' that aromatic nitro groups are characteristic of carcinogens. There are many types of machine learning algorithms. Decision trees like the one explained previously are probably the most simple and intuitive models. More sophisticated supervised learning approaches include support vector machines (Denis Fourches et al., 2010), Bayesian networks(Rong Liu et al., 2013a), and artificial neural networks (Mehdi Ghorbanzadeh, Fatemi, & Karimpour, 2012). See Table 1.6 for other methods.

Table 1.6. Supervised methods that are used to derive predictive models.

Method	Short description
Linear discriminant analysis (LDA)	Uses a linear combination of continuous descriptors to discriminate between two or more categorical variables
Classification algorithms and regression trees (CART)	Recursive process to partition the dataset in different subsets with respect to attributes values. The data is partitioned on the 'nodes' of a tree creating branches (subsets of data). Each branch is recursively partitioned until no added value in predicting a category is observed. Then, a leave corresponding to a category is created. This approach can use either continuous or categorical data in both, descriptors and category.
K-nearest neighbours (K-NN)	Local regression or classification method that uses the average of the K nearest neighbours to the test chemical in order to classify or predict the category or property of interest.
Support vector machines (SVM)	Model that represents the dataset as points in a space that maximises the separation between the different classes of the category. The test chemical is categorised or predicted with respect to the points in the space that are within a given threshold.
Bayesian networks (BN)	Graphical model that encodes probabilistic relationships (arcs) among random variables (nodes). The distribution of these variables with respect to the categories is used to assign a probability of pertinence to each category. The accumulated pertinence probability across all nodes, which are presumed independent, is used for categorisation. One of the advantages of this method is that not all descriptors (variables) are needed in order to have a prediction and that the influence of having additional data on the final classification can be tested beforehand.

Method	Short description
Artificial neural networks (ANN)	Set of interconnected nodes (neurons) that resemble biological neuronal networks. The neurons can compute values from inputs, and a hidden layer of neurons establishes self-adapted relations that derive in an output. Depending on the inputs values, different neurons are activated and outcome a result.

QSAR/QSPR

Quantitative structure-activity relationships (QSARs) are a specific type of *in silico* method. These methods are based on the assumption that the activity of the substances is related to its structure. The concept is well established (the first QSAR for biological activity can be dated back to 1868 (Fraser & Crum-Brown 1868) but in the past few decades it has been applied more efficiently and extensively due the availability of computational methods. The term structure-activity relationship (SAR) is sometimes used to express a simple qualitative association between an activity and the presence of a structural feature.

Quantitative structure-property relationships (QSPRs) are conceptually the same as QSARs but they relate structure to physicochemical properties (rather than biological activities) of the chemicals.

There are different ways to encode structural information in a QSAR/QSPR. The simplest way is identifying the presence or absence of certain chemical groups, i.e. fingerprints. These chemically-based encodings are usually not quantitative but discrete, giving rise to SAR. Alternatively, quantitative representations of chemical structures are used, which giving rise to QSAR. One of the most famous QSARs is the Hansch equation (Hansch & Fujita, 1964), which relates general bioactivity of compounds with their hydrophilicity. The extended Hansch (Hansch, 1969) equation 9) shows the curvilinear relationship between log1/C50 and hydrophobicity normally found in single dose tests:

$$log\ ^1/_{C_x} = -a(logP)^2 + blogP + \rho\sigma + \delta Es + K \tag{9}$$

Where C is the molar toxicant concentration producing a standard response (e.g. 50% mortality or effect) in a constant time interval, *logP* is the water-octanol partition coefficient, σ is the Hammett electronic parameter(Hammett, 1937), *Es* is Taft's steric factor (Taft, 1956), and ρ and δ are constants related to the sensitivity of the reaction to electron density.

A descriptor is a mathematical representation of chemical structure, (Todeschini & Consonni Viviana, 2009) and has been defined as

The molecular descriptor is the final result of a logic and mathematical procedure which transforms chemical information encoded within a symbolic representation of a molecule into a useful number or the result of some standardized experiment.

There are thousands of descriptors that can represent a chemical structure. The main families are presented in Table 1.7.

Table 1.7. List of chemical descriptors usually used in QSARs.

Descriptor family	Description
Zero-dimensional (0-D) / constitutional / information	These are derived from just the structural formula and only describe the composition, e.g. number of N atoms, number of double bonds, etc.
1-D / fingerprints	These describe the composition in terms of structural fragments, e.g. number of benzene rings.
2-D / topological	These include information on the connectivity of atoms and structural fragments. They are obtained from molecular graph theory (2D representations of chemical structure) and can differentiate molecules according to their size, degree of branching, flexibility, etc. Examples include atom connectivity indexes (Hall & Kier, 1977; Randic, 1975) which are derived from the bonds present in the molecule.
3-D / geometric	These are properties that depend on a three-dimensional representation of the chemical structures. They include size, shape, surface and volume-related descriptors, as well as electronic (quantum chemical) descriptors. They are typically derived from the coordinates of an energy-minimised conformation of the molecule. They may also take conformational flexibility into account, e.g. using molecular dynamic simulations (Hopfinger et al., 1997).
4-D	These are similar to 3-D descriptors but they also capture interaction energies. These energies are calculated in terms of the interaction of the molecule with a steric, electronic or hydrophobic probe (depending on the type of interaction energy). They are derived by various methods including Comparative Molecular Field Analysis (ComFA)(Cramer, Patterson, & Bunce, 1988). CoMFA is based on the rational that differences in a target property, e.g. biological activity, are often closely related to changes in shapes and strengths of non-covalent interaction fields surrounding the molecules. In CoMFA, field values are systematically calculated for ligands at each grid point of a regularly sampled 3-D grid box that extends 4 Å beyond the dimension of all molecules in the data set, using a sp3 carbon

Descriptor family	Description
	atom with +1 charge as probe molecules. Other 3-D programs are CoMSIA (Klebe, Abraham, & Mietzner, 1994), and GOLPE (Cruciani & Watson, 1994).
Physicochemical properties	These can also be used as 'descriptors' or more accurately as predictor variables. The property may be calculated from chemical structure or an experimental measurement may be used directly. A common example is logP.

Descriptors that have been found useful in the QPSR/QSAR modelling of particles (including NMs) are identified in Chapter 3.

Validation of QSARs for regulatory purposes

The first condition for using QSARs in regulatory decision making is the demonstration of model validity. Since the concept of validation is incorporated into legal texts and regulatory guidelines, it is important to clearly define what this means and to describe what the practical validation process might entail. According to the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2007a), the term validation is defined as follows: … *the process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose*.

This wide-ranging definition is intended to cover all kinds of traditional and alternative testing methods. In the context of QSARs, this definition is rather abstract and difficult to apply. However, in the case of QSARs, a set of five validation principles has been established by the OECD report (OECD, 2004). Thus, for a QSAR to be valid it must have:

- a defined endpoint
- an unambiguous algorithm
- a defined domain of applicability
- appropriate measures of goodness-of-fit, robustness and predictivity
- a mechanistic interpretation, if possible.

The 'appropriate documentation' for demonstrating model validity is recognised in the QSAR Model Reporting Format (QMRF), which is structured according to the OECD principles for QSAR validation. Information on QSAR model validity, including peer-reviewed documentation, is available from various sources, including the JRC QSAR Model Database (http://qsardb.jrc.it).

Expert systems

Expert systems have traditionally been associated with tools that capture and reproduce the knowledge of an 'expert'. One of the best known expert systems is Derek (Lhasa Ltd.), a knowledge-based expert system that predicts the toxicity of a chemical from its structure. Its predictions are based in part on structural alerts that describe structural features or

toxicophores associated with toxicity. These alerts are usually derived by human expert interpretation of toxicity data for known compounds within the same chemical class and an understanding of toxicological mechanisms.

A broader definition of the term 'expert system' was recommended during an ECVAM workshop (Dearden et al., 1997): *any formalized system, not necessarily computer-based, which enables a user to obtain rational predictions about the toxicity of chemicals*. According to this definition, any available software tool for toxicity prediction is considered an expert system, including those based entirely on statistical models (statistically-based systems), those based entirely on expert knowledge (knowledge-based systems), and those based on a combination of expert knowledge and statistical induction (hybrid systems). Widely used examples include Toxtree, TIMES-SS, TOPKAT, EPISuite, PASS, and Vega. A recent review of expert systems and their regulatory applicability (in the cosmetics sector) has been published by the International Cooperation on Cosmetics Regulation (ICCR; http://www.iccrnet.org/topics).

Applicability of QSAR/QSPR approaches to NMs

The challenge of developing QSARs for NMs has been discussed in the literature (Hristozov et al., 2014; Rong Liu et al., 2013a; Ratna Tantra et al., 2014; H. Zhou et al., 2008). Due to the novelty of applying the QSAR approach to NMs, the resulting models have sometimes been called quantitative nano-structure-activity relationships (QNARs) and the underlying descriptors nanodescriptors (Denis Fourches et al., 2010; Tomasz Puzyn, Leszczynska, & Leszczynski, 2009).

However, these terms are misleading since the main modelling challenges are not nano-specific, but rather they relate to the difference between modelling the behaviour of chemicals in solution ('classical' or 'bulk form') and in particulate form. Traditionally, QSPR and QSAR modelling has applied to substances in solution, typically undissociated molecules, but in principle, ionised molecules and soluble metal ions can be modelled too. This is sometimes complicated by the fact that the substance of interest can undergo abiotic or biotic (metabolic) conversions (in the case of molecules) or speciation (in the case of metal ions), creating a mixture whose composition can vary in time and space.

The modelling of particles, including nanoparticles, has been a challenge since relatively few theoretical descriptors are available for particles. In some cases, the particles have an amorphous (e.g. certain clays) rather than a crystalline structure, making it difficult to establish theoretical descriptors. Nevertheless, experimental descriptors (Table 1.1) may be available. In the case of NMs, it will be necessary to develop descriptors that are genuinely 'nanodescriptors' in the sense that they express the novel and size-dependent characteristics of NMs. A further complication is that a nano-sized (or micro-sized) particle does not form a homogeneous collection of species - it may undergo aggregation/agglomeration processes, adsorb and desorb macromolecules present in the surrounding medium, and may (partially) dissolve as well, leading to a distribution of masses/sizes/shapes (i.e. polydispersity). Rather than modelling a single species, it may therefore be necessary to model a distribution / mixture of species, which is increasingly difficult the more the material deviates from monodispersity. Furthermore, as with most 'classical' chemicals, the mode of toxicological action is often unknown, making it difficult to identify, *a priori*, the most relevant and predictive descriptors (Rallo et al., 2011; Shao et al., 2013).

As a consequence of these challenges, as well as the currently limited availability of systematic collections of physicochemical, *in vitro* and *in vivo* data (Oksel, Ma & Wang, 2015), there are relatively few QSARs for NMs in the scientific literature.

In principle, it should be easier to develop QSPRs (Martin, Maran, Sild, & Karelson, 2007) for NMs, since physicochemical (including magnetic and optical) properties are more closely linked to nanostructure than biological activity, and the property data is more likely to be generated in a systematic way (using standardised protocols). A few reviews of QSPRs and QSARs for NMs have been published elsewhere (Gallegos et al. 2009; Burello & Worth 2011; Gajewicz et al. 2012; Puzyn et al. 2010; Oksel, Ma, Liu et al, 2015) and a review on QSAR and QSPRs is reported in Chapter 3.

Physiologically Based Kinetic (PBK) modelling

Kinetics (sometimes referred to as toxicokinetics [TK] or pharmacokinetics [PK]) is the science that studies the fate of a substance (including NMs) in the body, whereas dynamics (sometimes referred to as toxicodynamics [TD] or pharmacodynamics [PD]) studies what a substance does to the body once it contacts the body (local toxicity) or enters the body (systemic toxicity). The usual OECD Test Guidelines for investigating TK are OECD TG 417 (toxicokinetics), TG 427 (*in vivo* skin absorption) and TG 428 (*in vitro* skin absorption). When studying TK using TG 417, separate studies are usually carried out and reported to study some 'ADME' parameters in isolation: one to determine relative (%) absorption and excretion (faeces, urine and sometimes exhaled air), one to establish organ distribution and one to establish metabolism. They are usually performed at two dose levels and with single and repeated (usually seven days, once per day) dosing. Indications of accumulation might be obtained when comparing tissue levels after seven days of exposure compared to a single exposure. Increasingly, concrete TK parameters like AUC, C_{max}, T_{max} and half-life ($t_{1/2}$) are also being investigated. In order to establish half-life ($t_{1/2}$), follow-up TK (tissue depletion) during a wash-out (no exposure) period following single or repeated dosing is needed.

Irrespective of whether a test substance is a NM or not, the TK is studied separately from the TD. It is therefore a challenge to evaluate whether data gathered in TK studies are relevant to the hazard characterisation information obtained from TD studies. One way of addressing this is to include some preliminary TK sampling in the TD studies. This is currently under discussion by the OECD Expert Group on the inhalation test guidelines 412 and 413 (28 and 90 days inhalation).

Traditionally, TK has been studied empirically in living organisms. In this approach, whole body parameters like absorption rate constant (k_a or k_{in}) and elimination rate constant (k_e or k_{out}) can be obtained using classical toxicokinetic (CTK) computer approaches to fit the empirical data (Figure 1.4A). CTK modelling can be used for interpolation between dose levels if measurements at these dose levels exist. However, it can be used for extrapolation only to a limited extent and care should be taken due to the possibility of non-linear relationships. Once k_a and k_e are established, CTK modelling can be used to assess the potential for $k_a > k_e$ and to determine the level of accumulation at given exposure levels.

A more recent approach, physiologically-based toxicokinetic (PBTK) modelling, is based on physiologically relevant compartments and processes (including bioavailability in the

relevant organs and the systemic blood flow). In contrast to CTK, PBTK allows various kinds of extrapolation, e.g. interspecies extrapolation, high-to-low dose extrapolation, route-to-route extrapolation and can take inter-individual variation into account. This has been used for non-NM substances. In addition, as shown in Figure 1.4B, PBTK modelling has been applied to simulate TK profiles by integrating independently obtained information from *in silico* (QSAR) and *in vitro* methods for absorption, distribution, metabolism and excretion (ADME). In that respect, it has more characteristics of a predictive modelling than the CTK modelling approach.

Although CTK modelling can be used to perform interpolation and assessment of accumulation based on at least some experimental *in vivo* data, it is generally considered as an empirical rather than predictive approach. Multi-compartmental classical empirical modelling (i.e. CTK) has provided some initial insights into the NM-specific (Ag and SiO_2) rates of absorption from the GI tract to the systemic circulation and into the distribution from the systemic circulation to specific organs (Bachler, von Goetz, & Hungerbuhler, 2014; D. Li et al., 2014; van Kesteren et al., 2014).

PBTK modelling approaches have greater potential to simulate the concentration-time profile. This is the especially the case for relatively simple chemicals for which the TK is (mainly) driven by passive diffusion across biological membranes. Incorporation of active processes such as active transport across external and internal membranes (Hagenbuch & Meier, 2004) is more demanding. Although for very small NMs, like quantum dots (<10 nm), diffusion might still be relevant, for NMs in general most processes that drive the absorption, tissue distribution and excretion (if any) are probably active processes such as macrophage uptake. These processes have not been mimicked *in vitro* nor modelled *in silico* in any detail.

An issue that is relevant not only for kinetic modelling but also for assessing the suitability of analogues for read-across (e.g. between bulk particles and nanoforms) is solubility (the amount that dissolves and the rate of dissolution) under physiologically relevant conditions. Typically, a considerable proportion of a NM does not dissolve[12] to a large extent (the NM is "durable"). With respect to kinetic modelling, it is much easier to model the behaviour of a NM that readily dissolves, i.e. becomes a 'classical readily soluble chemical' for which many of the usual assumptions hold. However, when it comes to insoluble particles, uptake by phagocytising cells needs to be considered, which is not trivial from a modelling point of view.

---

[12] Dissociation is to go from salt to dissociated ions (e.g. from AgCl to Ag^+ and Cl^-). To dissolve is to change from solid into 'dissolved in solution' (solute) status. For some chemicals, dissolving requires dissociation at the same time (from NaCl crystal into Na^+ and Cl^-. For other chemicals, dissociation does not occur when going into solution (e.g. from glucose crystals to glucose monomers in solution).
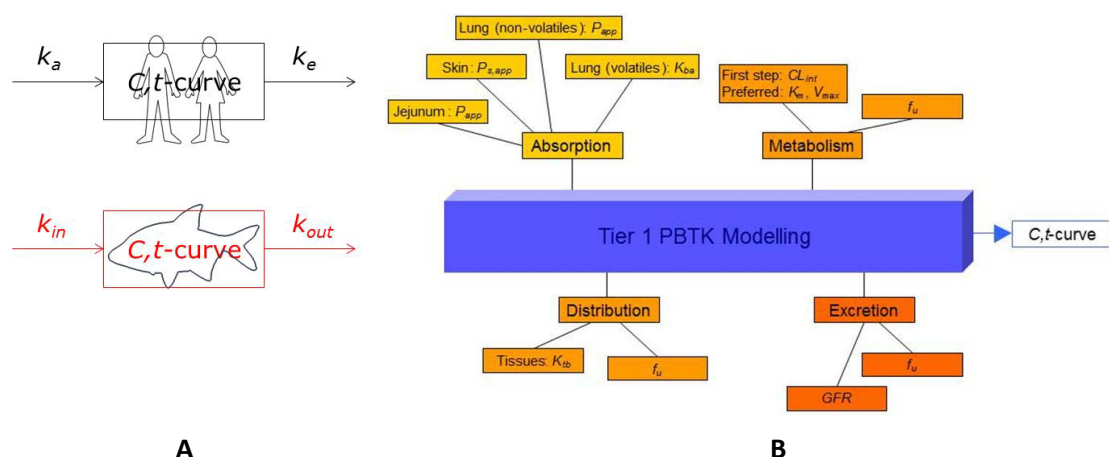
Figure 1.4. (A) Empirical classical toxicokinetic (CTK) modelling to fit the model to an experimental C,t-curve for humans or an environmental species *a posteriori*; (B) Physiologically-based toxicokinetic (PBTK) modelling to simulate a C,t-curve *a priori*. P_{app} = Apparent permeability, $P_{s,app}$ = apparent skin permeability, K_{ba} = blood:air partitioning coefficient, K_{tb} = tissue:blood partitioning coefficient, fu = fraction unbound to protein, CL_{int} = intrinsic clearance, K_m = Michaelis-Menten constant, V_{max} = maximum rate of metabolic conversion, GFR = glomerular filtration rate

The computational modelling of this type of biodistribution is more demanding than simulating simple diffusion using differential equations. As such, the development and use of PBTK modelling has not kept pace with experimental TKs and TDs. The number of papers covering PBTK modelling of NMs is currently very low, i.e. around 10-20. These are all hybrid PBTK models that simulate whole-body kinetics of NMs but rely heavily on calibration to experimental concentration-time course data, i.e. they have a strong empirical grounding (Bachler et al., 2014; Bachler, von Goetz, & Hungerbühler, 2013; D. Li et al., 2014; van Kesteren et al., 2014). This means that the physiological parameters such as blood flow and organ weights are measured data, but chemical-dependent parameters such as absorption rate are not known and are set by fitting to existing blood concentration-time course data.

Nevertheless, interesting results have been found from PBTK studies of NMs. For example, at dietary exposure to TiO_2 NMs between 15 to 150 nm, the size and crystalline structure of the particles had a minor influence on the biodistribution and at high internal exposure the particles agglomerate *in vivo* and are taken up by macrophages (Bachler et al., 2013; Bruinink et al., 2015). Agglomeration and de-agglomeration are very relevant processes for the exposure assessment as well as the hazard characterisation of NMs. High concentrations in an animal study might cause agglomeration, exposure to larger (maybe micron-sized) particles, and a blurred dose-response.

The future power of applying PBTK modelling to NMs lies in the possibility of simulating TK profiles *a priori* rather than fitting them *a posteriori*. In other words, the parameterisation of the equations in the model (e.g. giving values to absorption rates and transfer rates from blood to tissues) can be done by using QSPR predictions or *in vitro* measurements. This will require the further development of QSPR models and *in vitro* models for kinetic parameters. For example, the development of *in vitro* assays to measure active uptake by different types of phagocytising cells in different tissues may greatly enhance PBTK modelling approaches.

1.7.3 In vitro methods

In 2009, Stone et al. provided a first critical overview of *in vitro* test systems in nanotoxicology (V. Stone, Johnston, & Schins, 2009). They identified some general limitations of *in vitro* test systems, one of them being the fact that these test systems are limited to one or only a few different cell types and thus cannot fully represent the biological responses in a whole organism. Furthermore, these cell systems are usually derived from cancer or other long-lived cell lines, which can result in different outcomes when compared to *in vivo* tests (Joris et al., 2013). Single exposures are typically used in *in vitro* work which usually lasts from a few minutes up to a few days, depending on the tested endpoint. Therefore, chronic NM exposure cannot be tested sufficiently. Defining a suitable dose-range is a challenge for both *in vitro* and *in vivo* tests, as often unrealistically high doses are chosen in order to observe the effect of interest. *In vitro* studies often aim to acquire mainly mechanistic information, which are difficult or impossible to conduct in a whole body system.

The *in vitro* testing of NMs poses some specific technical challenges. The exposure medium plays an important role as it can affect the agglomeration or aggregation state of a NM and subsequently its uptake and toxicity (Joris et al., 2013). NMs can also interfere with the test system. For example, when using colorimetric assays such as the MTT assay (Table 1.8), one has to consider that a NM may generate an absorbance at the same wavelength as that used to quantify the coloured product, which will lead to an overestimation of the cell viability (V. Stone et al., 2009). Also, the NM might adsorb the coloured reagent due to its high specific surface area. When using fluorescent endpoints, one needs to consider the interference of the NM by for example physical blocking of the light emitted (e.g. carbon), reflection of the excitation light (e.g. TiO_2), and autofluorescence of the particle. Therefore, thorough care needs to be taken to choose the right systems, conduct appropriate pre-tests and include suitable controls (V. Stone et al., 2009).

Despite these technical challenges, *in vitro* models offer a number of advantages for nanotoxicity testing (Hirsch, Roesslein, Krug, & Wick, 2011). They can be used in mechanistic studies to investigate the potential of NMs to cross barriers, to enter cells, to influence cellular morphology and viability, to cause genotoxicity, to trigger cell signalling, gene expression and protein production. Table 1.8 lists *in vitro* systems that have been found useful for detecting cytotoxicity or cellular membrane damage. These assays can support the derivation of values for regulatory toxicity (e.g. LC_{50} or no observable adverse effect level - NOAEL), but also to determine sublethal concentrations for the assessment of mechanistic endpoints such as apoptosis, formation of ROS and oxidative stress, markers of pro-inflammatory enzymes and general gene-regulation assays.

High content imaging (HCI) systems, such as Cellomics, provide a means to upgrade traditional *in vitro* analyses to high throughput screening and offer a way to avoid interference of NM with colorimetric products, as in-cell fluorescence is analysed via an inverted microscope. Another advantage is the fact that it allows for live- and fixed-cell imaging, and multiple endpoints can be analysed in parallel in the whole-cell system. High throughput screening (HTS) is being an increasingly important means of detecting mechanistically relevant endpoints which can then serve for *in vivo* toxicity prediction (A. Nel et al., 2013).

At present, multiple efforts are ongoing to develop standardised *in vitro* methods for NM testing, but as yet, none have been formally validated by validation bodies such as the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM), which is hosted by the JRC. In the meantime, available and potentially useful methods have been summarised by the SCCS in their Guidance on the Safety Assessment of Nanomaterials in Cosmetics (SCCS 1484/12: SCCS 2012) and in the 9th revision of the Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation (SCCS 1564/15; SCCS 2015).

Table 1.8 Overview of the most commonly used *in vitro* test methods to detect nanoparticle-induced adverse effects *in vitro*.

General effect	Read-out	Assay
Cell viability	Determination of mitochondrial function by measuring the activity of mitochondrial enzymes; quantified by light absorbance	MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay
		MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay
		XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay
		WST (Water soluble Tetrazolium salts) assay
	ATP levels are an indicator of metabolically active, viable cells; quantified by luminescence	cellular ATP (adenosine triphosphate) content
Cell death via necrosis	Increase in the leakiness of the plasma membrane; quantified by light absorbance	LDH (lactate dehydrogenase) assay
	Cells with an intact cell membrane are able to prevent uptake of the dye; compromised cells however are colored blue within seconds of exposure; quantified by manual counting	Trypan Blue exclusion
		(PI) propidium iodide staining
Apoptosis	Staining of cleaved caspase 3/7, analysed by immunofluorescence staining or luminescence	caspase 3/7 staining
	Annexin V binds to phosphatidyl serine on the surface of apoptotic cells; quantified by flow cytometry	annexin V–FITC (fluorescein isothiocyanate) staining
ROS production	Cell-free or in the presence of cells, The presence of ROS converts DCFH to 2,7-dichlorofluorescein; analysis by fluorimetry or flow-cytometry	DCF(D)H (2,7-dichlorofluorescin) assay

General effect	Read-out	Assay
	Specific spin traps or probes allow for the quantification and specific identification of free radical species generated	EPR (Electroparamagnetic resonance) spectrometry
	Unwinding and linearization of a coiled bacterial DNA plasmid is used to estimate free radical and/or ROS exposure	plasmid assay
	For detection of superoxide, it exhibits blue-fluorescence in the cytosol until it's oxidized, where it intercalates within the cell's DNA, staining its nucleus a bright fluorescent red	DHE (dihydroethidium) assay
Oxidative stress	Its reduced form acts as an antioxidant by reacting directly with ROS to neutralize them, the oxidized glutathione (GSSG) is formed and accumulated; protein contents are analysed	GSH (glutathione) assay
	TBARS are formed as a byproduct of lipid peroxidation; colorimetrical quantification	lipid peroxidation (e.g thiobarbituric acid reactive substances (TBARS) assay)
	Quantification of mRNA expression changes of oxidative stress-dependent genes	e.g. HO-1 (heme oxygenase-1)
Inflammation	Detection of cytokine and/or chemokine protein production; colorimetrical quantification	ELISA (enzyme linked immunosorbent assay)
	Quantification of mRNA expression changes of cytokine regulating genes	e.g. TGFβ (tumor growth factor beta), IL-8 (Interleukin 8)
Genotoxicity	Evaluation of clastogenic (chromosome breaking) effects; microscopical analysis	alkaline comet assay
		micronucleus assay
	Specific comet assay: specific quantification of DNA double strand lesions	neutral comet assay
	Specific comet assay: quantification of oxidative DNA adducts	8-OHdG (8-hydroxydeoxyguanosine) formation
	Salmonella reverse mutation assay; quantification by counting of the mutants	Ames test*

*The Ames test has recently been described as not recommended for NMs by an OECD workshop (OECD, 2014d).

1.7.4 Grouping and read-across

The concept of chemical category has been defined within the REACH technical guidance (ECHA, 2008) and the OECD grouping guidance (OECD, 2007c, 2014e) as follows:

A chemical category is a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity.

In practical terms, this involves treating a group of similar substances as a category. Missing data on endpoints or properties within a category are predicted by read-across from data-rich analogues within the category.

The way in which similarity is defined within a group is essential to read-across. However, there is not a single way to measure/define it, and it can actually be endpoint-dependent. According to REACH and OECD guidance, similarities may be based on:

- common functional group(s) e.g. aldehyde
- common constituents or chemical classes, similar carbon range numbers e.g. UVCB substances (substance of Unknown or Variable composition, Complex reaction products or Biological materials)
- an incremental and constant change across the category e.g. a chain-length category for boiling point range;
- the likelihood of common precursors and/or breakdown products, via physical or biological processes, which result in structurally similar chemicals.

The terms 'category approach' and 'analogue approach' are used to describe techniques for grouping chemicals. An analogue approach is often used when a chemical grouping is based on a very limited number of chemicals, typically two substances. A chemical category is used to describe a grouping of three or more chemicals. The term read-across applies to both the category and analogue approaches.

One of the key considerations in grouping and read across is to demonstrate that the grouping method is not invalidated by 'extrinsic' factors such as bioavailability, metabolism, or reactivity. This is especially relevant for NMs. Although the development of chemical categories and QSARs are underpinned by the same principles of chemical similarity, there is no formal process for validating a category, i.e. there is no official body like EURL-ECVAM to determine the validity of chemical categories. Nevertheless, a robust justification to demonstrate the scientific robustness of the data gap filling approach needs to be presented. In other words, there is a need to justify the read-across argument. This should be done using a standard document known as the Reporting Formats for the Analogue and Category Approaches (section 6.2.6 in ECHA 2008b).

There is a preference for the use of interpolation within grouping approaches under REACH, presumably because this gives rise to less uncertainty than extrapolation. Extrapolation is therefore considered as less reliable due to this higher level of uncertainty associated with predictions. The exception to this is where an extrapolation from one substance to another leads to an equally severe or more severe hazard assessment for the target substance. Although it may seem logical to assume that interpolation is subject to less uncertainty than extrapolation, in reality the degree of uncertainty is not due to the interpolation or extrapolation of data, but rather the strength of the relationship forming the basis of the

category/analogue approach itself. This in turn is dependent on the size of the category and the amount and quality of the experimental data for the category members themselves. If the relationship underpinning the category is poorly defined then interpolation or extrapolation can result in significant uncertainty.

In addition to the available regulatory guidance, practical guiding principles and considerations for developing analogue and category approaches are described in several papers (ECETOC, 2012; Patlewicz, Roberts, Aptula, Blackburn, & Hubesch, 2013; Wu, Blackburn, Amburgey, Jaworska, & Federle, 2010).

It is important to bear in mind that there are no officially accepted chemical categories in the EU, so read-across arguments are presented (by the registrant) and evaluated (by the regulatory assessor) on a case-by-case basis. In order to ensure consistency in the evaluations of different assessors, ECHA is developing a Read-Across Assessment Framework (RAAF) for both human health and ecotoxicological effects. This is an attempt to identify the different kinds of arguments/hypotheses that can be made and the importance of confounding factors (e.g. differences in metabolism or mode of action between source and target chemicals). It also applies a scoring scheme to determine the validity of the read-across argument. Further information can be found at: http://echa.europa.eu/en/support/grouping-of-substances-and-read-across

While there is still considerable experience to be gained in the application of read-across to NMs, one can anticipate that the RAAF will need to be adapted at some point in order to better define and apply the concept of particle similarity.

Read across and categories of nanomaterials

In principle the development of categories of NMs should provide a valuable means of filling data gaps for the hazardous effects of NMs, especially in the absence of reliable QSARs. For the prediction of NM properties, three main kinds of read-across can be foreseen (even though strictly speaking the REACH legal text only refers to read-across between different substances): 1) from bulk to all nanoforms, 2) from bulk to specific nanoforms, 3) from one or more nanoforms to one or more other nanoforms (nanoforms of different chemical identity, or the same chemical identity but with differences in physicochemical characteristics).

As mentioned above, any grouping and read-across approach must be based on a robust scientific justification and the use of toxicologically relevant properties. It has been shown that the drivers for the toxicological effects of NMs include parameters such as composition (including the presence of impurities, coatings and surface treatment), shape and size, charge, surface reactivity, solubility, biological persistence, dispersibility, and the ability to translocate across biological barriers (Ken Donaldson & Poland, 2013). These properties are covered in Section 1.3..

1.7.5 *Weight of evidence*

According to the REACH legal text, weight-of-evidence (WoE) is one of the options for meeting the information requirements while arguing that testing is not scientifically necessary. In particular, according to REACH Annex XI:

"There may be sufficient weight of evidence from several independent sources of information leading to the assumption/conclusion that a substance has or has not a particular dangerous property, while the information from each single source alone is regarded insufficient to support this notion.

There may be sufficient weight of evidence from the use of newly developed test methods, not yet included in the test methods referred to in Article 13(3) or from an international test method recognised by the Commission or the Agency as being equivalent, leading to the conclusion that a substance has or has not a particular dangerous property.

Where sufficient weight of evidence for the presence or absence of a particular dangerous property is available:

- further testing on vertebrate animals for that property shall be omitted,

- further testing not involving vertebrate animals may be omitted.

In all cases adequate and reliable documentation shall be provided."

While weight-of-evidence is a widely used scientific concept, the way in which it is applied is very heterogeneous. According to a review carried out by Linkov et al (2011), the term WoE can be found in the scientific literature with a variety of meanings, ranging from the purely colloquial use of the word to structured approaches to data integration and interpretation. Focusing on the structured approaches, and building on previous work (Chapman, McDonald, & Lawrence, 2010; Weed, 2005), Linkov et al. proposed a taxonomy of WoE methods, ranging from mainly qualitative approaches to increasingly more quantitative ones (Table 1.9).

Table 1.9. Taxonomy of WoE methods (adapted from Linkov et al, 2012). The methods are listed in order of increasing quantitative rigour.

Method	Nature	Description
Listing evidence	Qualitative	Presentation of individual lines of evidence without attempt to integrate them
Best Professional Judgement	Qualitative	Qualitative integration of multiple lines of evidence, usually invoking a professional opinion
Causal Criteria	Semi-quantitative	Use of a consistent set of criteria for determining cause and effect
Logic	Semi-quantitative	Use of a systematic framework for evaluating multiple lines of evidence
Scoring	Quantitative	Quantitative integration of multiple lines of evidence by applying (different) weights and ranking them
Indexing	Quantitative	Weighting of multiple lines of evidence and integration into a single score (numerical value)
Quantification	Quantitative	Use of formal decision analytic methods, such as Bayesian statistics or Multi-Criteria Decision Analysis (MCDA)

Guidance on how to apply WoE in REACH is given in an ECHA practical guide (ECHA, 2010b). The guide recommends that WoE arguments should take into account the reliability, relevance, adequacy and quantity of the individual lines of evidence. While the ECHA guidance does not make reference to the Linkov taxonomy, from the examples presented, it would appear to fall under the Best Professional Judgement Category, although more quantitative approaches are not excluded.

EFSA has also developed guidance on "The Use of the Weight of Evidence Approach in Scientific Assessments", which at the time of writing (March 2017) was under public consultation (https://www.efsa.europa.eu/en/consultations/call/170306-0).

1.7.6 Integrated Approaches to Testing and Assessment (IATA)

Integrated assessment approaches are based on data generated by multiple alternative methods combined with other types of information (e.g. exposure considerations). These approaches have been developed to effectively and efficiently support decision making while reducing the reliance on animal testing. An IATA may include both formalised (e.g. *in vitro* prediction models and QSARs) and non-formalised methods (e.g. WoE and read-across).

The OECD (OECD, 2016c) defines an IATA as

> "An approach based on multiple information sources used for the hazard identification, hazard characterisation and/or safety assessment of chemicals. An IATA integrates and weights all relevant existing evidence and guides the targeted generation of new data, where required, to inform regulatory decision-making regarding potential hazard and/or risk. Within an IATA, data from various information sources (i.e. physicochemical properties, in silico models, grouping and read-across approaches, *in vitro* methods, *in vivo* tests and human data) are evaluated and integrated to draw conclusions on the hazard and/or risk of chemicals. "

Formalised methods within an IATA are referred to as "defined approaches", which consist of a defined set of information sources and data interpretation procedure that converts the results of the information sources into a prediction of the property of interest.

One of the first published IATA corresponds to skin corrosion and irritation (OECD, 2014f) and consists of three steps:

1) Existing information (*in vivo* & *in vitro* data, PC properties, and non-testing methods like QSAR and read-across);

2) WoE analysis;

3) Additional testing.

The WoE analysis (step 2) is carried out with the data obtained in step 1, which will mainly be obtained from literature, databases and other reliable sources of data. If the WoE is found conclusive, no more testing is needed. However, if it is found inconclusive step 3 will be used in order to obtain further data and re-evaluate step 2.

Essentially the same sequential strategy for skin irritation and/or corrosion is proposed in ECHA's guidance on information requirements and Chemical Safety Assessment (ECHA, 2014a), which also includes ITS for other human health and environmental effects.

More recently, with the advent of the AOP approach, there have been discussions on the development and evaluation of AOP-informed IATA (OECD, 2016b). It has been concluded that the degree to which an IATA needs to be populated by a full complement of methods addressing each of the key events will be dependent on the ultimate purpose it is being used for. For chemical categorisation purposes, e.g. to facilitate read-across, it is conceivable that using approaches to address the first key event, the molecular initiating event, might suffice. Whereas, if a risk assessment decision is being made where uncertainty needs to be minimized as far as possible, generating information to address a number of other key events and their quantitative relationship with the adverse outcome as well as information on the expected exposure may be necessary. Thus flexibility is foreseen in the choice of the various information sources depending on the purpose of the IATA and the substance under investigation.

1.8   Concluding remarks

In this chapter, we have summarised the REACH information requirements for NMs, with reference to the state-of-play in terms of possible amendment of the Annexes to the REACH Regulation and respective guidance. In addition, we have given an overview of the scientific basis for understanding the behaviour (fate and effects) of NMs, as well the different types of non-animal methods and approaches that are under development to determine the properties of NMs. This information provides the context and background information for the work presented in the rest of this report.

1.9 References

Aggarwal, P., J. B. Hall, C. B. McLeland, M. a Dobrovolskaia, and S. E. McNeil. 2009. Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. Advanced drug delivery reviews 61:428–37.

Aitken, R. ., A. Bassan, S. Friedrichs, S. M. Hankin, S. F. Hansen, J. Holmqvist, S. A. K. Peters, C. A. Poland, and C. L. Tran. 2011. Specific Advice on Exposure Assessment and Hazard / Risk Characterisation for Nanomaterials under REACH (RIP-oN 3) Final Project Report.

Alarifi, S., D. Ali, A. A. Al-Doaiss, B. A. Ali, M. Ahmed, and A. A. Al-Khedhairy. 2013. Histologic and apoptotic changes induced by titanium dioxide nanoparticles in the livers of rats. International journal of nanomedicine 8:3937–43.

American Society for Testing and Materials. 2010. ASTM B922 - 10 Standard Test Method for Metal Powder Specific Surface Area by Physical Adsorption.

Ankley, G. T., R. S. Bennett, R. J. Erickson, D. J. Hoff, M. W. Hornung, R. D. Johnson, D. R. Mount, J. W. Nichols, C. L. Russom, P. K. Schmieder, J. a Serrrano, J. E. Tietge, and D. L. Villeneuve. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environmental toxicology and chemistry / SETAC 29:730–41.

Arvidsson, R., S. Molander, B. A. Sandén, and M. Hasellöv. 2011. Challenges in Exposure Modeling of Nanoparticles in Aquatic Environments Rickard. Human and Ecological Risk Assessment 17:245–262.

ASTM. 1990. Evaluation of Airborne Dust Measurement Methods for Agricultural Chemical Carriers.

ASTM. 2009a. Standard Test Method for Airborne Asbestos Concentration in Ambient and Indoor Atmospheres as Determined by Transmission Electron Microscopy Direct Transfer (TEM).

ASTM. 2009b. Standard Test Method for Microvacuum Sampling and Indirect Analysis of Dust by Transmission Electron Microscopy for Asbestos Structure Number Surface Loading.

ASTM. 2010. Standard Test Method for Wipe Sampling of Surfaces, Indirect Preparation, and Analysis for Asbestos Structure Number Concentration by Transmission Electron Microscopy.

Asturiol, D., and A. Worth. 2012. D6.5 - Report on the development and applications of structure-activity models for toxicity prediction of ENP: Analysis of ENPRA dataset.

Bachler, G., N. von Goetz, and K. Hungerbuhler. 2014. Using physiologically based pharmacokinetic (PBPK) modeling for dietary risk assessment of titanium dioxide (TiO2) nanoparticles. Nanotoxicology:1–8.

Bachler, G., N. von Goetz, and K. Hungerbühler. 2013. A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. International journal of nanomedicine 8:3365–82.

Bakand, S., A. Hayes, and F. Dechsakulthorn. 2012. Nanoparticles: a review of particle toxicology following inhalation exposure.

Balasubramanian, S. K., K.-W. Poh, C.-N. Ong, W. G. Kreyling, W.-Y. Ong, L. E. Yua, and L. E. Yu. 2013. The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates. Biomaterials 34:5439–5452.

Benigni, R., and C. Bossa. 2006. Structural Alerts of Mutagens and Carcinogens. Current Computer Aided-Drug Design 2:169–176.

Borm, P. J. A., D. Robbins, S. Haubold, T. Kuhlbusch, H. Fissan, K. Donaldson, R. Schins, V. Stone, W. Kreyling, J. Lademann, J. Krutmann, D. Warheit, and E. Oberdörster. 2006. The potential risks of nanomaterials: a review carried out for ECETOC. Particle and fibre toxicology 3:11.

Braakhuis, H. M., M. V. D. Z. Park, I. Gosens, W. H. De Jong, and F. R. Cassee. 2014. Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. Particle and fibre toxicology 11:18.

Bruinink, A., J. Wang, and P. Wick. 2015. Effect of particle agglomeration in nanotoxicology. Archives of toxicology.

Buffle, J., K. J. Wilkinson, S. Stoll, M. Filella, and J. Zhang. 1998. A Generalized Description of Aquatic Colloidal Interactions: The Three-colloidal Component Approach. Environmental Science & Technology 32:2887–2899.

Burello, E. 2013. Profiling the biological activity of oxide nanomaterials with mechanistic models. Computational Science & Discovery 6:14009.

Burello, E., and A. P. Worth. 2011a. QSAR modeling of nanomaterials. Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology 3:298–306.

Burello, E., and A. P. Worth. 2011b. A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5:228–35.

Burello, E., and A. P. Worth. 2013. A rule for designing safer nanomaterials: do not interfere with the cellular redox equilibrium. Nanotoxicology:1–2.

Casarett, L., and J. Doull. 2008. Toxicology. Pages (C. D. Klaassen, Ed.). 7th edition. The McGraw-Hill Companies, Kansas City.

Cedervall, T., I. Lynch, S. Lindman, T. Berggård, E. Thulin, H. Nilsson, K. A. Dawson, and S. Linse. 2007. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proceedings of the National Academy of Sciences of the United States of America 104:2050–5.

CEN. 2002. CEN/TC 292 - Characterization of waste.

CEN. 2006. Workplace atmospheres - Measurement of the dustiness of bulk materials - Requirements and reference test methods. Brussels.

Chapman, P. M., B. G. McDonald, and G. S. Lawrence. 2010. Weight-of-Evidence Issues and Frameworks for Sediment Quality (And Other) Assessments. Human and Ecological Risk Assessment: An International Journal 8:1489–1515.

Chen, J., X. Dong, J. Zhao, and G. Tang. 2009. In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitioneal injection. Journal of applied toxicology : JAT 29:330–7.

Cheng, L.-C., X. Jiang, J. Wang, C. Chen, and R.-S. Liu. 2013. Nano-bio effects: interaction of nanomaterials with cells. Nanoscale 5:3547–69.

Cornelis, G., L. Pang, C. Doolette, J. K. Kirby, and M. J. McLaughlin. 2013. Transport of silver nanoparticles in saturated columns of natural soils. The Science of the total environment 463–464:120–30.

Cramer, R. D., D. E. Patterson, and J. D. Bunce. 1988. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. Journal of the American Chemical Society 110:5959–5967.

Cruciani, G., and K. A. Watson. 1994. Comparative molecular field analysis using GRID force-field and GOLPE variable selection methods in a study of inhibitors of glycogen phosphorylase b. Journal of medicinal chemistry 37: 2589–25601.

Cui, Y., H. Liu, M. Zhou, Y. Duan, N. Li, X. Gong, R. Hu, M. Hong, and F. Hong. 2011. Signaling pathway of inflammatory responses in the mouse liver caused by TiO2 nanoparticles. Journal of biomedical

materials research. Part A 96:221–9.

Dearden, J. C., M. D. Barratt, R. Benigni, W. Douglas, R. D. Combes, M. T. D. Cronin, P. N. Judson, M. P. Payne, A. M. Richard, M. Tichy, A. P. Worth, and J. J. Yourick. 1997. The Development and Validation of Expert Systems for Predicting Toxicity. Alternatives to laboratory animals 25: 223–252.

Desmet, C., Valsesia, A., Oddo, A. et al. (2017). Characterisation of nanomaterial hydrophobicity using engineered surfaces. Journal of Nanoparticle Research 19: 117. doi:10.1007/s11051-017-3804-z

DK EPA (2015). Dermal Absorption of Nanomaterials Titanium Dioxide and Zinc Oxide Based Sunscreen. ISBN 978-87-93352-53-7. Available from: http://www2.mst.dk/Udgiv/publications/2015/08/978-87-93352-53-7.pdf

Donaldson, K., P. J. a Borm, G. Oberdörster, K. E. Pinkerton, V. Stone, and C. L. Tran. 2008. Concordance between in vitro and in vivo dosimetry in the proinflammatory effects of low-toxicity, low-solubility particles: the key role of the proximal alveolar region. Inhalation toxicology 20:53–62.

Donaldson, K., and C. Poland. 2013. Nanotoxicity: challenging the myth of nano-specific toxicity. Current opinion in biotechnology 24:724–34.

Donaldson, K., and C. A. Poland. 2012. Inhaled nanoparticles and lung cancer - what we can learn from conventional particle toxicology. Swiss medical weekly 142:w13547.

Donaldson, K., A. Schinwald, F. Murphy, W.-S. Cho, R. Duffin, L. Tran, and C. Poland. 2013. The biologically effective dose in inhalation nanotoxicology. Accounts of chemical research 46:723–32.

Downs, T. R., M. E. Crosby, T. Hu, S. Kumar, A. Sullivan, K. Sarlo, B. Reeder, M. Lynch, M. Wagner, T. Mills, and S. Pfuhler. 2012. Silica nanoparticles administered at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not. Mutation research 745:38–50.

Duan, Y., J. Liu, L. Ma, N. Li, H. Liu, J. Wang, L. Zheng, C. Liu, X. Wang, X. Zhao, J. Yan, S. Wang, H. Wang, X. Zhang, and F. Hong. 2010. Toxicological characteristics of nanoparticulate anatase titanium dioxide in mice. Biomaterials 31:894–9.

Duffin, R., L. Tran, D. Brown, V. Stone, and K. Donaldson. 2007. Proinflammogenic Effects of Low-Toxicity and Metal Nanoparticles In Vivo and In Vitro: Highlighting the Role of Particle Surface Area and Surface Reactivity. Inhalation Toxicology 19:849–856.

EASAC, and JRC. 2011. Impact of engineered nanomaterials on health. Luxembourg.

EC. 2008. Nanomaterials in REACH. Follow-up to the 6th Meeting of the REACH Competent Authorities for the implementation of Regulation (EC) 1907/2006 (REACH). European Commission, Brussels 2008.

EC. 2011. Commission Recommendation of 18 October 2011 on the definition of nanomaterial. Pages Official Journal of the European Union.

EC. 2012a. Second Regulatory Review on Nanomaterials: COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL AND THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE.

EC. 2012b. D6.4 – Optimised PBPK/PD and Exposure Model.

ECETOC. 2012. Category approaches, Read-across, (Q)SAR. Brussels.

ECHA. 2008. Guidance on information requirements and chemical safety assessment Chapter R.6 : QSARs and grouping of chemicals. Helsinki.

ECHA. 2009. Guidance in a Nutshell - Chemical Safety Assessment. Pages ECHA Guidance. ECHA, Helsinki.

ECHA. 2010. Practical guide 2: How to report weight of evidence. Helsinki.

ECHA. 2011. Guidance on information requirements and chemical safety assessment; Chapter R.5: Adaptation of information requirements. Helsinki.

ECHA. 2012a. Guidance on information requirements and chemical safety assessment - Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance. Helsinki.

ECHA. 2012b. Guidance on information requirements and chemical safety assessment - Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7b Endpoint specific guidance.

ECHA. 2012c. Guidance on information requirements and chemical safety assessment - Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance:1–7.

ECHA. 2014. Guidance on information requirements and Chemical Safety Assessment Chapter R . 7a : Endpoint specific guidance. Helsinki.

ECHA. 2017. Appendix R . 6-1 : Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping.

EFSA. 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. Pages EFSA Journal 2011. Parma.

EFSA Scientific Committee. 2009. The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety- Scientific Opinion of the Scientific Committee. The EFSA Journal 958:1–39.

Elimelech, M., J. Gregory, X. Jia, and R. A. Williams. 1995a. Surface interaction potentials. Pages 33–68Particle deposition and aggregation: measurement, modelling and simulation. Butterworth-Heinemann Ltd.

Elimelech, M., J. Gregory, X. Jia, and R. A. Williams. 1995b. Colloidal hydrodynamics and transport. Pages 68–109Particle deposition and aggregation: measurement, modelling and simulation. Butterworth-Heinemann Ltd.

Elimelech, M., J. Gregory, X. Jia, and R. A. R. A. Williams. 1995c. Modelling of aggregation processes. Pages 157–202Particle deposition and aggregation: measurement, modelling and simulation. Butterworth-Heinemann, Woburn.

Epa, V. C., F. R. Burden, C. Tassa, R. Weissleder, S. Shaw, and D. A. Winkler. 2012. Modeling biological activities of nanoparticles. Nano letters 12:5808–12.

EU. 2007. Corrigendum to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending. Pages 278. Official Journal of the European Union L 396 of 30 December 2006, EU.

European Commission. 2012. Commission Staff working paper: Types and uses of nanomaterials, including safety aspects accompanying the Commission Communication on the Second Regulatory Review on Nanomaterials.

European Commission. 2013. Proposal for a Regulation of the European Parliament and of the Council on novel foods.

European Commission, EC, and IHCP JRC. 2011. REACH Implementation Project Substance Identification of Nanomaterials (RIP-oN 1). Pages (I. JRC, Ed.).

European Parliament and Council. 1997. Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. Pages 1–6(E. Union, Ed.)Official Journal of the European Communities.

European Parliament and Council. 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/4. Pages 849Official Journal of the European Union. Official Journal of the European Union, EC, EC.

European Parliament and Council. 2009. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Publications Office of the European Union, Luxembourg.

European Parliament and Council. 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union L 167:1–123.

European Parliament, and Council. 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union L342:59–209.

Fermin, D., and J. Riley. 2010. Charge in Colloidal Systems. Pages 23–44in T. Cosgrove, editor.Colloid Science: Principles, Methods and Applications. 2nd edition. Wiley.

Fourches, D., D. Pu, C. Tassa, R. Weissleder, S. Y. Shaw, R. J. Mumper, and A. Tropsha. 2010. Quantitative nanostructure-activity relationship modeling. ACS nano 4:5703–12.

Fraser, T. R., and A. Crum-Brown. 1868. ACTION OF THE SALTS OF THE AMMONIUM. J Anat Physiology 2:224–242.

Friedlander, S. K. 1977. Smoke, Dust, and Haze: Sheldon K. - Oxford University Press. Pages (K. E. Gubbins, Ed.). 2nd Editio. Oxford University press, New York, Oxford.

Gajewicz, A., B. Rasulev, T. C. Dinadayalane, P. Urbaszek, T. Puzyn, D. Leszczynska, and J. Leszczynski. 2012. Advancing risk assessment of engineered nanomaterials: application of computational approaches. Advanced drug delivery reviews 64:1663–93.

Gallegos, A., E. Burello, and A. Worth. 2009. Review of computational approaches for predicting the physicochemical and biological properties of nanoparticles. Ispra.

Geiser, M., C. Wigge, M. L. Conrad, S. Eigeldinger-Berthou, L. Künzi, H. Garn, H. Renz, and M. A. Mall. 2014. Nanoparticle uptake by airway phagocytes after fungal spore challenge in murine allergic asthma and chronic bronchitis. BMC pulmonary medicine 14:116.

Gerloff, K., B. Landesmann, A. Worth, S. Munn, T. Palosaari, and M. Whelan. 2017. The Adverse Outcome Pathway approach in nanotoxicology. Computational Toxicology 1:3–11.

Ghorbanzadeh, M., M. H. Fatemi, and M. Karimpour. 2012. Modeling the Cellular Uptake of Magneto fl uorescent Nanoparticles in Pancreatic Cancer Cells: A Quantitative Structure Activity Relationship Study. Industrial & Engineering Chemistry Research 51:10712–10718.

Gottardo et al.,2017. NANoREG framework for the safety assessment of nanomaterials, EUR 28550 EN, doi 10.2760/245972. http://publications.jrc.ec.europa.eu/repository/handle/JRC105651

Hagenbuch, B., and P. J. Meier. 2004. Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. Pflügers Archiv : European journal of physiology 447:653–65.

Hagens, W. I., A. G. Oomen, W. H. de Jong, F. R. Cassee, and A. J. A. M. Sips. 2007. What do we (need to) know about the kinetic properties of nanoparticles in the body? Regulatory toxicology and pharmacology 49:217–29.

Hall, L., and L. Kier. 1977. No Title. Journal of pharmaceutical sciences 66:642.

Hamaker, H. C. 1937. The London—van der Waals attraction between spherical particles. Physica 4:1058–1072.

Hammett, L. P. 1937. The Effect of Structure upon the Reactions of Organic Compounds. Benzene Derivatives. Journal of the American Chemical Society 59:96–103.

Hankin, S. M., S. A. . Peters, C. A. Poland, S. Foss Hansen, H. J., B. L. Ross, J. Varet, and R. J. Aitken. 2011. Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2) Final Project Report.

Hansch, C. 1969. Quantitative approach to biochemical structure-activity relationships. Accounts of Chemical Research 2:232–239.

Hansch, C., and T. Fujita. 1964. Additions and Corrections - ρ-σ-π Analysis. A Method for the Correlation of Biological Activity and Chemical Structure. Journal of the American Chemical Society 86:1616–1626.

Healy, T. W., A. Homola, R. O. James, and R. J. Hunter. 1978. Coagulation of amphoteric latex colloids: reversibility and specific ion effects. Faraday Discussions of the Chemical Society 65:156.

Hillery, A. M., P. U. Jan, and A. T. Florence. 1994. Comparative , Quantitative Study of Lymphoid and Non-Lymphoid Uptake of 60 nm Polystyrene Particles. Journal of Drug testing 2:151–156.

Hirsch, C., M. Roesslein, H. F. Krug, and P. Wick. 2011. Nanomaterial cell interactions: are current in vitro tests reliable? Nanomedicine (London, England) 6:837–47.

Ho, M., K.-Y. Wu, H.-M. Chein, L.-C. Chen, and T.-J. Cheng. 2011. Pulmonary toxicity of inhaled nanoscale and fine zinc oxide particles: mass and surface area as an exposure metric. Inhalation toxicology 23:947–56.

Hopfinger, A. J., S. Wang, J. S. Tokarski, B. Jin, M. Albuquerque, P. J. Madhav, and C. Duraiswami. 1997. Construction of 3D-QSAR Models Using the 4D-QSAR Analysis Formalism. Journal of the American Chemical Society 119:10509–10524.

Hornung, V., F. Bauernfeind, A. Halle, E. O. Samstad, H. Kono, K. L. Rock, K. A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nature immunology 9:847–56.

Hristozov, D. R., A. Zabeo, C. Foran, P. Isigonis, A. Critto, A. Marcomini, and I. Linkov. 2014. A weight of evidence approach for hazard screening of engineered nanomaterials. Nanotoxicology 8:72–87.

Ilinskaya, A., and M. Dobrovolskaia. 2013a. Nanoparticles and the blood coagulation system. Benefits of nanotechnology. Nanomedicine 8:773–84.

Ilinskaya, A., and M. Dobrovolskaia. 2013b. Nanoparticles and the blood coagulation system. Part II: safety concerns. Nanomedicine (London, England) 8:969–81.

ISO. 1996. Particle size analysis -- Photon correlation spectroscopy.

ISO. 2002. Surface chemical analysis -- High-resolution Auger electron spectrometers -- Calibration of energy scales for elemental and chemical-state analysis.

ISO. 2003. ISO 18757:2003 - Fine ceramics (advanced ceramics, advanced technical ceramics) -- Determination of specific surface area of ceramic powders by gas adsorption using the BET method.

ISO. 2004. Microbeam analysis -- Scanning electron microscopy -- Guidelines for calibrating image magnification.

ISO. 2005. Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 1: Mercury porosimetry.

ISO. 2006a. Surface chemical analysis -- Auger electron spectroscopy -- Derivation of chemical information.

ISO. 2006b. ISO 15901-2:2006 - Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 2: Analysis of mesopores and macropores by gas adsorption.

ISO. 2007a. Determination of particle size distribution -- Single particle light interaction methods -- Part 2: Light scattering liquid-borne particle counter.

ISO. 2007b. Determination of particle size distribution -- Single particle light interaction methods -- Part 3: Light extinction liquid-borne particle counter.

ISO. 2007c. Determination of particle size distribution -- Single particle light interaction methods -- Part 4: Light scattering airborne particle counter for clean spaces.

ISO. 2007d. ISO 15901-3:2007 - Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 3: Analysis of micropores by gas adsorption.

ISO. 2009a. Particle size analysis -- Laser diffraction methods.

ISO. 2009b. Determination of particle size distribution -- Differential electrical mobility analysis for aerosol particles.

ISO. 2010a. ISO 9277:2010 - Determination of the specific surface area of solids by gas adsorption -- BET method.

ISO. 2010b. ISO/TS 10867:2010 - Nanotechnologies -- Characterization of single-wall carbon nanotubes using near infrared photoluminescence spectroscopy.

ISO. 2010c. Surface chemical analysis -- X-ray photoelectron spectrometers -- Calibration of energy scales.

ISO. 2011a. ISO 22309:2011 - Microbeam analysis -- Quantitative analysis using energy-dispersive spectrometry (EDS) for elements with an atomic number of 11 (Na) or above.

ISO. 2011b. ISO/TS 10868:2011 - Nanotechnologies -- Characterization of single-wall carbon nanotubes using ultraviolet-visible-near infrared (UV-Vis-NIR) absorption spectroscopy.

ISO. 2011c. ISO/TS 10798:2011 - Nanotechnologies -- Charaterization of single-wall carbon nanotubes using scanning electron microscopy and energy dispersive X-ray spectrometry analysis.

ISO. 2011d. Nanotechnologies -- Characterization of single-wall carbon nanotubes using thermogravimetric analysis.

ISO. 2012a. ISO 9276-6:2008 - Representation of results of particle size analysis -- Part 6: Descriptive and quantitative representation of particle shape and morphology.

ISO. 2012b. ISO 13099-2:2012 - Colloidal systems -- Methods for zeta-potential determination -- Part 2: Optical methods.

ISO. 2012c. ISO/TS 10797:2012 - Nanotechnologies -- Characterization of single-wall carbon nanotubes using transmission electron microscopy.

ISO. 2012d. ISO/TR 10929:2012 - Nanotechnologies -- Characterization of multiwall carbon nanotube (MWCNT) samples.

ISO. 2013a. ISO/TR 13097:2013 - Guidelines for the characterization of dispersion stability.

ISO. 2013b. ISO 13099-3:2014 - Colloidal systems -- Methods for zeta potential determination -- Part 3: Acoustic methods.

ISO. 2102. ISO 13099-1:2012 - Colloidal systems -- Methods for zeta-potential determination -- Part 1: Electroacoustic and electrokinetic phenomena.

Jensen, K. A., Y. Kembouche, and S. H. Nielsen. 2013. Nanogenotox deliverable 4.7: Hydrochemical reactivity, solubility, and biodurability of NANOGENOTOX nanomaterials. Copenhagen.

Jiang, J., G. Oberdörster, and P. Biswas. 2008. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. Journal of Nanoparticle Research 11:77–89.

JIS. 2005. Method for particle size determination in metal catalysts by electron microscope.

JIS. 2007. Measurement of lattice parameters and crystallite sizes of carbon materials.

Joris, F., B. B. Manshian, K. Peynshaert, S. C. De Smedt, K. Braeckmans, and S. J. Soenen. 2013. Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap. Chemical Society reviews 42:8339–59.

Kermanizadeh, A. 2013. Engineered Nanomaterial Impact in the Liver following Exposure via an Intravenous Route–The Role of Polymorphonuclear Leukocytes and Gene Expression in the Organ. Journal of Nanomedicine & Nanotechnology 4:1–7.

van Kesteren, P. C. E., F. Cubadda, H. Bouwmeester, J. C. H. van Eijkeren, S. Dekkers, W. H. de Jong, and A. G. Oomen. 2014. Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology 0:1–10.

Klaine, S. J., P. J. J. Alvarez, G. E. Batley, T. F. Fernandes, R. D. Handy, D. Y. Lyon, S. Mahendra, M. J. McLaughlin, and J. R. Lead. 2008. NANOMATERIALS IN THE ENVIRONMENT: BEHAVIOR, FATE, BIOAVAILABILITY, AND EFFECTS. Environmental Toxicology and Chemistry 27:1825.

Klebe, G., U. Abraham, and T. Mietzner. 1994. Molecular Similarity Indices in a Comparative Analysis (CoMSIA) of Drug Molecules to Correlate and Predict Their Biological Activity. Journal of Medicinal Chemistry 37:4130–4146.

Knudsen, K. B., H. Northeved, P. K. Ek, A. Permin, T. L. Andresen, S. Larsen, K. M. Wegener, H. R. Lam, and J. Lykkesfeldt. 2014. Differential toxicological response to positively and negatively charged nanoparticles in the rat brain. Nanotoxicology 8:764–74.

Kreyling, W. G. 1992. Intracellular particle dissolution in alveolar macrophages. Environmental health perspectives 97:121–6.

Kreyling, W. G., M. Semmler-Behnke, and Q. Chaudhry. 2010. A complementary definition of nanomaterial. Nano Today 5:165–168.

Lai, D. Y. 2012. Toward toxicity testing of nanomaterials in the 21st century: a paradigm for moving forward. Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology 4:1–15.

Landsiedel, R., E. Fabian, L. Ma-Hock, B. van Ravenzwaay, W. Wohlleben, K. Wiench, and F. Oesch. 2012. Toxico-/biokinetics of nanomaterials. Archives of toxicology 86:1021–60.

Landsiedel, R., L. Ma-Hock, A. Kroll, D. Hahn, J. Schnekenburger, K. Wiench, and W. Wohlleben. 2010. Testing metal-oxide nanomaterials for human safety. Advanced materials (Deerfield Beach, Fla.) 22:2601–27.

Le, T., V. C. Epa, F. R. Burden, and D. A. Winkler. 2012. Quantitative structure-property relationship

modeling of diverse materials properties. Chemical reviews 112:2889–919.

Li, D., G. Johanson, C. Emond, U. Carlander, M. Philbert, and O. Jolliet. 2014. Physiologically based pharmacokinetic modeling of polyethylene glycol-coated polyacrylamide nanoparticles in rats. Nanotoxicology 8 Suppl 1:128–37.

Li, Y., C. Yang, X. Guo, Z. Dang, X. Li, and Q. Zhang. 2015. Effects of humic acids on the aggregation and sorption of nano-TiO2. Chemosphere 119:171–6.

Lidén, G. 2006. Dustiness testing of materials handled at workplaces. The Annals of occupational hygiene 50:437–9.

Lin, M. Y., H. Lindsay, D. A. Weitz, R. Ball, R. Klein, and P. Meakin. 1990. Universal reaction-limited colloid aggregation. Physical Review A 41:2005–2020.

Lin, S., and M. R. Wiesner. 2012. Theoretical investigation on the interaction between a soft particle and a rigid surface. Chemical Engineering Journal 191:297–305.

Linkov, I., P. Welle, D. Loney, A. Tkachuk, L. Canis, J. B. Kim, and T. Bridges. 2011. Use of multicriteria decision analysis to support weight of evidence evaluation. Risk analysis : an official publication of the Society for Risk Analysis 31:1211–25.

Liu, H. H., and Y. Cohen. 2014. Multimedia environmental distribution of engineered nanomaterials. Environmental science & technology 48:3281–92.

Liu, H., L. Ma, J. Liu, J. Zhao, J. Yan, and F. Hong. 2010. Toxicity of nano-anatase TiO 2 to mice: Liver injury, oxidative stress. Toxicological & Environmental Chemistry 92:175–186.

Liu, H., L. Ma, J. Zhao, J. Liu, J. Yan, J. Ruan, and F. Hong. 2009. Biochemical toxicity of nano-anatase TiO2 particles in mice. Biological trace element research 129:170–80.

Liu, R., H. Y. Zhang, Z. X. Ji, R. Rallo, T. Xia, C. H. Chang, A. Nel, and Y. Cohen. 2013. Development of structure-activity relationship for metal oxide nanoparticles. Nanoscale 5:5644–53.

Lynch, I., I. L. Feitshans, and M. Kendall. 2015. "Bio-nano interactions: new tools, insights and impacts": summary of the Royal Society discussion meeting. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 370:20140162-.

Ma, L., J. Zhao, J. Wang, J. Liu, Y. Duan, H. Liu, N. Li, J. Yan, J. Ruan, H. Wang, and F. Hong. 2009. The Acute Liver Injury in Mice Caused by Nano-Anatase TiO2. Nanoscale research letters 4:1275–85.

Mandelbrot, B. 1982. The Fractal Geometry of Nature. Holt, Henry & Company, Inc.

Martin, D., U. Maran, S. Sild, and M. Karelson. 2007. QSPR modeling of solubility of polyaromatic hydrocarbons and fullerene in 1-octanol and n-heptane. The journal of physical chemistry. B 111:9853–7.

Mast, J., and P.-J. De Temmerman. 2016. Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids.

Meesters, J., A. Koelmans, J. Quik, J. Hendriks, and D. van de Meent. 2014. Multimedia modeling of engineered nanoparticles with SimpleBox4nano: model definition and evaluation. Environmental science & technology 48:5726–36.

Metze, K. 2013. Fractal dimension of chromatin: potential molecular diagnostic applications for cancer prognosis. Expert review of molecular diagnostics 13:719–35.

Monopoli, M. P., C. Aberg, A. Salvati, and K. A. Dawson. 2012. Biomolecular coronas provide the biological identity of nanosized materials. Nature nanotechnology 7:779–86.

Monopoli, M. P., D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. B. Bombelli, and K. a Dawson. 2011.

Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles. Journal of the American Chemical Society 133:2525–34.

Moreno, P. A., P. E. Vélez, E. Martínez, L. E. Garreta, N. Díaz, S. Amador, I. Tischer, J. M. Gutiérrez, A. K. Naik, F. Tobar, and F. García. 2011. The human genome: a multifractal analysis. BMC genomics 12:506.

Mura, S., H. Hillaireau, J. Nicolas, B. Le Droumaguet, C. Gueutin, S. Zanna, N. Tsapis, and E. Fattal. 2011. Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. International journal of nanomedicine 6:2591–2605.

Nel, A., T. Xia, L. Mädler, and N. Li. 2006. Toxic potential of materials at the nanolevel. Science (New York, N.Y.) 311:622–7.

Nel, A., T. Xia, H. Meng, X. Wang, S. Lin, Z. Ji, and H. Zhang. 2013. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Accounts of chemical research 46:607–21.

Nohynek, G. J., and E. K. Dufour. 2012. Nano-sized cosmetic formulations or solid nanoparticles in sunscreens: a risk to human health? Archives of toxicology 86:1063–75.

Oberdörster, G., E. Oberdörster, and J. Oberdörster. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environmental health perspectives 113:823–39.

OECD. 2004. The Report From The Expert Group On (Quantitative) Structure-Activity Relationships [(Q)SARs] On The Principles For The Validation of (Q)SARs. Paris.

OECD. 2007a. Guidance document on the validation of (Quantitative) Structure-Activity Relationship [(Q) SAR] models. Paris.

OECD. 2007b. Guidance on grouping of chemicals. Series on testing and assessment No. 80. Paris.

OECD. 2009a. Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials. Paris.

OECD. 2009b. Guidance manual for the testing of manufactured nanomaterials. OECD's sponsorship programme. Paris.

OECD. 2010. Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; first revision.

OECD. 2011. Report of the WPMN expert meeting on inhalation toxicity testing for nanomaterials.

OECD. 2012a. Inhalation toxicity testing: expert meeting on potential revisions to OECD test guidelines and guidance document. Series on the Safety of Manufactured Nanomaterials No. 35. Paris.

OECD. 2012b. Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. Paris.

OECD. 2012c. The Adverse Outcome Pathway for skin sensitisation initiated by covalent binding to proteins. Part 2: Use of the AOP to develop chemical categories and integrated assessment and testing approaches. Series on testing and assessment No. 168. Paris.

OECD. 2012d. The Adverse Outcome Pathway for skin sensitisation initiated by covalent binding to proteins Part 1: Scientific evidence. Series on testing and assessment No. 168.

OECD. 2014a. Report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines. Series on the safety of manufactured nanomaterials No. 41. Paris.

OECD. 2014b. Ecotoxicology and environmental fate of manufactured nanomaterials: Test guidelines. Expert meeting report. Series on the safety of manufactured nanomaterials No. 40. Paris.

OECD. 2014c. Genotoxicity of manufactured nanomaterials: Report of the OECD expert meeting. Series on the safety of manufactured nanomaterials No. 43.

OECD. 2014d. Ecotoxicology and Environmental Fate of Manufactured Nanomaterials:Test Guidelines Expert Meeting Report. Paris.

OECD. 2014e. Guidance on grouping of chemicals. Second edition. Series on testing and assessment No. 194. Paris.

OECD. 2014f. New guidance document on the integrated approach on an integrated approach on testing and assessment (IATA) for skin corrosion and irritation. Series on Testing and Assessment No. 203. Paris.

OECD 2016. Toxicokinetic of manufactured nanomaterials: report from the OECD expert meeting. Series on the Safety of Manufactured Nanomaterials. ENV/JM/MONO(2016)24.

OECD. 2016a. Guidance Document on The Reporting of Defined Approaches to be used within Integrated Approaches to Testing and Assessment. ENV/JM/MONO(2016)28.

OECD. 2016b. Guidance Document for the Use of Adverse Outcome Pathways in Developing Integrated Approaches to Testing and Assessment (IATA). ENV/JM/MONO(2016)67. Series on Testing & Assessment No. 260

Osmond-McLeod, M. J., Y. Oytam, J. K. Kirby, L. Gomez-Fernandez, B. Baxter, and M. J. McCall. 2014. Dermal absorption and short-term biological impact in hairless mice from sunscreens containing zinc oxide nano- or larger particles. Nanotoxicology 8 Suppl 1:72–84.

Park, E.-J., J. Yi, K.-H. Chung, D.-Y. Ryu, J. Choi, and K. Park. 2008. Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicology letters 180:222–9.

Pashley, R., and M. Karaman. 2005. Applied Colloid and Surface Chemistry. John Wiley & Sons.

Patlewicz, G., N. Ball, E. D. Booth, E. Hulzebos, E. Zvinavashe, and C. Hennes. 2013a. Use of category approaches, read-across and (Q)SAR: general considerations. Regulatory toxicology and pharmacology : RTP 67:1–12.

Patlewicz, G., D. W. Roberts, A. Aptula, K. Blackburn, and B. Hubesch. 2013b. Workshop: use of "read-across" for chemical safety assessment under REACH. Regulatory toxicology and pharmacology 65:226–8.

Petosa, A. R., D. P. E. B. P. Jaisi, I. R. Quevedo, M. Elimelech, N. Tufenkji, and N. Haven. 2010. Aggregation and deposition of engineered nanomaterials in aquatic environments: role of physicochemical interactions. Environmental science & technology 44:6532–49.

Pippa, N., A. Dokoumetzidis, C. Demetzos, and P. Macheras. 2013. On the ubiquitous presence of fractals and fractal concepts in pharmaceutical sciences: a review. International journal of pharmaceutics 456:340–52.

Poater, A., A. Gallegos Saliner, M. Solà, L. Cavallo, and A. P. Worth. 2010. Computational methods to predict the reactivity of nanoparticles through structure-property relationships. Expert opinion on drug delivery 7:295–305.

Powell, J. J., N. Faria, E. Thomas-McKay, and L. C. Pele. 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. Journal of autoimmunity 34:J226-33.

Praetorius, A., M. Scheringer, and K. Hungerbühler. 2012. Development of environmental fate models for engineered nanoparticles-a case study of TiO2 nanoparticles in the Rhine River.

Environmental science & technology 46:6705–13.

Prasad, R. Y., K. Wallace, K. M. Daniel, A. H. Tennant, R. M. Zucker, J. Strickland, K. Dreher, A. D. Kligerman, C. F. Blackman, and D. M. Demarini. 2013. Effect of treatment media of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle. ACS Nano 7:1929–1942.

Puzyn, T., A. Gajewicz, D. Leszczynska, and J. Leszczynski. 2010. Nanomaterials - the next great challenge for QSAR modelers. Pages 383–403in T. Puzyn, J. Leszczynski, and M. T. D. Cronin, editors.Recent advances in QSAR studies. Springer, New York.

Puzyn, T., D. Leszczynska, and J. Leszczynski. 2009. Toward the development of "nano-QSARs": advances and challenges. Small (Weinheim an der Bergstrasse, Germany) 5:2494–509.

Puzyn, T., B. Rasulev, A. Gajewicz, X. Hu, T. P. Dasari, A. Michalkova, H.-M. Hwang, A. Toropov, D. Leszczynska, and J. Leszczynski. 2011. Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature nanotechnology 6:175–8.

Quik, J. T. K., M. C. Stuart, M. Wouterse, W. Peijnenburg, A. J. Hendriks, and D. van de Meent. 2012. Natural colloids are the dominant factor in the sedimentation of nanoparticles. Environmental toxicology and chemistry / SETAC 31:1019–22.

Quik, J. T. K., I. Velzeboer, M. Wouterse, A. A. Koelmans, and D. van de Meent. 2014. Heteroaggregation and sedimentation rates for nanomaterials in natural waters. Water Research 48:269–279.

Rallo, R., B. France, R. Liu, S. Nair, S. George, R. Damoiseaux, F. Giralt, A. Nel, K. Bradley, and Y. Cohen. 2011. Self-organizing map analysis of toxicity-related cell signaling pathways for metal and metal oxide nanoparticles. Environmental science & technology 45:1695–702.

Randic, M. 1975. Characterization of molecular branching. Journal of the American Chemical Society 97:6609–6615.

Rauscher, H., G. Roebben, V. Amenta, A. Boix Sanfeliu, L. Calzolai, H. Emons, C. Gaillard, N. Gibson, T. Linsinger, A. Mech, L. Quiros Pesudo, K. Rasmussen, J. Riego Sintes, B. Sokull-Klüttgen, H. Stamm, and E. Commission. 2014. Towards a review of the EC Recommendation for a definition of the term "nanomaterial"; Part 1: Compilation of information concerning the experience with the definition . Pages (H. Rauscher and G. Roebben, Eds.). European Commission Joint Research Centre.

Rivolo, P. 2012. Encyclopedia of Nanotechnology. Pages (B. Bhushan, Ed.). Springer Netherlands, Dordrecht.

Rivolo, P., J. Rose, M. Auffan, O. Proux, V. Niviere, and J. Y. Bottero. 2012. Encyclopedia of Nanotechnology. Pages (B. Bhushan, Ed.). Springer Netherlands, Dordrecht.

Roebben, G., H. Rauscher, V. Amenta, K. Aschberger, A. Boix Sanfeliu, L. Calzolai, H. Emons, C. Gaillard, N. Gibson, U. Holzwarth, T. Linsinger, K. Rasmussen, J. Riego Sintes, B. Sokull-Klüttgen, H. Stamm, and E. Commission. 2014. Towards a review of the EC Recommendation for a definition of the term "nanomaterial"; Part 2: Assessment of collected information concerning the experience with the definition. Pages (G. Roebben and H. Rauscher, Eds.). European Commission Joint Research Centre.

Salatin, S. and Yari Khosroushahi, A. (2017), Overviews on the cellular uptake mechanism of polysaccharide colloidal nanoparticles. Journal of Cellular and Molecular Medicine. doi:10.1111/jcmm.13110

Sargent, L. M., A. F. Hubbs, S. H. Young, M. L. Kashon, C. Z. Dinu, J. L. Salisbury, S. A. Benkovic, D. T. Lowry, A. R. Murray, E. R. Kisin, K. J. Siegrist, L. Battelli, J. Mastovich, J. L. Sturgeon, K. L. Bunker, A. A. Shvedova, and S. H. Reynolds. 2012. Single-walled carbon nanotube-induced mitotic disruption.

Mutation Research - Genetic Toxicology and Environmental Mutagenesis 745:28–37.

Savolainen, K., U. Backman, D. Brouwer, B. Fadeel, T. Fernandes, T. Kuhlbusch, R. Landsiedel, I. Lynch, and L. Pylkkänen. 2013. Nanosafety in Europe 2015-2025 : Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations Nanosafety in Europe Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations. EDITA, Helsinki.

SCCS. 2012. Guidance on the safety assessment of nanomaterials in cosmetics. Scientific Committee on Consumer Safety, Brussels.

SCCS. 2013. Memorandum on Relevance, Adequacy and Quality of Data in Safety Dossiers on Nanomaterials. Scientific Committee on Consumer Safety, Brussels.

SCCS. 2015. Notes of guidance for the testing of cosmetic ingredients and their safety evaluation. Scientific Committee on Consumer Safety, Brussels.

SCENIHR. 2009. Risk Assessment of Products of Nanotechnologies. Scientific Committee on Emerging and Newly Identified Health Risks, Brussels.

SCENIHR. 2010. Risks Scientific Basis for the Definition of the Term " nanomaterial ." Brussels.

Schultz, C., K. Powell, A. Crossley, K. Jurkschat, P. Kille, A. J. Morgan, D. Read, W. Tyne, E. Lahive, C. Svendsen, and D. J. Spurgeon. 2014. Analytical approaches to support current understanding of exposure, uptake and distributions of engineered nanoparticles by aquatic and terrestrial organisms. Ecotoxicology (London, England) 24:239–61.

Sellers, K., N. Deleebeek, M. Messiaen, M. Jackson, E. Bleeker, D. Sijm, and F. Broekhuizen. 2015. Grouping nanomaterials A strategy towards grouping and read-across.

Shao, C.-Y., S.-Z. Chen, B.-H. Su, Y. J. Tseng, E. X. Esposito, and A. J. Hopfinger. 2013. Dependence of QSAR models on the selection of trial descriptor sets: a demonstration using nanotoxicity endpoints of decorated nanotubes. Journal of chemical information and modeling 53:142–58.

Smoluchowski, M. V. 1917. Versuch einer mathematischen Theorie der Koagulationskinetik. Zeitschrift für Physikalische Chemie 92:129–168.

Soliman, M. M., H. F. Attia, M. M. Hussein, E. H. Mohamed, and T. A. Ismail. 2013. Protective effect of N-Acetylcystiene aganst titanium dioxide nanoparticles modulate immune responses in male albino rats. American Journal of Immunology 9:148–158.

Stefaniak, A., M. Hoover, G. Day, A. Ekechukwu, G. Whitney, C. Brink, and R. Scripsick. 2005. Characteristics of Beryllium Oxide and Beryllium Metal Powders for Use as Reference Materials. Journal of ASTM International 2:15.

Stern, S. T., and S. E. McNeil. 2008. Nanotechnology safety concerns revisited. Toxicological sciences : an official journal of the Society of Toxicology 101:4–21.

Stone, V., H. Johnston, and R. P. F. Schins. 2009. Development of in vitro systems for nanotoxicology: methodological considerations. Critical reviews in toxicology 39:613–26.

Sycheva, L. P., V. S. Zhurkov, V. V Iurchenko, N. O. Daugel-Dauge, M. A. Kovalenko, E. K. Krivtsova, and A. D. Durnev. 2011. Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo. Mutation research 726:8–14.

Taft, W. 1956. Steric Effects in Organic Chemistry. Pages (M. S. Newman, Ed.). John Wiley & Sons Inc., New York.

Tantra, R., C. Oksel, T. Puzyn, J. Wang, K. N. Robinson, X. Z. Wang, C. Y. Ma, and T. Wilkins. 2014. Nano(Q)SAR: Challenges, pitfalls and perspectives. Nanotoxicology:1–7.

Teeguarden, J. G., P. M. Hinderliter, G. Orr, B. D. Thrall, and J. G. Pounds. 2007. Particokinetics In Vitro: Dosimetry Considerations for In Vitro Nanoparticle Toxicology Assessments. Toxicological Sciences 97:614–614.

Todeschini, R., and Consonni Viviana. 2009. Molecular Descriptors for Chemoinformatics. Pages (R. Mannhold, H. Kubinyi, and G. Folkers, Eds.). Second edition. Wiley.

Tran, C. L., D. Buchanan, R. T. Cullen, A. Searl, and A. D. Jones. 2000. Inhalation of poorly soluble particles. II Influence of particle surface area on inflammation and clearence. Inhalation Toxicology 12:1113–1126.

Trouiller, B., R. Reliene, A. Westbrook, P. Solaimani, and R. H. Schiestl. 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer research 69:8784–9.

Tschopp, J., and K. Schroder. 2010. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nature reviews. Immunology 10:210–5.

Union, C. of the E., and Council of the European Union. 1991. Council Directive of 15 July 1991 concerning the placing of plant protection products on the market (91/414/EEC). Official Journal of the European Union L230:290.

Utembe, W., K. Potgieter, A. B. Stefaniak, and M. Gulumian. 2015. Dissolution and biodurability: Important parameters needed for risk assessment of nanomaterials. Particle and fibre toxicology 12:11.

Vandebriel, R. J., and W. H. De Jong. 2012. A review of mammalian toxicity of ZnO nanoparticles. Nanotechnology, science and applications 5:61–71.

Vilanova, O., G. Franzese, and M. Barnabei. 2014. BUBBLES User-friendly Bundle for Bio-nano Large-scale Efficient Simulations.

Vilaseca, P., K. A. Dawson, and G. Franzese. 2013. Understanding and modulating the competitive surface-adsorption of proteins through coarse-grained molecular dynamics simulations. Soft Matter 9:6978.

Vroman, L., and A. L. Adams. 1969. Findings with the recording ellipsometer suggesting rapid exchange of specific plasma proteins at liquid/solid interfaces. Surface Science 16:438–446.

Wang, F., L. Yu, M. P. Monopoli, P. Sandin, E. Mahon, A. Salvati, and K. A. Dawson. 2013. The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine : nanotechnology, biology, and medicine 9:1159–68.

Weed, D. L. 2005. Weight of evidence: a review of concept and methods. Risk analysis : an official publication of the Society for Risk Analysis 25:1545–57.

WHO. 2012. Nanotechnology and human health : Scientific evidence and risk governance. Bonn Germany.

Winter, M., H.-D. Beer, V. Hornung, U. Krämer, R. P. F. Schins, and I. Förster. 2011. Activation of the inflammasome by amorphous silica and TiO2 nanoparticles in murine dendritic cells. Nanotoxicology 5:326–40.

Wohlleben, W. 2012. Validity range of centrifuges for the regulation of nanomaterials: from classification to as-tested coronas. Journal of nanoparticle research : an interdisciplinary forum for nanoscale science and technology 14:1300.

Wolfgang Koch, W. S. 2008. A simple pulmonary retention model accounting for dissolution and macrophage-mediated removal of deposited polydisperse particles.

Wolfram, J., Y. Yang, J. Shen, A. Moten, C. Chen, H. Shen, M. Ferrari, and Y. Zhao. 2014. The nano-plasma interface: Implications of the protein corona. Colloids and surfaces. B, Biointerfaces.

Wu, S., K. Blackburn, J. Amburgey, J. Jaworska, and T. Federle. 2010. A framework for using structural, reactivity, metabolic and physicochemical similarity to evaluate the suitability of analogs for SAR-based toxicological assessments. Regulatory toxicology and pharmacology : RTP 56:67–81.

Xia, X.-R., N. a Monteiro-Riviere, and J. E. Riviere. 2010. An index for characterization of nanomaterials in biological systems. Nature nanotechnology 5:671–5.

Yang, S.-T., Y. Liu, Y.-W. Wang, and A. Cao. 2013. Biosafety and bioapplication of nanomaterials by designing protein-nanoparticle interactions. Small (Weinheim an der Bergstrasse, Germany) 9:1635–53.

Yang, W., J. I. Peters, and R. O. Williams. 2008. Inhaled nanoparticles--a current review. International journal of pharmaceutics 356:239–47.

van der Zande, M., R. J. Vandebriel, M. J. Groot, E. Kramer, Z. E. Herrera Rivera, K. Rasmussen, J. S. Ossenkoppele, P. Tromp, E. R. Gremmer, R. J. B. Peters, P. J. Hendriksen, H. J. P. Marvin, R. L. A. P. Hoogenboom, A. A. C. M. Peijnenburg, and H. Bouwmeester. 2014. Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Particle and fibre toxicology 11:8.

Zhang, H., Z. Ji, T. Xia, H. Meng, C. Low-Kam, R. Liu, S. Pokhrel, S. Lin, X. Wang, Y.-P. Liao, M. Wang, L. Li, R. Rallo, R. Damoiseaux, D. Telesca, L. Mädler, Y. Cohen, J. I. Zink, and A. E. Nel. 2012a. Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS nano 6:4349–68.

Zhang, W., J. Crittenden, K. Li, and Y. Chen. 2012b. Attachment efficiency of nanoparticle aggregation in aqueous dispersions: modeling and experimental validation. Environmental science & technology 46:7054–62.

Zhang, W., B. Rittmann, and Y. Chen. 2011. Size effects on adsorption of hematite nanoparticles on E. coli cells. Environmental science & technology 45:2172–8.

Zhou, H., Q. Mu, N. Gao, A. Liu, Y. Xing, S. Gao, Q. Zhang, G. Qu, Y. Chen, G. Liu, B. Zhang, and B. Yan. 2008. A nano-combinatorial library strategy for the discovery of nanotubes with reduced protein-binding, cytotoxicity, and immune response. Nano letters 8:859–65.

2 Review of computational tools and grouping approaches for nanomaterials

2.1 Introduction

This chapter discusses the practical considerations and opportunities in developing predictive models for the toxicity and fate of NMs. The first part presents an overview of software tools that are available, or under development, with emphasis on the different kinds of modelling approaches adopted. An in depth description of the literature-based models is given in Chapter 3. The second part of the chapter includes experience in the grouping of NMs for the purpose of read-across, and proposals for NM categorisation schemes.

2.2 Practical considerations in developing and applying predictive tools

Developing a predictive model consists of finding a (mathematical) relation between an endpoint (e.g. biological activity) and a more fundamental property (or set of properties) for a given group of substances. There are three key points that will determine the quality of the prediction tool: unequivocal identification of the substance, high quality data at both ends of the mathematical equation and "well behaved" data trends.

2.2.1 Unique identification

In order to have robust relationships between properties and activities, it must be assured that the property and activity that we are trying to relate, e.g. cytotoxicity and conduction band of nanomaterials, correspond to exactly the same substance. This is a relatively simple task in the chemicals world as the chemical structure uniquely defines a chemical identity. In addition, the IUPAC nomenclature provides chemical names that unequivocally define chemical structures and from which structural variations can be understood, e.g. methyl-, ethyl-, propyl-, etc. Even the smallest variations in structure can imply different activities as may occur between enantiomeric forms, which are compounds that have identical composition but different positioning of substituents. The most famous case is probably the different biological activities of the R and S forms of thalidomide(Lenz, 1988). Thus, it is clear that the identification of substances is crucial. In the case of NM there is not (yet) a nomenclature that unequivocally defines a NM entity of a given composition, i.e. a nanoform. In fact, the generation of such a nomenclature is a big challenge given that (in addition to composition) small variations in size, coating, impurities, synthetic route, or crystalline structure can lead to completely diverse biological activities. The main use of such a nomenclature will correspond to the distinction between bulk material and nanoforms, although the distinctions between nanoforms may also become crucial if different nanoforms show different activities (Gerloff et al., 2012a; Uboldi et al., 2016).

At present, and probably in the short- and mid-term future, the only way to determine whether two nanoforms are the same is to carry out an extensive characterization process. Characterization of nanoforms and/or nanomaterials is a complex issue. The NanoDefine FP7 project (NanoDefine, 2013) will produce guidance on the nanomaterial characterization process to identify/classify any substance or mixture in accordance with the EC

recommendation for the definition of nanomaterial (2011/696/EU). NanoDefine focuses on the following characteristics in order to identify a nanomaterial:

- Type of matrix (only for consumer products containing NMs)
- Chemical composition
- Nanoscaled dimensions and shape
- Presence of different sized particles
- Trade form and dispersibility
- Stability of particles during testing
- Specific properties

Each of these properties is further subdivided into more specific ones like the types of composite (core/shell, multiple coatings, mix of two or more materials) or whether the material can be dispersed in specific media. The reader is referred to the original source for further details (Gaillard, Mech, & Rauscher, 2015).

One of the main contributions to a nano-specific nomenclature that can allow for a unique identification of NMs is the one proposed in 2009 by Gentleman & Chan. It consists of a hierarchical codification system reminiscent of library classification taxonomies. In practice, it is a string of fields with typographic codes addressing composition, size, shape, and physicochemical properties. An example is shown below:

"Chemical Class"-"Size and shape"-"Core chemistry"-"Ligand chemistry"-"Solubility"

The full codification system is shown in Table 2.1. Such a format is designed to facilitate digital archiving and searching. The identity of the nanostructures can be directly read from the code, and the codes are relatively short compared to the complexity of the structures they define. It is reminiscent of the IUPAC chemical nomenclature. For instance, alcohols are indicated as –ol and a number indicates its position in a chain (i.e. 2-butanol). With Gentle and Chapman nomenclature, numbers can indicate the type of substance (i.e. organic or inorganic NM), as well as size, depending on the field in which the number belongs to. For instance, 2-90H(6)-(Pb,S) corresponds to:

- 2 indicates inorganic
- 90H(6) indicates size 90 nm of a polyhedron with 6 faces (a cube)
- (Pb,S) indicates the composition of the NM

This nomenclature allows the easy comparison of NMs by stripping its names. For instance, the third field (Core chemistry) can be used to group NMs that have a core that contains Au, or the last field (Solubility) can be used to group NMs by a measure of solubility (e.g. logD). More details and examples can be found in Table 2.2..

Another nomenclature presented for annotating NMs formulations and its material parts or entities based on computable string expressions was proposed by Thomas et al. (2012). The string expression also consists of numbered labels separated by hyphens, e.g. F1-N1-M1, F1-N2-M1, or F1-N2-S1 (see Table 2.2). The labels correspond to unique material parts and in practice indicate whether it is the whole nanoparticle formulation itself or one of its parts. The numbers are key in this nomenclature as they ultimately differentiate each of the parts, i.e. F1 corresponds to the first formulation of a database (e.g. Au NP), F2 to the second (e.g.

SiO$_2$ NM), and so on. Similarly, D1 would correspond to a specific medium like deionised water, D2 to another specific medium like glycerin, and so forth (see Table 2.3 for more examples). These numbers are predefined and follow the NanoParticle Ontology (NPO), which can be accessed at http://purl.bioontology.org/ontology/npo.

Another nomenclature that has a more atomistic character was proposed by Toropov and Leszczynski (2006). Most of their examples correspond to bulk material, but it can be applied to any NM. They proposed a SMILES-like nomenclature for nanomaterials that contain data on atom composition and technological conditions of the synthesis of the nanomaterials. The SMILES-like codes were used to define descriptors that were ultimately used to predict Young's modulus (elasticity) of NMs.

Table 2.1. Codification protocols of the nanomaterial nomenclature proposed by Gentleman and Chan.

Chemical class			Size and shape			core chemistry		ligand chemistry		Solubility	
XT1T2			r(re)M1M1b(m2)M3M4M5			(Z1,Z2,...,Zn)		[(fi,fe)1;(fi,fe)2;...;(fi,fe)n]		S[log D(pH)]	
X	1 if organic/ fullerene (contains no metals)	r	smallest defining dimension in nm			0	if no core	0	if no ligands	S	
	2 if inorganic/ organometallic	re	other defining size (if applicable)			Z1, Z2,..Zn	list core elements in conventional order; dopants can be included if known	fi	(see functional group code) on inside/adsorbed to core	O	if logD>1
T1	1=outermost chemistry	M1	B=ball			/	indicates inter-core boundary, for example (CD,Se/Zn,S) is a core/shell	fe	(see functional group code) outer functional group	W	if logD<-1
	D=dendrimer		H=polyhedron/faceted					/	indicates multilayer structures, for example [[(fi,fe)/(fi,fe)] is a bilayer for nested structures, only indicate outermost shell	OW	if -1<logD<1
	F=fullerene		R=rod/wire						bioconjugation[[(fi,fe/Bio)] or [(fi,Bio)] sheet structure, list twice, for example CNT [(Ful,Ful)]		indicate logD and pH of measurement (if known)
	L=liposome		P=plate/disc/well								
	P=polymer	M1b (M1 value not necessary)	A=astral(not after B)								
T2	N=nested		I=irregular								
		m2 (omit if unknown)	B(b),b=# radii: 1- spheroid 2-ellipsoid								
			H(h), h=# faces								
			R(r), r=# barrel faces, 0= cilinder								
			P(p),p=# sides, 0=circle								
			A(a),a=# arms								
		M3	L if elongated								
		M4	T if teethed/ jagged edges								
		M5	C if coiled/helical/twisted								

Abbreviations for functional groups used in ligand chemistry field

Functional Group	Abbrev								
acyl halide	Ach	haloalkane	Hak	phehyl/benzyl	Bnz	phosphine oxide	Pox	leucine	Leu
alanine	Ala	hydroxyperoxide	Hoo	carbonate	Cba	phosphodiester	Pde	lysine	Lys
acrylic	Acr	imine	Imn	cyanide	Cyd	phosphate	Pha	methionine	Met
arginine	Arg	imide	Imd	isocyanide	Icy	phosphonic acid	Poa	phenylalanin	Phe
alcohol	Alc	ketone	Ket	cyanate	Cya	pyridine	Pyr	proline	Pro
aldehyde	Ald	nitrate	Nta	isocyanate	Ica	sulphide	Sde	serine	Ser
alkyl	Alk	nitrile	Ntl	thiocyanate	Tca	sulphone	Soo	threonine	Thr
amide	Amd	nitro	Nto	isothiocyanate	Itc	sulphonic acid	Soa	tryptophan	Try

Some examples are:

NM	Code	NM	Code
~90nm wide PbS nanocubes aminoalkane cap	2-90H(6)-(Pb,S)-[(Amn,Alk)]-O	20-nm thick ZnO nanohelices, no cap	2-20P(4)LT-(Zn,O)-0-W
20-nm thick ZnO nanohelices, no cap	2-20P(4)LT-(Zn,O)-0-W	7-nm diameter fullerene MWNT	1FN-7RL-0-[(Ful,Ful)]-O
Gd atom inside hydroxylated buckyball [Gd@C82(OH)16] 7-nm	2F-1B(1)-(Gd)-[(Ful,Ful);(Ful,Alc)]-O	4-nm diameter CdSe NC capped with PAMAM dendrimer	2D-4H-(Cd,Se)-[(Amn,Amn)-O
57-nm diameter silica-coated Au NPs; poly(DMAEMA) cap biofunctionalized	2-57B(1)-(Au/Si,O)-P[(Amn,Acr/Bio)]-W	r-nm diameter star polymer 50% alkyl, 50% hydroxyl	1P-rA-(C)-[(Bnz,Alk);(Bnz,Alc)]-O

Table 2.2. Definition of the labels proposed by Thomas et al.

Label	Definition
F	Nanoparticle formulation (NPO_868)**Error! Bookmark not defined.***
N	Nanoparticle component (NPO_1496) or nanoparticle (NPO_707)
D	Medium (NPO_1853)
S	Structural parts of a nanoparticle like coat (NPO_1367), core (NPO_1617), and shell (NPO_760); structural parts of complex organic molecules (e.g., the core and branches of a dendrimer); other structural parts like a spacer molecule (NPO_485)
M	All chemical component parts other than the nanoparticle (N): molecular components of the nanoparticle (N), of the medium (D), and of any structural part (S)
L	Linkages (NPO_195) of a nanoparticle formulation: covalent linkage (NPO_563), encapsulation (NPO_138), and entrapment (NPO_471)

*NPO codes correspond to the labels of the Nanoparticle Ontology (http://www.nano-ontology.org/documentation/codes-for-npo-terms)

Table 2.3. Examples of strings annotating NMs.

| Label pairs	String examples	String interpretation
F#-N#	F1-N1, F1-N2	F1-N1 and F1-N2 are two types of nanoparticles in the nanoparticle formulation, F1.
N#-M#	F1-N1-M1, F1-N1-M2	F1-N1-M1 and F1-N1-M2 are molecular components of the nanoparticle, F1-N1
N#-S#	F1-N2-S1	F1-N2-S1 is a structural part of the nanoparticle, F1-N2
M#-S#	F1-M1-S1, F1-M1-S2, F1-M1-S3	F1-M1-S1, F1-M1-S2, F1-M1-S3 are structural parts of a molecular component (e.g., a complex organic molecule), F1-M1.
S#-M#	F1-N2-S1-M1, F1-N2-M1-S1-M1	F1-N2-S1-M1 is a molecular component of the structural part F1-N2-S1. F1-N2-M1-S1-M1 is a molecular component of the structural part F1-N2-M1-S1
F#-D#	F1-D1	F1-D1 is a medium of the nanoparticle formulation, F1.
D#-M#	F1-D1-M1,F1D1-M2	F1-D1-M1 and F1-D1-M2 are molecular components of the medium, F1-D1.
F#-M#	F1-M1,F1-M2	F1-M1 and F1-M2 are chemical component parts (other than the nanoparticle) of the nanoparticle formulation, F1.
M#-M#	F1-N2-S1-M1-M1, F1-N2-S1-M1-M2	F1-N2-S1-M1-M1 and F1-N2-S1-M1-M2 are molecular components of F1-N2-S1-M1.
F#-L#[...;...]	F1-L1[F1-N1;F1-N2], F1-L2[F1-N1-M2; F1-M2]	There is a linkage, F1-L1, existing between the two nanoparticles, F1-N1 and F1-N2. There is a linkage, F1-L2, existing between the molecular

Label pairs	String examples	String interpretation
		components, F1-N1-M2 and F1-M2.

The SMILES-like nomenclature contains information on atom composition (Al, Ti, Zr, O, …), type of substance (bulk or not), and the temperature of synthesis (coded by labels, i.e. a=20ºC, A=22ºC, B=25ºC, C=400ºC, …M=1500ºC)

For instance a ceramic nanomaterial composed of ZrO_2 synthesised at 1200 ºC would be coded as Zr,O,O,CER,%H. One of the shortcomings of this nomenclature is that it is restricted to encoding the available information on the synthesis of the NMs.

Another proposal for the nomenclature of nanomaterials is the Curly-braces enhanced Smart Material Input Line Entry Specification (CurlySMILES) (Drefahl, 2011). CurlySMILES is a chemical language for the specification of chemical materials and supramolecular structures. It provides a format to encode molecular details and extra-molecular features such as non-covalent interactions and attachment to a biomolecule as well as the surface of a substrate material or nanoparticle.

The CurlySmiles notation is a string of dot-separated component notations. For instance, $NaAlH_4$ would be represented as:

[Na+].[AlH4-]
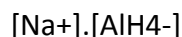
A component notation can consist of a plain SMILES, an annotated SMILES, or a special format notation. A plain notation maintains the grammar and rules of the known SMILES language and is modified by introducing attributes (e.g. structural variations, details and decorations) enclosed in curly braces. An annotation can be anchored to a particular atomic node or placed at the end of a SMILES component. For instance, oleic acid (cis form) would be represented as:

O=C(O)CCCCCCCC=C{Z}CCCCCCCC

A special format notation begins with an opening ({) and ends with a closing curly brace (}) and includes an alias or a notation for a structure that defies molecular-graph encoding. There are different types of special format notations. The most relevant for nanomaterials are the shape and state annotation markers (SSAM). These, correspond to a series of codes accounting for information like nanocrystal (nc), nanodisk (nd), nanoparticle (np), or nanowire (nw). They also encode information such as the crystal state (cr), allotrop name, or phase name (phn) (see http://www.axeleratio.com/csm/proj/stateshapeann.htm for a list of SSAM and definitions. For instance, diamond would be represented as:

[C]{crall=diamond}
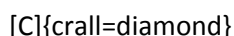
Materials with known atomic or substructural stoichiometry, but without a discrete pattern of finite atom connectivity (molecular structure), e.g. such as minerals are defined by stoichiometric formula notation (SFN), which is a variation of the special format notation as it begins with ({*) and ends with a curly brace (}). For instance, titanium dioxide (anatase) would be represented as:

{*TiO2}{crphn=anatase}

Unfortunately, besides the efforts put into developing nomenclatures, none of the ones presented here have been considered for the databases available today. However, not all the areas of nanochemistry are missing nomenclatures, for instance fullerenes and dendrimers have well-established nomenclatures (Friedhofen & Vögtle, 2006; Godly & Taylor, 1997; Goodson, Gladys, & Worst, 1995) and there is an ongoing IUPAC project dedicated to the nomenclature of CNTs and related substances (Project No.:2013-056-1-800).

Nomenclatures are implicitly linked to ontologies and databases since an ontology is a formalised system that captures the relationships between different concepts / terms (i.e. database objects). Ontologies are further discussed in chapter 2.2 together with databases for NMs.

2.2.2 High quality data at both ends of the mathematical model

One of the main problems that were encountered with the first data generated for nanomaterial was the variability. For instance, it was observed in the ENPRA project that some cell lines showed very high cytotoxicity for all NMs, while others showed no cytotoxic effects at all. Coefficient variations of >30% were not rare in ENPRA and NanoTest, and the Nanogenotox project showed some between laboratory variability when measuring particle size for the very same particles with a precise technique like TEM (c.a. 20nm).

The quality of a predictive model depends on the underlying quality (reliability and relevance) of the data from which the model is built. Any QSAR model, for example, is based on two sets of data: a) properties and b) activities. In relation to data reliability, it must be taken into account that all experimental measurements are subject to experimental variability, which is the consequence of the presence of systematic and/or random errors in the measurements. High quality data is thus data that is obtained by minimising this variability as far as practically possible. Assuming that it is impossible to remove all sources of variability, the practices listed below help to minimise them:

- Use data obtained with the same experimental protocol and preferably a standardised guideline
- Use data measured in the same laboratory and by the same personnel, if possible
- Use data on the very same endpoint
- Use data from compounds that are suitable for the test system, e.g. solubility, volatility, etc.

The reader is referred to the list of existing methods for the measurement of NM physicochemical (PC) properties (Table 1.2), and the list of endpoints and related methods (Table 1.3) that were presented in chapter 1. ISO standards and OECD TG for the specific methods are also included in the tables. Moreover, a detailed review of test methods to determine composition, size, shape, dissolvability, dispersibility, and stability including with performances, applicability domains is provided by Gaillard et al. (2015). The use of standardised tests should assure that the procedures used by two different persons in two different laboratories to measure the same property/endpoint are in fact the same and that the variability that arises from technicalities like time of exposure, bath temperature, or dispersion protocols, are minimised. These steps are important since NMs are suspensions of solids that are subject to many effects such as surface charge, aggregation/agglomeration, adsorption, or temperature that can have huge impact in final measurements. Only by using data that has been obtained with the same experimental

techniques on the very same endpoint increases the quality of the model, but it tends to reduce the number of data available for the model development (or for category formation). Therefore, it is necessary to decide on a suitable trade-off between the amount of data used and the overall data quality needed. It is not recommended to use data for modelling that has been generated with methods not suitable for testing the specific compounds. This is a problem with NMs as many of the testing methods are well stablished for chemicals and have just been adopted for NMs. The applicability or interference of the methods is not assured for all NMs as many of them are metals that may interfere with the test read-out. Due to the lack of standards it is not easy to identify such interferences. For example, in spectrophotometry based tests like MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann, 1983), the NM can mask the read-out (Kroll, Pillukat, Hahn, & Schnekenburger, 2009, 2012), or Ag NPs can interfere with the Griess reaction (Kaempfer et al., 2017).

Evaluation criteria to determine the quality of published experimental data, similar to the Klimisch classification (1997), has been proposed (Lubinski et al., 2013). It consists of a two-dimensional classification indicating the quality of the data and the usefulness for nanoQSAR modelling. The categories are shown in Table 2.4. Each category is determined based on a series of characteristics such as number of data points, data obtained from a single source, experiments carried out according to GLP, degree of characterisation of NPs, etc. The list of properties that determine each category can be found in the original work.

Table 2.4. Lubinski et al. categories to determine data quality and usefulness for QSPR and QSPR

Data quality		Data usefulness	
1+	1+ Reliable with additional information	A+	A+ useful for constructing a single nano-QSPR/nano-QSAR
1	1 Reliable	A	A Useful
2	2 Reliable with restrictions	B	B Useful with restrictions
3	3 Unreliable	C	C Limited use
4	4 Not assignable	D	D Not assignable

Test Guidelines will need to be more detailed for NMs as some of the aspects mentioned above are often not included in guidelines addressed for chemicals and even many details are often overlooked in NM publications. The OECD Working Party on Manufactured Nanomaterials (WPMN) has focused on developing appropriate methods and strategies to address potential safety concerns of NMs. Among others, OECD has been evaluating the guidelines for their adequacy to appropriately address the characterisation of NMs and the assessment of their toxicological properties. The guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials (ENV/JM/MONO(2012)40) (OECD, 2012a) refers specifically to water-insoluble manufactured nanomaterials, including those that can release soluble species, e.g. silver, as it is considered that soluble nanomaterials are unlikely to need different sample preparation techniques than other chemicals, apart from precautions dictated by the specific reactivity of each material. This document provides guidance relevant to sample preparation and dosimetry for

physicochemical characterisation, ecotoxicity studies, degradation, transformation and accumulation studies, and health effects. For example, concerning OECD TG 413 (Subchronic Inhalation Toxicity: 90-day Study), the OECD WPMN recommended modifications addressing the inclusion of BAL (broncho-alveolar lavage) studies for particles and NPs, the development of better guidance criteria for establishing a no observed adverse effect level (NOAEL) or benchmark dose (BMD) and consideration of cell proliferation (BrdU) of various components of the respiratory tract.

2.2.3 Well behaved data trends
"Well behaved" data translates into gradual behaviour (activity) with respect to specific properties or combination of properties (descriptors). When the activity of a test substance gradually increases with the gradual increase of another property, its behaviour is in general easier to predict than when there are sudden changes in activity. Well behaved data is often compared to a smooth mountain landscape, where the height is the activity and the space coordinates are a set of given properties. Unfortunately the real world, e.g. toxicology, drug discovery, or environmental toxicology is usually not similar to a smooth landscape but rather a harsh landscape plenty of cliffs. This implies that linear models, like regression QSARs, or models that are continuous have big difficulties in predicting landscapes that are not linear or continuous (Maggiora, 2006). Thus, trying to predict activities that do not show a gradual behaviour with rather simple linear equations is difficult and subject to a number of pitfalls. Some possibilities consist of defining smaller applicability domains for which the trends are kept, or transforming the continuous variables into discrete bins that allow for classification algorithm. Some authors have transformed specific dependent variables (e.g. cytotoxicity, membrane permeability, ROS) into more generic variables such as "toxicity", which turned out to be easier to model (Rong Liu et al., 2013b). Other possibilities include the use of different modelling techniques such as neural networks or support vector machines, which are usually considered to be "black boxes" as they are difficult to interpret, but that result more appropriate to deal with activity cliffs.

2.2.4 Lack of data and/or knowledge
In addition to uncertainties arising from data reliability (variability) described above, there is also uncertainty that results from lack of data and/or knowledge. In principle, data variability cannot be completely avoided, even though it can be minimised and characterised. In contrast, uncertainty can be reduced by obtaining more data and by better understanding its predictive relevance (WHO, 2014).

Thus, an important consideration in any predictive approach, including QSAR/QSPR, grouping for read-across, is the lack of knowledge on the specific modes of action that determine the ultimate outcome/activity. When dealing with chemicals, very specific chemical or atomic properties, usually computationally derived such as logP, electronegativity (Hansch, 1969), or the simple presence or absence of reactive groups, are used to predict toxicological outcomes. Due to the relative novelty of nanotechnology and the big challenge that NMs represent for computational scientists – nanomaterials do not have a well-defined structure and are too big to be easily calculated with traditional computational models that are meant for structures of less than 3000 atoms – are the main

causes for which there is not (yet) a well-established set of computational properties that can determine toxicological outcomes. The lack of computational properties does not preclude the modelling activity as measured PC properties can be used as model descriptors, although the availability of data is then significantly limited. In fact, there are a number of PC properties that can be experimentally measured on NMs (see Table 1.2 of chapter 1), however it is common practice in the literature to limit the PC characterization to measures of size, size distribution, zeta potential, and crystalline structure (Lubinski et al., 2013). Thus, in practice there is a lack of computationally derived descriptors and of experimental properties measured consistently for a diverse enough group of NMs. However, some advances are being made. For instance, heat of formation, which is a surrogate of redox activity (Burello & Worth, 2011a), seems to be one of the few properties that can be used to predict cellular effects like cytotoxicity (Tomasz Puzyn et al., 2011b). Its extrapolation to *in vivo* effects is more complex, although it has been shown that together with solubility it relates to pulmonary inflammation (Haiyuan Zhang et al., 2012a).

2.3 Models and tools for environmental behaviour and toxicity prediction

The different databases, tools, and models like QSARs, QSPRs, or PBTK that are available for NMs and that could be used in a modelling context are presented in this section.

2.3.1 Databases

The list of databases related to NMs is presented in the table below together with a short description and the links where they can be found. In general, most databases have fully or partially restricted access to data except for eNanomapper, NBI, and Nanomaterial registry, which can be accessed directly. Some of them offer free registration procedures that in principle allow access to data, but in practice, we encountered a number of difficulties (Table 2.5). Overall, the information that is readily available is scarce.

Table 2.5. Existing databases for NM properties

Name & Link	Description and comments on utility
NanoHub/Nano material registry https://nanohub.org/ https://www.nanomaterialregistry.org https://www.nanomaterialregistry.org/Search.aspx	The Nanomaterial Registry, sponsored by NIH, is a central registry and growing repository of publicly-available nanomaterial data which are fully curated based upon a set of Minimal Information about Nanomaterials (MIAN). Each nanomaterial curated into the Registry will provide the following information from a data source, when available: • Physical characteristics – values, protocols, metadata • Information on the related biological and/or environmental studies • Instance of Characterization information – preparation, synthesis, and time frame leading up to the nanomaterial characterization • Validation back to the Data Source – scholarly article Comments: • The system allows searches based on size, agglomeration states, size distribution, surface area, shape, composition, purity, surface charge,

Name & Link	Description and comments on utility
	surface chemistry and reactivity, solubility, stability, type of biological or environmental study, data source, and degree of compliance level. • Biological and environmental data present in the database cannot be downloaded, only physicochemical properties can be exported. • The data on physicochemical properties can be exported as an xls file. • The interface of the webpage is well designed but it is slow at showing the filtered data or browsing across pages. Only 3 nanoforms can be compared simultaneously.
NHECD http://www.nhecd-fp7.eu/	NHECD is a free access, robust and sustainable web based information system including a knowledge repository on the impact of nanoparticles on health, safety and the environment. It includes unstructured data (e.g., scientific papers and other relevant publications) and a mechanism for updating its knowledge repository, thus enabling the creation of a large and developing collection of published data on environmental and health effects following exposure to nanoparticles. The system allows for basic, advanced, intelligent and taxonomic level search features, depending on the specifics required during the retrieval process. This provides a comprehensive solution to wading through copious amounts of information, and provides the user with an option to make either a general or specialised search. Comments: • The system allows to search by model, experiment or nanoparticle attributes among others fields. • The database is not accessible (December '15)
caNanoLab https://caNanoLab.nci.nih.gov	caNanoLab is a data sharing portal that contains: • Nanotechnology protocols in biomedicine • Composition of nanomaterials • Functions of nanomaterials (for example, therapeutic, targeting, diagnostic imaging) • Physico-chemical characterizations including size, molecular weight, shape, physical state, surface chemistry, purity, solubility, and relaxivity • *In Vitro* characterizations such as cytotoxicity, blood contact properties, oxidative stress, and immune cell functions • Publications and reports from nanotechnology studies in biomedicine Comments: • The webportal is very comprehensive with a dedicated wiki • Some data is publicly available but most of it has restricted access • Data cannot be downloaded, only presence of data is shown on the record • Any person can ask for a user account that allows access to a personal restricted space and to upload data. Groups can also be created • The search queries are rather slow
PaFTox http://fraunhofer-repdose.de/	This is an extension of the already existing data for chemicals RepDose. In general, data on application route, nanoscale dimension, reliability, species and study duration are included in the database. In addition, each entry is given a quality criteria (Bitsch et al., 2006).

Name & Link	Description and comments on utility
	Membership is needed in order to access the database. The membership is free and can be obtained directly on the website, although it is not immediate, a couple of days may be necessary to obtain the login details. The dataset was filled with data preferentially obtained from inhalation studies. Regarding the data contained in the database: • Particle data contains: Specifications of primary object by producer/supplier and authors, and secondary object by authors. Size / distribution, aggregation/agglomeration state in the exposure media, shape, specific surface area, composition / purity, surface chemistry, solubility / dispersibility, and surface charge. • Study part contains: application type, exposure, instillation (number, frequency), post-exposure duration, species/strain/sex, number of animals, particle and fibre characteristic as administered (secondary object), reference, reliability, scope of study. • Toxicological data is aimed at in vivo studies, therefore the database is designed to accommodate organ related data. A complete review of the database can be found in Schröder et al. 2014 (Schröder et al., 2014). Comments: • Not accessible.
NBI http://nbi.oregon state.edu/	The NBI Knowledgebase is intended to offer industry, academia, the general public, and regulatory agencies a mechanism to rationally inquire for unbiased interpretation of nanomaterial exposure effects in biological systems. The knowledgebase serves as a repository for annotated data on nanomaterial characterization (*purity, size, shape, charge, composition, functionalization, agglomeration state*), synthesis methods, and nanomaterial-biological interactions (*beneficial, benign or deleterious*) defined at multiple levels of biological organization (molecular, cellular, organismal) Comments: • NMs can be searched by their composition, core, decorations, size, and other parameters. Concentration of NM in zebrafish after exposure is the main biological data found on the database. • The database is easily searchable, and the data is easily downloadable. The data is more abundant on dendrimers and nanocellulose than on metal and metal oxides.
NanoDatabank http://nanoinfo.o rg/nanodatabank /	NanoDatabank consists of a repository and database system of engineered nanomaterial (ENM) properties, experimental and simulation datasets of ENM fate and transport (F&T), as well as toxicity data. Login can be done as guest, lab administrator or system administrator. NanoDatabank contains a number of databases that include: • Physiocochemical properties • Toxicological properties • Experimental datasets of ENM toxicity • Experimental datasets of ENM fate and transport behaviour • Results of simulation predictions and estimation of ENM toxicity and fate behaviour

Name & Link	Description and comments on utility
	Comments: • Membership is required to access the data, and such a procedure is not completed after one week.
Nanotechnology Information Library http://nanoparticlelibrary.net/	The goal of the NIL is to help occupational health professionals, industrial users, worker groups, and researchers organize and share information on nanomaterials, including their health and safety-associated properties. NIOSH has released the NIL web resource in draft form for public review and feedback. Comments: • The database can be browsed and it mainly consists of summaries of publications indicating composition, size, and a link to the original source. • No activity/toxicity data is currently included in the database
Enanomapper https://apps.ideaconsult.net/enanomapper/	It is a public database hosting nanomaterials characterization data and biological and toxicological information. The database provides various possibilities to search and explore information, and to download data in various standard formats. The database supports data upload through configurable Excel templates. It currently contains data from MARINA and MODENA FP7 projects as well as from other sources such as the JRC repository or an inventory of Sigma-Aldrich nanomaterials. Comments: • The webpage allows searches of nanomaterials by identifier, citation, physicochemical properties, biological effects, composition, study purpose, protocol, guideline, or by free text. • The data can be browsed and viewed on the web without need of downloading it. The browsing experience is smooth and the data can be downloaded in different formats, i.e. xls, csv, json. • The dataset is fully open source and can be found on http://ambit.sourceforge.net/. A tool to download IUCLID 5 data is also available on the website. • At the moment the database content on biological data is a bit scarce, although probably projects of the Nanosafety cluster will upload their data once finished.
Modern Project http://modern-fp7.biocenit.cat/	• The modern project has set up a database in ISA-TAB-Nano standard format (Robinson, Cronin, Richarz, & Rallo, 2015) which can accommodate all types of data, e.g. toxicity, physicochemical properties. It is intended to accommodate non-structured data from various projects like PreNanoTox, QualityNano, MARINA, NanoFATE, and MODENA action COST. Comments: • The database is only accessible to members of the consortium.
Nanopuzzles http://nanopuzzles.eu/	A database on ISA-TAB-Nano format with data obtained from the literature including physicochemical properties of NMs and activities such as cytotoxicity or zebra fish mortality was collected by the different partners of the Nanopuzzles project and can be downloaded freely at: http://zenodo.org/record/35493#.Vp5z10YXzw0

Name & Link	Description and comments on utility
BAM http://www.nano.bam.de https://www.webshop.bam.de/default.php?cPath=2282	The web portal holds a registry of reference nanomaterials with data on composition, size, and data found on the safety data sheets. Comments: • The webportal is not a database per se, but includes a list of products with some properties. • No biological data is included in the list. • Some information is found in German, only.
Nanohub JRC http://www.nanohub.eu/	Nanohub JRC is a respository of all the data generated in a number of European and OECD projects on nanotechnology, i.e. ENPRA, MARINA, NANoREG, OECD-WPMN Gold, OECD-PROSPECT, etc. Comments: • The database is not web-based. It is necessary to run a java based client that requires the installation of Java Runtime Environment. • The address does not exist anymore (March 2017)
Nanomaterials registry https://www.healthdata.gov/dataset/nanomaterial-registry	This nanomaterial registry aims at providing consistent information on the biological and environmental interactions of NMs, as well as links to associated publications, modelling tools, computational results. Beyond the possibility to search for NMs on physicochemical properties and categories of NMs, it gives the possibility to identify similar NMs based on physicochemical properties.
Nano http://nano.nature.com/	Nano reports information extracted by indexed papers NMs and nanodevices. Each entry is linked to the source paper and the information is structured as follows: structure, size, composition, properties, characterisation methods, toxicity and biological effects, preparation/synthesis methods, applications, patents claims. Comments: • data present in the database cannot be downloaded

2.3.2 Database ontologies

An ontology can be defined as a framework of concepts related to a specific domain (e.g. nanotechnology). The main advantage of ontologies is that they facilitate the exchange of information between communities as they provide limited sets of terms (jargon) to refer to specific entities. Its main function is found in databases as clear and unique terms or concepts are needed in order to store data in a structured manner, and to deliver it in when needed. Concepts are usually called classes and have associated and unique IDs and labels, and may have synonym(s), definitions, and other associated "properties". For instance, some classes of the eNanomapper FP7 project (www.enanomapper.net) ontology are "material entity", "process", "quality", or "disposition".

Classes can also have subclasses such as "cell line" and "chemical substance" to "material entity"; or "adverse event" and "synthesis part" to "process" in the case of enanomapper. Subclasses can also have further sub-subclasses, and so on. The relation between classes and subclasses determines the structure of the ontology. In addition to properties, classes can have "mappings" to other ontologies, which indicate the classes of one ontology that correspond to the same class(es) of other ontologies. For instance the enanomapper class "nanoemulsion", which is a subclass of "material entity", is mapped to "Nanoemulsion" and "nanoemulsion" of the National Cancer Institute Thesaurus and the Nanoparticle Ontology, respectively. In fact, enanomapper is developing ontology for NMs by reusing and extending existing ontologies of relevance for the nanosafety domain. This exercise (Hastings et al., 2015) is intended to harmonise data from different fields and enable data sharing. This ontology covers among other areas NM classes, NMs physicochemical properties, biological characterisation of NMs, and environmental characterisation..

Thus, the way in which the ontology is built will determine the way entities or objects, nanoparticles in our case, are defined. The ontologies from which enanomapper ontology are derived are listed in Table 2.6.

Table 2.6. List of ontologies, acronyms and links included in the enanomapper ontology.

Acronym	Ontology	Link
NPO	NanoParticle Ontology	http://bioportal.bioontology.org/ontologies/NPO
ENM	eNanoMapper Ontology	http://purl.enanomapper.net/onto/enanomapper.owl
ENVO	Environmental Ontology	http://purl.bioontology.org/ontology/ENVO
IAO	Information Artifact Ontology	http://purl.bioontology.org/ontology/IAO
CHEMINF	Chemical Information Ontology	http://code.google.com/p/semanticchemistry/
OBI	Ontology for Biomedical Investigations	http://obi-ontology.org/
BAO	BioAssay Ontology	http://bioassayontology.org/
ChEBI	Chemical Entities of Biological Interest	http://www.ebi.ac.uk/chebi/
UO	Unit Ontology	http://code.google.com/p/unit-ontology/
OAE	Ontology of Adverse Events	http://purl.bioontology.org/ontology/OAE
PATO	Phenotype and Quality Ontology	https://code.google.com/p/pato/

Ontologies are not fixed and that they evolve in time by adopting new terms, properties and mappings. For instance, a nanoparticle in the nanoparticle ontology (NPO) is represented as shown in Figure 2.1 by properties accounting for particle size, shape, mass, chemical composition of coating(s), and surface related properties such as surface area, chemical composition, surface charge, and zeta potential.
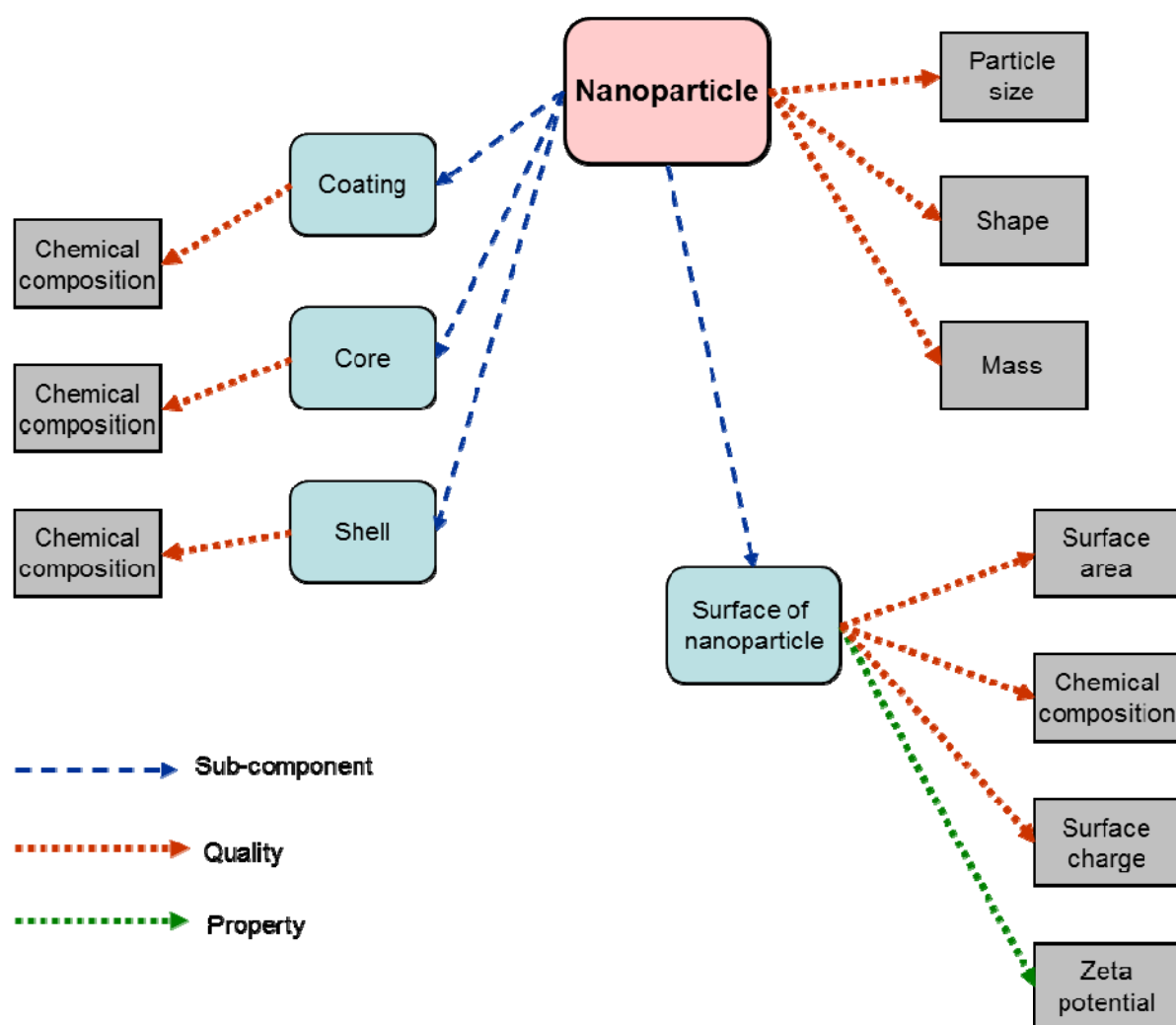
Figure 2.1. Representation of a NP in the NPO ontology. Adapted from http://www.nano-ontology.org/

2.4 Existing (proposals for) grouping approaches

The objective of this section is to report the state-of-the-art on grouping approaches for NMs. Various schemes for the categorisation of NMs have been proposed covering a variety of assessment goals, including: a) priority setting of NMs for further evaluation (including ranking based on level of concern) (e.g. Nel et al. 2013; Cockburn et al. 2012), b) guiding the choice of relevant endpoints and methods in testing strategies (Stone et al. 2013; Godwin et al. 2015) and c) grouping and read-across for the purpose of filling data gaps in regulatory submissions (e.g. Sellers et al. 2015), which is the application of particular interest in this report. It should be noted that terminology is not used consistently in different sources, so that "grouping" does not always correspond with the use of this term under REACH (European Parliament and Council, 2006b), where it is defined as follows:

"Substances whose physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern as a result of structural similarity may be considered as a group, or "category" of substances. Application of the group concept

requires that physicochemical properties, human health effects and environmental effects or environmental fate may be predicted from data for reference substance(s) within the group by interpolation to other substances in the group (read-across approach). "

2.4.1 Review of grouping approaches: search strategy

We have reviewed all published categorisation schemes and grouping for read-across approaches from literature and FP7 projects. Relevant approaches proposed by various FP7 projects are summarised in Appendix 5. The relevant contents on categorisation and grouping that are available are already taken into consideration. Other outputs are expected to be released from different projects in the coming months.

We performed a literature search in Scopus with the criteria presented in Table 2.7. Of the 293 papers resulting from the search, 28 were identified as relevant based on abstracts contents as relevant for categorisation and grouping approaches. Results considered of possible interest for NanoComput were also highlighted (23 results). From the selected 28 papers, 8 were proposing an original framework for categorisation of NMs, or approaches that may be useful for grouping or ranking (including a predictive approach). These papers are reported in the next paragraphs. The others are cited in the text, when relevant.

Table 2.7 Literature search strategy for grouping approaches

Keywords for nano-objects	Search for approaches
Nanopart*	Grouping
Nanomat*	Read-across
	Categorisation*
	Prioritisation*
	Ranking
Search results: 293 papers (last search: 30/11/2015)	
First selection on abstracts: 28 papers on categorisation or grouping approaches; 27 papers containing relevant information for NanoComput	*Selection criteria*: "categor", "group", "rank" or "predict" approaches were mentioned in the abstract, and abstract contents were relevant to our field of interest
Second selection of papers: of the 27 papers on categorisation and grouping, 3 were presenting categorisation frameworks and 5 are considered relevant as grouping for read-across (one is on read-across) and are reported in the respective tables (Table 2.8 and Table 2.10)	*Selection criteria:* the paper proposes a categorisation or grouping approach; ranking approaches considering similarities or relevant for our aim were also included

The papers identified as containing information on categorisation and grouping are presented next.

2.4.2 Grouping approaches for filling hazard data gaps by read-across

ECHA released in May 2016 the first draft of its guidance for REACH registrants on how to justify the use of hazard data between nanoforms of the same substance as an Appendix to Chapter R.6.7 of the Guidance on IR&CSA on QSARs and Grouping (ECHA, 2017b). This document proposes a revised version of the strategy for grouping of nanoforms presented earlier in a joint publication with RIVM and JRC (RIVM, JRC, & ECHA, 2016). Before the release of the draft ECHA guidance, the ECHA Group Assessing Already Registered Nanomaterials (GAARN) and the ECHA Nanomaterials working group (NMWG) had identified some key concepts and considerations related to NM grouping and read-across. It included the need to consider properties beyond chemical composition (e.g. aspect ratio, particle size, shape, or solubility), the reaffirmation of the similarity rules from REACH Annex XI for NMs, the relevance of toxicokinetic studies (and toxicokinetic proxies), in grouping, read-across, and for *in vitro* to *in vivo* tests extrapolation (ECHA, 2013, 2014b). The OECD (2014e) had acknowledged the need to develop frameworks for grouping of NMs. The European Food and Safety Agency identifies the relevance for read-across in risk assessment of NMs (EFSA Scientific Committee, 2011). Guidance on how to consider and integrate weight of evidence in scientific assessments is being finalised by EFSA, also taking into read-across (public consultation closed in May 2017).

An extensive review of the current concepts and approaches for grouping of NMs is given by Arts et al. (Arts et al., 2014) and in a report by the Dutch National Institute for Public Health and The Environment (RIVM) (Sellers et al., 2015).

The FP7 project ITS Nano has suggested that any approach adopted for grouping should take into account the changes occurring during the lifecycle (LC) of NMs (U. K. V. Stone, Balharry, Fernandes, Johnston, Munro, & Hartl, 2013). Key aspects are physicochemical (PC) characteristics of NMs (chemical composition, size, SSA, etc.), their behaviour and effects (ROS generation, electron transfer, photoreactivity, etc.) and their fate (e.g. hydrophobicity, agglomeration, zeta potential). The FP7 research project MARINA goes further in recommending that grouping be supported by information on kinetics (uptake, distribution, biopersistence) and early and apical biological effects (A. G. Oomen et al., 2014). NANOSOLUTIONS does a step forward in proposing a toxicity classifier to categorise NMs according to their toxicity, as presented in the next paragraph.

The US-Canada Regulatory Cooperation Council (RCC) has developed an approach based on chemical composition. In this approach, seven classes of NMs are defined: CNTs; inorganic carbon; metal and metalloid oxides; metals, metal salts and metalloids; semiconductor quantum dots; organics and other classes. In addition, toxicologically relevant PC properties are identified for each of the classes to support (sub-)classification (RCC, 2013a, 2013b). The Nanomaterial registry by the US National Institute of Health has defined similarity rules to support matching of NMs entries in the registry. Such rules determine similarity in the range 10%-85% depending on surface chemical composition, surface charge, shape and size. If the NMs were characterised in the same environment (defined taking into consideration both the kinetic and thermodynamic aspects) for size, then the NMs are in a 22.5-30% match; if the size values are within 10%, those two nanomaterials are an additional 15% match. If

both NMs have the same material type for their most outward chemistry, they are an additional 25% similar, and if the isoelectric point value is within 10% and the NMs were characterized in the same way, another 15% similarity can be added (NIH, 2014).

The German environment agency (UBA, BfR, & BAuA, 2011) has recommended that grouping be based on PC properties like primary particle size, surface properties, and water solubility. In addition to solubility, crystal structure, surface charge and coatings, conduction band energies are recommended as useful PC properties in grouping of NMs. They also recognise that one group should be identified by multiple types of parameters related to e.g. shape, biopersistence and toxicity; and that grouping could be based on the potential of NMs to cause inflammation. It was also admitted that nanotubes should be considered as a separate group (Schröder et al., 2014).

The International Cooperation on Cosmetics Regulation (ICCR) supports the application of the "bridging toxicity approach" in test waiving, which can be considered as implicit read-across. This consists in extrapolating (long term) toxicity data between nanoforms or from a non-nanoform to a nanoform when the properties of the (non)-nanoforms and the results of the (short term) toxicity studies are similar (Araki, Bose, Chaudhry, Dewan, & Dufour, 2013).

In occupational safety, the inhalation route is the exposure pathway of most concern. Some grouping schemes group NMs according to their PC properties. For example Gebel et al (2014) consider three categories according to the mode of action and exposure route: chemically mediated toxicity (e.g. soluble NMs), granular biodurable particles and fibrous NMs: Similarly, the German Bundesanstalt für Arbeitsschutz und Arbeitsmedizinsuch (BAuA) distinguishes (BAuA 2013):

- soluble

- granular biopersistent particles with specific toxicological properties

- granular biopersistent particles without specific toxicological properties, and

- biopersistent fibrous material.

The US National Institute on Occupational Safety and Health (NIOSH) has also followed this grouping approach and in addition has proposed a framework based on the mechanism causing the toxic effect: NMs are classified as (Kuempel, Castranova, Geraci, & Schulte, 2012)

- higher solubility particles that can reach systemic tissues (toxic ions reach systemic tissue)

- poorly soluble, low toxicity particles (toxicity is related to total deposited or retained particle dose in target respiratory tract region based on particle size)

- poorly soluble, high toxicity particles (same as above but with reactive surface)

- fibrous particles for which the toxicity is related to bioperistence and genotoxicity

The identification of these four classes of hazardous NMs is taken into account also by the British Standard Institute (https://nanohub.org/groups/gng/guidelines). The ECETOC task

force on Nanomaterials (Arts et al., 2015) defines a three-tier approach (DF4nanoGrouping) to group NMs for inhalation exposure in one of four main hazard classes following the above-mentioned categories (BAuA, 2013; BSI, 2007; Kuempel et al., 2012):

- soluble NMs

- biopersistent with high aspect ratio NMs

- passive NMs

- active NMs

This is done through a three-tier approach. Tier 0 precedes the DF4NanoGrouping by collecting intrinsic material properties to identify a NM. Tier 1 involves the assignment of a NM to the group of soluble NMs or to one of the other groups by means of its intrinsic properties. Tier 2 provides assignment of the NM to one of the three groups (i.e. biopersistent high aspect ratio, passive, or active NMs) depending on system-dependent properties. Toxicological information is used in Tier 3 to corroborate the assignment of the NM to a class and to support sub-grouping of active NMs depending on the outcome of short term *in vivo* studies. Applicability of the framework is addressed in Arts et al. (Arts et al., 2016). DF4NanoGrouping foresees read-across within the identified categories, which groups NMs with similar physicochemical and activity properties. For instance, group 1 may allow read-across between soluble NMs (even from bulk), group 2 for biopersistent and high aspect ratio NMs like CNTs, group 3 for non-fibrous passive NMs, and group 4 between reactive NMs. The case studies consist of 24 NMs of different classes of composition (carbonaceous, metal oxides and sulphates, amorphous silica, organic pigments). Each identified NM was assigned to one of the four pre-defined groups following the 3-tier approach. Assignment of NMs to groups i. to iii. does not need animal testing whereas group iv. represents specific hazards that are addressed with *in vivo* experiments. Although DF4nanoGrouping's framework defines qualifiers for grouping related to the use, release, and route of exposure, these considerations are missing in the practical example.

Other authors apply high throughput screening platforms together with computational methods for data evaluation to rank NMs and to guide *in vivo* testing. For example, Nel et al. (2013) have identified a set of *in vitro* assays reflecting toxicity pathways of NMs. The tests provide information about ROS, dissolution and release of toxic metal ions, cationic injury to surface membrane and organelles, pro-fibrogenic responses to CNTs, inflammasome activation by long aspect ratio materials, Photoactivation and influence of bandgap, Zebrafish embryo hatching interference, or cell membrane lysis by surface reactivity. The resulting data is claimed to support clustering based on similar biological responses or linkage to PC properties (e.g. shape, size, crystal structure, band gap, dissolution, surface chemistry, surface charge, and surface functionalization), but this is not translated into practical guidance.

The RIVM approach consists of different steps that aim at substantiate a hypothesis on the behaviour of the NM of interest depending on known information. A tiered testing strategy is presented where data are collected at different levels of complexity (tiers 0 to 2; some pieces of information are not required by REACH but are considered necessary for the assessment) and read-across is considered for each endpoint according to similarities

identified depending on collected information (mainly on PC properties and behaviour in environmental or biological media) (Sellers et al., 2015). The proposed strategy consists of a 4-step framework and on a 3-tiers data collection to evaluate NMs and decide on the applicability of read-across. The 4 steps comprise:

- collection of existing information (including NM characterisation and behaviour of the NM in different media)

- hypothesis formulation

- testing (3 tiers: PC properties, reactivity and *in vitro* toxicity, and *in vivo* toxicity)

- assessment (do data support the hypothesis, or is there need of new data?)

Step 1 is used to collect data to form a hypothesis (step 2) that eventually leads to experimental testing, which is used to issue a final assessment. The framework is illustrated by its application to two NM case studies. This approach does not aim primarily at assigning a NM to a predefined category, as hazard groups are eventually defined in a flexible manner after collection of information on PC properties and toxicological endpoints. In this approach, the LC of products containing NMs is considered as a step for identifying exposure routes when addressing specific case studies.

The proposals for hazard- and risk-based grouping approaches that propose a framework are presented in Table 2.8. Documents that merely provide or reiterate principles for grouping are not included in the table, and are only cited in the text. These documents focus on the testing of NMs and make reference to grouping approaches, identifying some key aspects a grouping approach should focus on, but do not propose a strategy or a framework. The aim of Table 2.8 is to systematically report the existing frameworks for grouping that are captured in the column "Approach", in order to easily compare the different approaches. To make this possible, the table captures information on the "assessment goal" of the approach, we extract the basic principle applied for grouping ("Basis for grouping ") and identify "predefined groups" when they are defined. If a "testing strategy" is supported, this is pinpointed in a dedicated column. We also report if the approach identifies the availability of standard methods like OECD test guidance or other standard operating procedures, based on the considerations made by the authors ("Practicality") and if applications to case studies are presented ("Applicability") and we comment on the applicability of the proposed method for REACH purposes.

Table 2.8. List of frameworks proposed for NM grouping.

Approach (reference)	Assessment goal(s)	Basis for grouping	Predefined groups	Testing strategy supported	Practicality (standard methods identified)	Applicability	Comments on applicability within REACH
RIVM grouping approach (Sellers et al., 2015)	Human health and environmental hazard and risk assessment	Similarity may be supported by information about chemical identity, particle characteristics, fundamental transport and behaviour, activity and reactivity (the so called Tier 0 testing) No threshold to determine similarity has been developed	No	Yes, read-across is considered for each endpoint to waive tests on animals	Standard methods are mostly not available	The approach is applied to TiO$_2$ and Ag NMs	Read-across is supported when PC properties of the NM under evaluation are similar to the ones reported in the reference studies (then test waiving is accepted)
DF4nanoGrouping (Arts et al., 2015)	Human health hazard assessment (inhalation exposure)	Assignment to one of the four pre-defined categories is based on water solubility, particle morphology and composition, dissolution rate, surface reactivity, dispersability. Confirmation of the categorisation of a NM comes through information from in vivo studies (biopersistence, biodistribution, genotoxicity). Use release and exposure information are applied as qualifiers to support grouping or appropriate testing	4 categories are identified: 1. Soluble NMs, 2. biopersistent high aspect ratio NMs, 3. Passive NMs, 4. Active NMs	Exposure-based test waiving is supported; toxicological tests are identified for the inhalation route	Preferred methods, protocols and existing test guidelines are identified	The approach is applied to carbonaceous, metal sulphate NMs, amorphous silica and pigments. Belonging of a NM to a pre-defined class determined testing strategy and risk management	The approach is based on REACH requirements. The proposed testing strategy is based on inhalation route of exposure (the other routes are only mentioned)

Approach (reference)	Assessment goal(s)	Basis for grouping	Predefined groups	Testing strategy supported	Practicality (standard methods identified)	Applicability	Comments on applicability within REACH
US National Institute for Occupational Safety and Health (NIOSH) (Kuempel et al., 2012)	Human health risk assessment in occupational settings (Inhalation)	Particle size, shape and density, surface area, reactivity, solubility	4 categories are identified: higher solubility particles that can reach systemic tissues; (ii) poorly soluble particles for which toxicity is related to the SSA, (iii) poorly soluble toxic NMs where reactive particle specific surface area dose determines toxicity and to the (iv) fibrous particles for which the toxicity is related to bioperistence and genotoxicity	A testing strategy is supported but not proposed	Specific standard methods are not identified	The approach is applied to fine and ultrafine particles (diesel exhaust particulate, carbon black in the ultrafine range). One NM falling in a predefined class would be compared to the identified reference materials and the risk estimate would be made according to identified PC properties	Occupational inhalation exposure is considered; the approach may support the selection of PC properties for hazard grouping but is not developed for REACH application
US-Canada Regulatory Cooperation Council	Human health and environmental	NMs are classified on their chemical composition in order to identify (dis)similarities with bulk chemicals and for	Seven classes are identified: CNTs; inorganic carbon; metal and	Yes. A flowchart is provided where,	Lack of standard methods	No case studies	A testing strategy is identified according to the

Approach (reference)	Assessment goal(s)	Basis for grouping	Predefined groups	Testing strategy supported	Practicality (standard methods identified)	Applicability	Comments on applicability within REACH
(RCC, 2013a, 2013b)	safety	supporting future read-across. PC properties for each group are identified to support grouping and read-across. Size, shape, surface chemistry, solubility, composition, crystal structure	metalloid oxides; metals, metal salts and metalloids; semiconductor quantum dots; organics and other classes	according to available information on exposure and PC properties, testing is suggested			exposure route PC properties for the definition of similarity for read-across are identified for each class
MARINA framework (A. Oomen et al., 2015)	Human health and environmental hazard and risk assessment	A group includes NMs with low variability in PC, exposure, (eco)toxicological kinetic or fate properties	Suggested predefined categories: quickly dissolving NMs, passive and active NMs, NMs with high aspect ratio	Yes: the Marina Risk Assessment Strategy is supported	Lack of standard methods	No case studies presented	Generic framework suitable for application within REACH

2.4.3. Hazard classes in control banding tools

Control Banding (CB) is a pragmatic approach that can be used for the control of the workplace exposure to agents with unknown or uncertain toxicological properties and for which there is a lack of quantitative exposure estimations. CB tools identify a range of control measures (such as general ventilation, and containment) according to the estimated range or "band" of hazard and of exposure based on combined hazard and exposure ranking.

For the purposes of this review, we were interested in investigating which PC properties or which toxicological endpoints are applied to rank the hazard of NMs in various nano-specific control banding tools, as this may in some cases also support the grouping NMs for read-across of hazardous (toxicological) properties.

Liguori et al. (2016) published an extensive review on all the available CB tools applicable to NMs, and comparing them in terms of the required inputs of PC properties, toxicology and exposure. Based on the information in this review, Table 2.9 summarises key features of the available CB tools specific to NMs. The aim of each CB tool is reported under "assessment goal", whereas "Information relevant for hazard classification" contains detail on the PC properties considered in the hazard banding (in case toxicological information is taken into account or required by the tool this is also reported in this column). Under the column "hazard classes" details on the number and type of hazard bands is reported and finally under "availability of case study" reference to any applications is reported. From the table it is evident that solubility, together with shape, are considered relevant PC properties for identifying the hazard group in all the tools except the Swiss precautionary matrix, where it was excluded because of lack of data (Höck et al. 2013). Solubility is taken into account in most tools as a screening property: the biological effects of highly soluble materials are considered similar to coarser particles and traditional risk assessment tools are considered suitable in these cases. For instance, in Stoffenmanager hazard banding is based on solubility and persistence (highly soluble particles are considered lower priority substances) (Van Duuren-Stuurman et al., 2012). On the other hand, the ANSES CB Tool requires solubility rate as an input and in case of low solubility rate, the assigned hazard band is higher (Liguori et al., 2016). Surface coating is a required input for hazard banding only in Nanosafer, and an optional input in the Swiss precautionary matrix. In some tools, (bio)persistence is considered as well (Liguori et al., 2016).

Interestingly, both NanoSafer and the ANSES CB tool consider the possibility to take into account data from the corresponding bulk material or analogous material. In the ANSES CB Tool an analogous material is defined as "*a substance or material with a similar composition and/or crystalline phase from the same chemical category and with similar documented physico-chemical properties (metal oxides, graphite, ceramics, etc.) as the substance of interest*" (Riediker et al. 2010).

NANOSOLUTIONS developed a classifier for of NM toxicity (ENM safety classifier) based on multiple data sources (intrinsic properties, omics data and in vitro toxicity data). Although the classifier is not developed as a control banding tool, it is reported in table 2.9 because it identifies three hazard classes that are defined according to physicochemical properties and toxicological tests (Fortino & Grevo, 2017).

In a data-poor context, Bayesian networks have been proposed by Marvin et al. (2017) for human hazard ranking of NMs. This approach consists in the selection of physicochemical

parameters relevant for hazard assessment of NMs by expert elicitation, and in the construction of a Bayesian network to classify NMs according to the information on exposure and hazard. A validation exercise shows that the ranking of hazard potential of NMs was satisfactory. As the authors state, the limit of this approach is that, there is no mechanistic evidence in hazard identification. Since this model is not applied as a control banding approach, it is not reported in Table 2.9.

Table 2.9. List of occupational banding tools containing a hazard module.

Approach (reference)	Assessment goal(s)	PC properties considered for hazard classification	Hazard classes	Availability of a case study
CB Nanotool (Zalk, Paik, & Swuste, 2009)	Oriented for nanotechnology researchers risk assessment and management	Chemical form, size, shape, surface reactivity, solubility; Information on parent material or NM: Toxicity (lowest OEL), LD50, mutagenicity, carcinogenicity, reproductive toxicology, dermal toxicity, asthmagen	4 bands for identified as severity scores (taking into account both exposure and hazard information by summing the identified factors)	The tool was applied to several activities and NMs (Paik, Zalk, & Swuste, 2008)
IVAM guidance (Cornelissen, Jongeneelen, Broekhuizen, & Broekhuizen, 2011)	Workers and occupational exposure	Shape (fibrous particle) and water solubility	Three hazard categories: (Water) soluble nanoparticles, Synthetic, persistent nanomaterials (non-fibrous) Fibrous, nonsoluble nanomaterials	NA
Swiss precautionary matrix (Höck et al., 2013)	Employers and employees prioritise health risks and implement control measures	Stability, redox activity, catalytic activity, ROS formation potential; induction potential for inflammation	Three classes of potential effects (low medium and high)	No
Stoffenmanager (Van Duuren-Stuurman et al., 2012)	Human health risk assessment in occupational settings (Inhalation)	Shape (fibre length). Inhalation hazard; water solubility; biopersistence	5 classes are identified	Fe powder. Falling in a risk band identifies the level of hazard priority
ANSES CB tool (Riediker et al., 2010)	Exposure prevention; for small to large enterprises	Reactivity, solubility rate, shape and biopersistence. Preliminary hazard band of the bulk material; is the material already classified ?	5 hazard bands are identified (from very low to very high)	No case study available in the guidance

Approach (reference)	Assessment goal(s)	PC properties considered for hazard classification	Hazard classes	Availability of a case study
NanoSafer (K. a Jensen et al., 2014)	SMEs; precautionary risk assessment	PC properties: size, shape, solubility, surface coating. Materials OEL; Risk	4 control banding classes are identified based on toxicity	No case study available
ENM safety Classifier (Fortino and Greco, 2017)	Group NMs according to toxicity	Intrinsic properties (nanospecific properties are not mentioned)	Three classes are identified as low, medium and high toxicity	The tool is developed for 31 NMs starting from the NANOSOLUTIONS dataset t and it is validated with data from MARINA

2.5 Experience in grouping for read-across of NMs

The research studies selected from the literature review consider different datasets and different methods to derive their conclusions. The only published study showing a example of read-across application for NMs for filling data gaps is presented by Gajewicz et al. (2015). They consider two case studies based on *in vitro* cytotoxic endpoints, and they calculated descriptors for activity (enthalpy of formation of a gaseous cation having the same oxidation state as that in the metal oxide structure and Mulliken's electronegativity). Euclidean distance was the similarity metric applied for the identification of groups of NMs with similar toxicity. Data was split into training and validation sets to perform read-across. The "prediction" was successful from both sets except for a few oxides for which toxicity is under predicted (SnO_2, Mn_2O_3 and V_2O_3) in the HaCaT cell line for TiO_2.

Zhang et al. (2012b) identify different hazard groups of NMs according to dissolution and energy (band gap) profiles. The band-gap prediction was successful in confirming the toxicity of the metal oxides whose band gap was overlapping with the cellular redox potential. Other metal oxides were exhibiting toxic effects but were outside the band gap range identified by the model (false negative predictions), and to solve this, a regression tree analysis taking into account both the effect of band gap and dissolution was successfully identifying three groups of NMs: non-toxic NMs, NMs toxic because of their solubility and NMs toxic because of their band gap energy.

Since there are only a few examples of grouping of NMs in the literature, in our analysis also other relevant approaches are included and summarised in Table 2.10. In particular, approaches that result in the ranking of NMs are potentially useful since a rank ordering of chemicals (or NMs) can be regarded as a group, and interpolation (i.e. read-across) of properties can be carried out between NMs of known toxicity and different rank order.

Table 2.10. Grouping for read-across: approaches identified in the literature and computational applications that aim at ranking NMs

Approach	Objective of the study	Methods	Dataset	Properties for grouping	Results	Comments
Gajewicz et al. (2015)	Read-across in dataset of NMs to fill data gaps for the cytotoxicity endpoint (reduction of cells viability, EC50) for E. coli and HaCaT cell line (human keratinocytes)	One calculated molecular descriptor is identified in each case study (endpoint) and is used to group NMs in the Euclidean space; NMs from the validation set are (qualitatively) predicted according to the euclidean distance	Two datasets with 17 and 18 metal oxide NMs	Euclidean distance is the similarity metric; properties that are considered are enthalpy of formation of a gaseous cation having the same oxidation state as that in the metal oxide structure (ΔH_{Me+}) and Mulliken's electronegativity (χ^C)	3 groups of NMs with increasing toxicity properties were identified in both case studies	This study represents an application of a qualitative read-across that could be relevant according to REACH Annex XI but the endpoint is not required by REACH
Zhang et al. (2012b)	Validate hazard ranking based on HTS output and on *in vivo* tests	Regression tree analysis for the effect of band gap and dissolution on metal oxides NMs toxicity (cell viability)	24 metal oxides	Cellular toxic NMs were identified depending on band gap. Dissolution was then addressed to assess the toxicological impact of NMs	Dissolution and band gap could predict the toxicity of 7 out of 8 NMs that were predicted (and tested) as toxic	This application identifies a group of non-toxic NMs and two groups of toxic NMs depending on dissolution and band gap. The authors show correlation between *in vitro* results and *in vivo* acute pulmonary inflammation
George et al. (2011)	Hazard ranking of a set of metal oxide NMs	Information on NM type, dose, duration of exposure, cellular targets, cytotoxicity events were extracted from	2 cell lines and 4 cytotoxicity responses	NMs were ranked according to (a) similarity between their lethal response	NMs were ranked in 4 groups according to the cytotoxicity endpoints. They were ranked in 5	Hazard-based ranking of NMs. A more extensive dataset on PC properties may help to identify a

Approach	Objective of the study	Methods	Dataset	Properties for grouping	Results	Comments
		HTS data and visualised in self organising maps and 4 groups of NMs were identified according to the observed effect	for 7 metal oxide NMs	outcome or (b) the cytotoxic response profile of each cell line (HTS data)	groups according to the in vivo tests	group of similar NMs (structural similarity)
Liu et al., (2015)	Consider dosimetry modelling in *in vitro* toxicity ranking	Hazard ranking was based on the EC50 and slope of the dose-response curves. Sedimentation of NMs was calculated via a fate model considering Brownian motion and gravitational settling	7 metal oxide NMs	Hazard ranking considering delivered dose was based on dose-response analyses and compared with administered dose ranking	The comparative ranking between administered and delivered dose did not show any difference	The approach would be useful if there was an extrapolation to in vivo studies
Chen et al., (2014)	Prediction of biological surface adsorption index(BSAI)	Calculation of the adsorption coefficient k as a function of 5 variables (describing molecular interactions); PCA for clustering	23 NMs (metal oxides, Ag, organic NMs)	Clustering using the 5 identified variables	The prediction of the adsorption index was improved compared to the previous model	The approach could be applied by using the five variables as the basis for ranking and read-across

2.5.1 Experience and activities of the European Commission on grouping of NMs

The EC launched the REACH Implementation Project on Nanomaterials (RIP-oN) in 2009 (EC, 2011b) with the aim of providing advice on key aspects of the implementation of REACH concerning Information Requirements (RIP-oN2) and Chemical Safety Assessment (RIP-oN3) of nanomaterials (Aitken et al., 2011; Hankin et al., 2011). The project recommended to invest in the extrapolation of information from studies conducted with bulk forms of the substance or modifications of the respective nanoforms to reduce further (*in vivo*) testing, taking into account particle size and surface area so that potential hazards are not underestimated. Read-across (nano-to-nano) on certain forms of NM such as low toxicity, low solubility particles was considered possible but still requiring further study and validation. The reports were finalised in July 2011 and are available from the DG ENV website: http://ec.europa.eu/environment/chemicals/nanotech/reach-clp/ripon_en.htm

The Nanosupport project (2010-2013) aimed to provide a scientific assessment of the information on nanomaterials contained in 2010 REACH registration dossiers, i.e. substances registered as nanomaterials or which based on their size could be considered nanomaterials or (may) contain a fraction in the nanosize range. The analysis showed that read-across was applied/claimed in almost half of the assessed dossiers for some (eco)toxicological endpoints based on the common parent compound or metal ion. The particle nature and/or different particle sizes as well as different solubility of such salts/oxides were usually not given special attention. Furthermore, read-across was not explicitly applied on a *form-to-form* basis in dossiers containing multiple forms of the substance. In general no argumentation was given for the robustness of the read-across.

The project recommended that

"A justification should be explicitly required when data from one form of a substance is used to cover the properties/hazards/risks associated with another form, especially for endpoints where particle size is known to have a significant impact on the outcome of the test. […] Read-across from one form to another should follow the same requirements as from one substance to another. The same is recommended for other non-testing approaches, […] e.g. QSAR models should only be used for nanomaterials if the materials are within the applicability range. […]

For NMs surface treated/functionalized or capped with (in)organic compounds (e.g. one or more (in)organic layers to prevent ion leaching and control dispersability), it should be taken into consideration that the properties and behaviour of the nanoparticle would be highly dependent on the properties (including biocompatibility etc.) of the surface treatment material (EC & ECHA, 2012)."

Since then, the Scientific Committee on Consumer Safety (SCCS) released an opinion on TiO_2 (SCCS, 2013b) where grouping was considered feasible for different forms of the same nanomaterial (NM with the same chemical identify). In the case of TiO_2 15 NMs were submitted for evaluation and three groups were distinguished according to crystallinity and photocatalytic activity.

Twenty three different materials were submitted for SCCS opinion on SiO_2. The applicants presented the 23 NMs in the following four groups: a. hydrophilic precipitated silica, b. hydrophilic pyrogenic silica, c. hydrophobic pyrogenic silica, and d. colloidal silica. The large variations in PC properties (VSSA, solubility and density) is not scientifically justified and

hence the proposal to apply data from one material to other materials within the same group is not accepted (Scientific Committee on Consumer Safety, 2015).

Other activities are being carried out within the ECHA NMWG which was established in 2012 as an informal advisory group consisting of experts from Member States, the European Commission, ECHA and accredited stakeholders organisations, with the mandate to "provide informal advice on any scientific and technical issues regarding the implementation of REACH and CLP legislation in relation to nanomaterials."

The following case studies for read-across have been presented (mainly by Industry) and discussed.

Zinc Oxide (Subgroup discussion CC 2013)

The presentation by the International Zinc Association illustrated that ZnO toxicity is driven by the Zn^{2+} ion, which is the same for the bulk, and the assessment of the bio-elution will therefore predict the overall toxicity. Nano sized and micro sized particles including coated materials gave comparable results in various simulated bio-fluids; however, the release of ions was dependent on pH.

Concerning penetration through (damaged) skin further information was requested about a new study presented within the OECD WPMN showing a bigger dermal uptake compared to previous studies.

ECHA informed the applicant that depending on the endpoint, bio-elution (alone) may not be sufficient to justify read across.

Calcium carbonate (Subgroup discussion CC 2013)

The presentation by the Industrial Minerals Association (IMA) explained that the nanoform of $CaCO_3$ did not have any new properties/functionalities but only an enhancement of already existing properties (fillers). No significant differences are expected in the phys-chem test results; Ca^{++} and CO_3^{2-} are the «biological agent» and these are physiologically abundant. A possible impact on the toxicity and hence on the difficulty to read across (between materials from different suppliers) may be due to impurities, which can be present in concentrations up to 20%.

Silver (Subgroup discussion CC 2013)

The presentation given by the European Precious Metals Federation (EPMF) concluded that all forms of Ag are essentially the same chemical with regard to the manufacturing process, uses and exposure control. Their effect is driven by the Ag ion; NanoAg, irrespective of particle size and coating type, was found less toxic or of equivalent toxicity to ionic Ag. Therefore, read-across from ionic Ag or nanoAg to non-nano Ag is suggested to be valid and conservative. New testing is therefore carried out with nano-Ag and read-across from the bulk form, as this is considered the more conservative.

Carbon based NM, Pigments and Me-oxides (5th NMWG 2014)

A presentation given by BASF showed different attempts to grouping nanomaterials based on the chemistry of carbon-based. Materials, covering: MWCNT, graphene, graphite nanoplatelets and carbon black, organic Diketopyrrololpyrrol-Pigments (DPP-Pigments) in bulk and nanoforms (DPP Orange 1 (bulk), DPP Orange 2 (nano), DPP Orange 3, Pigment Red 254-1 (bulk), Pigment Red 254-2 (nano), Pigment Yellow 74; Pigment Blue 15) and toxicity for metal oxides and silica using short term inhalation studies (STIS). A ranking of

nanomaterials according to their toxic potency in the STIS showed that chemistry and size did not correlate with toxicity for most of the tested materials and most were of low (NOAEC 10- 50 mg/m3) or medium (NOAEC ~10 mg/m3) toxicity. Higher toxicity ($\leq$ 0.5 mg/m3) was shown for some coated (CeO_2, ZnO, TiO_2) and uncoated nano-oxides (CeO_2, TiO_2) and MWCNTs. They concluded that various criteria need to be taken into consideration for the grouping of NMs, including exposure, release, solubility, uptake and these factors need to be considered in a testing strategy which should be concern-driven.

Pigments (5th NMWG 2014)
A presentation by Lanxess/Eurocolour outlined the close correlation between particle size/particle size distribution and characteristics such as colour, tinting strength and dispersibility, which are all dependent on the production process. These parameters are often used for production control and allow a reduction of the efforts employing electron microscopy. No correlation to toxicological effects were presented.

2.5.2 Pre-REACH experience on grouping and read-across

Before REACH entered into force, chemicals were regulated by a number of different regulations and directives including the Existing Substances Regulation No 793/93 (ESR)[13]. The risks of 141 priority ("existing") substances were assessed and the results were published as risk assessment reports (RAR). Some of these RARs covered several forms of a substance or groups of substances between which some of the endpoint data were read across. Read across and grouping approaches were also applied for classification and labelling. Lessons can be learned from such substances which exist as different salts and oxides, although at that time nanoforms were not dealt with. RARs of the substances listed below may therefore provide relevant examples for read across.

In general it may be concluded that for (inorganic) compounds the most relevant determinants for read across were based on solubility, bioaccessibility and absorption (measured as blood levels). However, it was also found that solubility was not always directly related to absorption. Usually the cation was considered responsible for the toxic effects, although this was not always the only determining factor.

Based on the particle size distribution in dustiness testing (mass median aerodynamic diameter: MMAD) the deposition pattern in human lungs of solids/metal powders was modelled by applying MPPI (Multiple-Path Particle Dosimetry Model Anjilvel 1995), e.g. for copper and lead.

Zinc: Zinc oxide, Zinc chloride, Zinc sulphate, Zinc stearate, Zinc phosphate
Large parts of the hazard section are identical in the risk assessment reports for the six zinc compounds. The grouping approach is based on the assumption that the zinc cation (for dissolved zinc species) is the determining factor for systemic toxicity. The RAR however also acknowledges that the bioavailability is affected by various physico-chemical parameters (ionic behaviour, solubility, pH, alkalinity etc.).

---

[13] http://echa.europa.eu/information-on-chemicals/information-from-existing-substances-regulation

Although there was some information on the solubility of the six zinc compounds (they are soluble in water (sulphate, chloride) or in diluted acids (phosphate, distearate and oxide) and elemental zinc is attacked by HCl to yield Zn^{2+}, adequate information was lacking how to quantitatively determine or estimate the bioavailable fraction of all the different zinc compounds in either laboratory animals or humans.

Cadmium

Data with other cadmium compounds (Cd chloride, Cd sulphide, other Cd compounds) were used as supporting data when no (or not enough) information on the effects of CdO/Cd metal was available and when the studies using cadmium compounds were mechanistically relevant.

Read-across was applied for oral, dermal, inhalation absorption, acute and repeated dose inhalation toxicity, mutagenicity, carcinogenicity, reproductive toxicity, developmental toxicity, bone and kidney.

The acute pulmonary toxicity of cadmium was considered to depend on the chemical and physical form of the administered compound, and therefore the question of the validity of an extrapolation to other CdO compounds was considered in the Cd RA.

The authors concluded that acute effects of Cd compounds in the lung cannot be predicted from their water solubility alone (G Oberdörster, Cox, & Baggs, 1987): small (insoluble) CdO particles and water soluble $CdCl_2$ for example were equally toxic in rats exposed by inhalation (G Oberdörster et al., 1987)– in another study CdO was even more toxic (Grose et al., 1987). The in vivo solubility in the lung after inhalation exposure is very high for CdO (G. Oberdörster & Cox, 1990).

Nickel: Nickel dinitrate, nickel sulfate, nickel carbonate, nickel dichloride

RARs were made separately for all 5 nickel compounds, however general conclusions on nickel compounds were drawn in a "Background document in support of the individual Risk Assessment Reports" which explains when results from other nickel compounds can be used (Larsen & Tyle, 2008).

The category approach was agreed for a large number of nickel compounds to fill data gaps. Depending on the endpoints nickel/nickel compounds were "source chemical" and/or "target chemical".

The determinants of nickel toxicity were considered to be: water solubility, bioaccessibility and bioavailability. The relationship between water solubility and bioavailability of nickel (II) at target sites was found to be more complex for nickel, with compounds of intermediate solubility having the highest bioavailability. The basic assumption was that after intake, nickel compounds (and metallic nickel) are changed and the nickel ion is the determining factor for toxicity. However, the potential release and absorption of nickel from metallic nickel is substantially lower than from the soluble compounds via all routes, which is why metallic nickel was not classified for the same endpoints as Nickel compounds (e.g. developmental toxicity).

The bioavailability depends on various characteristics of the individual nickel compounds, of which solubility is considered as being particularly important for the release of nickel ion and

thus the systemic bioavailability of the nickel ion. Ideally, data on the solubility of the nickel compounds in biological fluids are preferable; however, no data were available regarding the solubility of any of the five prioritised nickel compounds in biological fluids. For the purpose of risk characterisation the water solubility was used as a prediction of the solubility in biological fluids although it was acknowledged that such a prediction might not be correct as some data indicate that compounds insoluble or slightly soluble in water might be more soluble in biological fluids.

It was also recognised that with respect to local effects, the nickel ion may not be responsible for the toxic effects in all situations. Therefore, data on other nickel compounds in evaluations of local effects of an individual nickel compound were considered on a case-by-case basis.

Nickel, nickel sulphate, nickel dichloride, nickel carbonate and nickel nitrate were all classified for skin sensitisation (H317) and STOT Re1 (H372 Inhalation).

Metallic nickel was not classified (as the others) for Repr. 1B (H360) and mutagenicity (H341) and for carcinogenicity "only" in Cat 2 (H351), whereas the other compounds were Cat 1A (H350). It was agreed that metallic nickel should not be classified for this effect as the potential release and absorption of nickel from metallic nickel was substantially lower than from the soluble compounds via all routes. The nickel compounds were additionally classified for respiratory sensitisation (H334), skin irritation (H315) and only nickel dinitrate for serious eye damage (H318). The nickel compounds differed from each other with respect to acute toxicity via oral and inhalation exposure. Nickel dichloride was classified as toxic via these routes (H301, H331) while the others were classified as harmful (H302, H332).

Chromate sodium chromate, sodium dichromate, potassium dichromate, ammonium dichromate, chromium trioxide

All chromate compounds were covered in one RAR. They had the same proposal for classification and labelling, except chromium(IV)trioxide which was more toxic to skin (the others were harmful) and less toxic for reproductive effects (cat 3 instead of cat 2). Further differences concern the physical-chemical hazards such as explosion and fire. In relation to the potential for tumour induction after inhalation, it was noticed that the water solubility of Cr (VI) is inversely related to its bioavailability.

Lead

The voluntary RAR covered [Phthalato(2-)]dioxotrilead, basic lead carbonate, basic lead sulphate, basic lead sulphite, dioxobis(stearato)trilead, lead metal, lead monoxide (lead oxide), lead stabiliser compounds, neutral lead stearate, orange lead (lead tetroxide), pentalead tetraoxide sulphate, polybasic lead fumarate, trilead dioxide phosphonate.

Read-across has been based upon evaluation of physical properties (e.g. particle size in dustiness testing) and water solubility. This strategic approach has been made possible by a set of circumstances that include:

- all compounds under evaluation were sparingly soluble in water
- acute oral toxicity data were available for a number of compounds under evaluation
- indicated absence of toxicity up to the upper limit of ranges tested

- direct effects upon the skin or the lung (e.g. sensitization) were absent
- the particle size distribution of compounds was such that upper airway deposition was predicted to be followed by translocation to the gastrointestinal tract — systemic effects following acute inhalation exposure would thus largely be dictated by the oral exposure route.

Prediction of the toxic potential of different lead compounds based solely upon physical or chemical properties of individual lead compounds proved difficult due to discrepancies between parameters such as water solubility and the relative bioavailability of different lead compounds following the more common routes of exposure (e.g. ingestion). While extremely water insoluble lead compounds can have low bioavailability, some can exhibit bioavailability close to that of lead acetate. The failure of the water solubility of compounds to correlate with relative bioavailability is due to the complex acidic conditions within the stomach and variability of bioavailability of as a function of decreasing particle size. As particle size decreases, the resistance of otherwise stable lead compounds in the gastrointestinal tract will be diminished. Relatively oral bioavailability will further be modulated by "matrix" effects that can be exerted by lead-containing foods or soils that may be ingested.

Copper

The voluntary RAR covered copper (metal), copper (I) oxide, copper(II) oxide, copper sulphate pentahydrate, copper oxychloride. For the purposes of testing derogation and classification, read across was based upon evaluation of physical properties (e.g. particle size in dustiness testing), water solubility and oral absorption.

Read-across for classification and risk assessment was applied for:

1. acute inhalation toxicity for Cu(II)O, Cu-powder and CuS04.5H2O
2. acute oral toxicity for Cu-powder
3. mutagenicity
4. carcinogenicity
5. reproductive toxicity
6. repeated dose toxicity

2.6 References

Aitken, R. ., A. Bassan, S. Friedrichs, S. M. Hankin, S. F. Hansen, J. Holmqvist, S. A. K. Peters, C. A. Poland, and C. L. Tran. (2011). Specific Advice on Exposure Assessment and Hazard / Risk Characterisation for Nanomaterials under REACH (RIP-oN 3) Final Project Report.

Anjilvel, S. (1995). A Multiple-Path Model of Particle Deposition in the Rat Lung. Fundamental and Applied Toxicology 28:41–50.

Araki, D., R. Bose, Q. Chaudhry, K. Dewan, and E. Dufour. (2013). Report of the ICCR Working Group : Safety approaches to nanomaterials in cosmetics. ICCR/NANOSAFETY1/R/FINAL/.

Arts, J. H. E., M. Hadi, M.-A. Irfan, A. M. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, T. Petry, U. G. Sauer, D. Warheit, K. Wiench, W. Wohlleben, and R. Landsiedel. (2015). A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regulatory toxicology and pharmacology 71:S1–S27.

Arts, J. H. E., M. Hadi, M.-A. Irfan, A. M. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, T. Petry, U. G. Sauer, D. Warheit, K. Wiench, W. Wohlleben, and R. Landsiedel. (2016). Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regulatory toxicology and pharmacology 76:234–261.

Arts, J. H. E., M. Hadi, A. M. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, T. Petry, U. G. Sauer, D. Warheit, K. Wiench, and R. Landsiedel. (2014). A critical appraisal of existing concepts for the grouping of nanomaterials. Regulatory toxicology and pharmacology 70:492–506.

BAuA. (2013). Announcements on Hazardous Substances - Manufactured Nanomaterials - Announcement 527.

Bitsch, A., S. Jacobi, C. Melber, U. Wahnschaffe, N. Simetska, and I. Mangelsdorf. (2006). REPDOSE: A database on repeated dose toxicity studies of commercial chemicals--A multifunctional tool. Regulatory toxicology and pharmacology 46:202–10.

BSI. (2007). Nanotechnologies – Part 2: Guide to safe handling and disposal of manufactured nanomaterials. 71.100.99.

Burello, E., and A. P. Worth. (2011). A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5:228–35.

Chen, R., Y. Zhang, F. D. Sahneh, C. M. Scoglio, W. Wohlleben, A. Haase, N. A. Monteiro-Riviere, and J. E. Riviere. (2014). Nanoparticle surface characterization and clustering through concentration-dependent surface adsorption modeling. ACS nano 8:9446–56.

Cockburn, A., R. Bradford, N. Buck, A. Constable, G. Edwards, B. Haber, P. Hepburn, J. Howlett, F. Kampers, C. Klein, M. Radomski, H. Stamm, S. Wijnhoven, and T. Wildemann. (2012). Approaches to the safety assessment of engineered nanomaterials (ENM) in food. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 50:2224–42.

Cornelissen, I. R., F. Jongeneelen, P. Van Broekhuizen, and F. Van Broekhuizen. (2011). Guidance working safely with nanomaterials and nanoproducts, the guide for employers and employees.

Drefahl, A. (2011). CurlySMILES: A chemical language to customize and annotate encodings of molecular and nanodevice structures. Journal of Cheminformatics 3:1–7.

Van Duuren-Stuurman, B., S. R. Vink, K. J. M. Verbist, H. G. A. Heussen, D. H. Brouwer, D. E. D. Kroese, M. F. J. Van niftrik, E. Tielemans, and W. Fransman. (2012). Stoffenmanager Nano Version 10: A Web-Based Tool for Risk Prioritization of Airborne Manufactured Nano Objects. Annals of Occupational Hygiene 56:1–17.

EC. (2011). REACH Implementation Project Substance Identification of Nanomaterials (RIP-oN 1).

EC, and ECHA. (2012). NANO SUPPORT Project (I) - Scientific technical support on assessment of nanomaterials in REACH registration dossiers and adequacy of available information - Report on analysis and assessment and options for adapting REACH.

ECHA. (2013). Assessing human health and environmental hazards of nanomaterials- Best practice for REACH Registrants.

ECHA. (2014). Human health and environmental exposure assessment and risk characterisation of nanomaterials Best practice for REACH registrants.

ECHA. (2017). Appendix R 6-1 : Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping.

EFSA Scientific Committee. (2011). Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal 2011 9:1–36.

European Parliament and Council. (2006). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/4. Pages 849Official Journal of the European Union. Official Journal of the European Union, EC.

Foss Hansen, S., B. H. Larsen, S. I. Olsen, and A. Baun. (2007). Categorization framework to aid hazard identification of nanomaterials. Nanotoxicology 1:243–250.

Friedhofen, J. H., and F. Vögtle. (2006). Detailed nomenclature for dendritic molecules. New Journal of Chemistry 30:32–43.

Gaillard, C., A. Mech, and H. Rauscher. (2015). NanoDefine Development of an integrated approach based on Deliverable title : The NanoDefine Methods Manual Document Control Page.

Gajewicz, A., M. T. . Cronin, B. Rasulev, J. Leszczynski, and T. Puzyn. (2015). Novel approach for efficient predictions properties of large pool of nanomaterials based on limited set of species: nano-read-across. Nanotechnology 26:15701.

Gebel, T., H. Foth, G. Damm, A. Freyberger, P.-J. Kramer, W. Lilienblum, C. Röhl, T. Schupp, C. Weiss, K.-M. Wollin, and J. G. Hengstler. (2014). Manufactured nanomaterials: categorization and approaches to hazard assessment. Archives of toxicology 88:2191–211.

Gentleman, D. J., and W. C. W. Chan. (2009). A Systematic Nomenclature for Codifying Engineered Nanostructures. Small 5:426–431.

George, S., T. Xia, R. Rallo, Y. Zhao, Z. Ji, S. Lin, X. Wang, H. Zhang, B. France, D. Schoenfeld, R. Damoiseaux, R. Liu, S. Lin, K. a. Bradley, Y. Cohen, and A. E. Nel. (2011). Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. ACS Nano 5:1805–1817.

Gerloff, K., I. Fenoglio, E. Carella, J. Kolling, C. Albrecht, A. W. Boots, I. Förster, and R. P. F. Schins. (2012). Distinctive Toxicity of TiO2 Rutile/Anatase Mixed Phase Nanoparticles on Caco-2 Cells. Chemical research in toxicology 25:646–655.

Godly, E. W., and R. Taylor. (1997). Nomenclature and terminology of fullerenes: A preliminary study. Pure and Applied Chemistry 69:1411–1434.

Godwin, H., C. Nameth, D. Avery, L. L. Bergeson, D. Bernard, E. Beryt, W. Boyes, S. Brown, A. J. Clippinger, Y. Cohen, M. Doa, C. O. Hendren, P. Holden, K. Houck, A. B. Kane, F. Klaessig, T. Kodas, R. Landsiedel, I. Lynch, T. Malloy, M. B. Miller, J. Muller, G. Oberdorster, E. J. Petersen, R. C. Pleus, P. Sayre, V. Stone, K. M. Sullivan, J. Tentschert, P. Wallis, and A. E. Nel. (2015). Nanomaterial

Categorization for Assessing Risk Potential To Facilitate Regulatory Decision-Making. ACS nano.

Goodson, A. L., C. L. Gladys, and D. E. Worst. (1995). Numbering and Naming of Fullerenes by Chemical Abstracts Service. Journal of Chemical Information and Modeling 35:969–978.

Grose, E. C., J. H. Richards, R. H. Jaskot, M. G. Ménache, J. A. Graham, and W. C. Dauterman. (1987). A comparative study of the effects of inhaled cadmium chloride and cadmium oxide: pulmonary response. Journal of toxicology and environmental health 21:219–32.

Hankin, S. M., S. A. . Peters, C. A. Poland, S. Foss Hansen, H. J., B. L. Ross, J. Varet, and R. J. Aitken. (2011). Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2) Final Project Report. RNC/RIP-oN2/FPR/1/FINAL.

Hansch, C. (1969). Quantitative approach to biochemical structure-activity relationships. Accounts of Chemical Research 2:232–239.

Hastings, J., N. Jeliazkova, G. Owen, G. Tsiliki, C. R. Munteanu, C. Steinbeck, and E. Willighagen. (2015). eNanoMapper: harnessing ontologies to enable data integration for nanomaterial risk assessment. Journal of biomedical semantics 6:10.

Höck, J., T. Epprecht, H. Hofmann, H. K., K. H., L. C., L. L., G. P., N. B., R. M., S. K., S. B., S. C., S. W., S. C., U. A., von G. N., W. S., and W. P. (2013). Guidelines on the Precautionary Matrix for Synthetic Nanomaterials.

Jensen, K. a, a T. Saber, H. V Kristensen, B. Liguori, a C. Ø. Jensen, I. K. Koponen, and H. Wallin. (2014). NanoSafer vs 1 1 Nanomaterial risk assessment using first order modeling.

Kaempfer, A., R. La Spina, D. Gilliland, S. Valzacchi, D. Asturiol, V. Stone, and A. Kinsner-Ovaskainen. (2017). Silver nanoparticles and metallic silver interfere with the Griess reaction: reduction of the azo-dye formation via a competing Sandmeyer-like reaction. Chemical Research in Toxicology:acs.chemrestox.6b00280.

Klimisch, H. J., M. Andreae, and U. Tillmann. (1997). A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regulatory toxicology and pharmacology 25:1–5.

Kroll, A., M. H. Pillukat, D. Hahn, and J. Schnekenburger. (2009). Current in vitro methods in nanoparticle risk assessment: Limitations and challenges. European Journal of Pharmaceutics and Biopharmaceutics 72:370–377.

Kroll, A., M. H. Pillukat, D. Hahn, and J. Schnekenburger. (2012). Interference of engineered nanoparticles with in vitro toxicity assays. Archives of toxicology 86:1123–36.

Kuempel, E. D., V. Castranova, C. L. Geraci, and P. a. Schulte. (2012). Development of risk-based nanomaterial groups for occupational exposure control. Journal of Nanoparticle Research 14:1029.

Larsen, P. B., and H. Tyle. (2008). Nickel and Nickel Compounds: Report for the Danish Environmental Protection Agency.

Lenz, W. (1988). A short history of thalidomide embryopathy. Teratology 38:203–215.

Liguori, B., S. F. Hansen, A. Baun, and K. A. Jensen. (2016). Control banding tools for occupational exposure assessment of nanomaterials — Ready for use in a regulatory context?. NanoImpact 2:1–17.

Liu, R., H. H. Liu, Z. Ji, C. H. Chang, T. Xia, A. E. Nel, and Y. Cohen. (2015). Evaluation of Toxicity Ranking for Metal Oxide Nanoparticles via an in Vitro Dosimetry Model. ACS nano 9:9303–13.

Liu, R., H. Y. Zhang, Z. X. Ji, R. Rallo, T. Xia, C. H. Chang, A. Nel, and Y. Cohen. (2013). Development of structure–activity relationship for metal oxide nanoparticles. Nanoscale 5:5644–5653.

Lubinski, L., P. Urbaszek, A. Gajewicz, M. T. D. Cronin, S. J. Enoch, J. C. Madden, D. Leszczynska, J. Leszczynski, and T. Puzyn. (2013). Evaluation criteria for the quality of published experimental data on nanomaterials and their usefulness for QSAR modelling.

Maggiora, G. M. (2006). On outliers and activity cliffs - Why QSAR often disappoints. Journal of Chemical Information and Modeling 46:1535.

Marvin, H. J. P., Y. Bouzembrak, E. M. Janssen, M. van der Zande, F. Murphy, B. Sheehan, M. Mullins, and H. Bouwmeester. (2017). Application of Bayesian networks for hazard ranking of nanomaterials to support human health risk assessment. Nanotoxicology 11:123–133.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65:55–63.

NanoDefine. (2013). FP7-NMP-2013-LARGE-7 www.nanodefine.eu.

Nel, A., T. Xia, H. Meng, X. Wang, S. Lin, Z. Ji, and H. Zhang. (2013). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Accounts of chemical research 46:607–21.

NIH. (2014). Nanomaterial Registry https://www.healthdata.gov/dataset/nanomaterial-registry.

Oberdörster, G., and C. Cox. (1990). Carcinogenicity of cadmium in animals: What is the significance for man?. Toxicological & Environmental Chemistry 27:181–195.

Oberdörster, G., C. Cox, and R. Baggs. (1987). Long term lung clearance and cellular retention of cadmium in rats and monkeys. Journal of Aerosol Science 18:745–748.

OECD. (2012). Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. 36.

OECD. (2014). Guidance on grouping of chemicals Second edition Series on testing and assessment No 194. ENV/JM/MONO(2014)4.

Oomen, A., E. Bleeker, P. Bos, F. van Broekhuizen, S. Gottardo, M. Groenewold, D. Hristozov, K. Hund-Rinke, M.-A. Irfan, A. Marcomini, W. Peijnenburg, K. Rasmussen, A. Jiménez, J. Scott-Fordsmand, M. van Tongeren, K. Wiench, W. Wohlleben, and R. Landsiedel. (2015), October 26. Grouping and Read-Across Approaches for Risk Assessment of Nanomaterials. Multidisciplinary Digital Publishing Institute.

Oomen, A. G., P. M. J. Bos, T. F. Fernandes, K. Hund-Rinke, D. Boraschi, H. J. Byrne, K. Aschberger, S. Gottardo, F. von der Kammer, D. Kühnel, D. Hristozov, A. Marcomini, L. Migliore, J. Scott-Fordsmand, P. Wick, and R. Landsiedel. (2014). Concern-driven integrated approaches to nanomaterial testing and assessment--report of the NanoSafety Cluster Working Group 10. Nanotoxicology 8:334–48.

Paik, S. Y., D. M. Zalk, and P. Swuste. (2008). Application of a pilot control banding tool for risk level assessment and control of nanoparticle exposures. Annals of Occupational Hygiene 52:419–428.

Puzyn, T., B. Rasulev, A. Gajewicz, X. Hu, T. P. Dasari, A. Michalkova, H.-M. Hwang, A. Toropov, D. Leszczynska, and J. Leszczynski. (2011). Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature nanotechnology 6:175–8.

RCC. (2013a). Nanotechnology Initiative Work Element 2 , Priority Setting : Development of a Joint Nanomaterials Classification Scheme. Final report.

RCC. (2013b). Nanotechnology Initiative Work Element 3 : Risk Assessment / Risk Management. Final report.

Riediker, M., C. Ostiguy, J. Triolet, P. Troisfontaine, D. Vernez, G. Bourdel, N. Thieriet, and A. Cad??ne. (2010). Development of a control banding tool for nanomaterials. 2008-NaN-407.

RIVM, JRC, and ECHA. (2016). Usage of (eco)toxicological data for bridging data gaps between and grouping of nanoforms of the same substance Elements to consider.

Robinson, R. L. M., M. T. D. Cronin, A.-N. Richarz, and R. Rallo. (2015). An ISA-TAB-Nano based data collection framework to support data-driven modelling of nanotoxicology. Beilstein Journal of Nanotechnology 6:1978–1999.

SCCS. (2013). Opinion SCCS/1489/12 on Titanium Dioxide (nano form); COLIPA S75.

Schröder, K., C. Pohlenz-Michel, N. Simetska, J. U. Voss, S. Escher, and I. Mangersdorf. (2014). Carcinogenicity and Mutagenicity of Nanoparticles – Assessment of Current Knowledge as Basis for Regulation.

Scientific Committee on Consumer Safety. (2015). Safety Silica , Hydrated Silica , and Silica Surface Modified with Alkyl Silylates (nano form).

Sellers, K., N. Deleebeek, M. Messiaen, M. Jackson, E. Bleeker, D. Sijm, and F. Broekhuizen. (2015). Grouping nanomaterials A strategy towards grouping and read-across. 2015–61.

Stone, U. K. V., D. Balharry, T. Fernandes, H. Johnston, L. Munro, and M. Hartl. (2013). RESEARCH PRIORITISATION TO DELIVER AN INTELLIGENT TESTING STRATEGY FOR THE HUMAN AND ENVIRONMENTAL SAFETY OF NANOMATERIALS.

Thomas, D. G., S. Chikkagoudar, A. R. Chappell, and N. A. Baker. (2012). Annotating the structure and components of a nanoparticle formulation using computable string expressions. Proceedings. IEEE International Conference on Bioinformatics and Biomedicine 2012:889–894.

Toropov, A. A., and J. Leszczynski. (2006). A new approach to the characterization of nanomaterials: Predicting Young's modulus by correlation weighting of nanomaterials codes. Chemical Physics Letters 433:125–129.

UBA, BfR, and BAuA. (2011). Nanomaterials and REACH Background Paper on the Position of German Competent Authorities.

Uboldi, C., P. Urbán, D. Gilliland, E. Bajak, E. Valsami-Jones, J. Ponti, and F. Rossi. (2016). Role of the crystalline form of titanium dioxide nanoparticles: Rutile, and not anatase, induces toxic effects in Balb/3T3 mouse fibroblasts. Toxicology in vitro : an international journal published in association with BIBRA 31:137–45.

WHO. (2014). Guidance document on evaluating and expressing uncertainty in hazard characterization.

Zalk, D. M., S. Y. Paik, and P. Swuste. (2009). Evaluating the Control Banding Nanotool: a qualitative risk assessment method for controlling nanoparticle exposures. Journal of Nanoparticle Research 11:1685–1704.

Zhang, H., Z. Ji, T. Xia, H. Meng, C. Low-Kam, R. Liu, S. Pokhrel, S. Lin, X. Wang, Y.-P. Liao, M. Wang, L. Li, R. Rallo, R. Damoiseaux, D. Telesca, L. Mädler, Y. Cohen, J. I. Zink, and A. E. Nel. (2012a). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS nano 6:4349–68.

Zhang, H., Z. Ji, T. Xia, H. Meng, C. Low-Kam, R. Liu, S. Pokhrel, S. Lin, X. Wang, Y.-P. Liao, M. Wang, L. Li, R. Rallo, R. Damoiseaux, D. Telesca, L. Mädler, Y. Cohen, J. I. Zink, and A. E. Nel. (2012b). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS nano 6:4349–68.

3 Availability and regulatory relevance of literature-based models for NMs

3.1 Summary

This chapter describes the availability, and assesses the REACH relevance, of literature-based QSPR and QSAR models, as well as toxicokinetic (TK), toxicodynamic (TD), *in vitro* and *in vivo* dosimetry, and environmental fate models. The aim of the chapter is to identify models potentially useful for the fulfilment of REACH information requirements (Appendix III).

TK/TD/dosimetry/fate models are all kinetic models, i.e. they provide information on the (time-dependent) concentration or amount of NMs in a biological, technical or environmental compartment. TK models include relatively simple, classical toxicokinetic (CTK) models, as well as physiologically-based kinetic (PBK) models. Environmental fate models include material flow (MF) models, process-based fate models, and bioaccumulation models. TD models, including physiologically based dynamic (PBD) models, simulate the intensity and time-course of substance-induced effects on a biological system. These models are still in their infancy (only four such models were identified).

For each model domain (QSPR/QSAR, TK/TD/dosimetry/fate models), details of the paper search and selection strategy to compile the model inventory are first presented. To systematically capture information on TK and TD models, a structured template was developed (Appendix VII) Model inventories are stored as separate Excel files (Supplementary Materials S1 and S2). Secondly, a detailed analysis on the characteristics of available models is reported: emphasis is given to the nanomaterials covered by the models, the predicted endpoints, descriptors used and the statistical methods applied. In the third part of each section, we evaluate the extent to which available models are potentially useful for predicting REACH-required physicochemical properties, ecotoxicological and toxicological endpoints, or how available models could be applied supporting REACH submissions.

Some QSPR and QSAR models are indeed predicting REACH-relevant information. Under the physicochemical characterisation, models predicting water solubility, octanol-water partition coefficient, stability in organic solvents are available. For toxicological endpoints, there are some models for the Ames test, although the relevance of such models for mutagenicity assessment is questionable, as the OECD TG on the Ames test is not considered applicable to NMs. The majority of QSAR models are for cytotoxicity endpoints, which are not directly predictive of a REACH endpoint, but may be useful for screening purposes, or as the basis of grouping NMs for read across. For ecotoxicity endpoints, models are available on growth inhibition study, short term toxicity to fish and to invertebrates. Many other models have been developed using composite endpoints, which while related to REACH endpoints, are not directly predictive of them. Such models are therefore of limited utility for directly filling data gaps, but are potentially useful for screening purposes, or as the basis of grouping similar NMs for read across. Even where a model is directly predictive of a REACH endpoint, it does not necessarily follow that the prediction is adequate for filling a data gap. As with all QSPR and QSAR predictions, this needs to be evaluated on a case by case basis, in accordance with REACH guidance.

The utility of the established QSPR/QSAR model reporting format (QMRF) is evaluated by applying it to a QSAR model. On this basis, it is proposed to make minor adaptations to the QMRF in order to capture some NM-relevant physicochemical properties (Appendix VI).

The kinetic predictions provided by TK/dosimetry/fate models can be used for various purposes, which depending on the model type, include: a) the better interpretation of toxicity data; b) the design of toxicity studies; c) various types of extrapolation that support risk assessment (*in vitro* to in vivo, high dose to low dose, interspecies) and d) the prediction of toxicologically relevant internal concentrations at target organ/tissue level. The latter type of information could in principle be used to reduce uncertainty in read-across predictions for systemic endpoints.

3.2 QSPR/QSAR model inventory

A compilation of QSPRs and QSARs has been based on a comprehensive and systematic analysis of existing literature on computational models for nanomaterials. The aim of this compilation was to determine the models that are currently available and that could be used in a regulatory context (e.g. REACH) for the assessment of the hazardous properties of manufactured NMs. The models have been documented in a searchable Excel file (hereafter inventory). Parameters such as endpoint predicted, NMs modelled, descriptors used in the model, data source, algorithm, performance statistics, have been collected for each model. The detailed model information in the inventory was used to construct the "landscape" of available QSPR/QSARs, including the endpoints and NPs modelled, the descriptors that were mostly used in the models as well as the type of models and data sources used.

As explained in Chapter 1 (section 1.7.2), QSPRs and QSARs are models that mathematically relate a property (in the case of QSPRs) or (biological) activity (in the case of QSARs) of a given substance with its chemical structure (or other physicochemical properties). Such models are based on the assumption that the property/activity of a substance is related to its (chemical) structure. Independently of the method used to derive the QSPR/QSAR, the same 5 principles apply for their validation (OECD, 2007b), namely: 1) defined endpoint; 2) unambiguous algorithm; 3) defined domain of applicability; 4) appropriate measures of goodness-of-fit, robustness and predictivity; and 5) mechanistic interpretation.

In the following sections, the search strategy used to compile the QSPR/QSAR models is first described, followed by a detailed description of the parameters used to categorise the models and the methodology applied for the literature review. Finally, the current QSPR/QSAR model landscape will be described by analysing the endpoints, NPs, descriptors, statistical method, etc. of the QSAR-QSPR models that are included in the inventory.

3.2.1 *Search and selection strategy*

The steps followed from the selection of relevant papers to their inclusion in the inventory are summarized below:

1. Selection of papers by means of database websites
2. First selection of relevant publications
3. Removal of duplicates
4. Prioritisation of relevant papers based on the abstract
5. In-depth reading of the articles
6. Inclusion or exclusion of the model from the inventory

In order to select the initial set of publications, Scopus and WoS (Web of Science) database websites were used as search engine tools and the relevant terms included for the initial

screening were chosen. Specifically, the main terms were: the model itself (i.e. QSAR*[14], SAR*, "structure activity", QSPR*, "structure property", nano-QSAR and QNAR), the nanomaterial term (nanomaterial*, nanoparticle*, nanotube*, fullerene*), the desired endpoint to which the mathematical model is aiming to give answer (e.g. toxicity, ecotoxicity, cytotoxicity, solubility, adsorption, ROS production), and finally the species or human cell lines which are usually used in the experimental procedures for toxicity assessment (e.g. Daphnia, lung cells). In addition, due to sometimes the term QSAR is not used to define the models but the type of algorithm (e.g. decision tree, SVM, neural network, machine learning, random forest, linear regression and PCA), these terms were also included in the search parameters.

Different combinations of the terms included above led to the compilation of almost 1150 publications, which were included in the reference manager Mendeley program. After removal of duplicates, the set of publications was reduced down to a number close to 600. Then, a detailed evaluation of titles and abstracts further reduced the number of the final set, resulting in a total of 122 publications, from which 78 were assigned to QSAR and 44 to QSPR models. The screening performed to remove non-relevant papers in the last step was based mainly on the following criteria:

- Papers containing the SAR term but with other meanings (e.g. specific adsorption rate, also SAR, is associated with magnetic and hyperthermia effect)
- Papers describing the synthesis of NMs
- Very specific papers describing the behaviour of NMs for catalysis applications
- Scientific publications that state in the abstract "results provide the basis for a QSAR development", etc.

Most of the removed papers were experimental works dealing with nanomaterials, rather than publications developing or evaluating any model. In addition to the citation databases, new methodological approaches discussed in recent reviews (C Oksel, Ma, Liu, Wilkins, & Wang, 2015; R Tantra et al., 2015), and new scientific papers published during the execution of this work have also been taken into consideration to avoid missing any relevant QSPR-QSAR model.

Evaluation of the final set of 122 publications was performed following the OECD validation principles. This review was done in a chronological order starting from the oldest papers to be able to recognise the citations of already revised publications as well as identify possible new models, not captured using the strategy presented above.

A considerable number of papers, in which the developed models were the same or closely related to previous ones, were not reported in the inventory as (new) entries, but were included as associated references of the previous models. As an example, the paper entitled "Machine Learning for Nanomaterial Toxicity Risk Assessment"(Gernand & Casman, 2014b) is actually a brief summary of one of the models generated in a previous work of the same group (Gernand & Casman, 2014a). In this case, the first article (Gernand & Casman, 2014a) was used as main entry to describe the model while the paper (Gernand & Casman, 2014b) was included just as a reference of the previous model developed.

After an accurate review of the search results, a total of 59 publications for QSAR (out of around 800 found in the initial search) and 29 publications for QSPR models (out of around

---

[14] using "*", all terms containing QSAR are captured (e.g. QSAR, QSARs, QSAR-model)

350 found in the initial search) were included in the final inventory. Quantification of the papers that have been either included or excluded in the final inventory is shown in Figure 3.1.

In addition, Figure 3.2 shows the number of publications included in the inventory with respect to the year of publication. From the figure it is clear that the number of QSAR publications follows an increasing tendency over time since 2010 since a variety of datasets and methods have been made available, while the rate of QSPR publications remains almost constant. However, it must be stressed that due to the need of limiting the number of papers, QSPR models aiming at predicting endpoints that could have an influence on toxicity and on the bioavailability of the nanomaterials in the environment have been prioritized to others. For instance, a few papers developing models to predict the mechanical reinforcement of polymer nanocomposites by the addition of metal oxide particles have not been taken into consideration.
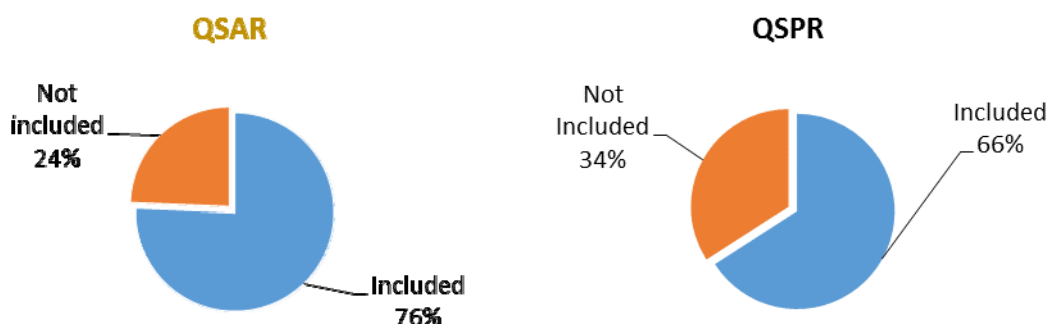
Figure 3.1. Percentage of QSAR (n=78) and QSPR (n=44) papers included and excluded from the inventory

	From 2000 to 2009	2010	2011	2012	2013	2014	2015	2016
QSAR	0	3	4	5	6	12	19	9
QSPR	13	0	3	0	4	3	4	2
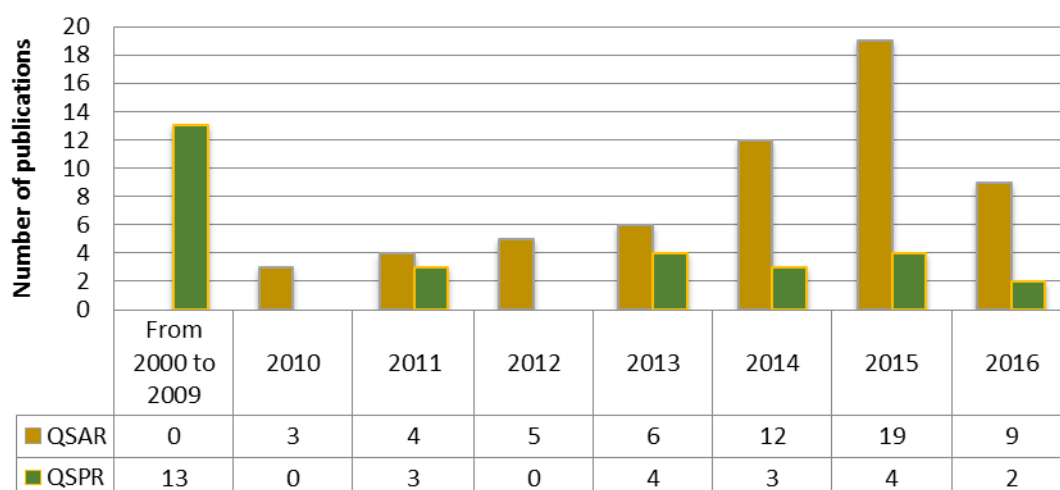
Figure 3.2. Number of publications included in the inventory as a function of publication year

Even though 59 QSAR and 29 QSPR publications were considered as relevant for the review, more than 88 rows were added to the inventory. This is because in some publications different cases are investigated, either because the same data is modelled by means of different statistical methods or because the same statistical method is applied to different NMs/endpoints. In such cases, all entries corresponding to the same paper are labelled with the same number followed by different letters in alphabetical order. The final number of entries was 152 and 52 for QSAR and QSPR models, respectively.

Figure 3.3 shows the structure of the inventory, covering all different sections (8) and corresponding parameters (35) used to categorize the relevant models. These sections are 1) source information, 2) predicted endpoint, 3) NMs, 4) data sets, 5) descriptors, 6) statistical methods, 7) model performance and 8) miscellaneous.
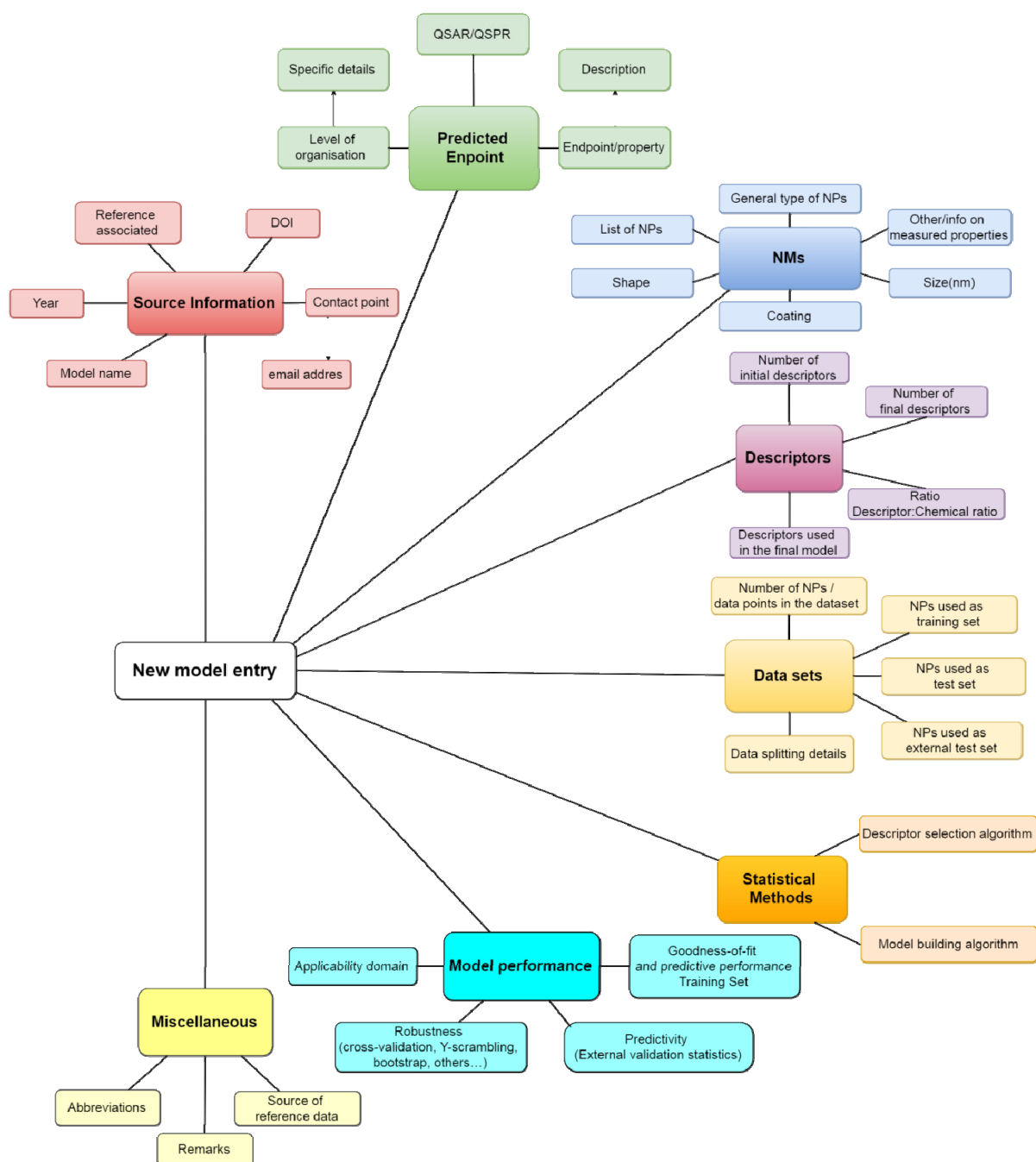
Figure 3.3. Structure of the QSPR/QSAR inventory

The content of each section is summarised below. It should be indicated that the structure of the inventory was inspired by the QSAR model reporting format templates (QMRF) which were developed for QSARs. The entries of the inventory can be easily transferred to the QMRF providing the latter is adapted to NMs by allowing the definition of physicochemical properties that define NMs.

1. **Source Information**: reference details of the publication, contact author and also the model name. To assign a model name, its aim and the method use to generate it have been used as main criteria.

2. **Predicted Endpoint**: information about the type of cells or organisms used in the study, the toxicity endpoint, and whether it is an *in vitro* or *in vivo* experiment (for QSAR), or information about the physicochemical properties evaluated (for QSPR).

3. **NMs:** in this section, first a more generic description of the type of NMs is provided, including metals, metal oxides, carbon-based or polymeric nanomaterials, and then the specific NMs are defined (i.e. Ag). Moreover, associated physicochemical properties are also indicated here, such as coating, size, shape, and any other relevant characterization performed.

4. **Descriptors**: this section specifies the number and which are the descriptors used (e.g. fingerprints, topological, geometric, physicochemical) in the models. It also includes the ratio between chemical compounds and final descriptors, which is a value used to assess the possible correlation by chance and overfitting due to a greater number of descriptors than the size of the training data set (Topliss & Edwards, 1979).

5. **Data sets**: size of the training, test and external validation data set are given. Details of data splitting are also specified when applicable.

6. **Statistical methods**: here, an explanation of the descriptor selection process (screening from an initial set to obtain a reduced final set of descriptors) as well the details of the statistical method applied to generate the model are provided. The software tools are also specified if they are provided in the publication.

7. **Model performance**: this section contains four inputs corresponding to the principles 3 and 4 of the OECD guideline, i.e. applicability domain, goodness-of-fit, robustness and predictivity.

8. **Miscellaneous**: definition of the abbreviations used is provided as well as the reference of data bases employed in the studies and relevant remarks, e.g. whether a mechanistic interpretation is presented which improves the reliability of a model (principle 5 of the OECD guideline).

3.2.2 Analysis of the available QSPRs and QSARs

To illustrate the current state of the art concerning the QSPR and QSAR model development applied to nanomaterials, we have selected four of the most representative inputs used to categorize such models in the inventory (Figure 3.4): 1) NMs, 2) descriptors 3) statistical methods and 4) endpoints predicted by the model. The figure also includes the quantification of the specific weight of each element within the same category assigned to either QSAR or QSPR models. These four inputs will be discussed separately below.
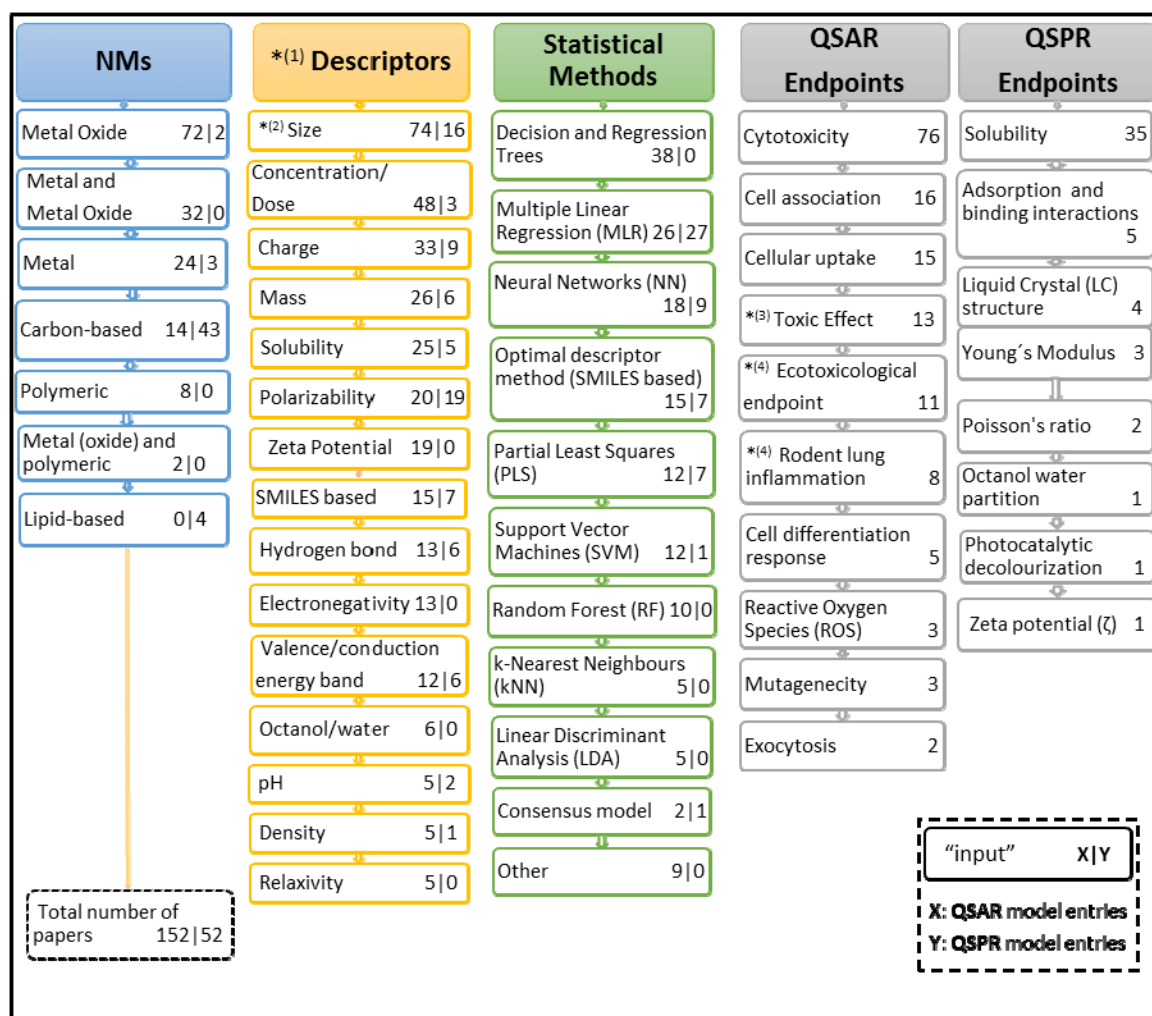
NMs

NMs	X|Y
Metal Oxide	72|2
Metal and Metal Oxide	32|0
Metal	24|3
Carbon-based	14|43
Polymeric	8|0
Metal (oxide) and polymeric	2|0
Lipid-based	0|4

Total number of papers 152\|52

*(1) Descriptors

Descriptors	X|Y
*(2) Size	74|16
Concentration/Dose	48|3
Charge	33|9
Mass	26|6
Solubility	25|5
Polarizability	20|19
Zeta Potential	19|0
SMILES based	15|7
Hydrogen bond	13|6
Electronegativity	13|0
Valence/conduction energy band	12|6
Octanol/water	6|0
pH	5|2
Density	5|1
Relaxivity	5|0

Statistical Methods

Method	X|Y
Decision and Regression Trees	38|0
Multiple Linear Regression (MLR)	26|27
Neural Networks (NN)	18|9
Optimal descriptor method (SMILES based)	15|7
Partial Least Squares (PLS)	12|7
Support Vector Machines (SVM)	12|1
Random Forest (RF)	10|0
k-Nearest Neighbours (kNN)	5|0
Linear Discriminant Analysis (LDA)	5|0
Consensus model	2|1
Other	9|0

QSAR Endpoints

Endpoint	X|Y
Cytotoxicity	76
Cell association	16
Cellular uptake	15
*(3) Toxic Effect	13
*(4) Ecotoxicological endpoint	11
*(4) Rodent lung inflammation	8
Cell differentiation response	5
Reactive Oxygen Species (ROS)	3
Mutagenecity	3
Exocytosis	2

QSPR Endpoints

Endpoint	X|Y
Solubility	35
Adsorption and binding interactions	5
Liquid Crystal (LC) structure	4
Young's Modulus	3
Poisson's ratio	2
Octanol water partition	1
Photocatalytic decolourization	1
Zeta potential (ζ)	1

"input" X\|Y

X: QSAR model entries
Y: QSPR model entries

Figure 3.4. Summary of the most relevant inputs obtained in the inventory analysis for QSAR and QSPR models. Numbers assigned to QSAR/QSPR models allow quantifying the specific weight of each element within the same type model of model. As an example, the number of models looking at carbon-based NMs corresponds to 14 and 43 for QSAR and QSPR models, respectively. Total number of models corresponds to the total number of entries in the inventory (152 and 52 for QSAR and QSPR, respectively). *(1): Most representative descriptors were included. *(2): Size descriptor includes size, radius, diameter, length, volume and aggregation/agglomeration *(3): Endpoint defined by the author. *(4): Endpoint defined in this work to group highly associated endpoints.

Nanomaterials

Models found in the literature search cover a total of 44 different NMs, including metals, metal oxides and carbon, polymeric and lipid-based particles. As shown in Figure 3.4, metal oxides account for about 72 out of 152 QSAR published models, and 32 models included both metals and metal oxides nanoparticles (NPs). Carbon-based and polymeric NMs are applied in only 14 QSAR models. In Table 3.1, all specific NMs are listed and grouped following the same division than in Figure 3.4.

Table 3.1. List of NMs reported in the inventory. The number in brackets indicates the how many times each NM appears among the different models, including both QSAR and QSPR studies.

GENERIC TYPE OF NANOMATERIAL	SPECIFIC TYPE OF NANOMATERIAL
Metal	Au(27), Ag(25),Cu(23), Al(18), Ni(16), Co(11), Ti(10), Fe(9), Sn(8), Zn(2), Si(1), Ge(1)
Metal Oxide	ZnO(73), Fe_2O_3(70), TiO_2(67), Fe_3O_4(53), Al_2O_3(50), SiO_2(45), CuO(43), La_2O_3(41), NiO(39), SnO_2(39),CoO(38), Y_2O_3(37), Sb_2O_3(35), Cr_2O_3(30), In_2O_3(30), CeO_2(24), WO_3(17), $(Fe_2O_3)_n(Fe_3O_4)_m$(16), Ni_2O_3(14), Mn_2O_3(12), Gd_2O_3(9), Yb_2O_3(9), Co_3O_4(7), HfO_2(7), $R-TiO_2$(5), ZrO_2(1)
Carbon-based	Fullerene(33), Nanotube(25)
Polymeric	p(NIPAm-co-AAc) (5), Streptokinase (SK) and Chitosan (3)
Lipid-based	Phytantriol (2), Monoolein (2)

As can be seen in Table 3.1, Au (27) and Ag (25) are frequently chosen as metal NPs (Papa, Doucet, Sangion, & Doucet-Panaye, 2016; Walkey et al., 2014), while ZnO (73), Fe_2O_3 (70) and TiO_2 (67) are the most repeated metal oxide NPs (T. C. Le et al., 2016; Papa, Doucet, & Doucet-Panaye, 2015; Toropova & Toropov, 2013). This is in line with the reported values for global production of nanomaterials, as detailed elsewhere, since TiO_2 and ZnO are two of the most produced nanomaterials over the world and with more range of applications (Arturo A Keller, Mcferran, Lazareva, & Suh, 2013). Furthermore, attention has been paid to evaluate the influence of TiO_2 particle size, and TiO_2 crystalline phase (i.e. anatase or rutile) which finally determines the reactivity of this material. For instance, E. Papa et al. investigated the membrane disruption (cytotoxicity) of TiO_2 and ZnO NPs onto immortalized rat L2 lung epithelial cells and rat lung alveolar macrophages (*in vitro* experiments) by different statistical methods such as Multiple Linear Regression (MLR) or Radial Basis Function Neural Networks (RBFNN). A significant number of models (21) were reported for both TiO_2 and ZnO NPs with different sizes in different media, as independent sets or including both NMs as part of the same data set (Papa et al., 2015).

The applicability domain of a model depends on the number and variety of NPs included in its development. The number of NMs[15] evaluated in each model differs considerably, ranging from 1 (TiO_2 with different sizes in Papa, Doucet, & Doucet-Panaye, 2015b) up to 32 (different metals and metal oxides having different sizes and coatings in Kleandrova, Luan, González-Díaz, Ruso, Speck-Planche, et al., 2014). For example, a model included CuO, Al_2O_3, Fe_2O_3, SnO_2, TiO_2, V_2O_3, Y_2O_3, Bi_2O_3, In_2O, Sb_2O_3, SiO_2, ZrO_2, CoO, NiO, Cr2O3 and La_2O_3 (T Puzyn et al. 2011).

Instead of using different sizes or different types of NMs, other authors have evaluated the effect of surface functionalization. Fourches et al. modelled the prediction of NMs cellular

---

[15] Nanomaterials having the same composition but different sizes are considered different NMs in the inventory

uptake by pancreatic human cancer cells (PaCa2) using a dataset of iron oxide particles coated with 109 different chemical groups. A k-Nearest Neighbour method was applied to two-dimensional descriptors (D Fourches et al., 2010).

Concerning the QSPR studies, carbon-based NMs (i.e. fullerenes and carbon nanotubes) account for an extremely high percentage of models (43 out of 52 QSPR models). This can be explained by the large number of papers (30 out of 52 QSPR models) reporting the solubility of C60 in different types of organic solvents (Huanxiang Liu et al., 2005; Petrova, Rasulev, Toropov, Leszczynska, & Leszczynski, 2011; Yousefinejad, Honarasa, Abbasitabar, & Arianezhad, 2013). Since C60 was discovered in 1985, there has been considerable interest in identifying possible applications for this highly symmetrical molecule.

CNTs have been evaluated in 12 (out of 52) of the QSPR models reported in the inventory. For instance, descriptors for prediction of mechanical properties were identified through QSPR models and evaluate the qualitative and quantitative results against computational and experimental data (Young's modulus and Poisson's ratio). CNTs of 10 nm long and 0.2-2.1 nm diameter were created with J Crystal Soft Nanotube Modeler version 1.6.1 and individually processed with Python scripts to create four types of surface defects: single vacancy, double vacancy, mixed single and double vacancy. The model was built with partial linear squares method and constitutional, topological, and physicochemical descriptors were used in this study. The final model contained the following critical descriptors: theoretical radius, chiral angle and ratio of non-sp^2 hybridized carbons to total number of carbons (Borders, Fonseca, Zhang, Cho, & Rusinko, 2013).

Significantly different to carbon-based nanomaterials, metal and metal oxide NPs only account for the 5 out of 52 QSPR models. From the studies reported in the inventory, only one attempt has been already applied to inorganic NMs to describe physicochemical properties. Zeta potential was predicted from a set of 18 metal oxides NPs applying 11 microscopic- image-based and 17 theory-based (calculated) descriptors (Mikolajczyk et al., 2015). In another study, the adsorption of methylene blue onto copper oxide NPs loaded on activated carbon (CuO-NP-AC) was modelled. Applied descriptors were pH, dye concentration, amount of NPs, time and amount of carbon active. After principal component analysis treatment, resulting principal components were used as independent variables into a MLR statistical method (Ghaedi et al., 2014a).

Regarding the used data sets, as few as 47 out of 204 reported models in the inventory generated their own experimental data (A Gajewicz et al., n.d.; T Puzyn et al., 2011; Christie Sayes & Ivanov, 2010), and therefore most of the studies were limited to small datasets, obtained by the same research group at matching conditions. Several datasets have been used extensively in a large number of publications, such as those generated in Fourches et al. 2010, T Puzyn et al. 2011, Sayes and Ivanov 2010, Shaw et al. 2008 and Weissleder et al. 2005. Such publications typically evaluate the same NMs as well as the same endpoints, but new models are implemented with different descriptors or different applied statistical methods. As a representative example, A. Toropov et al. 2012 retrieved cytotoxicity data on bacteria (*E.Coli*) from T Puzyn et al. 2011 for several metal oxide NPs, and developed a new model base based on SMILES (Simplified Molecular Input Line Entry Specification) notation and Monte Carlo optimization, compared to the MLR employed in the original reference. With the same data set (same NMs and endpoint) but different number and type of

descriptors (ΔH_{Me+}[16] in T Puzyn et al. 2011 and '['[17] , '='[18] and 'O'[19] in A. Toropov et al. 2012) the statistical fit for internal validation was slightly higher for the MLR (R^2=0.85) than for the SMILES-based model (R^2 ranging from 0.74-0.84). On the contrary, considerably better results were obtained with the SMILES-based model as regard as external validation (R^2 ranging from 0.84 to 0.96) compared to the MLR model (R^2=0.83). It is expected that in the following years, more data sets will become available resulting from the large effort done in the framework programmes FP7 and H2020, funded by the EU. An example is the dataset extracted from the data generated in the MODENA COST initiative (Cassano et al., 2016).

Another point that must be underlined is that only 137, 72 and 77 out of 204 models reported size, coating/functionalization and shape, respectively, revealing that physicochemical characterization of NMs is not reported as a routine basis. This could by reasoned by the fact that experimental descriptors might had been generated by computational resources, such as Molecular Dynamics simulations (Borders et al., 2013) or by means of different software tools, such as DRAGON (Gharagheizi & Alamdari, 2008). Therefore, in those publications the physicochemical characterization could have been considered not to be relevant and consequently not reported by the authors.

Size has been reported in 137 out of 204 works, while coating/surface functionalization, zeta potential and shapes are barely provided in literature. Another point to take into consideration is that ion dissociation, which is one the main processes driven toxicity of some NMs (e.g. Ag or CuO) is not generally included in the discussion of the models. Furthermore, some well-known technical challenges encountered when testing the effects of NMs on organisms (Love, Maurer-Jones, Thompson, Lin, & Haynes, 2012). For instance, the formation of protein coronas might lead to the modification of NMs properties compared to pristine NMs or the process of dispersing the particles might heavily influence the toxicity results.

Between the different applied characterization techniques, Dynamic Light Scattering (DLS) was applied in the 43 out of 204 models to characterize the size and the zeta potential of the NMs, while Transmission Electron Microscopy (TEM) was used in the 41 cases for size determination as well as to extract information about the shape, aspect ratio, corner count, curvature and aggregation state of the nanomaterials. Other techniques identified are nuclear magnetic resonance spectroscopy (12 models), photon correlation spectroscopy (8 models), or X-ray diffraction (8 models).

Endpoints

Most of the QSAR models published to date have attempted to predict the toxicological effects of NMs by means of cytotoxicity *in vitro* studies in different cell types. Such studies accounted for 152 out of 204 models, in fair agreement with the vast body of literature that exists examining the potential effect of NMs in *in vitro* experiments. Cytotoxicity endpoint is commonly (76 out of 152) evaluated in literature by the percentage of cellular viability (N

---

[16] ΔH_{Me+} represents the enthalpy of formation of a gaseous cation having the same oxidation state as that in the metal oxide structure.

[17] '[' : each non-hydrogen atom is specified independently by its atomic symbol enclosed in brackets [ ]

[18] '=' : double bond

[19] 'O' : oxygen

Sizochenko et al., 2014), i.e. LC$_{50}$, EC$_{50}$,[20] membrane damage measured by LDH release or by propidium iodide uptake (R Liu et al., 2011) among others. Due to the large variety of *in vitro* studies, some authors (6 out of 152) have generated a generic "endpoint" named biological activity (D Fourches et al., 2010; Winkler et al., 2014), which is obtained aggregating related response measures of different assays such as amount of ATP content, reducing equivalents, caspase-mediated apoptosis, or mitochondrial membrane potential for different cell types. This is an example of the trade-off between data availability and data quality – related endpoints are aggregated to obtain a generic prediction of biological activity.

In addition, cell association represented 16 out of 152 QSAR endpoints reported in the inventory, which is highly relevant for biodistribution and inflammatory response. For instance, several models arising from an original data set generated and modelled by Walkey et al. 2014, evaluated the cell association of A549 human lung epithelial carcinoma cells (ATCC) with Au NPs surrounded by a protein corona. Cell association was predicted using the protein corona fingerprint and/or the physicochemical properties of NMs as descriptors (e.g. single NM Volume [nm^3], molecular weight, percentage of polar/acidic amino acids and hydrodynamic diameter measured after exposure to serum) applying a wide variety of statistical methods, such as MLR, SVR, PLSR or k-NN (Kamath, Fernández, Giralt, & Rallo, 2015; R Liu, Jiang, Walkey, Chan, & Cohen, 2015; Papa et al., 2016).

As one of the cell association endpoints, cellular uptake[21] accounted for 15 out of 152 QSAR models). However, all relevant models reported in the inventory used the same original data set (Weissleder et al., 2005)[22] which describes the cellular uptake of cross-linked iron oxide NPs (coated (Fe$_2$O$_3$)n(Fe$_3$O$_4$)m) by pancreatic human cancer cells (PaCa2) (Chau & Yap, 2012; D Fourches et al., 2010; M Ghorbanzadeh, Fatemi, & Karimpour, 2012; R Liu, Rallo, Bilal, & Cohen, 2015; Melagraki & Afantitis, 2014; C Oksel, Winkler, Ma, Wilkins, & Wang, 2016; A A Toropov et al., 2013; Wen DAI, Xue-Ying SHAN, 2015). Finally, few researchers have addressed rodent lung inflammation[23] (8 models, e.g. immune response measured by macrophages (MAC) in Gernand and Casman 2014a), cell differentiation (5 models, e.g. Bygd, Forsmark, and Bratlie 2015), ROS (3 models, e.g. Le et al. 2016), mutagenicity (3 models, e.g. A. A. Toropov and Toropova 2015) and exocytosis (2 models, e.g. C Oksel et al. 2016) as predicted endpoints.

In analogy with the biological activity endpoint mentioned above, toxic effect (13 QSAR models) is an endpoint determined by aggregating related response measures, including mortality assays (e.g. cytotoxic concentration CC$_{50}$, EC$_{50}$, IC$_{50}$) for both cell lines (e.g. HEK293, HepG2) and organisms (e.g. *Danio rerio* - juvenile; *Daphnia pulex* - adults). The aggregation of different endpoints, resulted in a binary classification into toxic ("1") or non-toxic ("-1") classes (V V Kleandrova, Luan, González-Díaz, Ruso, Speck-Planche, et al., 2014).

---

[20] LC50 and EC50 are the effective concentration that kills or inhibits 50% of the living systems, respectively.

[21] Cell association is a wider term that also includes cellular uptake, membrane adsorption, etc.

[22] Some works reference D Fourches et al. 2010 instead of Weissleder et al. 2005.

[23] Rodent lung inflammation includes different endpoints such as cell death, cell membrane damage and immune response.

Regarding ecotoxicity studies, "Ecotoxicological endpoint" (11 models, Figure 3.4) was defined as an aggregation of different ecotoxicity-related endpoints such as LC_{50} (G. Chen, Peijnenburg, Kovalishyn, & Vijver, 2016), or percentage of mortality (Z. Zhou, Son, Harper, Zhou, & Harper, 2015a).

To summarize, the corresponding weight percentages of *in vitro* and *in vivo* endpoints assessed in the QSAR models, differentiating between human and ecotoxicity are shown in Figure 3.5. It is evident that the number of *in-vivo* experiments is significantly low, which clearly indicates that the ability of current models to predict *in vivo* toxicity is insufficient. This might be partially due to ethical and economical considerations, since large research efforts are dedicated to the development of toxicity test alternatives *in vitro*.
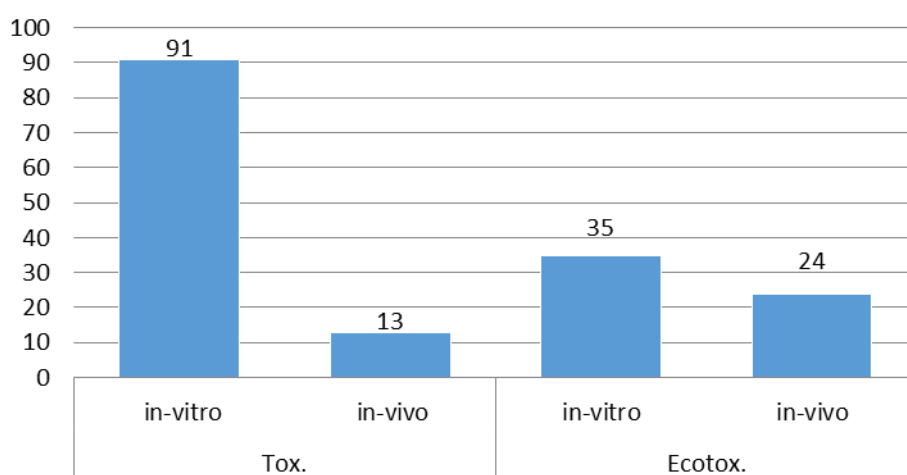

Figure 3.5. Number of QSAR models according to the different types of endpoints predicted. Differentiation between human and ecotoxicology is made as well as between *in vivo* and *in vitro*. Studies involving bacteria have been classified as *in vivo* ecotoxicological studies.

Concerning QSPR models, almost half of the studies were published between 2000 and 2010, and solubility of C_{60} in a large variety of organic solvents was the endpoint mostly evaluated (35 out of 52 models reported). This tendency has followed the same trend, as can be deduced from Figure 3.2. The reader is referred to a review which addressed this period (Saliner, Burello, & Worth, 2008). The solubility of a nanomaterial considers the interactions between both the nanomaterial and the solvent, which allows a modelling with information from both substances. It is to be noted that solubility studies including nanotubes and fullerenes C_{60}/C_{70}, accounted for the 43 out of 52 QSPR models. Solubility of carbon-based nanomaterials was modelled in different solvents. For instance, in Toropov et al., 2007, a model based on the parameters of chiral vectors *(n,m)* for CNT was generated by MLR to predict its water solubility. One representative example for fullerenes (C_{60}-C_{70}) was recently published by Sizochenko et al. (2016). The authors reported a prediction model for solubility in chlorobenzene, using atomic weight, partial charges, lipophilicity and polarization as physicochemical descriptors by means of a partial least squares method (PLS). From an environmental perspective, the solubility and different affinity for organic molecules is of extremely importance to determine the fate and transport of NMs in the different environmental compartments (A Praetorius, Arvidsson, Molander, & Scheringer, 2013).

Despite the large number of studies reported for carbon-based NMs, prediction of solubility for metal (e.g. Ag) or metal oxides NPs (e.g. Zn) remains unstudied by means of QSPR approaches.

Other endpoints have been evaluated in literature, but their contribution into the total number of studies is fairly small compared to solubility. Those endpoints are adsorption and binding interactions (5 models, e.g. Ghaedi et al. 2014), liquid crystal structure (4 models, e.g. Le et al. 2013),Young's modulus and Poisson's ratio (5 models, e.g. Borders et al. 2013), octanol water partition coefficient (1 model, A. A. Toropov, Leszczynska, and Leszczynski 2007), and zeta potential (ζ) (1, Mikolajczyk et al. 2015). As underlined before, this is one of the few attempts applied to inorganic nanomaterials to describe physicochemical properties. Interestingly, in this work, zeta potential predicted for aset of metal oxide NPs was described by the weighted energy of the highest occupied molecular orbital (quantum mechanical descriptor) and the spherical size of the NPs, which is a descriptor generated from electronic microscopy images. It is also worth recalling that zeta potential has been frequently used as a descriptor in different QSAR studies (Figure 3.4), and is represented in 19 out of 152 models (Epa et al. 2012; C Oksel et al. 2016; Silva et al. 2014).

Descriptors

Descriptors are the base of QSAR model frameworks. It has been already discussed in previous reviews that new specific descriptors for NMs are needed (C Oksel et al., 2015; T Puzyn, Leszczynska, & Leszczynski, 2009; Natalia Sizochenko & Leszczynski, 2016; Winkler et al., 2013; Ying, Zhang, & Tang, 2015). In this section an overview of the most applied descriptors as well as emergent useful "nano-descriptors" for different type of NMs are described. The most representative descriptors found in the inventory are shown in Figure 3.4.

Generally speaking, there are several descriptors which are most frequently applied in the models reported in the inventory. While for QSAR models, size (74), concentration/dose (48), charge[24] (33), mass (26), solubility (25) are the most representative, polarizability (19), size (16) and SMILES-based (7) are the most regularly applied descriptors in QSPR models. In this chapter, descriptors have been categorized into 1) experimental and 2) molecular. Some of these descriptors are novel, having been developed to capture the size-related properties of particles not captured by traditional descriptors.

Experimental descriptors

Experimental descriptors can be divided into, namely geometrical (e.g. size, radius, volume, shape, level of aggregation) and physicochemical (e.g. surface charge, pH, zeta potential, solubility). It should be pointed out that only 16 reported models included just experimental descriptors for model building.

NM size, either determined from electron microscopy images (i.e. real diameter) or determined in different relevant liquid exposure media as water, PBS or Dulbecco modified eagle medium (i.e. hydrodynamic diameter typically obtained by light diffraction scattering)

---

[24] Charge includes descriptors like atom charge, ion indexes and surface charge.

appears as the most common geometric descriptor (90 out of 204) used in the reviewed models (Chen et al. 2016; Papa et al. 2016), including primary or aggregated size (e.g. Pan et al. 2016) or volume (e.g. Le et al. 2016). Besides size, shape is represented in 10 models. This fact is not surprising since the high surface reactivity is mainly due to the nano-size of the particles. In a representative example, nanoparticle size of a set of 24 different metal oxide NPs (e.g. Al_2O_3, CuO, SiO_2)[25], with a particle size ranging from 10 to 100 nm (it should be noted that both Cr_2O_3 and Ni_2O_3 were outside of this range), was used as a descriptor to predict cytotoxicity on rat alveolar macrophage cells (RAW 264.7), by means of a random forest statistical method (N Sizochenko et al. (2015). In this work, particle size in Dulbecco modified eagle medium was quantified using a novel high-throughput DLS.

Concerning relevant physicochemical descriptors, solubility and/or the ability of NMs to disperse in a media were also repeatedly used (25 out of 152 models). Unlike in QSPR models (5 out of 52) including carbon-based NMs, where solubility was actually the predicted endpoint, in a large number of QSAR models (35 out of 52), dispersion/solubility is employed as descriptor to predict the activity of NMs. For instance, Chen et al. (2016) applied the water solubility of metal and metal oxide NPs as one of the descriptors to classify materials in active or inactive, based on a hazard ranking.

Furthermore, zeta potential has been frequently used in QSAR models (19 out of 152 models). For instance, zeta potential data obtained from measurements of Au NPs before and after Au NPs opsonisation by serum proteins in the culture media was used as a descriptor to predict the exocytosis of the particles on human macrophage-like U937 cells (Bigdeli, Hormozi-Nezhad, & Parastar, 2015). As far as the relationship between zeta potential and toxicity is concerned, Cassano et al. recently revealed that less negative zeta potential values (used as a descriptor) were associated with higher cytotoxicity of SiO_2 NPs (Cassano et al., 2016). All zeta potential values reported in this work were negative. These findings are in contrast with results from literature which suggested that cytotoxicity increased as a result of positive zeta potential. It was argued that this discrepancy could be due to the fact that zeta potential was measured in water, rather than in the exposure medium used for cytotoxicity testing, which could substantially modify the measured values.

To conclude with the experimental-based descriptors, attention should be paid to the concentration or exposure dose, which appeared in 48 QSAR models (Figure 3.4). This parameter is not a directly description of the structure or physicochemical property of a target NM, but rather is a variable in the experiment with direct influence on toxicity, since it is well known that toxicity is dose-dependent. Even though this descriptor is useful for predicting toxicity of NMs in QSAR models, it does not provide any information about the role that physicochemical properties or (molecular) structure play. For instance, Papa et al. (2015) published 16 models where TiO_2 and ZnO NPs membrane disruption was modelled by different statistical methods and all of them selected concentration as one of the final descriptors. To exemplify it with statistics, the specific case of classification tree model selected size in water and concentration from five initial descriptors, and significantly high external predictivity was reported (100% sensitivity, 86% specificity and 93%accuracy)[26]. In

---

[25] Original data set came from Zhang et al. 2012.
[26] According to the OECD guidance, sensitivity is defined as the fraction of active chemical correctly assigned, specificity as the fraction of non-active chemicals correctly assigned and accuracy as the fraction of chemicals correctly assigned.

line with this, other experimental conditions have been included as descriptors such as exposure time (e. g. Horev-Azaria et al. 2011) or temperature (e. g. Rispoli et al. 2010).

Molecular descriptors

Molecular descriptors are characterized by their theoretical and mathematical origin, and do not suffer from the inherent variability of experimental data. As explained in Chapter 1 (Table 1.6), molecular descriptors are normally classified according to their dimensionality (i.e. 0D, 1D, 2D, 3D and 4D).

The 0D and 1D molecular descriptors are directly extracted from the chemical formula of the NM, such as number of atoms or hydrogen bonds, functional groups, rings, etc. In a recent published study, the number of terminal primary C (sp3), number of atoms, number of bounds, number of non-H bonds and number of donor atoms for H-bonds were used to describe cross-linked iron oxide NPs, in order to predict NPs uptake by PaCa2 cells, using a genetic-program-based decision tree induction (GPTree) (C Oksel et al., 2016).

2D molecular descriptors are based on the topological information of a substance, which is related with the shape representation of the molecules through molecular graph theory. An example of these descriptors is the Simplex Representation of Molecular Structure (SiRMS). SiRMS considers that every molecule can be represented as a group of simplexes, where simplexes are fragments with fixed composition and structure. Then, descriptors become the number of identical simplexes that are present in a molecule (N Sizochenko et al., 2014, 2015). There were only few cases of 2D molecular descriptors classified in the inventory, which is consistent with the conclusions drawn in a recent published review (Natalia Sizochenko & Leszczynski, 2016).

With respect to 3D molecular descriptors, these are properties that depend on a three-dimensional representation of chemical structures, as introduced in Chapter 1 (Table 1.6). Size, shape and others, which are equal to some of the experimental descriptors listed above, are derived from theoretical sources, most of the times from molecular simulations (Pan et al., 2016). 3D molecular descriptors also include electronic descriptors, also known as quantum chemical descriptors. For instance, polarizability is frequently used in both QSAR and QSPR models. Because polarizability allows knowing how the molecular charge distribution responds to external electromagnetic fields, it is consistent to use this descriptor to predict a possible activity and/or property (Kleandrova, Luan, González-Díaz, Ruso, Melo, et al. 2014; Mu et al. 2016; Puzyn et al. 2011; Sivaraman et al. 2001; N Sizochenko et al. 2016; ...). Finally, 4D descriptors, which are closely related to 3D descriptors, include energies for hydrophobic or electronic interactions (e.g. Durdagi et al. 2008).

Most of the molecular descriptors reported in the inventory were generated by specific software programs. Such programs are listed in Table 3.2. It is worth recalling that currently, more than 1000 initial descriptors can be generated.

Table 3.2 List of identified molecular descriptor sources.

Software name	Description	Reference in the Inventory
Cerius	Graphical molecular modeling program which can be used to generate topographical and physicochemical molecular descriptors	(Harper et al., 2015)
Chemistry development kit (CDK)	Library programmed in Java for chemoinformatics and bioinformatics. Topological, electronic, geometrical and constitutional descriptors can be generated	(V C Epa et al., 2012)
CoMSIA/CoMFA	Comparative Molecular Field Analysis (CoMFA), and Comparative Molecular Similarity Indices Analysis (CoMSIA)	(Serdar Durdagi, Mavromoustakos, & Papadopoulos, 2008)
CORAL	CORrelation And Logic software that generates models under optimal descriptor based on SMILES strings	(A A Toropov et al., 2012)
DRAGON	Software employed to generate widely types of molecular descriptors and also allows analyzing them by pair-wise correlation, PCA, etc. The last version (DRAGON 7) can calculate up to 5270 molecular descriptors	(C Oksel et al., 2016)
HiTQSAR	Software used to generate Simplex-informational descriptors	(N Sizochenko et al., 2016)
MODESLAB	Software used to generate physicochemical descriptors	(A Poater, Saliner, Sol, Cavallo, & Worth, 2010)
MOE	Molecular Operating Environment has different fields of application, where "MOE Cheminformatics and QSAR" can generate up to 400 2D and 3D molecular descriptors	(R Liu, Rallo, et al., 2015)
MOLD2	From 2D chemical structure inputs, the program gives an output of diverse molecular descriptors	(Melagraki & Afantitis, 2014)
MOPAC	Molecular Orbital PACkage, is a semi-empirical quantum chemistry software, used for the optimization of substance geometry simulations and or to obtain quantum mechanical descriptors	(A Gajewicz et al., n.d.)
PaDEL-Descriptor	Software used to compute molecular descriptors and fingerprints. Up to 1875 descriptors, where 1444 are 1D and 2D descriptors and 431 are 3D descriptors. PaDEL uses the library CDK to generate part of its descriptors	(Chau & Yap, 2012)
Pentacle	From a set of structures, energy interaction in 3D maps encoded into GRID based Molecular Interaction Fields, or MIFs are generated	(Rofouei, Salahinejad, & Ghasemi, 2014a)

Software name	Description	Reference in the Inventory
Chemspider	Database holding 58 million chemical structures, properties, and associated information. It also integrates ChemAxon (allowing access to chemicalize, online platform for chemical calculations, search and text processing). It is owned by the Royal Society of Chemistry.	(Z. Zhou et al., 2015a)
Online Chemical Database (OCHEM)	The website (www.ochem.eu) allows the users either to upload their own data or to use updated data by other users. With the different data sets, molecular descriptors can be computed from different descriptor packages. It includes almost all libraries and descriptors listed in this table and other packages which were not identified in the reviewed models. In addition, the program provides the possibility to build new models by means of a large set of possible statistical methods (e.g. neural networks, kNN, PLS).	(G. Chen et al., 2016)

Novel descriptors

Following a novel approach, Gajewicz et al. (2015) derived a set of initial descriptors from images obtained with a Hitachi H-7600 TEM, having a 0.35 nm point-to-point resolution. Each image was converted to numerical format, by converting pixels to certain values. In the 8-bit monochrome image (called gray scale image), each pixel had an assigned value ranging from 0 to 255, depending on the image gray levels. This method and also the data generated by Gajewicz et al. were used in further works to generate initial descriptors just as area, volume, surface diameter, aspect ratio, porosity, sphericity and circularity (Kar, Gajewicz, Roy, Leszczynski, & Puzyn, 2016; Mikolajczyk et al., 2015; C Oksel et al., 2016). In the same pioneer work, quantum mechanical descriptors were generated by (i) optimization of the cluster geometry with respect to the decreasing energy gradient and (ii) calculation of the descriptors on the basis of the optimized geometry.

Kleandrova et al. (2014) investigated new descriptors based on a perturbation approach. The aim of this technique was to overcome the problem about the different experimental conditions used by different researchers when performing toxicity studies. Generating random pairs of NPs, one par is defined as the reference NM and the other one is defined as the new NMs in the new experimental condition. This increases considerably the number of data points, since 85 initial NMs lead to 4133 data points, which were generated by NM-NM random pairs. Linear Discriminant Analysis (LDA) was implemented using the reference NP toxicity (EC50, IC50, TC50 or LC50) and the differences between NMs (size, shape, experimental condition and biological targets) as descriptors. To predict the toxicity of new NMs, these are paired with the training NMs which are set as reference NMs. Further details about the model equations are given in the original publication Kleandrova et al. (2014).

Similarly, considerable efforts have been devoted by other authors to expand the number of descriptors. Optimal descriptor method (applied in 22 out of 204), developed by Toropov

and co-workers, is based on SMILES notation. SMILES notation represents the molecular structure of a chemical compound in different fragments and the combination of the correlation weights (correlation with the endpoint) of each fragment generates a new descriptor which will be used in a linear regression to predict the endpoint. Monte Carlo optimization is used to select the most correlated fragments among the different possibilities (A. a Toropov, Leszczynska, and Leszczynski 2007; A A Toropov et al. 2008, 2009, 2012; A A Toropov and Toropova 2015a; Toropova et al. 2016; Toropova and Toropov 2013;). One of the first works was applied to the data set generated by Puzyn et al. (2011), to predict the cytotoxicity of metal oxide NPs in bacteria (*E. Coli*). The advantage of this method is that descriptors can easily be generated. However, the main disadvantage is the lack of mechanistic interpretation in the final model. Studies by A.A. Toropov and co-workers account for a 21 of the total reported models in the inventory.

There is a variant of SMILES-based descriptors named pseudo- or quasi- SMILES-based descriptors, which were included the same optimal-based descriptors category (Figure 3.4). This variant of SMILES notation also uses a string of characters to represent a substance that can be fragmented as input for optimal descriptors method. These strings hold categorical variables and continuous variables (transformed into categorical variables) which are not molecular structure descriptions. An example of quasi-SMILES based descriptors within optimal descriptor method is the work reported by Toropov and Toropova to predict the mutagenicity of multi-walled carbon-nanotubes and fullerene (A A Toropov & Toropova, 2015b). In this study, the quasi-SMILES were categorical variables were fullerene (X) or MWCNT (Z), dark condition (0) or irradiation condition (1), with (Y) or without preincubation (N) and presence (+) or absence of S9[27] (-). In addition to categorical variables, continuous variables were dose (g/plate) of C60 (50, 100, 200, 400 and 1000, defined by letters) and dose (μg/plate) of MWCNT which was defined in a similar way than dose of C60. As an example of the combination of such variables, the string "X0+A" would mean C60 in dark conditions in the presence of S9 at dose 50 g/plate.

There is also a recent study were pseudo-SMILES were tested as descriptors for random forest method and it was compared with the linear regression based on optimal descriptor method (Cassano et al., 2016). Interestingly, results showed that pseudo-SMLIES can be translated into descriptors for different statistical methods.

Another interesting approach for descriptor generation is the Liquid Drop Model (LDM) in combination with SiRMS (N Sizochenko et al., 2014). In the LDM, NPs are densely grouped in clusters with a minimum radius of interaction defined by the Wigner-Seitz radius, which represents the idea of the use a drop of spherical shape. The molecules inside the drop do not behave as the external ones because of the particles located inside the drop are surrounded by other particles and the interactions are not equal to the surface particles where the number of interactions is lower. Thus the forces are not equally compensated as for the internal particles. The associated models in the *Inventory* are the predictive classification of metal oxide NPs cytotoxicity on Bacteria Escherichia Coli (E. Coli) and another for human keratinocyte cell line (HaCaT). Both models used random forest as statistical method.

---

[27] product of an organ tissue homogenate which contains important enzymes used for metabolic activation in order to get a mutagenesis in Ames test (Mortelmans & Zeiger, 2000)

Finally, the parameters n and m, which define the chiral vector of carbon nanotubes, were used in a model as only descriptors to predict the water partition coefficient using MLR as statistical method (Andrey A Toropov et al., 2007). The chiral vector (C_h) of carbon nanotubes is defined as following:

$$C_h = n \cdot a_1 + m \cdot a_2$$

Since a nanotube can be regarded as a "rolled up" graphene sheet, the direction of roll defined by linear combination of units vectors (a1,a2) of hexagonal lattice and the coefficients n and m are the integers which multiply such vectors. Different combinations results in so-called "armchair" ($n=m$), "zigzag" ($m=0$) or "chiral" nanotubes. Since the electronic band structure will be set with metallic and semiconductor behaviour depending on the coefficients n and m properties as it is explained in Charlier et al. (2007). Nanotubes defined by the n and m indices will be metallic if $n-m=3l$ and I is an integer and semiconductor if I is a non-integer ($n-m=3l\pm1$). Since different electronic structures can result in different catalytic activity and behaviour, it chiral vector coefficient parameters have been used as descriptors.

Statistical Methods

As pointed out in the introduction, the most common supervised learning methods used to derive predictive models and a short description for each of them can be found in Table 1.5 (Chapter 1). Regarding the specific contribution of every statistical method (Figure 3.4), decision and regression trees (38 and 0 models for QSAR and QSPR, respectively) and MLR (26 and 27 models for QSAR and QSPR, respectively) were the most frequently used methods.

Even though MLR is also frequently applied in QSAR models, classification methods, including Support Vector Machines (SVM)[28] (e.g. Rong Liu et al. 2013), Random Forest (RF) (e.g. N Sizochenko et al. 2015) or LDA (e.g. Kleandrova, Luan, González-Díaz, Ruso, Speck-Planche, et al. 2014) have been also regularly employed to categorize the data points into several groups or classes. For instance, in Melagraki et al. (2015) and Oksel et al. (2016), biological response was categorized into active and inactive by means of a decision tree (DT) model.

The highest proportion of regression methods in QSPR models can be attributed to the type of endpoints and properties usually included in the studies. These are continuous variables, such as adsorption (X. R. Xia et al., 2011) or solubility (Huanxiang Liu et al., 2005), rather than categorical variables. On the other hand, most of QSAR models aim at providing categorization for dependent variables, such as level of damage, toxic or not toxic, or active or not active.

Neural networks have also been used in a large number of both QSAR (18 out of 152) and QSPR (9 out of 52) models. NN can be applied in both (non-)linear regression and classification models, and have shown better statistical results when compared with other methods. As an example, Le et al. (2016) predicted membrane damage by ZnO NPs using MLR with Expectation Maximization (MLREM) and Bayesian Regularization Artificial Neural

---

[28] Support Vector Regression (SVR) is also considered as SVM for the count, since SVR uses the same principles as SVMs with only slight differences to generate a regression analysis.

Network applying a sparse Laplacian Prior (BRANNLP). The external validation increased from R^2=0.57 in MLREM to R^2=0.86 in BRANNLP.

Model performance is defined by:

(i)   Applicability domain.
(ii)   Goodness-of-fit and predictive performance (training set).
(iii)   Robustness (cross-validation, Y-scrambling, bootstrap …)
(iv)   Predictivity (external validation).

As shown in Figure 3.6, over half of QSAR/QSPR models (122 out of 204) did not specify the applicability domain of the built model. The basic methodology used to report the applicability domain was to indicate the type of NMs and the range of values for the final descriptors. The most frequently mathematical approach applied (26 out of 204) was the leverage and Williams plot (included in the OECD (2007)).
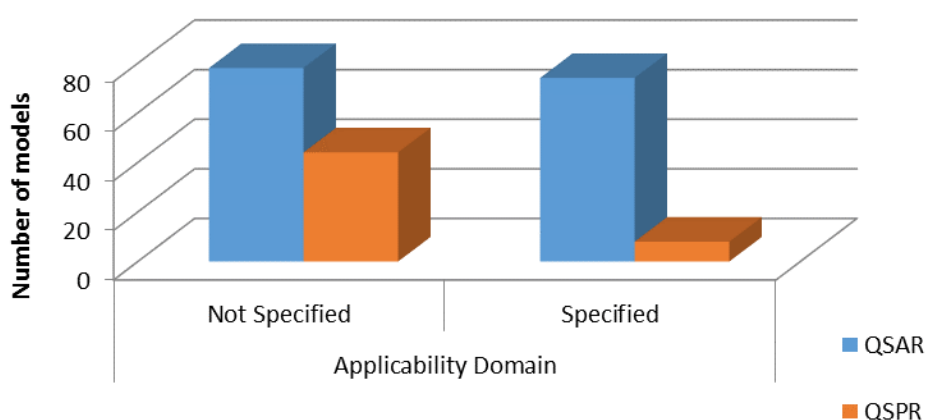

Figure 3.6. Number of models in which applicability domain was specified. Models were differentiated into QSAR and QSPR

Regardless of the relevance of model validation, one of the common weaknesses in the reviewed models is the lack of external validation. 28 out of 88 reviewed publications did not applied an external validation in their models, which is one the important key aspects covered by the fourth OECD principle, and determines the level of model reliability. The reasons behind this fact are associated to the small size of data sets (not allowing data splitting) and also to what the authors consider as predictivity assessment.

In this regard, cross-validation techniques are used to evaluate the robustness and avoid problems as overfitting in the models. Commonly, the authors apply cross-validation techniques, which split the data into training and test set several times, to evaluate the "predictivity" of a model. The result of repeating the splitting process several times, derives in the use of the training dataset to also evaluate the model "predictivity". According to the OECD guideline, only external data which was not involved in the model building can be considered as valid external validation. Thus, there are models that only apply internal validation when they actually aim at reporting an external validation. For instance, this was observed in D Fourches et al (2010) and Papa et al. (2015).

Comparison between studies with and without external validation is shown in Figure 3.7. It can be concluded that there is no clear trend towards the reduction of the percentage of papers without external validation over time.

Good examples of models in which a validation process has been applied covering the OECD principles are Puzyn et al. (2011)[29] , Melagraki et al. (2014), Wen DAI et al. (2015), Yousefinejad et al. (2013), Rofouei et al. (2014) and Papa et al. (2016).
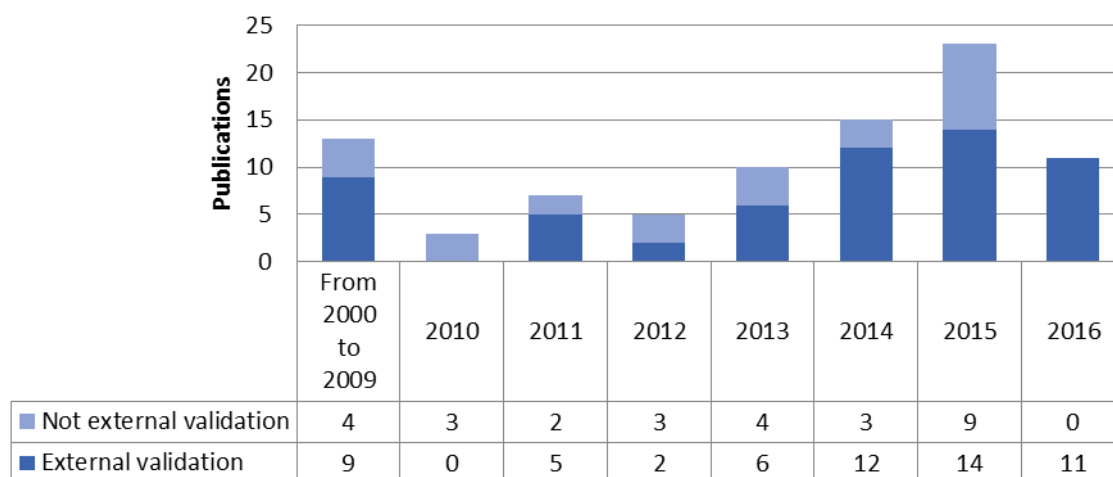

	From 2000 to 2009	2010	2011	2012	2013	2014	2015	2016
Not external validation	4	3	2	3	4	3	9	0
External validation	9	0	5	2	6	12	14	11

Figure 3.7. Number of publications with and without external validation over time

3.2.3 Applicability of available QSPR and QSAR models

In this section, the models most closely predict a REACH-related endpoint are discussed in more detail.

As already reported, the most investigated endpoints in QSAR models is cytotoxicity to different cell lines. Some models are predicting cell uptake, exocytosis, immune response, cell differentiation, and oxidative stress. These models are not directly relevant for filling data gaps but could be useful for screening or supporting grouping and read-across. In the following paragraphs models have been associated where possible to REACH requirements listed in Appendix III of this report.

The applicability domain of the collected models is not always explicitly defined; where it was not identified, the range of NMs to which the model was applied is reported instead.

Physicochemical properties
There are only 6 QSPRs that predict properties of NMs that are required for REACH, i.e. 1 for water solubility (Andrey A Toropov et al., 2007), 1 for octanol-water partition coefficient (Andrey A Toropov et al., 2007), 4 dispersion in organic solvents (Salahinejad and Zolfonoun 2013, Rofouei et al. 2014, Yilmaz et al. 2015), and 1 adsorption/sorption (Ghaedi et al., 2014b). These models are reported in Table 3.3. Mapping of REACH required

---

[29] Methodology followed in this work can be found in the supporting information file (S1)

physicochemical properties to REACH requirements. OECD TG are identified as for studies on NMs existing TGs are not always considered applicable.

Table 3.3. Mapping of REACH required physicochemical properties to REACH requirements. OECD TG are identified as for studies on NMs existing TGs are not always considered applicable

REACH requirement	OECD test guideline	REACH relevance	QSAR endpoint	Applicability domain	Reference
Annex VII Water solubility	105, water solubility. But only soluble NMs or NMs with high dissolution rates. Measurement of rate and extent of dissolution is recommended (OECD guidance 62).	Yes	$logS=-5.1041\ -3.5075n\ -3.5941m$ where n and m form the chiral vector of a given CNT.	Carbon nanotubes ($5<=n<18$ and $0<=m<=10$ not specified in the publication)	(Andrey A Toropov et al., 2007)
Annex VII n-octanol-water partition coefficient	107, 117, 123 n-octanol-water partition coefficient but only for water soluble or with high dissolution rate NMs.	The property is considered in REACH but the corresponding TGs are in general not applicable to NMs.	$logS=-3.9193\ +3.7703n\ -3.6001m$ where n and m form the chiral vector of a given CNT	Not specified. Carbon nanotubes ($5<=n<18$ and $0<=m<=10$ not specified in the publication)	(Andrey A Toropov et al., 2007)
Annex IX stability in organic solvents	-	The study does not need to be conducted if the substance is inorganic. Grouping, read-across and QSARs are not applicable at present	Dispersibility[1] = f((a_IC, diameter), hydrogen bonding ability and polarizability (BCUT_SMR), molecular flexibility (b_rotR) and electrostatic (Q_VSA_FPOS, AM1_Eele)	Not specified. SWCNTs in organic solvents	(Salahinejad and Zolfonoun 2013[1], Rofouei et al. 2014[2], Yilmaz et al. 2015[3])

REACH requirement	OECD test guideline	REACH relevance	QSAR endpoint	Applicability domain	Reference
			interactions) Dispersibility[3] = f(SRW09, ATS6m, Dipole Z, and X0Av) steric Dispersibility[2] = f(TIP, DRY and N1 probes)		
Annex VIII & IX	312, Leaching in Soil Columns 303A Aerobic Sewage Treatment Simulation Test may be used as indirect measurement to predict sorption of NMs into sludge	It is considered in REACH.	Adsorption of methylene blue onto CuO-NP-AC= f(pH, contact time, amount of adsorbent, and temperature)	Not specified. Methylene blue onto CuO nanoparticles on activated carbon (CuO-NP-AC)	(Ghaedi et al., 2014b)

Ecotoxicity endpoints

The inventory reported 5 models aimed at calculating ecotoxicological endpoints for nanomaterials. Of these, only 1 paper reports algorithms predicting REACH-relevant endpoints. The others are suggesting new biological metrics that integrate multiple toxicological endpoints. For instance, Liu et al. (2013) proposes an embryonic zebrafish (EZ) metric that combines endpoint information on mortality, delayed development, morphological malformations.

Zhou et al. (2015b) focuses on whole animal evaluations using the embryonic zebrafish (*Danio rerio*) embryos. A total of 21 endpoints were observed during development at 24 and 120 hours post-fertilization (hpf) that included mortality as well as morphological, behavioural and developmental endpoints in sub-lethal exposures. This model is not predictive of a REACH endpoint.

Kleandrova et al. (2014; 2014) develop a model to classify NMs as toxic and non toxic taking into consideration different measures (EC50, IC50, TC50, LC50). Harper et al. (Harper et al., 2015) develop a model that integrates different endpoints of toxicity to zebra fish embryo (e.g. mortality, malformation, developmental progression) though a weighted hazard score. Although these applications allow NMs to be grouped according to a broad set of ecotoxicity endpoints, they are not directly applicable for predicting REACH endpoints.

Chen et al. (2016) report a series of global and species-specific models that could indeed be applied to predict REACH endpoints. QSAR models are built on EC50 and LC50 values for *Danio rerio*, *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Staphylococcus aureus*; the species relevant to REACH and the respective models are reported in Table 3.4.

Table 3.4 QSAR models for ecotoxicological endpoints required by REACH, from (G. Chen et al., 2016)

REACH requirement	OECD test guideline	QSAR endpoint	Applicability domain
Annex VII Growth inhibition study (aquatic plants, algae preferred)	201, inhibition of algal growth (based on EC50)	NMs are categorised as active or inactive ; the decision tree models are built on EC50 values	Metal and metal oxide nanomaterials
Annex VII Short term toxicity (invertebrates, preferred species Daphnia)	OECD 202 *Daphnia magna* Acute immobilization (including EC50 acute immobilisation test)	NMs are categorised as active or inactive; the decision tree models are built on LC50 values	Metal nanoparticles
Annex VIII Short term toxicity fish	OECD TG 203: Fish, Acute Toxicity Test	NMs are categorised as active or inactive; the decision tree models are built on LC50 values for *Danio Rerio*	Metal, metal oxide nanoparticles
Annex IX Long term ecotoxicity (invertebrates, e.g. Daphnia)	OECD TG 211. *Daphnia magna* Reproduction Test	EC50	Metal nanoparticles

Toxicological endpoints

There are no models covering the toxicological endpoints on acute toxicity, repeated dose toxicity, (skin and respiratory) sensitisation, carcinogenicity, and reproductive toxicity. The only endpoint that is covered from available QSARs being also an endpoint of REACH relevance is "*In vitro* – Mutagenicity Bacterial Reverse Mutation Test (*Salmonella typhimurium*)", required under REACH Annex VII.

The three different models build on two available datasets on *Salmonella typhimurium* (Shinohara, Matsumoto, Endoh, Maru, & Nakanishi, 2009; Wirnitzer, Herbold, Voetz, & Ragot, 2009), that was exposed in different test conditions to fullerene or multi-walled carbon nanotubes (MWCNTs). These tests were considered for model development; descriptors were related to test conditions (dark condition or irradiation, cells preincubation, presence or absence of metabolic activation, dose). The three models differ only in relation to the database used and, as a consequence, the descriptors used for the prediction. The reference OECD TG is the n. 471 (Ames test), providing indication on how to perform the test and to evaluate for mutagenicity depending on the count of revertant colonies. However, as reported in the ECHA guidance on REACH information requirements (ECHA, 2012b), the Ames test is not applicable to NMs, and Kumar and Dhawan (2013) explain that the tester strains would need to be modified with deep rough mutation so to improve cell permeability

to NMs. It is not mentioned in the source reference if this adaptation was applied, hence the data available and the resulting model simulations may not be relevant.

3.2.4 Documentation of QSPR and QSAR models

The suitability of QMRFs to report QSPRs and QSARs was evaluated by applying the reporting format to a QSAR for NMs (Burello & Worth, 2011a). In general it is concluded that the QMRF is adequate to report QSPRs and QSARs although the characteristics of NMs might not be covered in sufficient detail. The current QMRF only considers the presence of chemical name, CAS numbers, SMILES, INCHI, MOL, and formula as information on the training and test set compounds. Clearly, these data types are not all relevant or sufficient to unequivocally distinguish NMs, therefore it was found necessary to expand the properties section, at least until a standard nomenclature for NMs is created and generally adopted.

In particular, the following parameters should be added to the QMRF to make it applicable to NMs:

- NP composition: Yes

- NP size: Yes

- NP agglomeration/aggregation: No

- NP crystalline phase: No

- NP crystalline and grain size: No

- NP aspect ratio/shape: No

- NP specific surface area: No

- NP Zeta potential: No

- NP surface chemistry: No

- NP dustiness: No

- NP porosity: No

- NP pour density: No

- NP photocatalytic activity: No

- NP radical formation potential: No

- NP catalytic activity: No

The full QMRF is provided in Appendix VI.

3.3 PBK, PBD and dosimetry model inventory

3.3.1 Introduction

A compilation of relevant papers describing toxicokinetic (CTK and PBK), toxicodynamic (PBD) models and dosimetry models (*in vitro* & respiratory tract models) has been performed based on a comprehensive state of the art analysis of existing literature on computational models for nanomaterials (NMs). The aim of this compilation is to document such models in searchable inventory templates and to provide an overall analysis of the "model landscape".

This will give insight into the availability and applicability of computational approaches that are potentially useful in the assessment of the hazardous properties of the manufactured NMs under REACH. Each relevant papers collected has been reported in an Excel file, and have been characterized with respect to several parameters, such as endpoint of the model, NMs evaluated, the parameters involved in the modelling process among other inputs that will be explained below. To capture information in a structured way, a template was first developed (Appendix VII). The Excel file (Supplementary Material S3) in which this template was implemented will hereafter be referred as the model inventory.

Unlike QSAR models (compilation reported in a separate document), there is not an established OECD guidance on how to assess the quality of the above mention models. Within the drug development regulatory field, there are some recommendations on best-practice methods but neither the FDA (Food and Drug Administration) nor EMA (European Medicine Agency) have adopted any formal guidance yet. In July 2016, the EMA released a draft guidance ("Guideline on the qualification and reporting of physiologically based pharmacokinetic (PBPK) modelling and simulation"; EMA/CHMP/458101/2016, CMPH 2016). This guidance has been not taken into consideration given that at the time of writing this report the guidance was still under revision and highly focused on processes related to traditional chemicals (such as metabolism). The applicability or the potential adaption of this guidance to evaluate NMs has not been addressed in this report.

In the following sections, the search strategy used to compile the models will be first described, followed by a detailed description of the parameters used to characterize the models and the methodology applied for the literature review. Finally, an overall analysis, i.e. model landscape, of the current state of the above mentioned models will be given based on the outcomes of such inventory.

3.3.2 Search and selection strategy

A methodology approach has been defined in order to perform the characterization of available computational models including PBK, PBD and dosimetry models either in *in vivo* or *in vitro* systems. The work has been performed in three different steps:

1 Selection of relevant papers, from robust and leading databases,

2 Review of collected papers

3 Reporting of the main characteristics in structured and searchable inventory

4 Overall analysis of the model landscape.

In order to select the initial set of publications, the Scopus and WoS (Web of Science) database websites were employed as search engine tools and the relevant terms included for the initial screening were chosen. Specifically, the main terms were:

- The model itself (i.e. PBK, PBD, PBPK, PBPD, "physiologically based", "kinetic, "compartmental model", "numerical", "computational" and "modelling")[30]

- The NM term (nanomaterial*, nanoparticle*, fullerene)

- The desired endpoint to which the mathematical model is aiming to give answer (e.g. "accumulation")

- Finally, the terms that generally describe the test systems used in the experimental procedures for toxicity assessments ("*in vitro*", "*in vivo*").

Different combinations of the terms included above led to the compilation of almost 690 publications which were included in the reference manager Mendeley. After removal of duplicates, the set of publications was reduced down to a number close to 474 papers. Then, a detailed revision of titles and abstracts was performed. To discard those publications related to cancer or drug research and therefore out of the scope of the project, papers including "tumour", "cancer", "drug", "delivery system" or "thermal" in the title, abstract or keywords, where selected. This selection resulted in a large number of articles (247) that were checked and generally discarded once the general topic was identified. Literature references about imaging systems based on NMs (including the terms "imaging" or "magnetic" in the title, abstract of keywords) were also removed. The final set resulted in a total of 176 publications.

The screening performed to remove non-relevant papers in the next step was based on the identification on those papers describing experimental data with NMs but not actually developing or evaluating any computational model were also excluded. As a result, 48 publications were considered as relevant.

In a next step, an additional search in Scopus including the term "CNT" in combination with other relevant search terms previously described was performed. A specific search on ATLA (Alternatives to Laboratory Animals) in Pubmed was also conducted given that this journal is not considered within the Scopus database. This search retrieved 8 papers but none of them was considered relevant. In addition, some papers previously identified by JRC and not included at this point were manually added to the final set of publications to be included in the inventory.

As a result of this process, 62 papers were compiled. Eighteen publications were discarded after an accurate reading of the abstracts. The main reasons to discard them were:

1) Articles using or evaluating a relevant model but not developing a new one,

2) Articles considering QSAR methodologies

3) Articles performing experimental toxicology evaluations.

The final set of articles to be reported was reduced to 48 publications.

---

[30] "*": It is applied in the search engine to include all terms which include the "word" previous the "*"

3.3.3 Review and reporting

A review of the final set of 48 publications was performed in a chronologic order starting from the oldest papers to be able to recognise the citations of already reviewed publications as well as identify possible new models, not captured using the strategy presented above.

It must be also underlined that there were some papers which the developed models were in fact the same or closely related to previous models reported in the inventory. Even though these papers have not been included in the inventory as new entries, they are referred in the table as it will be explained below, in the following sections. After an accurate identification of this kind of publications and the inclusion of two new relevant ones, 35 papers were included in the final inventory.

The detailed reporting of the whole set of papers provided and overview of the different types of models considered. As a result, the following classification was adopted:

1   Toxicokinetic (PBK) models: Numerical models commonly derived from physiologically relevant compartments and processes (physiologically based kinetics models (PBK)) and constructed from mass-balance equations (i.e. accounting for material entering and leaving a system). Classical toxicokinetic (CTK) models are also included within this classification.

2   Toxicodynamic (PBD) models: Models that simulate the intensity and time-course of NM effects on a biological system (e.g. prediction of the inflammatory response of macrophages under exposure to NMs).

3   Dosimetry models: Computational models that predict the fate and the local concentration/dose of NMs in a defined *in vitro* or *in vivo* system. The models in this section have been divides in two different categories:

   3.1 Respiratory tract dosimetry: biologically-based mechanistic approaches to predict the fate of inhaled particles, by describing the physical and physiological factors that influence the deposition, clearance, and retention of inhaled particles.

   3.2 *In vitro* dosimetry: models that calculate the dose-rates and target cell doses based on particle kinetics and transport prediction of NMs to cells in liquid-based *in vitro* systems.

It should be pointed that the Search Strategy was stressed more on physiologically based models due to their broader use and potential relevance for risk assessment purposes (they allow interspecies extrapolation, route-to-route extrapolation, dose extrapolation, etc). Although the search strategy did not explicitly include "dosimetry" as a search term, both *in vitro* and *in vivo* computational dosimetry models have been included in the inventory and thoroughly reviewed from the literature. On the other hand, toxicodynamic models were not covered in such wide manner and just a few examples (n=4) were incorporated and reported in the inventory.
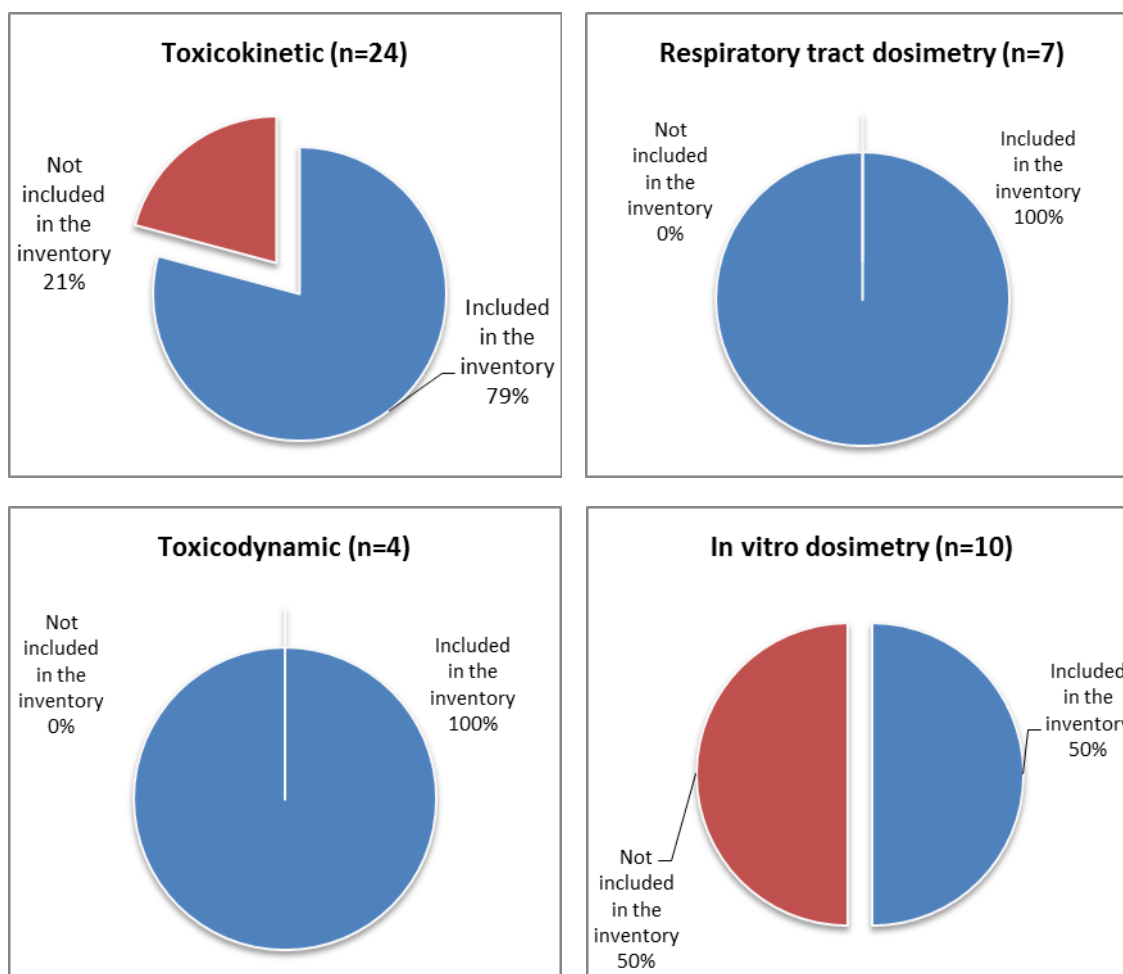
Figure 3.8. Percentage of included and excluded papers for each model

Figure 3.8 shows a graphical representation of the percentage of reviewed papers that have been either included or excluded in the final inventory and Figure 3.9 shows from the included papers the number of papers for each model in detail.

In addition, Figure 3.10 depicts the number of publications included in the inventory with respect to the year of publication. Based on this figure, the number of PBK publications considered as relevant remains almost constant over the time. A peak of PBK publications (n=5) was observed in 2015. A constant number of publications is also observed for the Respiratory tract dosimetry models. For the rest of models there is some variability depending on the year of publication. It should be noted that this figure reflects the criteria adopted to include the publications as "relevant models" (principally new developed models) but it does not reflect the actual number of publications using these models in the nanotoxicology field.
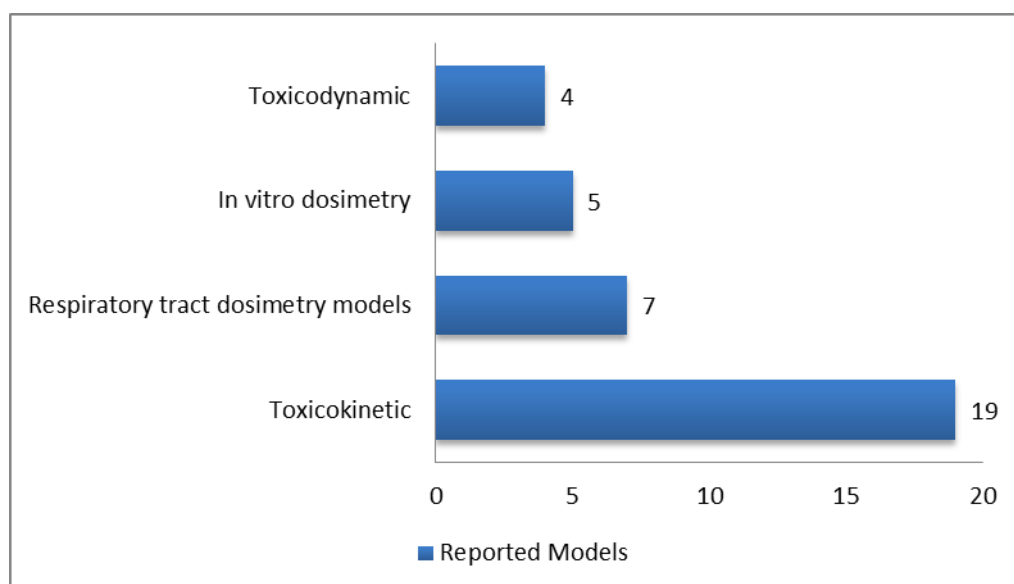
Figure 3.9. Number of publications of each model included in the Inventory

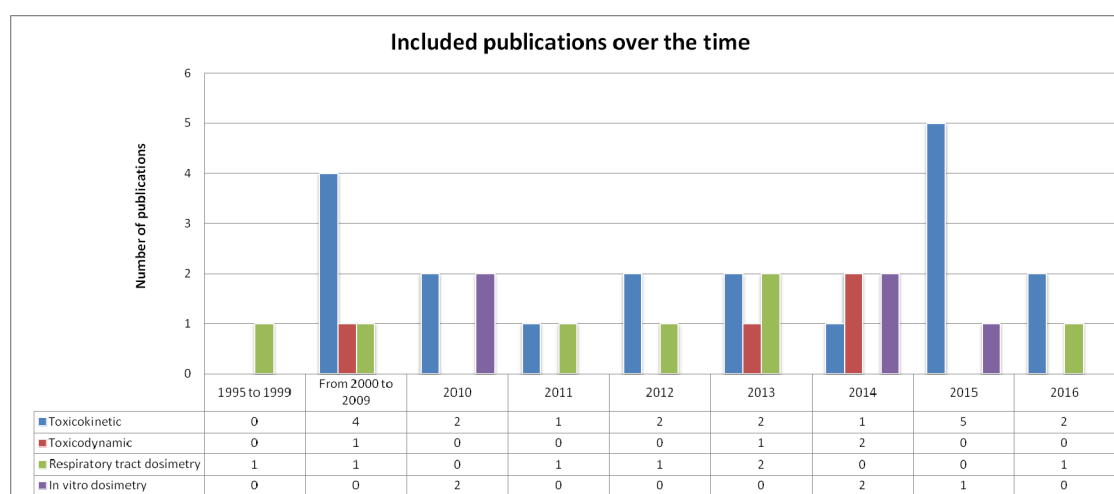|	1995 to 1999	From 2000 to 2009	2010	2011	2012	2013	2014	2015	2016
■Toxicokinetic	0	4	2	1	2	2	1	5	2
■Toxicodynamic	0	1	0	0	0	1	2	0	0
■Respiratory tract dosimetry	1	1	0	1	1	2	0	0	1
■In vitro dosimetry	0	0	2	0	0	0	2	1	0

Figure 3.10. Number of publications included in the Inventory as a function of publication year

The diagram depicted in Figure 3.11 represents the inventory structure, covering the different sections (n=5) and corresponding parameters (n=39) used to characterize the relevant models. These sections are 1) Model meta data, 2) Model description, 3) Inputs and outputs, 4) NP description and 5) Model domain. The contents of each one is summarized below:

- Model metadata: includes model details (name, version, homepage) information about the model owner (ownership, contact point, email address, license), the reference (associated literature references and DOI). To assign a model name, its aim and a generic term to define the type of model have been used as main criteria.

- Model description: this section gather the main characteristics of the model (i.e. a generic description of the model output(s), the level of organisation considered (i.e. compartments, tissues, cells), the model type), information about the processes

151

considered within the model (including units, level of description/definition). This section also allows the possibility to include free text to add any comment considered of importance.

- Inputs and Outputs: Information on the (nano)particle-specific or chemical-specific parameters that the model use as input or output is reported in this section (parameter, symbol, units, protocol for measured values). Assumptions or key information on the protocol related to the inputs are also covered by this section.

- NPs description: in this section the type of NMs used to build the model or to evaluate it are described (i.e TiO2, Ag, CeO2, metal oxide, carbon-based, polystyrene, etc). Then other associated physic-chemical properties, such as coating, size, shape, and any other relevant characterization performed. This also contains the description of the NMs used in other literature references evaluating or using the same model. The information about these references is placed in the subsection "Used in reference".

- Model domain: in this section the applicability domain stated by the author or inferred from the description and the outcome(s) of the model is provided. It also state if one or more physicochemical properties of the NMs (i.e size, density, agglomeration state, etc) are used as input model parameters. General adopted assumptions by the model are also detailed at this level.

3.3.4 *Results of the analysis of the available PBK models*

The parameters considered most relevant in the characterization of a toxicokinetic model are summarised in Figure 3.12. These include: the type of generic NMs used in the model (either to develop or evaluate the model); the species and the exposure routes used to build the model and those physiologically relevant compartments (organs or tissues) considered.
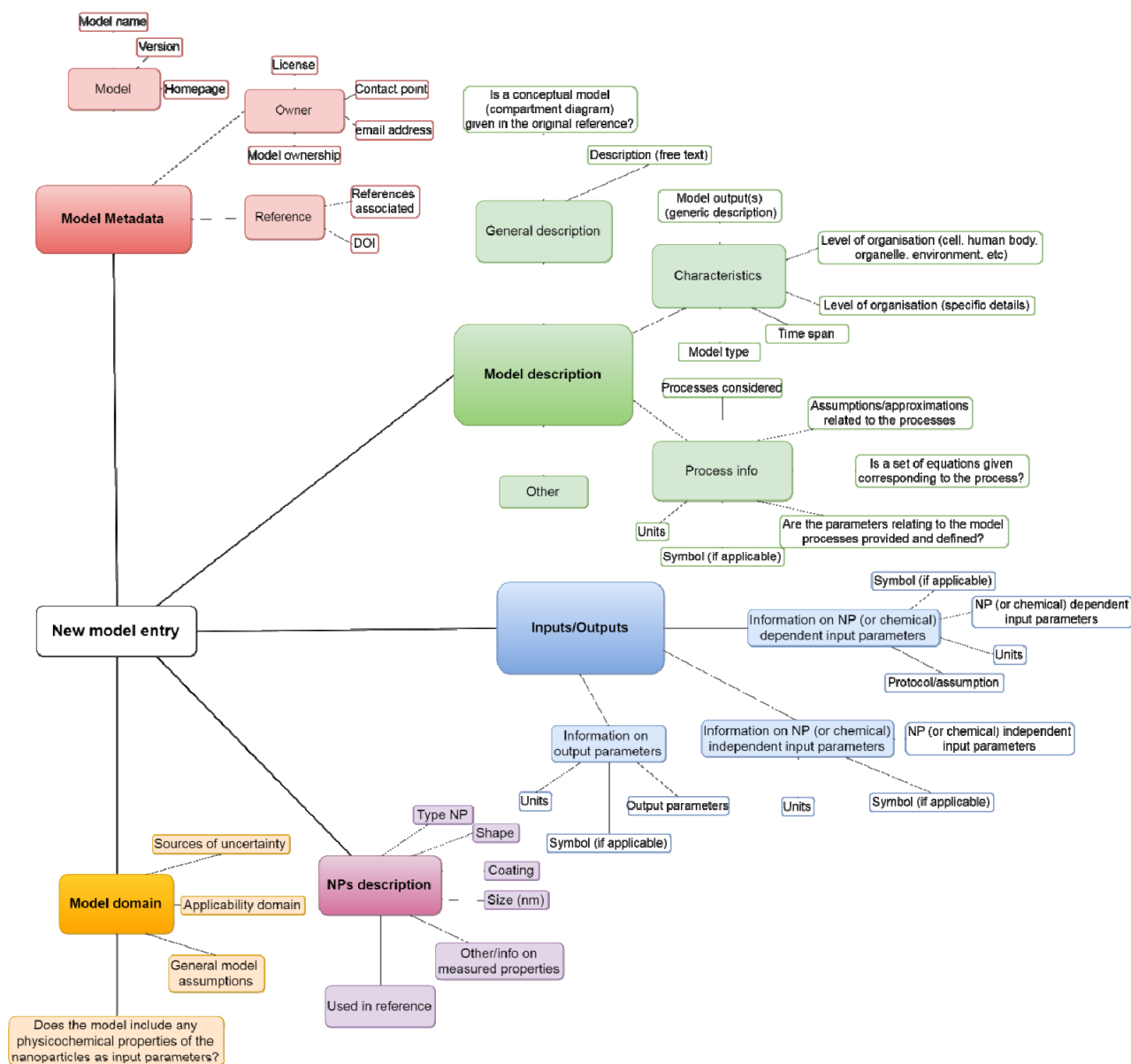
Figure 3.11. Inputs included in the inventory for characterizing the different models
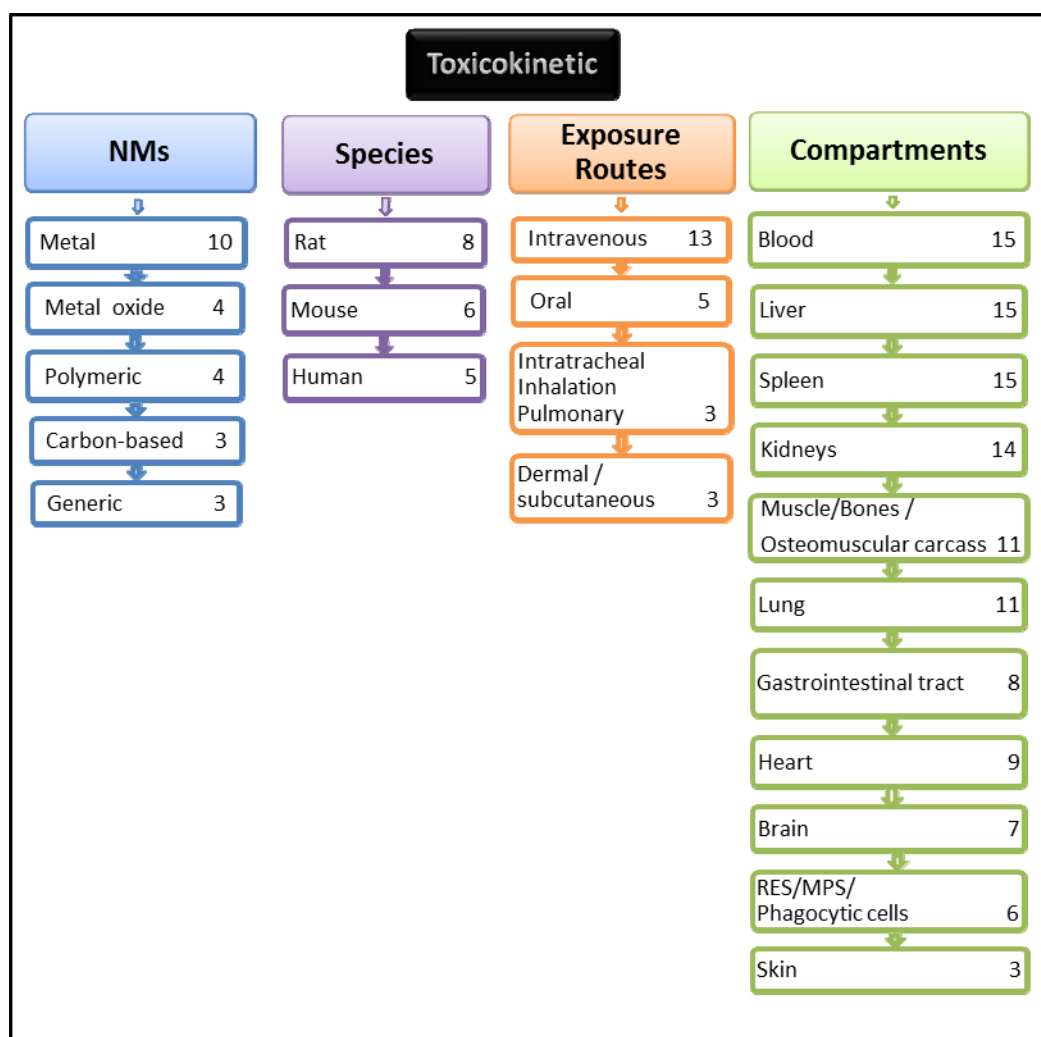
Figure 3.12. Summary of the type of NMs, species, exposure routes and compartments used in the toxicokinetic (PBK) models for NMs. The numbers represent the times that each specific parameter has been identified.

Most of the models included in the inventory are PBK models (PBK) except six of them (Zhu et al., 2009, Tien et al., 2010., Li et al 2011, Denim et al., 2015; van Kesteren et al., 2015 & Sahneh et al., 2015). These models are not stated as physiologically based models since they do not use any physiological parameter (i.e. organ weight, tissue volume, blood volume, blood flow) as direct input parameters. They generally follow classical toxicokinetic (CTK) modelling approaches and use empirically calculated physiological factors (i.e absorption, transportation, elimination rate constants). Despite their limited capability to extrapolate between species or exposure routes they are considered of relevance to predict the transport and concentration of NMs in several tissues.

Nanomaterials

Models found in the literature search cover a total of 15 different NMs including metals, metal oxides, polymeric and carbon-based nanomaterials. Metal NMs are the most common materials (covered by 10 out of 19 models). As seen in Table 3.5, Ag (4) and Au (3) are the most frequently evaluated metals. Metal oxides, polymeric and carbon-based nanomaterials are represented at a similar extent. It has to be pointed out that three of the models (Fallon

154

et al., 2004; Tien et al., 2010 & Sahneh et al., 2015) are not focused on the evaluation of a specific type of nanomaterial. They can be applied to nanoparticles in broad generic sense, independently of their chemical composition. Table 3.5 shows a list with the specific type of NM for each group:

Table 3.5. List of NMs reported in the Inventory for kinetic models. The number in brackets indicates the how many times each NM appears among the different models, including both PBK and CTK studies.

Generic type of NMs	Specific type of NMs
Metal	Silver (4), Gold (3), CdSeTe (1), Iridium (1), CdTe(1), CdS(1)
Metal oxide	Fe_2O_3 (1), TiO_2(1), ZnO (1), SiO_2(1)
Polymeric	Polystyrene (1), Poly(amidoamine(1), PLGA(1) , polyacrylamide (1)
Carbon-based	Carbon (3)
Generic NM	(3)

Exposure routes & Compartments

As can be observed in Figure 3.12, the intravenous route is the most common route of administration taken into consideration (13 out of 19 models). Other administration routes such as those relevant for the oral route, the inhalation route (e.g. intratracheal administration), the dermal or the subcutaneous route are represented at a lesser extent.

The PBK models compiled within this report vary on complexity from full PBK models where all of the distribution organs and tissues included are represented as separate perfused compartments (i.e. Péry et al. 2009 includes more than 20 compartments) to more simplified, minimal PBK models in which tissues with similar kinetics are lumped (i.e. van Kesteren et al. 2015 includes 3 compartments). Blood, liver, spleen and kidneys are the most represented compartments among the different models (see Figure 3.12). Liver and spleen are one of the major targets of nanoparticle accumulation, especially after intravenous administration and together with blood, the main compartments containing Reticuloendothelial system (RES; also called mononuclear phagocyte system or MPS) cells. Nanoparticles are rapidly captured and retained by these cells (monocytes circulating in the blood, reticular cells in the spleen and Kupffer cells in the liver). The RES has been considered as a separate compartment in six models (Bachler et al., 2013; Li et al., 2014, Bachler et al., 2015; Sahneh et al., 2015; Lin et al., 2016, Liang et al., 2016).

Model inputs & outputs

The input parameters considered by the models compiled in this section are physiological parameters generally used by PBK models not focused on NMs (i.e. body weight, organ weight, blood flow, organ and tissue volumes, blood flow to organs, etc). It should be noted that only one of the models (Bachler et al. 2013) uses a NP physicochemical property

(diameter) as a direct input parameter (to calculate the MPS uptake rate of NMs from the blood circulation). Other physicochemical properties such as surface area, specific surface area, density or agglomeration state are not specifically considered as input parameters.

As other computational kinetic models, the estimation of the concentration in tissues, organs or other specific compartments along the time is the main predicted model outcome of the compiled kinetic models for NMs.

Applicability domain
Some authors attempt to define the applicability domain of their models to those particles used to build or evaluate the model (i.e. Bachler et al. 2003 : "The model could successfully predict the biodistribution of ionic silver and 15–150 nm silver nanoparticles, which were not coated with substances designed to prolong the circulatory time (e.g., polyethylene glycol"). Some others expand the applicability domain to NMs with similar generic physicochemical properties (Li et al., 2011 develops and evaluates a model using a variety of different NPs (silver, gold, quantum dots, polystyrene, and carbon NPs) and states that the model can be applied to "Non-degradable/non-metabolizable nanoparticles". However, most of the authors do not explicitly define the applicability domain of their model. In these particular cases, it has been assumed that the applicability domain should be limited to nanoparticles within the range of those used to develop or evaluate the model.

Assumptions
Some relevant model assumptions have been identified during the reviewing process. They can be considered as critical factors responsible for part of the uncertainty of the compiled models:

Related to the NMs:

- nanoparticles are insoluble

- physicochemical properties of nanoparticles do not affect the bio-kinetics

- no agglomeration of nanoparticles

- no nanoparticle-overload effects in the lung.

Related to the model structure and processes:

- all compartments are well mixed (homogeneous), i.e., no spatial gradients

- The concept of partitioning between tissues and blood is clear for conventional substances and is based on the chemical potential of molecules in different phases such as water, fat and protein phase. It is less clear what determines the partition of nanoparticles but we can assume that hydrophilicity and lipophilicity are important factors. The hydrophobicity of particle coatings is well addressed for cosmetics and pharmaceuticals, for example, but not in REACH. Anyway, an equilibrium of concentrations of exchangeable moieties of nanoparticles in tissue and blood can be described by a steady concentration ratio referred to as partition (Lankveld, et al., 2010).

- The rates of mechanical transport are independent of the chemical composition and crystal form of the nanoparticles (Tien et al., 2010).

156

- All organs have the same blood over tissue partition coefficient value to facilitate parameters estimation (Péry et al., 2009)

REACH relevance

In general toxicokinetic data can be used for: "… further acceptability and applicability of quantitative structure-activity relationships, read-across or grouping approaches in the safety evaluation of substances. Kinetic data may also be used to evaluate the toxicological relevance of other studies (e.g. *in vivo*/*in vitro*)." (OECD TG 417).

REACH allows the use of any scientifically justified information as weight of evidence (Annex XI) supporting read-across approach.

As stated in REACH (Chapter R.8: Characterisation of dose [concentration]-response for human health) for conventional chemicals), PBK can support the derivation of DNEL (Derived non-effect level) from animal data to account for human health risk. PBK models may potentially be used to determine some specific assessment factors (AFs): 1) route-to-route; 2) interspecies and 3) high-dose-low-dose extrapolation. In addition PBK modelling data can aid in the quantification of intraspecies variability, which may be caused by variation in anatomical, physiological and biochemical parameters with, age, gender, genetic predisposition and health status. PBK models can be used to quantify these, which would result in possible modification of additional AFs. However, risk assessors, who are using these models, should be able to interpret them and their outputs.

3.3.5 *Results of the analysis of the available PBD models*

Four toxicodynamic models have been included in the inventory. Given the limited number of compiled publications, a comprehensive overview of this kind of models was not feasible. Alternatively, a general description has been provided for each of them:

1) Shelley et al., 2008 developed a model to simulate the cell population dynamics (including toxic effects and functional viability along time) of rat alveolar macrophages under exposure to 80 nm aluminium NP. A system of mechanistically ordinary differential equations was derived based on the following primary state variables: macrophage population, nanoparticle concentration and macrophage phagocytosis function level.

The model demonstrates how *in vitro* data can be used within a simulation setting of *in vivo* cell dynamics.

2) Maher et al., 2014 used a phenomenological rate equation model that numerically simulates uptake and cellular responses to polyamidoamine dendrimer (PAMAM) nanoparticles of different generations (number of initial branching points). Nanoparticle uptake and the subsequent cellular response measured by change in cellular markers of oxidative stress, mitochondrial damage, inflammatory response and apoptosis where the processes simulated.

The model is intended to be used as a tool to interpolate and visualise the range of dose and temporal dependences and elucidate the mechanisms underlying the *in vitro* cytotoxic response to nanoparticle exposure.

3) Mukherjee et al., 2013 developed a multiscale toxicodynamic model to quantify and predict pulmonary effects due to uptake of NMs in mice. The kinetics of surfactant and

pulmonary function due to interactions of NMs at the alveolar microenvirontment are simulated. Collection of toxicodynamic modules to describe the dynamics of tissue focused on cells and the alveolar surfactant chemicals that regulate the process of breathing, as well as the response of the pulmonary system to xenobiotics. It is worth mentioning that the model uses some nanoparticles properties (i.e. zeta potential, diameter and surface area) as input parameters.

The model predictions were compared with *in vivo* lung function response measurements in mice and the analysis of mice lung lavage fluid following exposures to citrate-stabilized 10-20nm Ag NPs and carbon black nanoparticles.

4) Mukherjee et al., 2014 (b) developed a mathematical model that predicts the *in vitro* inflammatory response (i.e. expression levels of cytokines) of immune cells exposed to citrate-coated and PVP-coated Ag in a culture system. Additionally, the model was executed with and without the inclusion of the NP agglomeration-diffusion-sedimentation-reaction model (ADSRM; Mukherjee et al., 2014a), to determine the extent of effects due to *in vitro* cellular dosimetry of NPs.

REACH relevance
Interspecies differences result from variation in the sensitivity of species due to differences intoxicokinetics but also in toxicodynamics. Toxicodynamic models can potentially be coupled with toxicokinetic models and be used to calculate interspecies assessment factors during the human risk assessment process.

3.3.6 Results of the analysis of the available resrpiratory tract dosimetry models
The respiratory tract dosimetry models compiled in this section are based on modelling fluid and particle dynamics in subject-specific respiratory geometry tracts. Among these modelling techniques, computational fluid dynamics (CFD) models allow for simulations of airflow patterns and particle deposition efficiencies in complex geometries such as those found in the upper respiratory tract of laboratory animals and humans and provide a valuable supplement to experimental work in evaluating dose-response.

The parameters considered most relevant in the characterization of the respiratory tract dosimetry models are summarised in Figure 3.13. These include: the type nanomaterial (NM) used in the model (either to develop or evaluate the model); the species considered to build the model and the input parameters (nanoparticle-independent or nanoparticle-dependent parameters) needed to run the model.
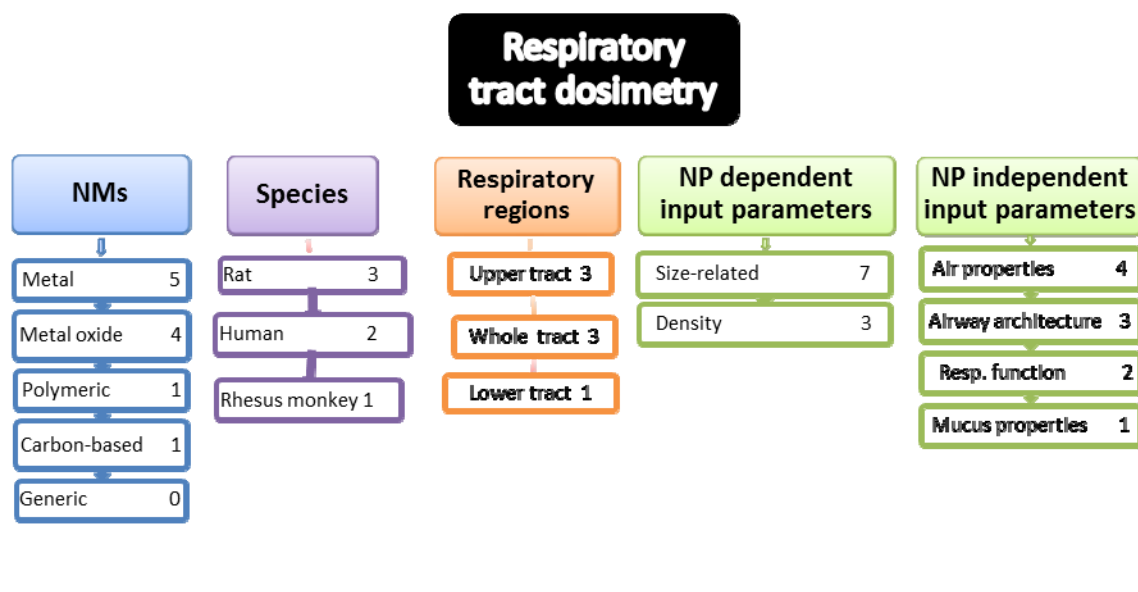
Figure 3.13. Summary of the type of NMs, species/system, and input parameters (NP-dependent or NP-independent) used in the Respiratory tract dosimetry models for NMs. The numbers represent the times that each specific parameter has been identified.

Nanomaterials and applicability domain

Computational fluid and particle dynamic simulation models have been developed to study airflow, gas uptake and deposition fractions of particles that cover the nano-size range but also the micro-size range. Hence, this type of models can simulate the behaviour of a "generic" particle including but not limited to particles in the nano-size range. For instance, Schroeter et al., 2013, uses a CFD model based on the architecture of the nasal passage of an adult and an infant rhesus monkey to simulate inhaled airflow and particle deposition for inhaled nanoparticles (0.5 – 1000 nm) and microparticles (1 to 20μm). Other authors (Anjilvel et al., 1995, Garcia et al., 2009, Zhang et al., 2011 and Henry et al., 2016) also use similar approaches that can be used to evaluate "generic" particles from 10 nm to 10μm. Other authors have evaluated their model with particles in the nano-sized range. For instance, Kolanjiyil et al., 2013, compared the predicted nasal depositions with experimental results using polymeric (polystyrene latex) and metal (silver wool) nanoparticles ranging from 3.6 to 100 nm, for validation purposes.

A complementary approach to these models was followed by Kirch et al., 2012. This author investigated the fate of inhaled particles after deposition onto the pulmonary mucosa. This study applied ex vivo and computational approaches to investigate the dependency of mucociliary clearance on size, shape, charge and surface chemistry of nano and microparticles. Polymeric particles (polystyrene particles ranging from 200 nm to 6μm) and metal oxide particles (maghemite (Fe_2O_3) particles ranging from 146 to 555 nm) were under investigation.

The compiled Multiple-Path Particle Dosimetry (MPPD) Mode developed by Anjilvel et al 1995 is freely available from https://www.ara.com/products/multiple-path-particle-dosimetry-model-mppd-v-304

Species

Assessment on human health risk from exposure to inhaled materials often relies on extrapolation of dose-response data from laboratory animals. Inhalation toxicological studies are mainly conducted in rodents such as mice or rats. Due to the differences in their respiratory tract architecture and other physiological parameters, determining lung deposition fraction becomes critical for being able to use the animal toxicity data to evaluate the potential human health effects associated with exposure to the inhaled materials.

Three out of six models found in the literature (Anjilvel et al., 1995, Garcia et al., 2009, Schroeter et al., 2013) developed a respiratory tract dosimetry model for rats. Zhang et al., 2011 and Kolanjiyil et al., 2013 build their deposition models based on the human airway geometry. Finally, as mentioned above Schroeter et al., 2013 uses a model based on the nasal architecture of the rhesus monkey.

Model inputs & outputs

Unlike the majority of PBK models, the input parameters used in the respiratory tract dosimetry models include some of the physicochemical properties of the NMs. As can be seen in Figure 3.13, two main NM-dependent properties have been identified: Size related parameters (e.g. diameter, radius & diffusion coefficient) and particle density. Four groups of NP-independent parameters were also identified. These are related to the air properties (e.g. air density, viscosity, flow rate, etc), the airway architecture (e.g. airway length, diameter, volume, and area), respiratory function parameters (e.g. tidal volume and breathing frequency) and mucus properties (e.g. thickness and viscosity).

Particle deposition (deposited mass or deposited fraction (as percentage)) in different respiratory sections of the upper respiratory tract (URT; olfactory region i.e. Garcia et al., 2009 Schroeter et al., 2013 and Henry et al., 2016), the lower respiratory tract (LRT: lungs, bronchi, bronchioles, and alveoli; i.e. Anjilvel et al., 1995) or the whole respiratory tract (i.e. Zhang et al., 2011 & Kolanjiyil et al., 2013) are the main outcomes of the compiled models.

Assumptions

Some relevant model assumptions have been identified during the reviewing process. They can be considered as critical factors responsible for part of the uncertainty of the compiled models:

Related to the architecture of the airway:

- Homogeneity in the airway geometry (e.g. alveolar volume was assumed to be equally distributed among all alveolar ducts; Anjilvel et al., 1995)

- Disturbances in the air flow caused by the presence of the nanoparticles are neglected (Garcia et al., 2009)

- Constant and homogeneous parameters (e.g., constant velocity of ciliary beating; Zhang et al., 2011)

- Related to the particles:

- Uniform concentrations of monodisperse particles (Schroeter et al., 2013)

REACH Relevance

Respiratory tract dosimetry models can be used to determine the internal dose following inhalation. However, care must be taken since the most sensitive endpoint may vary for different durations or routes of exposure resulting in different internal doses from the same external inhaled concentration. These models can also be to extrapolate from animal toxicological data to humans, e.g. calculation of a human equivalent dose (HED).

3.3.7 Results of the analysis of the available in vitro dosimetry models

Commonly, *in vitro* dosimetry models are based on mathematical approaches that describe the dynamics of particles in liquids. The parameters considered most relevant in the characterization of the respiratory tract dosimetry models are summarized in Figure 3.14.
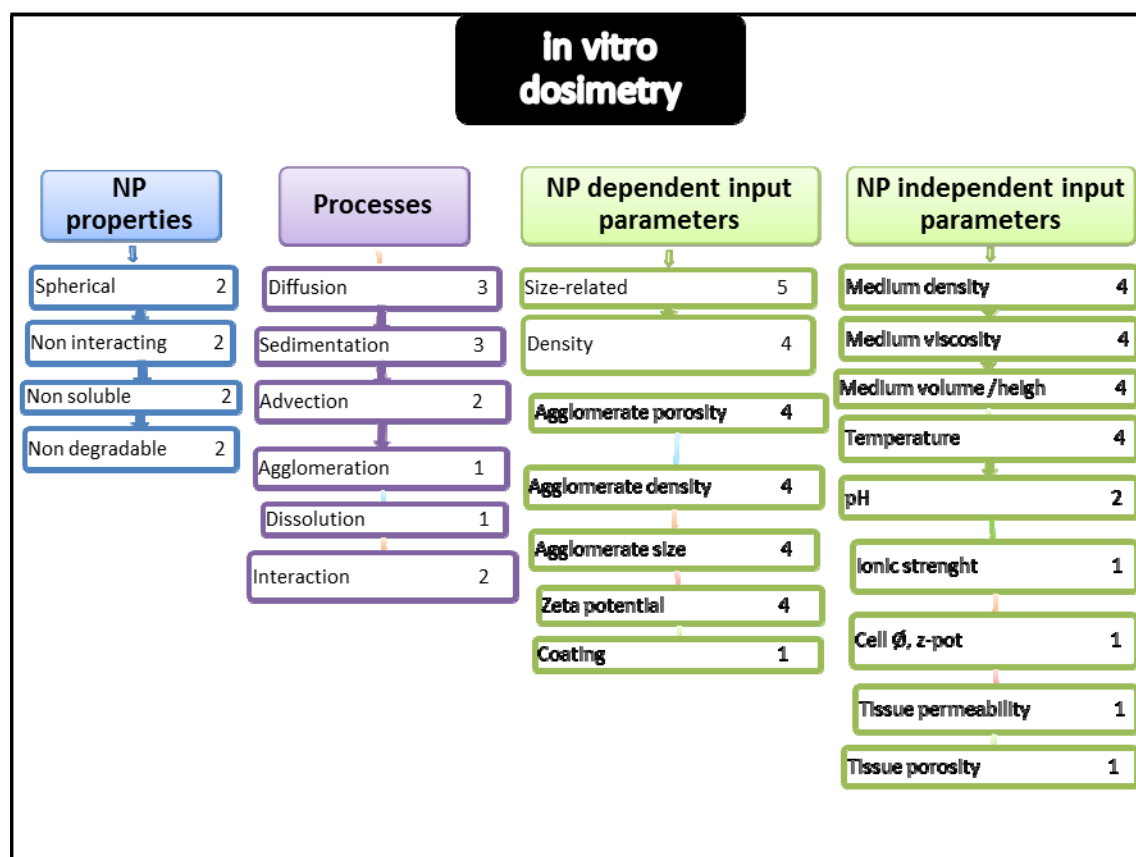

Figure 3.14. Summary of the type of NMs, processes and input parameters (NP-dependent or NP-independent) identified in the *in vitro* dosimetry models. The numbers represent the times that each specific parameter has been identified.

Processes

Kinetic processes such as diffusion (e.g. based Stokes-Einstein equation), sedimentation (e.g. based on Stokes law) and advection (transfer by motion of the fluid) are some of the processes that are considered in these models (e.g. i*n vitro* Sedimentation, Diffusion and Dosimetry model – ISDD – by Hinderliter et al., 2010). Mukherjee et al., 2014 (a) also includes in their agglomeration-diffusion-sedimentation-reaction model (ADSRM), dynamic transformation processes important for nanoparticles, specifically dissolution. Neither the ISDD nor ADSRM models consider the interaction of nanoparticles with molecules present in

the test system media. To overcome this limitation in the ASDRM model, Mukherjee et al., 2015 extended of the ADSRM model to enabling the assessment of ENM interaction with various fractions of lipids and surfactant proteins. Another enhancement worth mentioning is the semi-analytical solution for the ISDD model developed by Mahnama et al., 2014. Based on a generalized integral transform technique (GITT) the predictions concerning the advection-diffusion processes are improved and consequently the accuracy of the ISDD modelling. The above mentioned models are mainly designed for *in vitro* supporting systems such as tubes or cell culture well plates.

A different approach included in this section was developed for NP injected in tissues by Su D et al., 2010. This model aims to predict the spatial distribution of nanoparticles in tissues after nanofluid injection into the extracellular space of tissues. In this particular case, interactions of the NPs with the surrounding media are also considered. To this aim van der Waals interactions, electrostatic forces and attachment of nanoparticles to solid structures among others are incorporated into the model.

Model inputs & outputs
Physicochemical properties of the nanoparticles are relevant input parameters in the *in vitro* dosimetry models. Size, density and zeta potential of the primary nanoparticles as well as properties related to the agglomeration state (e.g. agglomeration density, size and porosity) are the main representative "nanoparticle-dependent inputs" and have been depicted in Figure 3.14.

It is important to note that, the effect of the nanomaterial coating was also addressed by Mukherjee et al., 2014 (a) that included an estimation of the fraction of citrate adhered to the NP and the fraction of NP surface area that is available for reaction.

The properties of the assay media are also required model input parameters. Media characteristics identified in the models and classified in the inventory as "nanoparticle-independent inputs" have been also summarized in Figure 3.14.

The estimation of the fraction of administered particles that would deposit on cells as a function of time (Hinderliter et al., 2010, Mahnama et al., 2014, Mukherjee et al. 2014 (a) and Mukherjee et al., 2015) or the spatial distribution of the NPs in the extracellular space (Su et al. 2010) are the main output parameters identified in the inventory.

Nanomaterials & applicability domain
As mentioned above, the computational models compiled in this section generally predict the dynamics of particles in liquids or predict the transport of colloids through a porous medium (i.e. Su et al. 2010). Similarly to the Respiratory tract dosimetry, the models include but are not limited to particles in the range of 0-100 nm. For instance, Hinderliter et al. 2010 states that the ISDD model can be used for non-interacting spherical particles and their agglomerates. The model was tested with multiple sizes of polystyrene spheres (20-1100 nm), 35 nm amorphous silica and large agglomerates of 30 nm iron oxide particles. Other authors (Cohen et al., 2013, Cohen et al., 2014, Deloid et al 2014 & Teeguarden et al 2014, using the ISDD have evaluated a variety of different nanomaterials (<100nm) including metal (Au, Ag) metal oxides (Al$_2$O$_3$, CeO$_2$, CoO, Cr$_2$O$_3$, Fe$_2$O$_3$, Fe$_3$O$_4$, Gd$_2$O$_3$, Mn$_2$O$_3$, SiO$_2$, TiO$_2$, ZrO$_2$) and carbon nanoparticles.

Similarly to the ISDD model, Su et al., 2010 assumes that the model can evaluate spherical, chemically inert and solid particles, but in this case restricted to particles within the nano-size range. The model used 10nm Fe_3O_4 particles for simulation purposes.

Mukherjee et al., 2014 (a) evaluated the ADSRM model with different AgNP sizes (from 1 to 110 nm) and surfaces chemistries (i.e. citrate, PEG and PVP). It has to be noted that the author assumes that despite of using different surface chemistries, the model cannot adequately capture solution interactions (e.g. those involving polymer chains) due to the lack of information regarding the coating chemistry. However, the model deals at some extend with the dissolution of AgNP. A further publication from the same author (Mukherjee et al., 2015) enabled the assessment of NM interaction with various fractions of lipids and surfactant proteins of the alveolar lining fluid. In addition to the same AgNP the author also used 600nm SiO_2 particles.

Assumptions
Some examples of assumptions taken by the *in vitro* dosimetry models have been identified and listed. They can be considered as critical factors responsible for part of the uncertainty of the compiled models:

- Single average particle hydrodynamic diameter (Hinderliter et al., 2010)

- Size, number and effective density of agglomerates remain constant over time (Hinderliter et al., 2010)

- Non buoyant particles (particles immediately and permanently adhere to cells and are thereby removed from further influencing transport) (Hinderliter et al., 2010; Mukherjee et al., 2014).

- Deposition of nanoparticles is assumed to be irreversible (Su et al., 2010).

- Oxidation of AgNPs coatings has been assumed to be zero for PEG and PVP coatings (Mukherjee et al., 2014).

REACH relevance
REACH allows the use of any scientifically justified information as weight of evidence (Annex XI) supporting read-across approach.

In vitro dosimetry models may also be helpful in the *in vitro-in vivo* extrapolation (IVIVE), and they are mentioned in ECHA guidance for estimating biotransformation rates in bioaccumulation assessments.

3.4 Fate model inventory

3.4.1 *Search and selection strategy*

The steps followed from the selection of relevant papers to their inclusion in the inventory are summarized below and are the same than those followed during the QSAR/QSPR and PBK model revision, with some modifications.

1. Selection of papers by means of database websites
2. First selection of relevant publications
3. Removal of duplicates
4. Prioritization of relevant papers based on the abstract
5. Addition of relevant papers from other sources (EU projects, reviews)
6. Reading of the articles
7. Inclusion or exclusion of the model from the inventory

In order to select the initial set of publications, Scopus and WoS (Web of Science) database websites were used as search engine tools and the relevant terms included for the initial screening were chosen (point 1 above). This case is substantially different than the QSAR example since the fate and transport of colloids in environmental media has been evaluated since long ago, and the number of terms related to the topic is rather broad. On the other hand, environmental exposure assessment to NMs is a more recent field and the number of papers and groups working on them is quite limited. Specifically, the main search components (SC) were:

- SC1: model, modelling, simulation tool, fate, multimedia
- SC2: nanomaterial* (therefore also including engineered nanomaterials), nanoparticle*
- SC3: exposure, environmental concentration, environmental distribution, environmental exposure, human exposure, exposure assessment, flow, exposure to nanomaterial* and nanomaterial* exposure (also with nanoparticles*), transport, dissolution, aggregation, deposition, transformation and dissolution.
- SC4: porous media, aquatic or water or aquatic.

Different combinations of the terms included above led to the compilation of almost 1100 publications, which were included in the reference manager Mendeley program (point 2 above). After removal of duplicates (point 3 above), the set of publications was reduced down to a number close to 900. Then, a detailed evaluation of titles and abstracts further reduced the number of the final set (point 4 above). The main criteria used to remove non-relevant papers and classify relevant papers were:

- A large amount of papers describing measurements and transport modelling of particles generated from diesel engines were found, which are traditionally called ultrafine particles in literature. Modelling of ultrafine NMs in outdoor air and also modelling of NMs in indoor air have been not included in the environmental fate inventory.
- Numerous papers evaluating inflammation in *in vitro* or *in vivo* experiments have been removed (specially related to the human exposure term).
- Experimental environmental fate studies have been also removed, e.g. dissolution, stability or transport in porous media studies.

As the typology of studies found is rather broad, it was decided to divide the papers by three different generic categories:

1) Material flow (MF) models: these models typically track the materials from production and manufacturing to use and further to end-of-life stages and identify at each stage how much

materials are released into which technical or environmental compartment. Depending on the model different assumptions are made, different environmental compartments are included and different transfer factors are used that describe the amounts of mass flowing from one compartment into another.

2)   Environmental process-based fate models: these models determine the fate (partitioning between compartments) and transport (advection and deposition fluxes) of the materials in a system by modelling physicochemical processes, such as agglomeration, or sedimentation.

3)   Bioaccumulation models: these models address the uptake or accumulation of NMs in aquatic organisms.

As can be expected, some studies combining MF and process-based fate modelling approaches were found since available fate models rely strongly on input data to the environmental compartments that are provided by MFA models. Therefore, this division is justified by the fact that main focus of the different models was put in either MF or in process-based fate models. From the literature search also some bioaccumulation models were available, and are distinguished as a separate type of approach.

In addition to the citation database, new methodological approaches discussed in recent reviews (Baalousha et al., 2016; Dale, Lowry, & Casman, 2015), reports being drafted between NANoREG (FP7) and ProSafe (H2020) projects as well as new scientific papers published during the execution of this work have also been taken into consideration to expand the search (point 5 above). As a result of the search process, a total of 141 relevant publications were compiled, from which 38 corresponded to MFA models and 100 corresponded to process-based environmental fate models, plus 3 bioaccumulation models. Review of papers was done in a chronologic order starting from the oldest papers to be able to recognise the citations of already revised publications as well as identify possible new models, not captured using the strategy presented above (point 6 above).

A considerable number of papers, in which the developed models were the same or closely related to previous ones, were not reported in the inventory as (new) entries, but were included as associated references of the original models (point 7 above).

After an accurate review of the search results, a total of 27 publications for MFA models (out of 38) and 54 publications for process-based environmental fate models (out of 100) were included in the final inventory. Figure 3.15 shows the number of publications included in the inventory with respect to the year of publication.
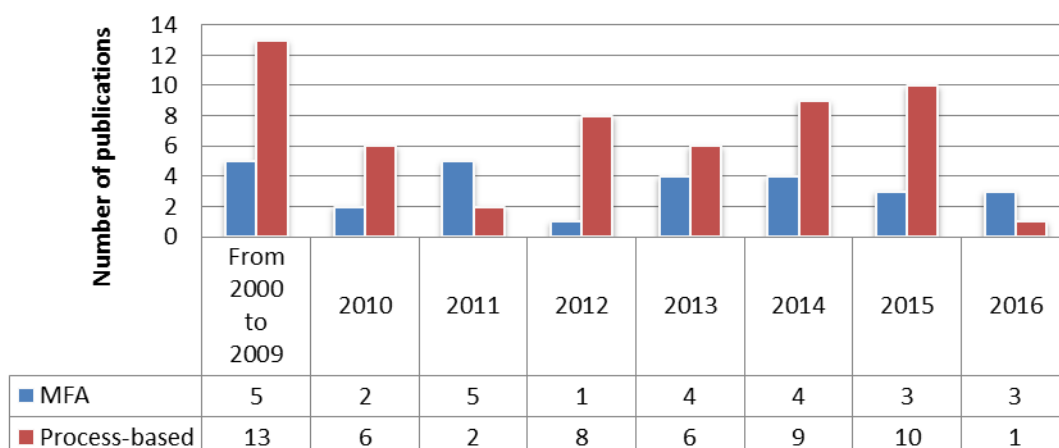
	From 2000 to 2009	2010	2011	2012	2013	2014	2015	2016
■ MFA	5	2	5	1	4	4	3	3
■ Process-based	13	6	2	8	6	9	10	1

Figure 3.15. Number of publications included in the inventory as a function of publication year

In the next section MF models, environmental fate process-based models (in either water or porous media) and bioaccumulation models will be discussed separately. The field has been extensively covered by several reviews recently published (Baalousha et al., 2016; Caballero-Guzman & Nowack, 2016a; Cornelis, Hund-Rinke, Kuhlbusch, van den Brink, & Nickel, 2014; W. Peijnenburg, Praetorius, Scott-Fordsmand, & Cornelis, 2016).

3.4.2 Analysis of the available fate models - material flow models

MF models track NMs from one compartment to the other and identify at each stage the NM quantity (mass) that is released to the environmental (e.g. freshwater, soil, air) and technological compartments (e.g. waste water treatment plant). This is realised through the identification of NMs applications and the definition of transfer coefficients. The final aim of these models (the same as in process-based environmental fate models), is to estimate predicted environmental concentrations (PECs) (F Gottschalk, Lassen, Kjoelholt, Christensen, & Nowack, 2015), or to provide qualitative analysis by means of scores or rankings (Niall O'Brien & Cummins, 2010), therefore to assess environmental exposure. In some cases, however, PEC has been used to estimate consumer exposure via the air compartment (Royce et al., 2014).

Depending on the scope of the published studies, there is a large variety in terms of the number of compartments evaluated, the number of product life cycle stages considered, the environmental/technological compartments identified as well as the extent to which some fate processess are evaluated. It has been already discussed in literature that MF models are meant to provide a first step in NMs environmental exposure estimation, but such estimates are in general not based on fundamental multimedia fate and transport analysis (Fadri Gottschalk, Scholz, & Nowack, 2010). Figure 3.16 shows an overview of the available models to predict the concentration of NMs in the environment (in some cases also exposure to consumers thorough the environment) with respect to some parameters used to report the models in the inventory: 1) NMs, 2) Environmental/technological compartments, 3) Life

cycle stages, 4) Regional (e.g. city) / national scale and static /dynamic modelling[31], 5) Deterministic or stochastic approach.

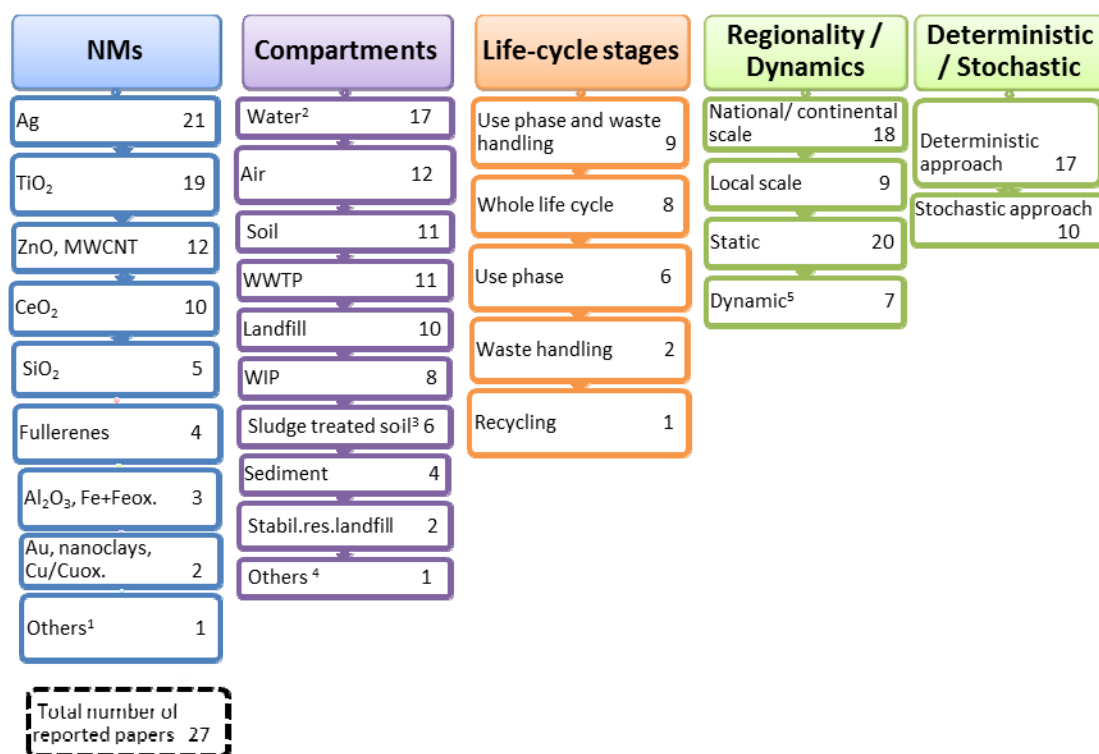| NMs		Compartments		Life-cycle stages		Regionality / Dynamics		Deterministic / Stochastic	
Ag	21	Water[2]	17	Use phase and waste handling	9	National/ continental scale	18	Deterministic approach	17
TiO$_2$	19	Air	12	Whole life cycle	8	Local scale	9	Stochastic approach	10
ZnO, MWCNT	12	Soil	11	Use phase	6	Static	20		
CeO$_2$	10	WWTP	11	Waste handling	2	Dynamic[5]	7		
SiO$_2$	5	Landfill	10	Recycling	1				
Fullerenes	4	WIP	8						
Al$_2$O$_3$, Fe+Feox.	3	Sludge treated soil[3]	6						
Au, nanoclays, Cu/Cuox.	2	Sediment	4						
Others[1]	1	Stabil.res.landfill	2						
		Others[4]	1						

Total number of reported papers 27

Figure 3.16. Summary of several relevant inputs obtained in the inventory analysis for mass-flow models. Number of models allows quantifying the specific weight of each element within the same type of model. Total number of models corresponds to the total number of entries in the inventory (27). 1Include organo-silica, hydroxyapatite, latex, CuCO$_3$, quantum dots, carbon black, Ca peroxide, keratin fibers and Al. These NMs only appear once in the inventory. 2 Includes surface water, rivers, natural freshwater and drinking water. 3Also including agricultural soil. 4Include incinerated ash landfill, groundwater, drinking water plant, environment, human body, lungs, swimming pools and bioactive landfill (slag). 7The dynamic modeling of release intends to describe the evolution over time of the amounts of NMs released to the environment. Includes models with dynamic consideration on NMs production, release from stocks or release coefficients.

Nanomaterials and nano-enabled products
Nanomaterials (NMs) covered by current MFA are limited to silver, metal oxides and carbon-based NMs. As seen in Figure 3.16, Ag was included in 21 out of the 27 publications reported. TiO$_2$ (19), ZnO (12) and CeO$_2$ (10) were the most frequent evaluated metal oxides and the most studied carbon-based NM was MWCNT, which was represented in 10 studies. The total number of NMs covered in the inventory is 21. However, 10 of these NMs were only evaluated once, either in Boxall et al. (2007), Keller et al. (2013) or Tiede et al. (2016).

---

[31] Dynamic modeling of release intends to describe the evolution over time of the amounts of NMs released to the environment

Although the number of papers on this subject is relatively large, very little information about the incorporation of NMs in commercial products is available from the manufactures, thus it is difficult to determine the real degree to which NMs have penetrated the market. It is worth pointing out that the EU has recently approved synthetic amorphous silicon dioxide as an existing substance for use in biocidal products for product-type 18 (insecticides). This is the first product registered as a nanomaterial form, tough the approval covers stable aggregated particles with sizes above 1 μm, with primary particles at the nano-scale.

Different approaches have been followed in literature to determine how these NMs can reach the different environmental / technological compartments, being some models only applied for certain material in selected applications (Arvidsson, Molander, & Sandén, 2014) while others have the aim to be comprehensive (Arturo A Keller et al., 2013; Mueller & Nowack, 2008). On one hand, some studies have evaluated market size of specific products, market penetration and concentration of NMs in products to determine the total amount of NMs mass in different product families, like in the first quantitative approach for assessing NMs release and concentrations for environmental media (Boxall et al., 2007). In this particular case, the focus of the studies was put either on specific products such as CeO_2-based diesel additives (B. Park et al., 2008) and cosmetics (Lorenz, Von Goetz, Scheringer, Wormuth, & Hungerbühler, 2011) or by contrast, in a wide range of products (UK specific case in Tiede et al., 2016).

On the other hand, another approach, firstly proposed by Nowack and co-workers, have been widely used by different authors with slightly different methodologies: 1) worldwide (an at a country level when available) production volume is allocated to different countries/regions by means of the population of industrialized world (Mueller & Nowack, 2008), in proportion to Gross Domestic Product (Sun, Gottschalk, Hungerbühler, & Nowack, 2014) or by the Inequality-adjusted Human Development Index (IHDI, which gives an idea of human development achievement)(A A Keller & Lazareva, 2014); 2) then production volume is allocated to different product categories (e.g. paints, coatings, electronics, textile) based on internet sources and also from knowledge about NMs concentrations in the different nano-enabled products. Some of the internet sources correspond to developed nano-inventories trying to map different products containing NMs that are currently in the market[32]. In Keller et al. (2013), product allocation was performed following the indications of a market study, based on manufacturer information gathered through surveys and interviews. In another work reporting the flow of TiO_2, ZnO, Ag and MWCNT trough recycling processes, 33 different product categories containing NMs were defined, which significantly expanded the range of nano-enabled products (Caballero-Guzman, Sun, & Nowack, 2015).

Since it is well recognized that information about production volumes and product allocation is not available[33], allocation of different NMs to products has been assumed to follow the same pattern for some NMs (Arturo A Keller et al., 2013; Mueller & Nowack, 2008). It is important to take into account that all these assumptions bring uncertainty to the final

---

[32] Examples include the Woodrow Wilson Centre for Scholars' Project on Emerging Nanotechnologies (WWI, 2012), the ANEC/BEUC Inventory (ANEC/BEUC, 2010) and the BUND inventory (BUND, 2011)

[33] Information on production volumes may be available in REACH registration dossiers through ECHA but this information is not available to the public

assessment as it will be discussed below. Most of the current models assume that in a given region, population is using products containing NMs distributed in certain applications, based on inventories or market reports, but with a lack of real evidence (Luoma, 2008).

Two of the few existing studies assessing exposure to NMs applied in real products were aiming to estimate emissions to air (B. Park et al., 2008) and to soil and water compartments (A. C. Johnson & Park, 2012) of a CeO_2-based fuel additive (Envirox). These studies relied on industry-provided figures, and in Park et al. the impact of Envirox on existing ambient particulate matter levels was assessed in real terms by atmospheric monitoring and also by the use of modelling with two emission models (COPERT and TRENDS). Results obtained showed that it was highly unlikely that exposure to CeO_2 at the environmental levels will elicit pulmonary inflammation. Moreover, this product showed positive environmental effects associated with the increased fuel efficiency and low hydrocarbon emissions. This work also constitutes the only example which provides physicochemical characterization of the particles included in the product.

On the whole, the MF models reported in the inventory worked with mass of NMs, and as a rule physicochemical properties were not taken into account (e.g. size, zeta potential, surface reactivity). However, one model reported a simple particle flow analysis approach (PFA) (Arvidsson, Molander, & Sandén, 2011) using three different case studies: TiO_2 in sunscreen and Ag in textiles and circuit electronics. To determine particle number, representative sizes obtained from literature / company websites were assigned to both TiO_2 and Ag and release to the environment (compartments were not specified) was assessed qualitatively based on different factors such as technology diffusion, consumption per capita of nano-products or product lifetime. The study only concluded that most nano-TiO_2 was released from sunscreen use.

Release scenarios

In MF models, each release scenario defines the type of process causing NMs release (or waste) in each of the life cycle stages of a nano-enabled product (Caballero-Guzman & Nowack, 2016b). For instance, considering a antimicrobial textile containing Ag NMs as a reference, the following release scenarios could be defined: 1) wearing, 2) washing 3) drying, 4) ironing 5) recycling, 6) incineration and 7) landfill (Wigger et al., 2015).[34]

 In MF models, release of NMs during the different steps of the value-chain of a product is defined by means of release factors, which determines amount of NMs entering into the different environmental / technological compartments. This is a critical step in exposure assessment since depending on the flows that NMs follow, potential risks will be taken place in water or in soil for instance. This concept is currently applied in chemical environmental exposure assessment by the ECHA (ECHA, 2010a). Release factors or default parameters to estimate release rates are linked to different environmental release categories (industrial production, formulation and wide dispersive uses and associated release factors are based on the highest release factors available for representative use patterns). Importantly release factors are common to all products, regardless of its composition. In absence of experimental data some authors have adopted some of these factors to define worst-case scenarios, typically to estimate release of NMs during the synthesis /manufacturing stages

---

[34] Release scenarios from 1-4 correspond to the use phase of the product and from 5-7 to disposal phase.

(Fadri Gottschalk, Sonderer, Scholz, & Nowack, 2010). Release of NMs at industrial settings has to be assumed since this information is not provided by industry because of confidentially issues as a routine basis. In Shinohara et al. (2009), release of fullerenes to air outside the factory was estimated in 0.03 %, based on the efficiency of HEPA filters for gases[35].

In contrast, release factors defining release amounts of NMs from products during their use have been established by numerous authors. It is well known that release from products depends on factors such as NMs distribution into the product (i.e. surface or embedded), NMs stock in the article or whether the intended use is either indoors or outdoors (Mitrano, Motellier, Clavaguera, & Nowack, 2015). Typically, release factors are defined by means of personal judgment or when available, from release experiments described in literature (Boldrin, Hansen, Baun, Hartmann, & Astrup, 2014; Fadri Gottschalk, Scholz, et al., 2010). These studies typically mimic conditions that resemble real use of products, as textile washing (Mitrano et al., 2014) or polymer weathering (Nguyen et al., 2012). These experiments give insight into both the quantification of the released material per time unit in each product life cycle stage, and also on the characteristics of the NMs released. In some models complete release into the environment is assumed, like release from textiles in Boxall et al. (2007) or Arvidsson et al. (2014). In other cases, complete release into air or water is more evident, when products are deliberately releases into the environment such as cleaning spray agents (Royce et al., 2014) or cosmetics products (Musee, 2011).

It is also common to explore different scenarios, including realistic and worst case scenarios (Blaser, Scheringer, Macleod, & Hungerbühler, 2008). In O'Brien and Cummins (2010) it was assumed that Ag release from textiles into WWTP was 50% in NM form while 50% as ionic form. In other works it is also assumed that 100% of the NMs produced will be eventually released, but differentiating between environmental compartments (e.g. water) or technological compartments in which NMs will end up at certain point (e.g. landfill) (Arturo A Keller et al., 2013; Sun et al., 2014). As an example, TiO_2 release from cement in Europe was assumed to be distributed between WWTP (1%), landfill (29%), and recycling compartments (70%) (Sun et al., 2014). Therefore, in these studies long-term accumulation as stock is not considered since current models assume that NMs are produced and released to waste streams and environmental compartments during the same period of time, commonly one year. Consequently, resulting NMs concentrations are proportional to the NMs concentrations values used as input in the model. This issue will be introduced below because recently some studies have introduced time-dependent processes in material flow exposure assessment.

Besides release factors, one point that has not being currently addressed is the exposure forms that NMs might exhibit when they are released into the environment. Only few studies have included transformations taken place during the use phase of the products, specifically for dissociation of Ag into Ag^+ in water contact scenarios (Blaser et al., 2008; Mueller & Nowack, 2008), and also for sulfidation of Ag and ZnO once these NMs are released into the environment (F Gottschalk et al., 2015). This is of extremely importance for two reasons: 1) exposure forms (e.g. free or aggregated particles, particles embedded

---

[35] CEN standards on the efficiency of NMs filtering are under development in the frame of the EC mandate M461

matrices) determine further fate and transport (and toxicity) of particles in the environment, and 2) if NMs transform into non-nanoforms, predicted NMs concentrations in the environment might be actually overestimating real concentrations.

Environmental / Technological compartments

As can be seen in Figure 3.16, aquatic compartment has been the most evaluated environmental compartment in the reported papers, and examples of PECs in surface water (N. J. O'Brien & Cummins, 2011), river water (F Gottschalk, Ort, Scholz, & Nowack, 2011) and drinking water (N O'Brien & Cummins, 2010; Tiede et al., 2016) can be found. Water, air, soil, WWTP, landfill and WIP compartments are habitually incorporated in the MFA with a product life-cycle perspective (Sun et al., 2015; Y Wang, Kalinina, Sun, & Nowack, 2016), with only few cases considering the sediment compartment. Among these, landfills, soil and water (or sediment) are defined as sink compartments, and from such compartments no downstream flows are modelled. Transformation products account as a removal (e.g. Ag_2S generated from Ag in WWTP is considered as a degradation product and hence as a mass loss from the system Sun et al. 2016). It is worth recalling that models generally consider well mixed compartments, thus not being space oriented.

Some authors have predicted concentrations in more specific compartments such as swimming pools (TiO_2 NMs from different sources in David Holbrook et al., 2013). Other studies have modeled NMs flows during waste handing (different kind of landfills and WIP Mueller et al. 2013) and recycling process in Switzerland for large variety of nano-enabled products and possible treatments (Caballero-Guzman et al., 2015). Products considered for recycling were cars, cooling devices, electronic waste (e-waste), metals and batteries, mineral material coming from the construction and demolition waste, textiles and wood and the NMs evaluated were TiO_2, ZnO, Ag and MWCNT. Results obtained revealed that the largest sink compartments for NMs were incineration plants and landfills. Moreover, flows were small compared to the overall flows in the whole system described in (Sun et al., 2014).

Although MFA attempt to estimate NMs concentrations, such estimates are in general not based on fundamental fate and transport analysis. For instance, sedimentation, resuspension, surface erosion or water-sediment partitioning of NPs are based on very crude assumptions, since currently there is no data available concerning NP transfer to and from these compartments (Fadri Gottschalk, Scholz, et al., 2010). A worst case scenario of no sedimentation, i.e. all particles remained in the water phase, was considered by Arvidsson et al. (2011), and Gottschalk et al. (2011) reported a (i) a conservative scenario without any NMs transformation/deposition in rivers and (ii) an optimistic scenario with rapid NMs removal. These different scenarios try to address the current uncertainty associated to the behaviour of NMs in the aquatic environment. Similarly, in Sun et al., (2014) it was assumed that nanoparticles eventually deposited in water (40 days) and soil (10 days). Therefore 10/365 and 40/365 of the total input flows into these compartments were considered as the fraction of NMs remaining in the two compartments.

Besides fate processes in the different environmental compartment, (dimensionless) transfer coefficients have been applied to determine NMs amount that remained in waste water treatment plants as sludge and the fraction remaining in the effluent, and therefore reaching natural waters. As for release factors, such coefficients have been taken from literature sources when available or from personal judgment. In a representative example,

the lowest reported specific removal efficiencies in waste water treatment plants were incorporated into the calculations for CeO_2 (95% removal), and when no information was available on removal, two scenarios were considered: (1) a conservative scenario where the removal efficiency was assumed to be 0% and (2) a more realistic scenario, where a removal efficiency of 97% for particles in packed-bed filters was applied (Tiede et al., 2016).

Regarding the PECs, MF models considering the whole life cycle of nano-enabled products in a more comprehensive way report that in general the highest concentrations of NM are found in sediments, followed by waste-water sludge treated soils and then surface water. Regarding technological compartments, NMs were more accumulated in waste water sludge, followed by landfills, waste incineration plants bottom and fly ashes compartments (Sun et al., 2016, 2014). These results are in reasonable good agreement with the values reported in Keller et al. (2013), but it should be pointed out that different results are motivated due to differences in scope (global vs. regional or single country) or types of NMs. It must be also underlined that waste practices might be region-specific, and therefore this could affect whether sludge produced in waste water treatment plants is deposited in landfills or burned in waste incineration plants, for instance. In any case, from both representations it is evident that higher PECs of nano-TiO_2 are expected than the other studied NMs in the different environmental compartments. This has been attributed to the large production and application of TiO_2. To finalize, another important assumption adopted by current models is that NMs background concentration is in most cases neglected.

Uncertainty, temporal and spatial scales
It can be stated that current reference MF models are: 1) probabilistic mass flow models (PMFA), developed by Nowack and co-workers (Fadri Gottschalk, Scholz, et al., 2010) and deterministic models developed by UC CEIN (Fadri Gottschalk, Scholz, et al., 2010; Arturo A Keller et al., 2013). Both models are static and therefore determine steady-state concentrations. The main difference therefore relies in the fact PMFA applies a stochastic approach to computing probability distributions of mass flows and PEC, by means of Monte Carlo simulations and Markov Chain Monte Carlo (MCMC) modelling. This allows the model to cope with the uncertainties and inherent variability of its parameters (e.g. NMs production volumes, transfer coefficients etc.), since for such parameters all available information is collected and probability distributions are produced. Type of applied probability distribution depends on the amount of information available. For instance, uniform distributions are applied when data is lacking (Sun et al., 2014). In the case of deterministic models, values (such as production volumes) are only considered from single sources. Consequently uncertainty is not assessed for highly uncertain parameters such as production amounts, product allocation, and market penetration, amount of NMs in the products, release factors or removal efficiencies in waste water treatment plants or water incineration plants. Unfortunately, the absence of analytical methods able to quantify trace concentrations of NMs has made impossible to validate the outcomes of such MFA models.

Recently, PMFA modelling has been updated by the addition of a dynamic component (Bornhöft, Sun, Hilty, & Nowack, 2016; Sun et al., 2016). This improvement allows predicting the former, current and future mass-flows of NMs to the different compartments over time, by incorporating into the model the lifetime of a product, how many years the NMs release events take place for one product and how much of the fraction is released every year. In

contrast to previous reported models, these studies consider long-term NMs accumulation as stock.

Finally, regarding the spatial scale of the models, as deduced from Figure 3.16, most models (n=18) have assessed NMs exposure at country (e.g. Germany, Wigger et al. 2015) or continent level (e.g. USA Mahapatra et al. 2015), while a lower number of studies (n=7) have focused on small regions such as Gothenburg city (Arvidsson et al., 2014).

3.4.3 Process-based environmental fate models

Process-based environmental fate models describing the behaviour of nanomaterials (NMs) take into consideration transformation and degradation (e.g. dissolution), interaction with suspended particulate matter (i.e heteroaggregation) and transport processess (e.g. sedimentation). These models have been splitted between two groups:

1. Models describing the fate and transport of NMs in aquatic media (including multimedia box models)

2. Models describing the fate and transport of NMs in soil media

For details regarding colloidal theories underlying environmental and biological fate (e.g. DLVO and Smoluchowsky-Friedlander), the reader is refered to section 1.5.1 in Chapter 1. Moreover, some reviews have been recently published covering nanomaterials fate in the environment (Baalousha et al., 2016; Cornelis et al., 2014; W. J. G. M. Peijnenburg et al., 2015; W. Peijnenburg et al., 2016).

Fate in aquatic environments

NMs covered by current environmental fate process-based models in aquatic media are limited to a restrict number of metal oxides (12, 9 and 5 out of 24 studies for CeO_2, TiO_2, and ZnO, respectively) and Ag which was included in 11 out of the 24 publications reported. This is in fairly good agreement with the numbers corresponding to the NMs evaluated in the MF models described above. The PEC of other NMs (CNT, Cu, Cu oxides, Fe, Al_2O_3, nanoclays and SiO_2) in different environmental compartment was also evaluated in only single study (H H Liu & Cohen, 2014).

Table 3.6 shows some representative examples of different types of models that have been included in the inventory. Two different categories have been established, in a similar way than reported previously by (Baalousha et al., 2016):

1) Spatiotemporally averaged fate multimedia box models
2) Spatiotemporally explicit fate models[36]

Regarding the first type of studies, two large environmental fate models have been reported so far: i) the SimpleBoxNano (SB4N) (J. A. J. Meesters, Quik, Koelmans, Hendriks, & van de Meent, 2016; J. Meesters et al., 2014) and the ii) MendNano model from the US (H H Liu, Bilal, Lazareva, Keller, & Cohen, 2015; Haoyang Haven Liu & Cohen, 2014a). Both models consider the environment as a collection of well-mixed compartments, each representing a specific medium or biological entity, with intermediate mass transport between adjacent

---

[36] Spatially and temporally resolved models, especially needed for site-specific higher tier NM exposure assessments on the scale of small rivers or hydrological units. Spatial (e.g. m or km) and temporal (e.g. months, weeks) resolutions depends on each model.

compartments. Models include aggregation/agglomeration [37], hetero-aggregation, sedimentation, dissolution and transformation reactions in addition to transport affecting NMs bound to particulate matter. While SB4N has been solved at steady state, MendNano determines the dynamic environmental multimedia mass distribution and concentrations of NMs. However, both models are spatially unresolved, averaging concentrations over large regions (e.g. country scale). One of the main differences is that SB4N considers first order rate constants to model mechanistically transport and transformations processes, while MendNano assumes time independent partitioning ratios for processes of aggregation and attachment, which control the environmental fate of colloidal systems.

These two models carried out simulations including emission release rates of different NMs obtained as the outputs in mass flow models (MFA), based on life cycle inventory assessment (Arturo A Keller et al., 2013; Mueller & Nowack, 2008). As mentioned in the previous section, fate and transport NMs processes in MFA models are generally based on assumptions, rather than defined mechanistically by physicochemical equations. Differences in the PEC estimated by MFA and multimedia box environmental fate models were different depending on the environmental compartments considered. For instance, SB4N showed that atmospheric deposition is a relatively effective removal process since PEC for TiO_2 was 170 times smaller than PEC calculated in (Mueller & Nowack, 2008). It was also shown that steady-state was reached within one year of study. On other hand, PECs estimated in the water compartment were in the same order of magnitude, revealing that removal by sedimentation of NMs did not lead to significant differences between both models.

It is worth recalling that SB4N (J. Meesters et al., 2014) is an adaption of the SimpleBox model, and provides as an output PECs at the steady state. SimpleBox has been used as a regional distribution module in the EU system for Evaluation of Substances (EUSES) model, which is supported for application in environmental exposure assessment in REACH (ECHA, 2016c). However, since thermodynamic equilibrium does not apply to NMs, (Markus, Parsons, Roex, de Voogt, & Laane, 2015), SB4N proposes different forms (species) for NMs in the different compartments: i) (1) freely dispersed, (2) hetero-agglomerated with natural colloidal particles (<450 nm), or (3) attached to larger natural particles (>450 nm), which are subjected to gravitational forces (sedimentation). Apart from this concept, other two elements are actually included in SB4N compared to the model addressing conventional chemicals: 1) transformations processes are considered as altered species of the same NMs (i.e. not consider a removal process) and 2) dissolution (release of ions from the NM surface) is applied as a removal process. In air, behaviour of NMs is interpreted via the aerosol coagulation, where first-order rate constant for "aggregation" and "attachment" in air are applied.

SB4N model also stresses the need of more experimental data, since experimental values are required for some of the parameters not fully covered by existing colloidal theory (e.g. hetero-attachment efficiency) (Baalousha et al., 2016). Recently, Monte Carlo simulations were performed on the environmental fate, concentrations and speciation of three different

---

[37] Agglomeration is defined as collection of weakly or medium strongly bound particles whereas aggregation refers to a particle comprising strongly bonded or fused particles held together by strong forces such as covalent bonds. In fact, modelling language does not distinguish between the two because often it is assumed that once an agglomerate is formed, it does not de-agglomerate (Avilov, Lamon, Hristozov, & Marcomini, 2017). As a result, the two terms are used interchangeably.

NMs by a probabilistic modelling approach in order to reflect realistic distributions of variability and uncertainty of the original deterministic S4BN model (J. A. J. Meesters et al., 2016).

Other fate models in aquatic media with different degrees of complexity have been developed. Some of them explored the possibility to apply colloidal chemistry kinetic equations to describe particle aggregation and sedimentation, such in Arvidsson et al. (2011), one of the first studies on the topic. In this work, a kinetic model was presented, based on kinetic laws that describe changes in particle concentration in a homogeneous fluid, and collision efficiency was identified as the most important parameter in understanding NM environmental fate. These laws were described by Smoluchowski (1917) and Friedlander (1977). The importance of fractal dimension and the assumption of irreversible agglomeration were also discussed. Hetero-aggregation was defined but not taken into consideration for the modelling exercise.

 In another example, experimental data produced by other authors was used to explore the possibilities and limitations on the implementation of first-order rate constants for the sedimentation and dissolution processes (J T K Quik, Vonk, Hansen, Baun, & Van De Meent, 2011). The outcome of this study was NMs concentration in water resulting from in lab-based water column experiments. Reasonable correlations were found in this study. In order to further explore these findings, the same authors verified such model with experimental data of CeO_2 NMs in natural river water containing natural colloids. By monitoring CeO_2 concentration in the water column at different initial NMs concentration, and with filtered /unfiltered river water, it was found that when hetero-aggregation was dominant (low initial NMs concentration) a first-order kinetic apply (k_{sed}), since NMs deposit onto natural colloids, and then these underwent sedimentation. It is currently assumed that environmental relevant NMs concentrations are very low. Therefore the presence of NMs will lead to hetero-aggregation, instead of homo-aggregation (Antonia Praetorius, Scheringer, & Hungerbühler, 2012). Actually, in a recent reported model, NMs were assumed to hetero-aggregate completely in all media, therefore sedimentation only accounted for solid particulate material (Amy L. Dale, Lowry, & Casman, 2015). On the other hand, when homo-aggregation is dominant (high initial NMs concentrations), this process is faster than what first order kinetics describes (Petosa, Jaisi, Quevedo, Elimelech, & Tufenkji, 2010).

Hetero-aggregation is the main process to include in the modelling of fate for nanomaterials, with the hetero-attachment efficiency as main parameter (α_{het}, see Table 1.1 in Chapter 1). In Praetorius et al. (2012), attachment efficiency is treated as the probability that two colliding particles stick to each other and is always between 0 and 1, and as mentioned above the way on how to define this value differs between models. It is already known that such parameters depend on both NMs surface properties as well as the properties of the surrounding medium, so for one NM it might be different if the NOM concentration in the media is different, for instance. In last few years, different approaches have been published on how to measure this parameter experimentally (L E Barton, Therezien, Auffan, Bottero, & Wiesner, 2014; A Praetorius et al., 2014).

Other important processes extensively evaluated in literature are dissolution (e.g. ZnO) and transformation (e.g. from Ag to Ag_2S). Several examples have been included in the inventory evaluating Ag dissociation with respect to pH and natural organic matter (NOM) concentration (J. Liu & Hurt, 2010) or nanoparticles size (W Zhang, Yao, Sullivan, & Chen,

2011). There is a current discussion in the scientific community on how to incorporate both hetero-aggregation and dissolution processes in the existing environmental fate models.

Praetorius et al. (2012) reported a river multimedia model that calculated the predicted environmental steady-state concentrations of TiO_2 NMs in water and sediment in Rhine River. The model takes into account relevant processes for NM fate such as hetero-aggregation (expressed with a pseudo-first-order rate constant), sedimentation, sediment resuspension and burial. Attachment efficiency is the parameter governing aggregation rates and fractal dimension is considered for the prediction of sedimentation rates. Due to the relative abundance of particulate matter compared to NMs in the system, homo-aggregation was considered negligible. The initial size distribution of the aggregated TiO_2 NPs was set to be log- normal with a mode at 300 nm. Attachment efficiency for hetero-aggregation was varied from 0.001 to 1 (different values) to evaluate the effect on hetero-aggregation rate constant. Uniform conditions throughout the model were assumed (varying parameters in different runs) and it was found that concentration in the sediment was several order of magnitude higher than in the moving water in all scenarios evaluated (in both free and hetero-aggregated) (Antonia Praetorius, Scheringer, & Hungerbühler, 2012).

In another study, a Monte Carlo method was applied to a simple model for TiO_2, Ag, ZnO and CeO_2, considering both aerobic and anaerobic conditions in waste water treatment (L E Barton et al., 2015). WWTP was compartmentalized, compared to a previous work published by the same group (Hendren, Badireddy, Casman, & Wiesner, 2013), into sewer system, primary clarifier, secondary treatment (aeration and secondary clarifier) and sludge processing (anaerobic digestion). Particularly, sedimentation was assessed indirectly by determining experimentally NMs attachment to suspended matter. As a result, distribution coefficients (ratio of NMs concentration in the supernatant and the settled material) allowed assigning NMs fraction ending up in the sludge and NMs fraction remaining in the WWTP effluent. Moreover, redox transformations of Ag and ZnO were assumed to be first order in the absence of more complete rate information.

More recently, some spatiotemporal explicit models have been published (Amy L. Dale et al., 2015; De Klein, Quik, Bäuerlein, & Koelmans, 2016a; Dumont, Johnson, Keller, & Williams, 2015; Sani-Kast et al., 2015), allowing in some cases to predict hotspots of exposure (in certain regions or sections of a river for instance) and also consider the feedbacks between hydrology and nanoparticle behaviour.

In a first attempt, in the framework of NANOFATE FP7 EU-funded project, Dumont et al. used a GWVA model to simulate expected concentrations of ZnO and Ag, which are likely to be emitted to surface waters across the whole Europe. GWAVA's main component simulates river discharge and a number of other hydrological variables, such as volumes of lake water and human water abstractions, in a spatially and temporally explicit manner. NMs loss in river water was assumed to follow first order-kinetics (Quik et al 2012). Different assumptions were made by the authors: 1) households were the only source of NMs loading from sewage effluent, 2) NMs input was assumed to be constant in time and represents the current situation, 3) dissolution losses of nano-Ag were not modelled because experimental results from literature showed these are negligible compared to the modelled sedimentation losses. As general conclusion it was found that concentrations tended to be higher further downstream in river systems because in this section of the rivers, STP discharges were more common (Dumont et al., 2015). As recognized by the authors, risk was overestimated since

transformation processes like sulfidation were not taken into account, which hence could reduce risk in surface waters.

In a follow-up approach (Joris T K Quik, de Klein, & Koelmans, 2015), a model coupled NMs (CeO$_2$ and Ag) fate processes to the hydrology of subsections of the modelled system for the first time (Dommel catchment river, The Netherlands). The basic input parameters of this model were the characteristics of the natural particles and the NMs (different size classes), which were used for the calculation of aggregation and sedimentation rates (input concentrations at flow time zero were 1,10 and 100 ng/L Ag or CeO$_2$ NMs, with 10 ng/L). Main outputs were concentrations of free, homo- and hetero- aggregated NMs in the water column and in the sediment. Importantly, the shear rate (G) in the term for orthokinetic aggregation (see Section 1.5.1 for definition) was calculated from the flow rate as calculated by the DUFLOW hydrological model, thus providing a direct link between river morphometry, hydrology and aggregation behaviour. Attachment efficiencies were defined by taking into account variability and uncertainty of the hetero-aggregation rate constants by Monte Carlo probabilistic modelling. Moreover, different scenarios were proposed: default spatially explicit (different river sections) and spatially homogeneous scenario (one cross section is defined, with uniform flow velocity).

Results from modelling over 40.3 km and simulated 9 days of flow, showed different total retentions of NMs in the whole river section (ranging from 10 to 50%), depending on the abundance or large NMs-suspended solids aggregates. For both Ag and CeO$_2$, concentration of pristine NMs and homoaggregates decreased almost zero after only 5 km (Joris T K Quik et al., 2015), because they transformed into hetteroaggregates quickly. Actually, only aggregates with radius below 1.5 microns were stable in the water column and dominate at the end of the river. By contrast, removal of NMs was due to sedimentation of (hetero-)aggregates, mainly of sizes larger than 5 microns. In other areas, resuspension was found to be the dominant process in the model, leading to negligible NMs concentrations in the sediment. It was concluded that the inclusion of details, e.g. heterogeneity of sizes of both pristine NMs and suspended solids, is highly recommended to avoid simplifications.

Recently, NanoDUFLOW model has been evaluated against data, by comparting measured and modelled concentrations of <450 nm Ce, Al, Ti and Zr-based particles for river Dommel (De Klein et al., 2016a). Ce validation showed very good results, demonstrating as in the original paper than hetero-aggregates formed rapidly. Therefore, final concentration of NMs was rather unaffected for different values of attachment efficiency parameter (α_{het}). However, the authors stressed the fact that for a full model validation, longer field campaigns covering different season and years would be required.

Finally, another model considering spatiotemporal variability was recently published in which the effect of stream dynamics and chemical transformations on the environmental fate of ZnO and Ag NMs in a watershed-scale model was evaluated (Amy L. Dale et al., 2015). ZnO and Ag NPs and their transformation by-products entered the model via wastewater treatment facility effluent and biosolids applications to row crops, hay, and pasture land. The river simulation calculated sediment transport rates as a function of spatiotemporal variability in streamflow, and tracked temperature-dependent chemical reactions including ZnO NP dissolution, oxygen-dependent dissolution of Ag NPs, sulfide-dependent sulfidation of metal ions, and metal ion complexation with particulate phases. NPs were assumed to heteroaggregate completely in all media, therefore initial particle size was not taken into

consideration. Zn in WWTP effluent was modelled as 7.5% ZnO NPs and 92.5% Zn^{2+}. All Zn in soils was assumed to be complexed (particle-associated) Zn^{2+}. Ag NPs were assumed to be over 50% sulfidized in biosolids and effluent, as observed experimentally. Interestingly, very low retention compared to the model reported by Quick and co-workers (Joris T K Quik et al., 2015) was found and it was shown that mobility was due to flow-dependent sediment transport. Again, different concentrations between water and sediment compartments were explained by difference in regional stream velocities. Zn accumulated less than Ag because Zn^{2+} was moving with the water phase along the river. In this work, higher Ag concentrations could also be linked to areas in which agricultural runoff was a significant fraction of stream loads.

It should be pointed out that in all models reported above (with the exception of the NanoDUFLOW model validation in De Klein et al., 2016), it was not the intention of the authors to estimate real PEC in a region over a specific period of time, since the input (mass loading entering into the system) was uncertain in all cases (e.g. taken from other MF studies, scale down from country estimations to local scale.). These models intended, however, to evaluate the influence of natural variability in the environmental fate and transport of NMs in aquatic environment.

Table 3.6 Summary of representative spatiotemporally averaged and explicit environmental fate models reported in the inventory. The most complete models in terms of citations, comprehensive studies.

Spatiotemporally average fate multimedia box models				
Reference	**Compartments**	**Processes[1]**	**NMs**	**Approach /Assumptions**
(Haoyang Haven Liu & Cohen, 2014a)	Air Water Sediment Soil Biota	Dry and wet deposition, aerosolization, wind resuspension, sedimentation, resuspension, burial, dissolution	TiO_2, Ag CNT, Cu, Cu oxides, ZnO Fe, Fe oxide, Al_2O_3, CeO_2 Nanoclay, SiO_2	The approach considers the environment as a collection of well-mixed compartments each representing a specific medium or biological entity, with intermediate mass transport between adjacent compartments. Processes are defined as rates. Particle size distribution is discretized into a number of size fractions. It does not consider agglomeration kinetics (an attachment factor of 1 was chosen , i.e. 100% of NPs are bound to the suspended particulate matter), therefore the model assumes fixed (time independent) partitioning ratios for processes of aggregation and attachment, which control the environmental fate of colloidal systems
(J. Meesters et al., 2014)	Air Soil Water Sediment	Aggregation and attachment[2] of NMs in air, water, soils and sediments. Atmospheric deposition, sedimentation, dissolution, resuspension sediment burial soil run-off	TiO_2[3]	Environmental fate model that uses first order kinetics (transport and transformation processes for colloids) to estimate environmental background concentrations for nanocolloids in an environmental system. The model solves simultaneous mass balance equations using simple matrix algebra. SimpleBox4nano (SB4N) is a modified version of the Simple Box model, which has served as a regional distribution module in the European Union System for Evaluation of Substances (EUSES) model. Homo-aggregation is not considered in the model. Within each compartment, NMs can occur in different physicochemical form (species): (1) freely dispersed, (2) hetero-aggregated with natural colloidal particles (<450 nm), or (3) attached to larger natural particles (>450 nm) that are prone to gravitational forces in aqueous media
(J. A. J. Meesters et al., 2016)	Same than above	Same than above	CeO_2, TiO_2, ZnO	Same than above, applies a probabilistic approach. Monte Carlo simulations were performed on the environmental fate, concentrations and speciation of 3 different nanomaterials. For all inputs and model parameters data was collected, reflecting realistic distributions of variability and uncertainty
(Antonia Praetorius, Scheringer, &	Water	Hetero-aggregation	TiO_2	Multimedia box model. Processes affecting NM behaviour and transport are parameterized and combined in a system of coupled mass-balance

		Spatiotemporally average fate multimedia box models		
Reference	**Compartments**	**Processes[1]**	**NMs**	**Approach /Assumptions**
Hungerbu, 2012)	Sediment	Sedimentation Resuspension Exchange of water from moving to stagnant compartment Burial Bed Load transport Water flow in the moving water		equations. The aim is to estimate the distribution of NPs along the Rhine River. SPM in the model are represented as a log- normal particle size distribution with a mode of 5 μm (particle diameter) and spanning from 1.5 to 80 μm. It is assumed that particles do not reach the river in their original form. The initial size distribution of the aggregated TiO_2 NPs was set to be log- normal with a mode at 300 nm. The nanoparticles in the distribution were assigned to five size classes (the model is run separately for each size class). Homoaggregation and dissolution are not taken into account. Attachment efficiency for heteroaggregation was varied from 0.001 to 1 (different values) to evaluate the effect on heteroaggregation rate constant
(Amy L Dale, Lowry, & Casman, 2013)	Freshwater sediment	Ag oxidation Ag sulfidation Particle mixing due to bioturbation Diffusive mixing of dissolved species	Ag	Mass balance sediment model for metals, describes the speciation of cadmium in sediments. Total sediment Ag (concentration profiles of Ag^+ ions, Ag and Ag_2S nanoparticles, free Ag_2S and Ag linked to organic matter as a function of sediment depth) concentration as a function of sediment depth. The model was calibrated to experimental data collected from nano-Ag dosed artificial freshwater wetland mesocosms. Constant for Ag oxidation decreases exponentially in response to Ag NP sulfidation. In general, reaction rates were assumed to exhibit a linear dependence on the concentrations of all reactants. Aggregation is not taken into account in this study, neither particle size variation. By omitting aggregation, the model assumes that Ag NPs mix in the sediment at approximately the same rate as the sediment particles themselves. Complexation between Ag^+ and Cl^- is neglected
(J T K Quik et al., 2011)	Water	Homo-aggregation Hetero-aggregation Sedimentation Dissolution	MWCNT CeO_2 Fe^0 ZnO TiO_2	Model aims to estimate concentration of NMs in water, based on the current approach to environmental risk characterization for chemicals in the EU. Possibilities and limitations on the implementation of first order rate constants for the processes sedimentation and dissolution of NMs are discussed. It is accepted, that sedimentation and dissolution of NMs can be explained by first order reactions / removal models (approximations are made based on experimental data, see Figure 2 and Figure 3 in the paper)

Spatiotemporally average fate multimedia box models				
Reference	**Compartments**	**Processes**[1]	**NMs**	**Approach /Assumptions**
			Ag Al_2O_3	
(Money, Reckhow, & Wiesner, 2012)	Water Sediment	Aggregation Dissolution Deposition	Ag	Bayesian networks is used as a Tool for nanomaterials risk forecasting and develop a baseline probabilistic model that incorporates nanoparticle specific characteristics and environmental parameters, along with elements of exposure potential, hazard, and risk related to nanomaterials. The tool utilizes a combination of expert and empirical knowledge bases related to the aquatic behaviour and exposure of nano-Ag as a function of environmental conditions and particle characteristics. Continuous variables were discretized into states based on environmental-relevant thresholds. Connections presented in the model represent conditional relationships and do not explicitly use mechanistic or physical models to describe how one variable affects another. These conditional relationships are represented as probability distributions for the different parameters in the whole range of values considered (e.g. different size intervals; qualitative collision rates defined as low, intermediate, high).
(Lauren E. Barton et al., 2015)[4]	WWTP effluent WWTP sludge	Sedimentation in WWTP Transformation in WWTP	Ag CeO_2 TiO_2 ZnO	Monte Carlo methods were applied to a simple model for aerobic wastewater treatment (Hendren et al., 2013). Previous model is expanded by incorporating experimental data, obtained in environmentally relevant media, into a waste watee treatment plant model that considers both aerobic and anaerobic processes (waste water treatment plant is here compartmentalized) and redox transformations of NMs that may occur during treatment. Sedimentation is measured indirectly by monitoring NP attachment to suspended matter, which is assumed to be further removed by sedimentation. Distribution coefficients, (ratio of NPs concentration in the supernatant and the settled material, see inputs section) was determined experimentally. Redox transformations of Ag and ZnO were assumed to be first order in the absence of more complete rate information.

Spatiotemporally explicit fate models				
Reference	**Compartments**	**Processes**	**NMs**	**Approach /Assumptions**
(Dumont et al., 2015)	Freshwater	Sedimentation (Hetero-aggregation)	Ag ZnO	This work describes the use of the GWVA model to simulate expected concentrations of ZnO and Ag, which are likely to be emitted to surface water. GWAVA's main component simulates river discharge and a number of other hydrological variables, such as water volumes and human water abstractions, in a spatially and temporally explicit manner. The model is deterministic. NMs are routed down the river network, during which concentrations are calculated by accounting for any NMs losses and dilution by river discharge. NMs loss in river water follows first order-kinetics (Quik et al 2012). Transformations (after production) do not make the particle size increase beyond 100 nm nor to individual molecules are not considered as NMs losses. It is assumed that households are the only source of NMs loading from sewage effluent. NMs input is assumed to be constant in time and represents the current situation. Dissolution losses of nano-Ag were not modelled because experimental results from literature showed these are negligible compared to the modelled sedimentation losses
(Joris T K Quik et al., 2015)	Water (river) Sediment	Homo-aggregation Hetero-aggregation Sedimentation Dissolution Degradation Burial to deeper sediment layers Resuspension	CeO_2 Ag	This model allows for the first time coupling of NMs fate processes to the hydrology of subsections of the modelled system (Dommel catchment river). Coupling is done with DUFLOW Modelling Studio (v3.8.7) that is a software package for simulating one-dimensional unsteady flow in open-channel systems. This enables the modelling of feedbacks between flow conditions and water quality processes, which has been recognized as a key feature in realistic system models. The basic input parameters of this model are the characteristics of the natural particles and the NMs (different size classes), which are used for the calculation of aggregation and sedimentation rates (input concentrations at flow time zero were 1,10 and 100 ng/L of nano-Ag or nano-CeO_2). Main outputs are concentrations of free, homo- and hetero- aggregated NMs in the water column and in the sediment. The shear rate (G) in the term for orthokinetic aggregation is calculated from the flow rate as calculated by the DUFLOW hydrological model, thus providing a direct link between river morphometry, hydrology and aggregation behaviour. Authors used a measured value for the attachment efficiency. Different scenarios have been proposed: default spatially explicit (different river sections) and spatially

Spatiotemporally explicit fate models				
Reference	**Compartments**	**Processes**	**NMs**	**Approach /Assumptions**
				homogeneous scenario (one cross section is defined, with uniform flow velocity)
(Amy L. Dale et al., 2015)	Water (river) Sediment	Surface run-off during rain Sediment run-off Deposition/sedimentation Resuspension Burial Dissolution Transformation (complexation) Transformation (sulfidation)	ZnO Ag	Model to evaluate the effect of stream dynamics and chemical transformations on the environmental fate of NMs in a watershed-scale model. James River Basin portion is coupled to the EPA publically available water quality modelling suite WASP7 and configured both to model NMs fate. ZnO and Ag NPs and their transformation by-products entered the model via wastewater treatment facility effluent and biosolids applications to row crops, hay, and pasture land. The river simulation calculated sediment transport rates as a function of spatiotemporal variability in streamflow; distinguished between oxic and anoxic sediment bed layers; and tracked temperature-dependent chemical reactions including ZnO NP dissolution, oxygen-dependent dissolution of Ag NPs, sulfide-dependent sulfidation of metal ions, and metal ion complexation with particulate phases. NPs were assumed to hetero-aggregate completely in all media (therefore initial particle size was not taken into consideration). Zn in WWTP effluent was modelled as 7.5% ZnO NPs and 92.5% Zn^{2+}. All Zn in soils was assumed to be complexed (particulate-associated) Zn^{2+}. Ag NPs were assumed to be over 50% sulfidized in biosolids and effluent, as observed experimentally.

[1] Only the main processes affecting NMs are included

[2] Heteroaggregation of NMs s with colloidal particles is referred to as "aggregation", whereas association of NMs with larger particles is referred to as "attachment"

[3] The model is tested with values for TiO_2 nanomaterials derived from Mueller, N. C., & Nowack, B. (2008). Exposure modeling of engineered nanoparticles in the environment. Environmental Science & Technology, 42(12), 4447–4453

[4] This work proposes a second conceptual model of a land application unit, where NMs are impacted by runoff, uptake, leaching and transformation processes. Parameters were obtained from literature. Where error estimates were unavailable, an error of 10% was applied.

Fate in the soil compartment

The categorization of models of colloid transport in porous media is complex as: i) analytical solution of transport equations is not possible and, so, apart from different transport equations we encounter different approaches for the numerical solution; ii) most models are for 1D transport in saturated packed-bed columns, which use to fail when predicting colloid fate in real environments.

All the models analysed, but two, are based in the Colloid Filtration Theory (CFT). Since its beginnings in the 1970s, several processes affecting colloid filtration have been defined and used for colloid transport and retention in porous media. These processes are summarised in Table 3.7.

Yao, Habibian and O'Melia published in 1971 the most cited model of colloid filtration in porous media (Yao, Habibian, & O'Melia, 1971), which derived from Friedlander model of air filtration (Friedlander, 1958) and collected the different mechanisms affecting colloid transport and colloid-collector attachment developed until the moment: settling, interception and diffusion. This model is usually referred in literature as YHO model. This model defines the colloid transport as a sum of three processes that define the traditional advection-diffusion equation with a sink term that is the irreversible colloid-collector attachment. This last depends on an attachment rate (k_{att}), which was analytically determined. Attachment rate depends on collision efficiency factor (α) and the single-collector efficiency (η), among others. YHO model derives single-collector efficiency from the sum of the contributions of the mechanisms of interception (η_I), diffusion (η_D) and gravity (η_G). However, no derivation of collision efficiency factor was done and it was assumed complete effectiveness ($\alpha=1$).

The analytical determination of the single-collector efficiency has been improved since then. In 1976, Rajagopalan and Tien included surface interactions (London forces and double layer interaction forces) in which is known as RT model (Rajagopalan & Tien, 1976). In 1996, Bai and Tien included different electrokinetic parameters to determine single-collector efficiency under unfavourable attachment conditions (R. B. Bai & Tien, 1996). In 2004, Tufenkji and Elimelech improved RT model for the determination of single-collector efficiency for Brownian dominated colloids, thus for nanomaterials (Tufenkji & Elimelech, 2004), in which is known as TE model. TE model has been the most accepted and used model for the analytical determination of single-collector efficiency of nanomaterials since 2004. Wei et al reported a modification of the model for submicroparticles with Lagrangian path instead of traditional Happel sphere-in-cell trajectories (Wei & Wu, 2010). Lately, in 2015, Bradford and Torkzaban has published a new work including in the single-collector efficiency determination non-DLVO mechanisms, such as nano- and micro-roughness or nanoscale chemical surface heterogeneity (Bradford & Torkzaban, 2015).

While, as seen, important research has been done for analytical determination of single-collector efficiency (η), collision efficiency (α) has received little attention and or it is supposed to be 1 (full contact efficiency) or it is fitted using experimental results. Taghavy et al derived dependence of collision efficiency on ionic strength (Taghavy, Mittelman, Wang, Pennell, & Abriola, 2013).

Anyway, although several works with nanomaterials use the TE model for deriving attachment rate (Bradford, Torkzaban, Kim, & Simunek, 2012; Y Li, Wang, Pennell, & Briola,

2008; Taghavy, Pennell, & Abriola, 2015; Yonggang Wang et al., 2008), most of models directly determine attachment rate by fitting of the transport equation with breakthrough curves (Babakhani, Fagerlund, Shamsai, Lowry, & Phenrat, 2015; Bradford, Simunek, Bettahar, van Genuchten, & Yates, 2003; Neukum, Braun, & Azzam, 2014; Raychoudhury, Tufenkji, & Ghoshal, 2012; Tosco & Sethi, 2009).

Another important consideration on colloid-collector attachment is that traditional CFT consider attachment as the sink part of the function, so, irreversible attachment (Rajagopalan & Tien, 1976; Tufenkji & Elimelech, 2004; Yao et al., 1971). It was demonstrated that ionic strength (IS) strongly influences colloid-collector attachment, so, variations in IS may convert collector surfaces from favourable to unfavourable deposition sites (Bradford et al., 2012). This fact shows the reversibility of the process and, so, it justifies considering attachment as a reversible process (Bradford et al., 2003; Xueying Liu, O'Carroll, Petersen, Huang, & Anderson, 2009; Raychoudhury et al., 2012; Taghavy et al., 2015). A last possibility is the combination of irreversible and reversible attachment as two separated processes as it is has been proved that even a change to unfavourable deposition does not force complete release of deposited colloids (Babakhani et al., 2015). Detachment rates are always fitted based on experimental breakthrough curves.

Traditional CFT has been used in the colloid transport and retention, but breakthrough curves show profiles that need extra phenomena to be considered. The first and most important one is blocking, so, once a deposition site is occupied by a particle it is unutilized for attachment (deeper explanation in Table 3.7). This blocking directly affects attachment and is always considered as a function that "multiplies" the attachment part of transport function. Johnson and Elimelech compared in 1995 RSA and Langmuirian approaches for blocking (P. R. Johnson & Elimelech, 1995) and, despite RSA showing better fitting to experimental results, Langmuirian approach is the most used in the literature. An important point in colloidal filtration models evolution occurred in 2008 when Wang et al combined TE model for attachment determination with Langmuirian blocking reported by Johnson and Elimelech (Y Li et al., 2008; Yonggang Wang et al., 2008). The opposite of blocking would be ripening, so, colloid deposition catalyzes other colloid deposition, but it has never been included in the CFT functions.

Another phenomenon that affects colloid retention is straining, that is the retention of a colloid in a pore or due to collector surface roughness. Straining is somewhere between attachment and physical filtration, and, at the moment, it has been always considered an irreversible process. The first important CFT model considering straining was the one presented by Bradford et al in 2003, which also considered attachment, detachment and exclusion (Bradford et al., 2003). This has been the only model at the moment considering exclusion (deeper explanation in Table 3.7).

Finally, there are two important phenomena that are usually considered in fate in water, but their incorporation in CFT has only occurred lately: colloid aggregation or agglomeration and colloid dissolution. Homo-aggregation is an important phenomenon in water systems, but not as important in water as hetero-aggregation is more probable. Anyway, in highly concentrated systems and in especial cases showing high colloid-colloid contact efficiency it is important to consider homo-aggregation as colloid size is determinant in transport (Raychoudhury et al., 2012; Taghavy et al., 2015). Colloid dissolution is important in reactive

particles, as metal can be released and transported as cations, that in some cases we should consider (Taghavy et al., 2013).

Table 3.7 gives summary of the most relevant CFT models.

The main problem with CFT model is that they predict quite well colloid filtration in laboratory column experiments under controlled and homogeneous conditions though it becomes difficult to know in advance the correct model for each nanomaterial (Goldberg, Scheringer, Bucheli, & Hungerbühler, 2014), but it is rather difficult to predict their behaviour in real environment with non-saturated porous media and conditions that change with heavy rain periods, so, their use in long time fate prediction is still questioned.

Two other approaches have been proposed. First, Bai and Li presented in 2014 a work based on an autoregressive integrated moving average (ARIMA) model (C. Bai & Li, 2014). Such a model was proven very useful to predict immediate transport and retention of nanomaterials as it would happen during an accidental spill. And, second, Goldberg et al. presented in 2015 a work based on machine learning, much more similar to a QSAR model, which predicts transport and retention based on multiple previous studies (Goldberg, Scheringer, Bucheli, & Hungerbühler, 2015).

3.4.4 Accumulation / depuration models

Three models have been included in the inventory and a summary is provided for each of them:

1) Tervonen et al., 2010 fitted accumulation and depuration data of 235 nm fullerene_C_{60} nanoparticles in *Daphnia Magna* to first order one-compartment kinetic model and a first-order decay model respectively (Tervonen, Waissi, Petersen, Akkanen, & Kukkonen, 2010). The same approach was employed by Fan et al., 2016 to predict the bioconcentration factors of different TiO_2 nanoparticles (i.e. anatase and rutile crystalline forms, ranging from 30 to 200 nm and with different hydrophobic and hydrophilic coatings)

2) Zhao and Wang (2010) developed a model for the uptake in *Daphnia Magna* of 20nm carbonate-coated AgNPs from the environment (water and food). The study provides the first quantitative estimate of the fraction of AgNP uptake from the water.

3) Piccapietra et al., (2012) described the kinetics of intracellular silver nitrate and carbonate-coated AgNPs (average diameter 29nm) over the time in *Chlamydomonas reinhardtii.* Based on the estimated uptake and release constants, non-linear and linear equations were built to model the intracellular accumulation of silver over the time.

Table 3.7 Summary of phenomena affecting colloidal transport in porous media.

Process considered	Explanation	Variables	First reference in the table	Important models that consider the process
Advection	It is the transport of the colloid by bulk motion	It depends on the local velocity of water (v), which is usually assumed as constant	Yao K-M, Habibian MT, O'Melia CR (1971) Water and waste water filtration. Concepts and applications. Environ Sci Technol 5:1105–1112.	All
Hydrodynamic dispersion	Dispersivity quantifies the colloids that stray away from the carrying water	Depends on the position-dependent diffusion coefficient (D), which is usually determined using the Einstein's equation		All
Irreversible colloid attachment or deposition	It is the process that accounts for colloid-collector aggregation, known as heteroaggregation in water media processes. In traditional CFT is considered irreversible, later was considered reversible and, finally, both reversible and irreversible attachments are considered simultaneously	Attachment rates depend, among others, on colloid collision efficiency factor (α) and single-collector efficiency (η). The last depends on diffusion, interception, sedimentation, DLVO and non-DLVO phenomena. Models may be single-site or dual-site, the last considering favourable and unfavourable deposition sites		YHO model, RT model, TE model, Wang 2008-LiY 2008 model, Babakhani 2015 mode (reversible and irreversible)
Reversible colloid attachment or deposition			Bradford SA, Simunek J, Bettahar M, et al. (2003) Modeling Colloid Attachment, Straining, and Exclusion in Saturated Porous Media. Environ Sci Technol 37:2242–2250	Bradford 2003 model, MNM1D (Tosco 2010), Liu 2009 model (for non-spherical), Raychoudhury 20012 model, Torkzaban 2013 model, Vitorge 2014 model, Neukum 2014 model (for fractured stones),

Process considered	Explanation	Variables	First reference in the table	Important models that consider the process
Colloid detachment or release or remobilization	It accounts for the number of colloids that return to the fluid flux after being deposited onto a collector	It can be considered together with attachment as an attachment/detachment reversible process, or isolated as if irreversible attachment under favourable conditions and detachment under unfavourable conditions		Taghavy 2015 model, Babakhani 2015 model (reversible and irreversible)
Blocking or exclude area effect	It is the phenomena that considers monolayer coverage of collectors and, so, that the porous media has a maximum capacity of attached colloids	Blocking is considered [$B(\theta)$ or ψ_b] in the attachment component of the transport equation. As it is only relevant once the attachment approaches the solid maximum capacity, it is very often not considered. Random sequential adsorption (RSA) and Langmuirian blocking are the most common approaches	Johnson PR, Elimelech M (1995) Dynamics of Colloid Deposition in Porous Media: Blocking Based on Random Sequential Adsorption. Langmuir 11:801–812	Johnson 1995, Johnson 1996 (heterogeneous collectors), Wang 2008-LiY 2008 model, Liu 2009 model, Torkzaban 2013 model, Vitorge 2014 model

Process considered	Explanation	Variables	First reference in the table	Important models that consider the process
Straining or depth-dependent retention	Similar to mechanical retention, it is the retention of the colloid in a pore, being in contact with, at least, two points (two collectors or due to surface roughness). But opposite to mechanical retention, a strained colloid does not block the fluid flux through the pore and is governed by the same mechanisms that govern colloid attachment.	It depends on pore length, distance and shape of colloid spatial distribution. Differently to blocking, straining is usually considered as a different process, independent of attachment	Bradford SA, Simunek J, Bettahar M, et al. (2003) Modeling Colloid Attachment, Straining, and Exclusion in Saturated Porous Media. Environ Sci Technol 37:2242–2250	Bradford 2003 model, Vitorge 2014 model
Filter ripening	It occurs when colloid-colloid attachment is more favorable than colloid-collector attachment and, so, colloid deposition is enhanced over time, oppositely to blocking.	-	None	None
Exclusion	It considers that only a portion of the pores are accessible to the colloids due to size limitations or to charge repulsions.	To account for all type of exclusions, Darcy water velocity and volumetric water content are corrected.	Bradford SA, Simunek J, Bettahar M, et al. (2003) Modeling Colloid Attachment, Straining, and Exclusion in Saturated Porous Media. Environ Sci Technol 37:2242–2250	Bradford 2003.

Process considered	Explanation	Variables	First reference in the table	Important models that consider the process
Colloid dissolution	It is the reactive or dissolvable particles dissolution into ions	It is usually considered as a one-way process and dissolved ions transport modelled simultaneously	Taghavy A, Mittelman A, Wang Y, et al. (2013) Mathematical modeling of the transport and dissolution of citrate-stabilized silver nanoparticles in porous media. Environ Sci Technol 47:8499–8507	Taghavy 2013 model
Colloid aggregation or agglomeration	Homoaggregation of particles in the water medium, which directly affects their transport due to changes in particle size	It is usually considered as a one-way process	Raychoudhury T, Tufenkji N, Ghoshal S (2012) Aggregation and deposition kinetics of carboxymethyl cellulose-modified zero-valent iron nanoparticles in porous media. Water Res 46:1735–1744	Raychoudhury 2012 model, Taghavy 2015 model, Babakhani 2015 model

3.4.5 Assumptions made in fate models

Some relevant model assumptions have been identified during the reviewing process and have been discussed in the main body text. They can be considered as critical factors responsible for part of the uncertainty of the compiled models and are summarized below:

- Very little information about the NMs production volumes and the incorporation of NMs in commercial products is publicly available and this leads to high uncertainties in emissions estimates. This uncertainty has been addressed by some authors by using probabilistic mass-flow modelling using more than one source to define a value (this applies to production amounts, product allocation, and market penetration, amount of NMs in the products, release factors and transfer coefficients)(Sun et al., 2014).

- Commonly, allocation of different NMs to different products has been assumed to follow the same pattern for several NMs.

- MF models reported in the inventory worked with the mass of NMs, and as a rule physicochemical properties are not taken into account (e.g. size, zeta potential, surface reactivity). Moreover, it is assumed that particles keep their properties during their whole life cycle (i.e. they do not exhibit transformations).

- In absence of experimental data some authors have adopted worst case release factors to define worst-case scenarios, typically to estimate release of NMs during the synthesis /manufacturing stages into the different environmental compartments (e.g. ERC from ECHA Guidelines). On the other hand, release factors defining release amounts of NMs from products during their use have been established by numerous authors since there is more available data in literature.

- In general, environmental fate models consider well mixed compartments, thus not being space oriented. Few examples have been already published which are spatially resolved (A L Dale, Lowry, & Casman, 2015b).

- Transfer coefficients in MF models determining for instance NMs amount that remained in waste water treatment plants as sludge and the fraction remaining in the waste water treatment plants effluent, are most of the times defined from personal judgment and also from literature when available (similar to release factors).

- MF models determine typically steady state-concentrations. Recently, probabilistic MF modelling has been updated by the addition of a dynamic component (Bornhöft et al., 2016; Sun et al., 2016). This improvement allows predicting the former, current and future mass-flows of NMs to the different compartments over time, by incorporating into the model the lifetime of a product.

- Generally speaking, process-based environmental fate models consider different NM forms (species) in the different environmental compartments, which is in contrast with traditional chemicals that reach thermodynamic equilibrium: (1) freely dispersed, (2) hetero-aggregated with natural colloidal particles (e.g. <450 nm), or (3) attached to larger natural particles (e.g. >450 nm) (J. A. J. Meesters, Koelmans, Quik, Hendriks, & Van De Meent, 2014).

- It is currently assumed that environmental relevant NMs concentrations are very low, thus the presence of NMs will lead to hetero-aggregation, instead of homo-aggregation. Aggregation is regarded as irreversible process and consequently the break-up of such aggregates is not considered. It is also well accepted that particles reach the aquatic environment already hetero-aggregated, and therefore sedimentation kinetics is determined by the dynamics of suspended matter sedimentation.

- In process-based environmental fate models the input (mass loading entering into the system) is highly uncertain because it is taken from other MF studies and sometimes they scale down the PEC value from bigger regions to local scale. The primary aim of these models is, however, to evaluate the influence of natural variability in the environmental fate and transport of NMs in aquatic environment.

- Application of colloidal models is also associated to high uncertainty because the values for the relevant constants are not generally defined or calculated for NMs and generally the information is not available (e.g. for the Hamaker constants, and for attachment coefficients).

3.4.6 Conclusions on fate models

After state of the art analysis, some general conclusions regarding how environmental fate models can give answer to regulatory needs can be drawn:

- Multimedia mass-balance modelling has already been adopted as the basis for the risk assessment and chemical exposure model EUSES, and is based on a life-cycle perspective (Den Hollander, van Eijkeren, & van de Meent, 2004). This provides a firm scientific basis for regulators to consider the applicability of mass flow analysis to nanomaterials.
- In addition, some of the assumptions made to define release factors, which determine the amount of NMs entering into the different environmental/technological compartments, is currently being applied in chemical environmental exposure assessment by the ECHA (ECHA, 2016b). Such release factors could substitute for the current environmental release categories (ERCs) used to determine chemical concentrations in the environment.
- In order to assess uncertainty in production/release estimates, probabilistic assessment is highly recommended (EFSA, 2012; US EPA, 2014).
- Some of the nano-considerations currently included in the models to predict the fate and transport of nanomaterials in the environment are widely accepted by the scientific community (e.g. definition of hetero-aggregation, transformation and dissolutions processes, etc.). These processes and also other physicochemical properties specific to NMs are summarized in a document published by ECHA, developed in order to provide advice to registrants for use when preparing registration dossiers that cover nanoforms (ECHA, 2017). In the latter document, details of some of the existing environmental fate models are given in its Appendix 1 (J. Meesters et al., 2014; Antonia Praetorius, Scheringer, & Hungerbu, 2012; Joris T K Quik et al., 2015). For example SB4N as an adaption of the SimpleBox model has been used as a regional distribution module in the EU system for EUSES model, used for environmental exposure assessment in REACH (J. Meesters et al., 2014). An important drawback is that the lack of analytical techniques able to both measure trace concentration of NMs and differentiate between background and NMs hinders the validation of such models. Research on detection of engineered nanomaterials from backgprund material is ongoing (Antonia Praetorius et al., 2017).

3.5 References

3.5.1 QSPR and QSAR models

Aggarwal, P., Hall, J. B., McLeland, C. B., Dobrovolskaia, M. a, & McNeil, S. E. (2009). Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. Advanced Drug Delivery Reviews, 61(6), 428–37. http://doi.org/10.1016/j.addr.2009.03.009

Aitken, R. ., Bassan, A., Friedrichs, S., Hankin, S. M., Hansen, S. F., Holmqvist, J., … Tran, C. L. (2011). Specific Advice on Exposure Assessment and Hazard / Risk Characterisation for Nanomaterials under REACH (RIP-oN 3) Final Project Report.

Alarifi, S., Ali, D., Al-Doaiss, A. A., Ali, B. A., Ahmed, M., & Al-Khedhairy, A. A. (2013). Histologic and apoptotic changes induced by titanium dioxide nanoparticles in the livers of rats. International Journal of Nanomedicine, 8, 3937–43. http://doi.org/10.2147/IJN.S47174

Aldieri, E., Fenoglio, I., Cesano, F., Gazzano, E., Gulino, G., Scarano, D., … Fubini, B. (2013). The role of iron impurities in the toxic effects exerted by short multiwalled carbon nanotubes (MWCNT) in murine alveolar macrophages. J Toxicol Environ Health A, 76(18), 1056–1071. http://doi.org/10.1080/15287394.2013.834855

Allegri, M., Perivoliotis, D. K., Bianchi, M. G., Chiu, M., Pagliaro, A., Koklioti, M. A., … Charitidis, C. A. (2016). Toxicity determinants of multi-walled carbon nanotubes: The relationship between functionalization and agglomeration. Toxicology Reports, 3, 230–243. http://doi.org/10.1016/j.toxrep.2016.01.011

American Society for Testing and Materials. (2010). ASTM B922 - 10 Standard Test Method for Metal Powder Specific Surface Area by Physical Adsorption (Refractory Metal Powders No. ASTM B922-10). Retrieved from http://www.astm.org/Standards/B922.htm

Anjilvel, S. (1995). A Multiple-Path Model of Particle Deposition in the Rat Lung. Fundamental and Applied Toxicology, 28(1), 41–50. http://doi.org/10.1006/faat.1995.1144

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., … Villeneuve, D. L. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environmental Toxicology and Chemistry / SETAC, 29(3), 730–41. http://doi.org/10.1002/etc.34

ANSES. (2016). CLH report Proposal for Harmonised Classification and Labelling Substance Name : Titanium dioxide. Retrieved from http://echa.europa.eu/harmonised-classification-and-labelling-previous-consultations/-/substance-rev/13832/term

Araki, D., Bose, R., Chaudhry, Q., Dewan, K., & Dufour, E. (2013). Report of the ICCR Working Group : Safety approaches to nanomaterials in cosmetics (No. ICCR/NANOSAFETY1/R/FINAL/).

Armand, L., Tarantini, A., Beal, D., Biola-Clier, M., Bobyk, L., Sorieul, S., … Carriere, M. (2016). Long-term exposure of A549 cells to titanium dioxide nanoparticles induces DNA damage and sensitizes cells towards genotoxic agents. Nanotoxicology, 10(7), 913–923. http://doi.org/10.3109/17435390.2016.1141338

Arts, J. H. E., Hadi, M., Irfan, M.-A., Keene, A. M., Kreiling, R., Lyon, D., … Landsiedel, R. (2015). A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regulatory Toxicology and Pharmacology, 71(2), S1–S27. http://doi.org/10.1016/j.yrtph.2015.03.007

Arts, J. H. E., Hadi, M., Irfan, M.-A., Keene, A. M., Kreiling, R., Lyon, D., … Landsiedel, R. (2016). Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regulatory Toxicology and Pharmacology, 76, 234–261. http://doi.org/10.1016/j.yrtph.2015.03.007

Arts, J. H. E., Hadi, M., Keene, A. M., Kreiling, R., Lyon, D., Maier, M., … Landsiedel, R. (2014). A critical appraisal of existing concepts for the grouping of nanomaterials. Regulatory Toxicology and Pharmacology, 70, 492–506. http://doi.org/10.1016/j.yrtph.2014.07.025

Arvidsson, R., Molander, S., & Sandén, B. A. (2011). Impacts of a silver-coated future: Particle flow analysis of silver nanoparticles. Journal of Industrial Ecology, 15(6), 844–854. http://doi.org/10.1111/j.1530-9290.2011.00400.x

Arvidsson, R., Molander, S., & Sandén, B. A. (2014). Assessing the Environmental Risks of Silver from Clothes in an Urban Area. Human and Ecological Risk Assessment, 20(4), 1008–1022. http://doi.org/10.1080/10807039.2012.691412

Arvidsson, R., Molander, S., Sandén, B. A., & Hassellöv, M. (2011). Challenges in Exposure Modeling of Nanoparticles in Aquatic Environments Rickard. Human and Ecological Risk Assessment, 17(1), 245–262. Retrieved from http://www.tandfonline.com/doi/abs/10.1080/10807039.2011.538639#.U-nq6BAlqic

ASTM. (1990). Evaluation of Airborne Dust Measurement Methods for Agricultural Chemical Carriers (E35.22 No. STP25370S). Retrieved from http://www.astm.org/DIGITAL_LIBRARY/STP/PAGES/STP25370S.htm

ASTM. (2009a). Standard Test Method for Airborne Asbestos Concentration in Ambient and Indoor Atmospheres as Determined by Transmission Electron Microscopy Direct Transfer (TEM) (No. D6281-9). Retrieved from http://www.astm.org/Standards/D6281.htm

ASTM. (2009b). Standard Test Method for Microvacuum Sampling and Indirect Analysis of Dust by Transmission Electron Microscopy for Asbestos Structure Number Surface Loading (No. D5755–09(2014)e1). Retrieved from http://www.astm.org/Standards/D5755.htm

ASTM. (2010). Standard Test Method for Wipe Sampling of Surfaces, Indirect Preparation, and Analysis for Asbestos Structure Number Concentration by Transmission Electron Microscopy (No. D6480–05(2010)). Retrieved from http://www.astm.org/Standards/D6480.htm

Asturiol, D., & Worth, A. (2012). D6.5 - Report on the development and applications of structure-activity models for toxicity prediction of ENP: Analysis of ENPRA dataset (No. NMP4-SL-2009-228789).

Avilov, S., Lamon, L., Hristozov, D., & Marcomini, A. (2017). Improving the prediction of environmental fate of engineered nanomaterials by fractal modelling. Environment International, 99, 78–86. http://doi.org/10.1016/j.envint.2016.11.027

Azqueta, A., & Dusinska, M. (2015). The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetics, 6, 239. http://doi.org/10.3389/fgene.2015.00239

Baalousha, M., Cornelis, G., Kuhlbusch, T. A. J., Lynch, I., Nickel, C., Peijnenburg, W., & Van Den Brink, N. W. (2016). Modeling nanomaterial fate and uptake in the environment: Current knowledge and future trends. Environmental Science: Nano, 3(2), 323–345. http://doi.org/10.1039/c5en00207a

Babakhani, P., Fagerlund, F., Shamsai, A., Lowry, G. V, & Phenrat, T. (2015). Modified MODFLOW-based model for simulating the agglomeration and transport of polymer-modified Fe0 nanoparticles in saturated porous media. Environmental Science and Pollution Research. Department of Hydrology Engineering, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran. http://doi.org/10.1007/s11356-015-5193-0

Bachler, G., von Goetz, N., & Hungerbuhler, K. (2014). Using physiologically based pharmacokinetic (PBPK) modeling for dietary risk assessment of titanium dioxide (TiO2) nanoparticles. Nanotoxicology, 1–8. http://doi.org/10.3109/17435390.2014.940404

Bachler, G., von Goetz, N., & Hungerbühler, K. (2013). A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. International Journal of Nanomedicine, 8, 3365–82. http://doi.org/10.2147/IJN.S46624

Bai, C., & Li, Y. (2014). Time series analysis of contaminant transport in the subsurface: applications to conservative tracer and engineered nanomaterials. Journal of Contaminant Hydrology, 164, 153–62. http://doi.org/10.1016/j.jconhyd.2014.06.002

Bai, R. B., & Tien, C. (1996). A New Correlation for the Initial Filter Coefficient under Unfavorable Surface Interactions. Journal of Colloid and Interface Science, 179(2), 631–634. http://doi.org/10.1006/jcis.1996.0259

Bakand, S., Hayes, A., & Dechsakulthorn, F. (2012). Nanoparticles: a review of particle toxicology following inhalation exposure. Retrieved from http://informahealthcare.com/doi/abs/10.3109/08958378.2010.642021

Balasubramanian, S. K., Poh, K.-W., Ong, C.-N., Kreyling, W. G., Ong, W.-Y., Yua, L. E., & Yu, L. E. (2013). The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates. Biomaterials, 34(22), 5439–5452. http://doi.org/10.1016/j.biomaterials.2013.03.080

Ball, N., Cronin, M. T. D., Shen, J., Adenuga, M. D., Blackburn, K., Booth, E. D., … Hartung, T. (2016). Toward Good Read-Across Practice (GRAP) guidance. ALTEX. http://doi.org/10.14573/altex.1601251

Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Mayne-L'Hermite, M., Reynaud, C., Cassio, D., … Carrière, M. (2010). Toxicological consequences of TiO2, SiC nanoparticles and multi-walled carbon nanotubes exposure in several mammalian cell types: an in vitro study. Journal of Nanoparticle Research, 12(1), 61–73. http://doi.org/10.1007/s11051-009-9694-y

Barton, L. E., Auffan, M., Durenkamp, M., McGrath, S., Bottero, J.-Y., & Wiesner, M. R. (2015). Monte Carlo simulations of the transformation and removal of Ag, TiO2, and ZnO nanoparticles in wastewater treatment and land application of biosolids. Science of the Total Environment, 511, 535–543. http://doi.org/10.1016/j.scitotenv.2014.12.056

Barton, L. E., Auffan, M., Durenkamp, M., McGrath, S., Bottero, J.-Y., & Wiesner, M. R. (2015). Monte Carlo simulations of the transformation and removal of Ag, TiO2, and ZnO nanoparticles in wastewater treatment and land application of biosolids. Science of The Total Environment, 511, 535–543. http://doi.org/10.1016/j.scitotenv.2014.12.056

Barton, L. E., Therezien, M., Auffan, M., Bottero, J.-Y., & Wiesner, M. R. (2014). Theory and Methodology for Determining Nanoparticle Affinity for Heteroaggregation in Environmental Matrices Using Batch Measurements. Environmental Engineering Science, 31(7), 421–427. http://doi.org/10.1089/ees.2013.0472

BAuA. (2013). Announcements on Hazardous Substances - Manufactured Nanomaterials - Announcement 527 (Vol. 527). Retrieved from http://www.baua.de/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/Announcement-527.html;jsessionid=5208C23A837B260D7B2F355EBF9ED81E.1_cid353

Benigni, R., & Bossa, C. (2006). Structural Alerts of Mutagens and Carcinogens. Current Computer Aided-Drug Design, 2(2), 169–176. http://doi.org/10.2174/157340906777441663

Bigdeli, A., Hormozi-Nezhad, M. R., & Parastar, H. (2015). Using nano-QSAR to determine the most responsible factor(s) in gold nanoparticle exocytosis. RSC Advances, 5(70), 57030–57037. http://doi.org/10.1039/c5ra06198a

Binderup, M.-L., Bredsdorff, L., Beltoft, V. M., Mortensen, A., Löschner, K., Larsen, E. H., & Eriksen, F. D. (2013). Systemic Absorption of Nanomaterials by Oral Exposure; Part of the "Better control of nano" initiative 2012-2015. Copenhagen.

Birkedal, R., Shivachev, B., Dimova, L., Petrov, O., Nikolova, R., Mast, J., … Jensen, K. A. (2012). Nanogenotox deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials. Copenhagen. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

Bitsch, A., Jacobi, S., Melber, C., Wahnschaffe, U., Simetska, N., & Mangelsdorf, I. (2006). REPDOSE: A database on repeated dose toxicity studies of commercial chemicals--A multifunctional tool. Regulatory Toxicology and Pharmacology, 46(3), 202–10. http://doi.org/10.1016/j.yrtph.2006.05.013

Blaser, S. A., Scheringer, M., Macleod, M., & Hungerbühler, K. (2008). Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. The Science of the Total Environment, 390(2–3), 396–409. http://doi.org/10.1016/j.scitotenv.2007.10.010

Boldrin, A., Hansen, S. F., Baun, A., Hartmann, N. I. B., & Astrup, T. F. (2014). Environmental exposure assessment framework for nanoparticles in solid waste. Journal of Nanoparticle Research, 16(6). http://doi.org/10.1007/s11051-014-2394-2

Borders, T. L., Fonseca, A. F., Zhang, H., Cho, K., & Rusinko, A. (2013). Developing descriptors to predict mechanical properties of nanotubes. Journal of Chemical Information and Modeling, 53(4), 773–782. http://doi.org/10.1021/ci300482n

Borm, P. J. A., Robbins, D., Haubold, S., Kuhlbusch, T., Fissan, H., Donaldson, K., … Oberdörster, E. (2006). The potential risks of nanomaterials: a review carried out for ECETOC. Particle and Fibre Toxicology, 3, 11. http://doi.org/10.1186/1743-8977-3-11

Borm, P. J. A., Schins, R. P. F., & Albrecht, C. (2004). Inhaled particles and lung cancer, part B: Paradigms and risk assessment. International Journal of Cancer. http://doi.org/10.1002/ijc.20064

Bornhöft, N. A., Sun, T. Y., Hilty, L. M., & Nowack, B. (2016). A dynamic probabilistic material flow modeling method. Environmental Modelling and Software, 76, 69–80. http://doi.org/10.1016/j.envsoft.2015.11.012

Boxall, A., Chaudry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., & Watts, C. (2007). CURRENT AND FUTURE PREDICTED ENVIRONMENTAL EXPOSURE TO ENGINEERED NANOPARTICLES. York.

Boyles, M. S. P., Young, L., Brown, D. M., MacCalman, L., Cowie, H., Moisala, A., … Stone, V. (2015). Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. Toxicology in Vitro, 29(7), 1513–1528. http://doi.org/10.1016/j.tiv.2015.06.012

Braakhuis, H. M., Oomen, A. G., & Cassee, F. R. (2015). Grouping nanomaterials to predict their potential to induce pulmonary inflammation. Toxicology and Applied Pharmacology. http://doi.org/10.1016/j.taap.2015.11.009

Braakhuis, H. M., Park, M. V. D. Z., Gosens, I., De Jong, W. H., & Cassee, F. R. (2014). Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. Particle and Fibre Toxicology, 11(1), 18. http://doi.org/10.1186/1743-8977-11-18

Bradford, S. A., Simunek, J., Bettahar, M., van Genuchten, M. T., & Yates, S. R. (2003). Modeling Colloid Attachment, Straining, and Exclusion in Saturated Porous Media. Environmental Science & Technology, 37(10), 2242–2250. http://doi.org/10.1021/es025899u

Bradford, S. A., & Torkzaban, S. (2015). Determining Parameters and Mechanisms of Colloid Retention and Release. Langmuir : The ACS Journal of Surfaces and Colloids, 31(44), 12096–12105. http://doi.org/10.1021/acs.langmuir.5b03080

Bradford, S. A., Torkzaban, S., Kim, H., & Simunek, J. (2012). Modeling colloid and microorganism transport and release with transients in solution ionic strength. Water Resources Research, 48(9), n/a-n/a. http://doi.org/10.1029/2012WR012468

Bruinink, A., Wang, J., & Wick, P. (2015). Effect of particle agglomeration in nanotoxicology. Archives of Toxicology. http://doi.org/10.1007/s00204-015-1460-6

BSI. (2007). Nanotechnologies – Part 2: Guide to safe handling and disposal of manufactured nanomaterials (ICS 13.100 No. 71.100.99).

Buffle, J., Wilkinson, K. J., Stoll, S., Filella, M., & Zhang, J. (1998). A Generalized Description of Aquatic Colloidal Interactions: The Three-colloidal Component Approach. Environmental Science & Technology, 32(19), 2887–2899. http://doi.org/10.1021/es980217h

Burello, E. (2013). Profiling the biological activity of oxide nanomaterials with mechanistic models. Computational Science & Discovery, 6(1), 14009. http://doi.org/10.1088/1749-4699/6/1/014009

Burello, E., & Worth, A. P. (2011a). A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology, 5(2), 228–35. http://doi.org/10.3109/17435390.2010.502980

Burello, E., & Worth, A. P. (2011b). QSAR modeling of nanomaterials. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology, 3(3), 298–306. http://doi.org/10.1002/wnan.137

Burello, E., & Worth, A. P. (2013). A rule for designing safer nanomaterials: do not interfere with the cellular redox equilibrium. Nanotoxicology, (July), 1–2. http://doi.org/10.3109/17435390.2013.828109

Bygd, H. C., Forsmark, K. D., & Bratlie, K. M. (2015). Altering invivo macrophage responses with modified polymer properties. Biomaterials, 56, 187–197. http://doi.org/10.1016/j.biomaterials.2015.03.042

Caballero-Guzman, A., & Nowack, B. (2016a). A critical review of engineered nanomaterial release data: Are current data useful for material flow modeling? Environmental Pollution, 213, 502–517. http://doi.org/10.1016/j.envpol.2016.02.028

Caballero-Guzman, A., & Nowack, B. (2016b). A critical review of engineered nanomaterial release data: Are current data useful for material flow modeling? Environmental Pollution, 213, 502–517. http://doi.org/10.1016/j.envpol.2016.02.028

Caballero-Guzman, A., Sun, T., & Nowack, B. (2015). Flows of engineered nanomaterials through the recycling process in Switzerland. Waste Management, 36, 33–43. http://doi.org/10.1016/j.wasman.2014.11.006

Cai, X., Ramalingam, R., Wong, H. S., Cheng, J., Ajuh, P., Cheng, S. H., & Lam, Y. W. (2013). Characterization of carbon nanotube protein corona by using quantitative proteomics. Nanomedicine: Nanotechnology, Biology, and Medicine, 9(5), 583–593. http://doi.org/10.1016/j.nano.2012.09.004

Casarett, L., & Doull, J. (2008). Toxicology. (C. D. Klaassen, Ed.) (7th ed.). Kansas City: The McGraw-Hill Companies. http://doi.org/10.1036/0071470514

Cassano, A., Marchese Robinson, R. L., Palczewska, A., Puzyn, T., Gajewicz, A., Tran, L., … Cronin, M. T. D. (2016). Comparing the CORAL and Random Forest approaches for modelling the in vitro cytotoxicity of silica nanomaterials. Alternatives to Laboratory Animals : ATLA, 44(6), 533–556.

Cassee, F., Lynch, I., Valsami-jones, É., Falk, A., Svendsen, C., Fransman, W., … Stone, V. (2017). ACTION PLAN 2017 – 2021, 1–16.

Catalán, J., Siivola, K. M., Nymark, P., Lindberg, H. K., Suhonen, S., Järventaus, H., … Norppa, H. (2015). In vitro and in vivo genotoxic effects of straight versus tangled multi-walled carbon nanotubes. Nanotoxicology, 5390(January 2016), 1–47. http://doi.org/10.3109/17435390.2015.1132345

Cedervall, T., Lynch, I., Lindman, S., Berggård, T., Thulin, E., Nilsson, H., … Linse, S. (2007). Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proceedings of the National Academy of Sciences of the United States of America, 104(7), 2050–5. http://doi.org/10.1073/pnas.0608582104

CEN. (2002). CEN/TC 292 - Characterization of waste (292 No. 12457–1). Retrieved from http://standards.cen.eu/dyn/www/f?p=204:110:0::::FSP_PROJECT,FSP_ORG_ID:14486,6273&cs=1F4 6F9399C700ACD45A3EB04FE4A2BE1B

CEN. (2006). Workplace atmospheres - Measurement of the dustiness of bulk materials - Requirements and reference test methods (No. EN 15051:2006). Brussels.

Chapman, P. M., McDonald, B. G., & Lawrence, G. S. (2010). Weight-of-Evidence Issues and Frameworks for Sediment Quality (And Other) Assessments. Human and Ecological Risk Assessment: An International Journal, 8(7), 1489–1515. http://doi.org/10.1080/20028091057457

Charlier, J. C., Blase, X., & Roche, S. (2007). Electronic and transport properties of nanotubes. Reviews of Modern Physics, 79(2), 677–732. http://doi.org/10.1103/RevModPhys.79.677

Chau, Y. T., & Yap, C. W. (2012). Quantitative Nanostructure-Activity Relationship modelling of nanoparticles. RSC Advances, 2(22), 8489–8496. http://doi.org/10.1039/c2ra21489j

Chen, G., Peijnenburg, W. J. G. M., Kovalishyn, V., & Vijver, M. G. (2016). Development of nanostructure-activity relationships assisting the nanomaterial hazard categorization for risk assessment and regulatory decision-making. RSC Advances, 6(57), 52227–52235. http://doi.org/10.1039/c6ra06159a

Chen, J., Dong, X., Zhao, J., & Tang, G. (2009). In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitioneal injection. Journal of Applied Toxicology : JAT, 29(4), 330–7. http://doi.org/10.1002/jat.1414

Chen, R., Zhang, Y., Sahneh, F. D., Scoglio, C. M., Wohlleben, W., Haase, A., … Riviere, J. E. (2014). Nanoparticle surface characterization and clustering through concentration-dependent surface adsorption modeling. ACS Nano, 8(9), 9446–56. http://doi.org/10.1021/nn503573s

Chen, W.-Y., Cheng, Y.-H., Hsieh, N.-H., Wu, B.-C., Chou, W.-C., Ho, C.-C., … Lin, P. (2015). Physiologically based pharmacokinetic modeling of zinc oxide nanoparticles and zinc nitrate in mice. International Journal of Nanomedicine, 10, 6277–92. http://doi.org/10.2147/IJN.S86785

Cheng, L.-C., Jiang, X., Wang, J., Chen, C., & Liu, R.-S. (2013). Nano-bio effects: interaction of nanomaterials with cells. Nanoscale, 5(9), 3547–69. http://doi.org/10.1039/c3nr34276j

Cho, H. H., Huang, H., & Schwab, K. (2011). Effects of solution chemistry on the adsorption of ibuprofen and triclosan onto carbon nanotubes. Langmuir, 27(21), 12960–12967. http://doi.org/10.1021/la202459g

Clift, M. J. D., Raemy, D. O., Endes, C., Ali, Z., Lehmann, A. D., Brandenberger, C., … Rothen-Rutishauser, B. (2012). Can the Ames test provide an insight into nano-object mutagenicity? Investigating the interaction between nano-objects and bacteria. Nanotoxicology, 7(February), 1–13. http://doi.org/10.3109/17435390.2012.741725

Cockburn, A., Bradford, R., Buck, N., Constable, A., Edwards, G., Haber, B., … Wildemann, T. (2012). Approaches to the safety assessment of engineered nanomaterials (ENM) in food. Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association, 50(6), 2224–42. http://doi.org/10.1016/j.fct.2011.12.029

Cornelis, G., Hund-Rinke, K., Kuhlbusch, T., van den Brink, N., & Nickel, C. (2014). Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. Critical Reviews in Environmental Science and Technology, 44(24), 2720–2764. http://doi.org/10.1080/10643389.2013.829767

Cornelis, G., Pang, L., Doolette, C., Kirby, J. K., & McLaughlin, M. J. (2013). Transport of silver nanoparticles in saturated columns of natural soils. The Science of the Total Environment, 463–464, 120–30. http://doi.org/10.1016/j.scitotenv.2013.05.089

Cornelissen, I. R., Jongeneelen, F., Broekhuizen, P. Van, & Broekhuizen, F. Van. (2011). Guidance working safely with nanomaterials and nanoproducts, the guide for employers and employees. Retrieved from http://www.etui.org/content/download/3553/39999/file/Guidance+on+safe+handling+nanomats&products.pdf

Cortez, B. A., & Machado-Santelli, G. M. (2008). Chrysotile effects on human lung cell carcinoma in culture: 3-D reconstruction and DNA quantification by image analysis. BMC Cancer, 8(1), 181. http://doi.org/10.1186/1471-2407-8-181

Cramer, R. D., Patterson, D. E., & Bunce, J. D. (1988). Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. Journal of the American Chemical Society, 110(18), 5959–5967. http://doi.org/10.1021/ja00226a005

Creutzenberg, O. (2013). Toxic Effects of Various Modifications of a Nanoparticle Following Inhalation. Retrieved from www.baua.de/dok/4430542

Cruciani, G., & Watson, K. A. (1994). Comparative molecular field analysis using GRID force-field and GOLPE variable selection methods in a study of inhibitors of glycogen phosphorylase b. Journal of Medicinal Chemistry, 37(16), 2589–601. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8057302

Cui, Y., Liu, H., Zhou, M., Duan, Y., Li, N., Gong, X., … Hong, F. (2011). Signaling pathway of inflammatory responses in the mouse liver caused by TiO2 nanoparticles. Journal of Biomedical Materials Research. Part A, 96(1), 221–9. http://doi.org/10.1002/jbm.a.32976

Cveticanin, J., Joksic, G., Leskovac, A., Petrovic, S., Sobot, A. V, & Neskovic, O. (2010). Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells. Nanotechnology, 21(1), 15102. http://doi.org/10.1088/0957-4484/21/1/015102

Dale, A. L., Lowry, G. V., & Casman, E. A. (2015). Stream Dynamics and Chemical Transformations Control the Environmental Fate of Silver and Zinc Oxide Nanoparticles in a Watershed-Scale Model. Environmental Science and Technology. http://doi.org/10.1021/acs.est.5b01205

Dale, A. L., Lowry, G. V, & Casman, E. A. (2013). Modeling nanosilver transformations in freshwater sediments. Environmental Science & Technology, 47(22), 12920–8. http://doi.org/10.1021/es402341t

Dale, A. L., Lowry, G. V, & Casman, E. A. (2015a). Much ado about α: Reframing the debate over appropriate fate descriptors in nanoparticle environmental risk modeling. Environmental Science: Nano, 2(1), 27–32. http://doi.org/10.1039/c4en00170b

Dale, A. L., Lowry, G. V, & Casman, E. A. (2015b). Stream Dynamics and Chemical Transformations Control the Environmental Fate of Silver and Zinc Oxide Nanoparticles in a Watershed-Scale Model. Environmental Science and Technology, 49(12), 7285–7293. http://doi.org/10.1021/acs.est.5b01205

David Holbrook, R., Motabar, D., Quiñones, O., Stanford, B., Vanderford, B., & Moss, D. (2013). Titanium distribution in swimming pool water is dominated by dissolved species. Environmental Pollution, 181, 68–74. http://doi.org/10.1016/j.envpol.2013.05.044

De Klein, J. J. M., Quik, J. T. K., Bäuerlein, P. S., & Koelmans, A. A. (2016a). Towards validation of the NanoDUFLOW nanoparticle fate model for the river Dommel, the Netherlands. Environmental Science: Nano, 3(2), 434–441. http://doi.org/10.1039/c5en00270b

De Klein, J. J. M., Quik, J. T. K., Bäuerlein, P. S., & Koelmans, A. A. (2016b). Towards validation of the NanoDUFLOW nanoparticle fate model for the river Dommel, the Netherlands. Environmental Science: Nano, 3(2), 434–441. http://doi.org/10.1039/c5en00270b

Dearden, J. C., Barratt, M. D., Benigni, R., Douglas, W., Combes, R. D., Cronin, M. T. D., … Yourick, J. J. (1997). The Development and Validation of Expert Systems for Predicting Toxicity. Alternatives to Laboratory Animals : ATLA, 25, 223–252.

de Temmerman, P., Mast, J., Guiot, C., Spalla, O., Rousset, D., Shivachev, B., & Tarassov, M. (2012). Nanogenotox deliverable 4.2. Transmission electron microscopic characterization of NANOGENOTOX nanomaterials Final report. Copenhagen. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

Den Hollander, H. A., van Eijkeren, J. C. H., & van de Meent, D. (2004). SimpleBox 3.0: multimedia mass balance model for evaluating the fate of chemical in the environment, (RIVM report 601200003).

Di Giorgio, M. L., Bucchianico, S. Di, Ragnelli, A. M., Aimola, P., Santucci, S., & Poma, A. (2011). Effects of single and multi walled carbon nanotubes on macrophages: Cyto and genotoxicity and electron microscopy. Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 722(1), 20–31. http://doi.org/10.1016/j.mrgentox.2011.02.008

Dobrzyńska, M. M., Gajowik, A., Radzikowska, J., Lankoff, A., Dušinská, M., & Kruszewski, M. (2014). Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats in vivo. Toxicology, 315, 86–91. http://doi.org/10.1016/j.tox.2013.11.012

Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forrest, G., & Alexander, A. (2006). Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety. Toxicological Sciences. http://doi.org/10.1093/toxsci/kfj130

Donaldson, K., Borm, P. J. a, Oberdörster, G., Pinkerton, K. E., Stone, V., & Tran, C. L. (2008). Concordance between in vitro and in vivo dosimetry in the proinflammatory effects of low-toxicity, low-solubility particles: the key role of the proximal alveolar region. Inhalation Toxicology, 20, 53–62. http://doi.org/10.1080/08958370701758742

Donaldson, K., Duffin, R., Murphy, F. A., & Poland, C. A. (2012). The Toxicology of Carbon Nanotubes. (K. Donaldson, C. Poland, R. Duffin, & J. Bonner, Eds.)The Toxicology of Carbon Nanotubes. Cambridge: Cambridge University Press. http://doi.org/10.1017/CBO9780511919893

Donaldson, K., Murphy, F. a, Duffin, R., & Poland, C. a. (2010). Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Particle and Fibre Toxicology, 7, 5. http://doi.org/10.1186/1743-8977-7-5

Donaldson, K., & Poland, C. (2013). Nanotoxicity: challenging the myth of nano-specific toxicity. Current Opinion in Biotechnology, 24(4), 724–34. http://doi.org/10.1016/j.copbio.2013.05.003

Donaldson, K., & Poland, C. A. (2012). Inhaled nanoparticles and lung cancer - what we can learn from conventional particle toxicology. Swiss Medical Weekly, 142, w13547. http://doi.org/10.4414/smw.2012.13547

Donaldson, K., Poland, C. A., & Wargo, E. (2009). Nanotoxicology: new insights into nanotubes. Nature Nanotechnology, 4(11), 708–710. http://doi.org/10.1038/nnano.2009.327

Donaldson, K., Poland, C. a, & Schins, R. P. F. (2010). Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies. Nanotoxicology, 4(December), 414–20. http://doi.org/10.3109/17435390.2010.482751

Donaldson, K., Schinwald, A., Murphy, F., Cho, W.-S., Duffin, R., Tran, L., & Poland, C. (2013). The biologically effective dose in inhalation nanotoxicology. Accounts of Chemical Research, 46(3), 723–32. http://doi.org/10.1021/ar300092y

Downs, T. R., Crosby, M. E., Hu, T., Kumar, S., Sullivan, A., Sarlo, K., … Pfuhler, S. (2012). Silica nanoparticles administered at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not. Mutation Research, 745(1–2), 38–50. http://doi.org/10.1016/j.mrgentox.2012.03.012

Drefahl, A. (2011). CurlySMILES: A chemical language to customize and annotate encodings of molecular and nanodevice structures. Journal of Cheminformatics, 3(1), 1–7. http://doi.org/10.1186/1758-2946-3-1

Duan, Y., Liu, J., Ma, L., Li, N., Liu, H., Wang, J., … Hong, F. (2010). Toxicological characteristics of nanoparticulate anatase titanium dioxide in mice. Biomaterials, 31(5), 894–9. http://doi.org/10.1016/j.biomaterials.2009.10.003

Duffin, R., Tran, L., Brown, D., Stone, V., & Donaldson, K. (2007). Proinflammogenic Effects of Low-Toxicity and Metal Nanoparticles In Vivo and In Vitro: Highlighting the Role of Particle Surface Area and Surface Reactivity. Inhalation Toxicology, 19, 849–856. Retrieved from http://informahealthcare.com/doi/abs/10.1080/08958370701479323

Dumont, E., Johnson, A. C., Keller, V. D., & Williams, R. J. (2015). Nano silver and nano zinc-oxide in surface waters - exposure estimation for Europe at high spatial and temporal resolution. Environmental Pollution (Barking, Essex : 1987), 196, 341–349.

Durdagi, S., Mavromoustakos, T., Chronakis, N., & Papadopoulos, M. G. (2008). Computational design of novel fullerene analogues as potential HIV-1 PR inhibitors: Analysis of the binding interactions between fullerene inhibitors and HIV-1 PR residues using 3D QSAR, molecular docking and molecular dynamics simulations. Bioorganic and Medicinal Chemistry, 16(23), 9957–9974. http://doi.org/10.1016/j.bmc.2008.10.039

Durdagi, S., Mavromoustakos, T., & Papadopoulos, M. G. (2008). 3D QSAR CoMFA/CoMSIA, molecular docking and molecular dynamics studies of fullerene-based HIV-1 PR inhibitors. Bioorganic and Medicinal Chemistry Letters, 18(23), 6283–6289. http://doi.org/10.1016/j.bmcl.2008.09.107

EASAC, & JRC. (2011). Impact of engineered nanomaterials on health. Luxembourg.

EC. (2008). Nanomaterials in REACH. Follow-up to the 6th Meeting of the REACH Competent Authorities for the implementation of Regulation (EC) 1907/2006 (REACH). Brussels 2008: European Commission. Retrieved from http://ec.europa.eu/enterprise/sectors/chemicals/files/reach/nanomaterials_en.pdf

EC. (2011a). Commission Recommendation of 18 October 2011 on the definition of nanomaterial (No. L 275/38). Official Journal of the European Union (Vol. 2011/696/E). Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:275:0038:0040:EN:PDF

EC. (2011b). REACH Implementation Project Substance Identification of Nanomaterials (RIP-oN 1). Retrieved from http://ec.europa.eu/environment/chemicals/nanotech/pdf/report_ripon1.pdf

EC. (2012a). Commission Staff Working Paper: Types and uses of nanomaterials, indcluding safety aspects. European Commission.

EC. (2012b). D6.4 – Optimised PBPK/PD and Exposure Model (ENPRA No. NMP4-SL-2009-228789). Retrieved from http://www.enpra.eu/MemberArea/Projectdocuments/Finaldeliverables.aspx

EC. (2012c). Second Regulatory Review on Nanomaterials: COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL AND THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE (No. COM(2012) 572).

EC, & ECHA. (2012). NANO SUPPORT Project (I) - Scientific technical support on assessment of nanomaterials in REACH registration dossiers and adequacy of available information - Report on analysis and assessment and options for adapting REACH. (E. Commission, Ed.). Brussels: JRC, ECHA. Retrieved from http://ec.europa.eu/environment/chemicals/nanotech/pdf/jrc_report.pdf

ECETOC. (2012). Category approaches, Read-across, (Q)SAR (No. 116). Brussels.

ECHA. (2008). Guidance on information requirements and chemical safety assessment Chapter R.6 : QSARs and grouping of chemicals (Guidance for the implementation of REACH). Helsinki. Retrieved from http://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf

ECHA. (2009). Guidance in a Nutshell - Chemical Safety Assessment (No. ECHA-09-B-15-EN). ECHA Guidance. Helsinki: ECHA. Retrieved from http://echa.europa.eu/documents/10162/13632/nutshell_guidance_csa_en.pdf

ECHA. (2010a). Guidance on information requirements and chemical safety assessment, Chapter R.16: Environmental Exposure Estimation.

ECHA. (2010b). Practical guide 2: How to report weight of evidence. Helsinki. Retrieved from http://echa.europa.eu/documents/10162/13655/pg_report_weight_of_evidence_en.pdf

ECHA. (2011). Guidance on information requirements and chemical safety assessment; Chapter R.5: Adaptation of information requirements (No. ECHA-2011-G-15-EN). Helsinki. Retrieved from http://echa.europa.eu/documents/10162/13643/information_requirements_r5_en.pdf

ECHA. (2012a). Guidance for identification and naming of substances under REACH and CLP.

ECHA. (2012b). Guidance on information requirements and chemical safety assessment - Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance (No. ECHA-12-G-03-EN). Helsinki. Retrieved from http://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf

ECHA. (2012c). Guidance on information requirements and chemical safety assessment - Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7b Endpoint specific guidance, (April).

ECHA. (2012d). Guidance on information requirements and chemical safety assessment - Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance, (April), 1–7.

ECHA. (2013). Assessing human health and environmental hazards of nanomaterials- Best practice for REACH Registrants. Retrieved from http://echa.europa.eu/documents/10162/5399565/best_practices_human_health_environment_nano_en.pdf

ECHA. (2014a). Guidance on information requirements and Chemical Safety Assessment Chapter R . 7a : Endpoint specific guidance. Helsinki. Retrieved from http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf

ECHA. (2014b). Human health and environmental exposure assessment and risk characterisation of nanomaterials Best practice for REACH registrants.

ECHA. (2015a). Guidance on information requirements and chemical safety assessment Chapter R . 5 : Adaptation of information requirements (Version 4.). Helsinki: ECHA. Retrieved from https://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf

ECHA. (2015b). Read-Across Assessment Framework (RAAF) (No. ECHA-15-R-07-EN). Helsinki. Retrieved from http://echa.europa.eu/documents/10162/13628/raaf_en.pdf

ECHA. (2016a). Appendix 4 : Recommendations for nanomaterials applicable to the Guidance on Registration Draft (Public) Version 1.0.

ECHA. (2016b). Guidance on information requirements and chemical safety assessment Chapter R.16: Environmental exposure assessment.

ECHA. (2016c). Guidance on information requirements and chemical safety assessment Chapter R . 17 : Estimation of exposure from articles. Guidance on IR and CSA. Helsinki. Retrieved from https://echa.europa.eu/documents/10162/13632/information_requirements_r16_en.pdf

ECHA. (2017a). Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance and Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance. Helsinki. Retrieved from https://echa.europa.eu/documents/10162/23047722/nano_ir_human_health_caracal_en.pdf/dcee7 ddc-fc2d-28bf-ebce-0fdf270cf878

ECHA. (2017b). Appendix R . 6-1 : Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping. Retrieved from https://echa.europa.eu/documents/10162/13564/appendix_r_6-1_nano_caracal_en.pdf/a07d989c-99ec-d87f-0f4d-b198022f4744

EFSA. (2011). Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (Scientific Opinion No. 9(5);2140). EFSA Journal 2011 (Vol. 9). Parma. Retrieved from http://www.efsa.europa.eu/en/efsajournal/doc/2140.pdf

EFSA. (2012). Guidance on the Use of Probabilistic Methodology for Modelling Dietary Exposure to Pesticide Residues. EFSA Journal, 10(10), 1–95. http://doi.org/10.2903/j.efsa.2012.2839.

EFSA Scientific Committee. (2009). The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety- Scientific Opinion of the Scientific Committee. The EFSA Journal, 958, 1–39.

EFSA Scientific Committee. (2011). Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal 2011, 9(5), 1–36. http://doi.org/10.2903/j.efsa.2011.2140.

Elimelech, M., Gregory, J., Jia, X., & Williams, R. A. (1995a). Colloidal hydrodynamics and transport. In Particle deposition and aggregation: measurement, modelling and simulation (Vol. 1, pp. 68–109). Butterworth-Heinemann Ltd.

Elimelech, M., Gregory, J., Jia, X., & Williams, R. A. (1995b). Surface interaction potentials. In Particle deposition and aggregation: measurement, modelling and simulation (pp. 33–68). Butterworth-Heinemann Ltd.

Elimelech, M., Gregory, J., Jia, X., & Williams, R. A. R. A. (1995c). Modelling of aggregation processes. In Particle deposition and aggregation: measurement, modelling and simulation (pp. 157–202). Woburn: Butterworth-Heinemann.

Environment , Health & Safety News. (2017), (35).

Epa, V. C., Burden, F. R., Tassa, C., Weissleder, R., Shaw, S., & Winkler, D. A. (2012). Modeling biological activities of nanoparticles. Nano Letters, 12(11), 5808–5812. http://doi.org/10.1021/nl303144k

Epa, V. C., Burden, F. R., Tassa, C., Weissleder, R., Shaw, S., & Winkler, D. A. (2012). Modeling biological activities of nanoparticles. Nano Letters, 12(11), 5808–12. http://doi.org/10.1021/nl303144k

Esch, R. K., Han, L., Foarde, K. K., & Ensor, D. S. (2010). Endotoxin contamination of engineered nanomaterials. Nanotoxicology, 4(1), 73–83. http://doi.org/10.3109/17435390903428851

EU. Corrigendum to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending, Pub. L. No. 1907/2006 (2007). EU: Official Journal of the European Union L 396 of 30 December 2006.

Eugenia Valsami-Jones, I. L. and C. A. C. (2016). Nanomaterial Ontologies for Nanosafety : A Rose by any Other Name …. Journal of Nanomedical Research, 3(5), 4–6. http://doi.org/10.15406/jnmr.2016.03.00070

European Commission. (2012). Commission Staff working paper: Types and uses of nanomaterials, including safety aspects accompanying the Commission Communication on the Second Regulatory Review on Nanomaterials (Vol. COM(2012)).

European Commission. (2013). Proposal for a Regulation of the European Parliament and of the Council on novel foods. COM(2013) 894 Final.

European Commission, EC, & IHCP JRC. (2011). REACH Implementation Project Substance Identification of Nanomaterials (RIP-oN 1). (I. JRC, Ed.). Retrieved from http://ec.europa.eu/environment/chemicals/nanotech/pdf/report_ripon1.pdf

European Industry. (2016). COMMENTS AND RESPONSE TO COMMENTS ON CLH ON TITANIUM DIOXIDE: PROPOSAL AND JUSTIFICATION Comments. Retrieved from https://echa.europa.eu/documents/10162/13626/clh_comments_titanium_dioxide_en.pdf

European Parliament and Council. Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. (E. Union, Ed.), L43Official Journal of the European Communities 1–6 (1997).

European Parliament and Council. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/4, L396/1 Official Journal of the European Union 849 (2006). EC, EC: Official Journal of the European Union. http://doi.org/http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:396:0001:0849:EN:PDF

European Parliament and Council. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/4, L396/1 Official Journal of the European Union 849 (2006). EC: Official Journal of the European Union. Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:396:0001:0849:EN:PDF

European Parliament and Council. (2009). Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union. Luxembourg: Publications Office of the European Union.

European Parliament and Council. (2012). Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union, L 167, 1–123.

European Parliament, & Council. (2009). Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union, L342, 59–209.

European Parliament, & Council. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, re, L 304 Official Journal of the European Union 46 (2011). Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1169&from=en

Fadeel, B., Fornara, A., Toprak, M. S., & Bhattacharya, K. (2015). Keeping it real: The importance of material characterization in nanotoxicology. Biochemical and Biophysical Research Communications, 468(3), 498–503. http://doi.org/http://dx.doi.org/10.1016/j.bbrc.2015.06.178

Falck, G., Lindberg, H., Suhonen, S., Vippola, M., Vanhala, E., Catalan, J., … Norppa, H. (2009). Genotoxic effects of nanosized and fine TiO2. Human & Experimental Toxicology, 28(6–7), 339–352. http://doi.org/10.1177/0960327109105163

Fenoglio, I., Aldieri, E., Gazzano, E., Cesano, F., Colonna, M., Scarano, D., … Fubini, B. (2012). Thickness of multiwalled carbon nanotubes affects their lung toxicity. Chemical Research in Toxicology, 25(1), 74–82. http://doi.org/10.1021/tx200255h

Fermin, D., & Riley, J. (2010). Charge in Colloidal Systems. In T. Cosgrove (Ed.), Colloid Science: Principles, Methods and Applications (2nd ed., pp. 23–44). Wiley. Retrieved from http://eu.wiley.com/WileyCDA/WileyTitle/productCd-1444320203.html

Fortino, V., & Grevo, D. (2017). Molecular signatures and the computational classifier capable to predict ENM safety, 11.

Foss Hansen, S., Larsen, B. H., Olsen, S. I., & Baun, A. (2007). Categorization framework to aid hazard identification of nanomaterials. Nanotoxicology, 1(3), 243–250. http://doi.org/10.1080/17435390701727509

Fourches, D., Pu, D., Tassa, C., Weissleder, R., Shaw, S. Y., Mumper, R. J., & Tropsha, A. (2010). Quantitative nanostructure-activity relationship modeling. ACS Nano, 4(10), 5703–12. http://doi.org/10.1021/nn1013484

Fourches, D., Pu, D., Tassa, C., Weissleder, R., Shaw, S. Y., Mumper, R. J., & Tropsha, A. (2010). Quantitative nanostructure - Activity relationship modeling. ACS Nano, 4(10), 5703–5712. http://doi.org/10.1021/nn1013484

Fraser, T. R., & Crum-Brown, A. (1868). ACTION OF THE SALTS OF THE AMMONIUM. J Anat Physiology, 2(2), 224–242.

Friedhofen, J. H., & Vögtle, F. (2006). Detailed nomenclature for dendritic molecules. New Journal of Chemistry, 30(1), 32–43. http://doi.org/10.1039/b513437b

Friedlander, S. K. (1958). Theory of Aerosol Filtration. Industrial & Engineering Chemistry, 50(8), 1161–1164. http://doi.org/10.1021/ie50584a036

Friedlander, S. K. (1977). Smoke, Dust, and Haze: Sheldon K. - Oxford University Press. (K. E. Gubbins, Ed.) (2nd Editio). New York, Oxford: Oxford University press. Retrieved from http://ukcatalogue.oup.com/product/9780195129991.do

Gaillard, C., Mech, A., & Rauscher, H. (2015). NanoDefine Development of an integrated approach based on Deliverable title : The NanoDefine Methods Manual Document Control Page.

Gajewicz, A., Cronin, M. T. ., Rasulev, B., Leszczynski, J., & Puzyn, T. (2015). Novel approach for efficient predictions properties of large pool of nanomaterials based on limited set of species: nano-read-across. Nanotechnology, 26(1), 15701. http://doi.org/10.1088/0957-4484/26/1/015701

Gajewicz, A., Rasulev, B., Dinadayalane, T. C., Urbaszek, P., Puzyn, T., Leszczynska, D., & Leszczynski, J. (2012). Advancing risk assessment of engineered nanomaterials: application of computational approaches. Advanced Drug Delivery Reviews, 64(15), 1663–93. http://doi.org/10.1016/j.addr.2012.05.014

Gajewicz, A., Schaeublin, N., Rasulev, B., Hussain, S., Leszczynska, D., & Puzyn, T. (n.d.). Towards Understanding Mechanisms Governing Cytotoxicity of Metal Oxides Nanoparticles : Hints from Nano-QSAR Studies Supplementary material.

Gallegos, A., Burello, E., & Worth, A. (2009). Review of computational approaches for predicting the physicochemical and biological properties of nanoparticles (JRC Scientific and Technical Reports). Ispra. Retrieved from http://publications.jrc.ec.europa.eu/repository/bitstream/111111111/5155/1/eur 23974 en.pdf

Gebel, T., Foth, H., Damm, G., Freyberger, A., Kramer, P.-J., Lilienblum, W., … Hengstler, J. G. (2014). Manufactured nanomaterials: categorization and approaches to hazard assessment. Archives of Toxicology, 88(12), 2191–211. http://doi.org/10.1007/s00204-014-1383-7

Geiser, M., Wigge, C., Conrad, M. L., Eigeldinger-Berthou, S., Künzi, L., Garn, H., … Mall, M. A. (2014). Nanoparticle uptake by airway phagocytes after fungal spore challenge in murine allergic asthma and chronic bronchitis. BMC Pulmonary Medicine, 14, 116. http://doi.org/10.1186/1471-2466-14-116

Gentleman, D. J., & Chan, W. C. W. (2009). A Systematic Nomenclature for Codifying Engineered Nanostructures. Small, 5(4), 426–431. http://doi.org/10.1002/smll.200800490

George, S., Xia, T., Rallo, R., Zhao, Y., Ji, Z., Lin, S., … Nel, A. E. (2011). Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. ACS Nano, 5(3), 1805–1817. http://doi.org/10.1021/nn102734s

Gerloff, K., Fenoglio, I., Carella, E., Kolling, J., Albrecht, C., Boots, A. W., … Schins, R. P. F. (2012a). Distinctive Toxicity of TiO2 Rutile/Anatase Mixed Phase Nanoparticles on Caco-2 Cells. Chemical Research in Toxicology, 25, 646–655.

Gerloff, K., Fenoglio, I., Carella, E., Kolling, J., Albrecht, C., Boots, A. W., … Schins, R. P. F. (2012b). Distinctive Toxicity of TiO2 Rutile/Anatase Mixed Phase Nanoparticles on Caco-2 Cells.

Gerloff, K., Landesmann, B., Worth, A., Munn, S., Palosaari, T., & Whelan, M. (2017). The Adverse Outcome Pathway approach in nanotoxicology. Computational Toxicology, 1, 3–11. http://doi.org/10.1016/j.comtox.2016.07.001

Gernand, J. M., & Casman, E. A. (2014a). A meta-analysis of carbon nanotube pulmonary toxicity studies-how physical dimensions and impurities affect the toxicity of carbon nanotubes. Risk Anal, 34(3), 583–597. http://doi.org/10.1111/risa.12109

Gernand, J. M., & Casman, E. A. (2014b). Machine learning for nanomaterial toxicity risk assessment. IEEE Intelligent Systems, 29(3), 84–88. http://doi.org/10.1109/MIS.2014.48

Ghaedi, M., Ghaedi, A. M., Hossainpour, M., Ansari, A., Habibi, M. H., & Asghari, A. R. (2014a). Least square-support vector (LS-SVM) method for modeling of methylene blue dye adsorption using copper oxide loaded on activated carbon: Kinetic and isotherm study. Journal of Industrial and Engineering Chemistry, 20(4), 1641–1649. http://doi.org/10.1016/j.jiec.2013.08.011

Ghaedi, M., Ghaedi, A. M., Hossainpour, M., Ansari, A., Habibi, M. H., & Asghari, A. R. (2014b). Least square-support vector (LS-SVM) method for modeling of methylene blue dye adsorption using

copper oxide loaded on activated carbon: Kinetic and isotherm study. Journal of Industrial and Engineering Chemistry, 20(4), 1641–1649. http://doi.org/10.1016/j.jiec.2013.08.011

Gharagheizi, F., & Alamdari, R. F. (2008). A molecular-based model for prediction of solubility of C60 fullerene in various solvents. Fullerenes Nanotubes and Carbon Nanostructures, 16(1), 40–57. http://doi.org/10.1080/15363830701779315

Ghorbanzadeh, M., Fatemi, M. H., & Karimpour, M. (2012). Modeling the Cellular Uptake of Magneto fl uorescent Nanoparticles in Pancreatic Cancer Cells: A Quantitative Structure Activity Relationship Study. Industrial & Engineering Chemistry Research, 51(32), 10712–10718. http://doi.org/10.1021/ie3006947

Ghorbanzadeh, M., Fatemi, M. H., & Karimpour, M. (2012). Modeling the cellular uptake of magnetofluorescent nanoparticles in pancreatic cancer cells: A quantitative structure activity relationship study. Industrial and Engineering Chemistry Research, 51(32), 10712–10718. http://doi.org/10.1021/ie3006947

Gini, C. (1912). Variabilità e mutabilità. Memorie di metodologica statistica. (E. Pizetti & T. Salvemini, Eds.) (1955th ed.). Rome: Libreria Eredi Virgilio Veschi.

Godly, E. W., & Taylor, R. (1997). Nomenclature and terminology of fullerenes: A preliminary study. Pure and Applied Chemistry, 69(7), 1411–1434. http://doi.org/10.1351/pac199769071411

Godwin, H., Nameth, C., Avery, D., Bergeson, L. L., Bernard, D., Beryt, E., … Nel, A. E. (2015). Nanomaterial Categorization for Assessing Risk Potential To Facilitate Regulatory Decision-Making. ACS Nano. http://doi.org/10.1021/acsnano.5b00941

Golbamaki, N., Rasulev, B., Cassano, A., Marchese Robinson, R. L., Benfenati, E., Leszczynski, J., & Cronin, M. T. D. (2015). Genotoxicity of metal oxide nanomaterials: review of recent data and discussion of possible mechanisms. Nanoscale, 7(6), 2154–2198. http://doi.org/10.1039/C4NR06670G

Goldberg, E., Scheringer, M., Bucheli, T. D., & Hungerbühler, K. (2014). Critical assessment of models for transport of engineered nanoparticles in saturated porous media. Environmental Science and Technology, 48(21), 12732–12741. http://doi.org/10.1021/es502044k

Goldberg, E., Scheringer, M., Bucheli, T. D., & Hungerbühler, K. (2015). Prediction of nanoparticle transport behavior from physicochemical properties: Machine learning provides insights to guide the next generation of transport models. Environmental Science: Nano, 2(4), 352–360. http://doi.org/10.1039/c5en00050e

Goodson, A. L., Gladys, C. L., & Worst, D. E. (1995). Numbering and Naming of Fullerenes by Chemical Abstracts Service. Journal of Chemical Information and Modeling, 35(6), 969–978. http://doi.org/10.1021/ci00028a004

Gottschalk, F., Lassen, C., Kjoelholt, J., Christensen, F., & Nowack, B. (2015). Modeling flows and concentrations of nine engineered nanomaterials in the Danish environment. International Journal of Environmental Research and Public Health, 12(5), 5581–5602. http://doi.org/10.3390/ijerph120505581

Gottschalk, F., Ort, C., Scholz, R. W., & Nowack, B. (2011). Engineered nanomaterials in rivers e Exposure scenarios for Switzerland at high spatial and temporal resolution. Environmental Pollution, 159(12), 3439–3445. http://doi.org/10.1016/j.envpol.2011.08.023

Gottschalk, F., Scholz, R. W., & Nowack, B. (2010). Probabilistic material flow modeling for assessing the environmental exposure to compounds: Methodology and an application to engineered nano-TiO2 particles. Environmental Modelling & Software, 25(3), 320–332. http://doi.org/10.1016/j.envsoft.2009.08.011

Gottschalk, F., Sonderer, T., Scholz, R. W., & Nowack, B. (2010). Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. Environmental Toxicology and Chemistry / SETAC, 29(5), 1036–1048. http://doi.org/10.1002/etc.135

Greim, H., Borm, P., Schins, R., Donaldson, K., Driscoll, K., Hartwig, A., … Speit, G. (2001). TOXICITY OF FIBERS AND PARTICLES. Inhalation Toxicology, 13(9). Retrieved from http://www.tandfonline.com/doi/abs/10.1080/08958370118273#aHR0cDovL3d3dy50YW5kZm9ubGl uZS5jb20vZG9pL3BkZi8xMC4xMDgwLzA4OTU4MzcwMTE4MjczP25lZWRBY2Nlc3M9dHJ1ZUBAQDA=

Grose, E. C., Richards, J. H., Jaskot, R. H., Ménache, M. G., Graham, J. A., & Dauterman, W. C. (1987). A comparative study of the effects of inhaled cadmium chloride and cadmium oxide: pulmonary response. Journal of Toxicology and Environmental Health, 21(1–2), 219–32. http://doi.org/10.1080/15287398709531014

Grosse, Y., Loomis, D., Guyton, K. Z., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., … Straif, K. (2014). Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. The Lancet. Oncology, 15(13), 1427–8. http://doi.org/10.1016/S1470-2045(14)71109-X

Gu, Z., Yang, Z., Chong, Y., Ge, C., Weber, J. K., Bell, D. R., & Zhou, R. (2015). Surface Curvature Relation to Protein Adsorption for Carbon-based Nanomaterials. Scientific Reports, 5, 10886. http://doi.org/10.1038/srep10886

Guichard, Y., Schmit, J., Darne, C., Gaté, L., Goutet, M., Rousset, D., … Binet, S. (2012). Cytotoxicity and genotoxicity of nanosized and microsized titanium dioxide and iron oxide particles in Syrian hamster embryo cells. The Annals of Occupational Hygiene, 56(5), 631–44. http://doi.org/10.1093/annhyg/mes006

Guiot, C., Spalla, O., Jensen, K. A., Kembouche, Y., Witschger, O., & Rousset, D. (2012). Nanogenotox D4.5: Surface charge, hydrodynamic size and size distributions by zetametry, dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) in optimized aqueous suspensions for titanium and silicon dioxide. Copenhagen. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

Hagenbuch, B., & Meier, P. J. (2004). Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. Pflügers Archiv : European Journal of Physiology, 447(5), 653–65. http://doi.org/10.1007/s00424-003-1168-y

Hagens, W. I., Oomen, A. G., de Jong, W. H., Cassee, F. R., & Sips, A. J. A. M. (2007). What do we (need to) know about the kinetic properties of nanoparticles in the body? Regulatory Toxicology and Pharmacology, 49(3), 217–29. http://doi.org/10.1016/j.yrtph.2007.07.006

Hall, L., & Kier, L. (1977). No Title. Journal of Pharmaceutical Sciences, 66, 642.

Hamaker, H. C. (1937). The London—van der Waals attraction between spherical particles. Physica, 4(10), 1058–1072. http://doi.org/10.1016/S0031-8914(37)80203-7

Hamilton, R. F., Wu, Z., Mitra, S., Shaw, P. K., & Holian, A. (2013). Effect of MWCNT size, carboxylation, and purification on in vitro and in vivo toxicity, inflammation and lung pathology. Particle and Fibre Toxicology, 10(1), 57. http://doi.org/10.1186/1743-8977-10-57

Hammett, L. P. (1937). The Effect of Structure upon the Reactions of Organic Compounds. Benzene Derivatives. Journal of the American Chemical Society, 59(1), 96–103. http://doi.org/10.1021/ja01280a022

Hankin, S. M., Peters, S. A. ., Poland, C. A., Foss Hansen, S., J., H., Ross, B. L., … Aitken, R. J. (2011). Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2)

Final Project Report (No. RNC/RIP-oN2/FPR/1/FINAL). Retrieved from http://ec.europa.eu/environment/chemicals/nanotech/pdf/report_ripon2.pdf

Hansch, C. (1969). Quantitative approach to biochemical structure-activity relationships. Accounts of Chemical Research, 2(8), 232–239. http://doi.org/10.1021/ar50020a002

Hansch, C., & Fujita, T. (1964). Additions and Corrections - ρ-σ-π Analysis. A Method for the Correlation of Biological Activity and Chemical Structure. Journal of the American Chemical Society, 86(24), 1616–1626. http://doi.org/10.1021/ja01062a035

Hansen, S. F., Hjorth, R., Skjolding, L. M., Bowman, D. M., Maynard, A., Baun, A., … Nickel, C. (2017). A critical analysis of the environmental dossiers from the OECD sponsorship programme for the testing of manufactured nanomaterials. Environ. Sci.: Nano, 74, 147–160. http://doi.org/10.1039/C6EN00465B

Harper, B., Thomas, D., Chikkagoudar, S., Baker, N., Tang, K., Heredia-Langner, A., … Harper, S. (2015). Comparative hazard analysis and toxicological modeling of diverse nanomaterials using the embryonic zebrafish (EZ) metric of toxicity. Journal of Nanoparticle Research, 17(6), 1–12. http://doi.org/10.1007/s11051-015-3051-0

Hastings, J., Jeliazkova, N., Owen, G., Tsiliki, G., Munteanu, C. R., Steinbeck, C., & Willighagen, E. (2015). eNanoMapper: harnessing ontologies to enable data integration for nanomaterial risk assessment. Journal of Biomedical Semantics, 6, 10. http://doi.org/10.1186/s13326-015-0005-5

Healy, T. W., Homola, A., James, R. O., & Hunter, R. J. (1978). Coagulation of amphoteric latex colloids: reversibility and specific ion effects. Faraday Discussions of the Chemical Society, 65, 156. http://doi.org/10.1039/dc9786500156

Hendren, C. O., Badireddy, A. R., Casman, E., & Wiesner, M. R. (2013). Modeling nanomaterial fate in wastewater treatment: Monte Carlo simulation of silver nanoparticles (nano-Ag). The Science of the Total Environment, 449, 418–425. http://doi.org/10.1016/j.scitotenv.2013.01.078

Hendren, C. O., Powers, C. M., Hoover, M. D., & Harper, S. L. (2015). The Nanomaterial Data Curation Initiative: A collaborative approach to assessing, evaluating, and advancing the state of the field. Beilstein Journal of Nanotechnology, 6(1), 1752–1762. http://doi.org/10.3762/bjnano.6.179

Heringa, M. B., Geraets, L., van Eijkeren, J. C. H., Vandebriel, R. J., de Jong, W. H., & Oomen, A. G. (2016). Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations. Nanotoxicology, 1–11. http://doi.org/10.1080/17435390.2016.1238113

Hillery, A. M., Jan, P. U., & Florence, A. T. (1994). Comparative , Quantitative Study of Lymphoid and Non-Lymphoid Uptake of 60 nm Polystyrene Particles. Journal of Drug Testing, 2(1992), 151–156.

Hirsch, C., Roesslein, M., Krug, H. F., & Wick, P. (2011). Nanomaterial cell interactions: are current in vitro tests reliable? Nanomedicine (London, England), 6(5), 837–47. http://doi.org/10.2217/nnm.11.88

Ho, M., Wu, K.-Y., Chein, H.-M., Chen, L.-C., & Cheng, T.-J. (2011). Pulmonary toxicity of inhaled nanoscale and fine zinc oxide particles: mass and surface area as an exposure metric. Inhalation Toxicology, 23(14), 947–56. http://doi.org/10.3109/08958378.2011.629235

Höck, J., Epprecht, T., Hofmann, H., K., H., H., K., C., L., … P, W. (2013). Guidelines on the Precautionary Matrix for Synthetic Nanomaterials. Version 3. Retrieved from http://www.bag.admin.ch/themen/chemikalien/00228/00510/05626/index.html?lang=en

Hopfinger, A. J., Wang, S., Tokarski, J. S., Jin, B., Albuquerque, M., Madhav, P. J., & Duraiswami, C. (1997). Construction of 3D-QSAR Models Using the 4D-QSAR Analysis Formalism. Journal of the American Chemical Society, 119(43), 10509–10524. http://doi.org/10.1021/ja9718937

Horev-Azaria, L., Kirkpatrick, C. J., Korenstein, R., Marche, P. N., Maimon, O., Ponti, J., … Villiers, C. (2011). Predictive toxicology of cobalt nanoparticles and ions: Comparative in vitro study of different cellular models using methods of knowledge discovery from data. Toxicological Sciences, 122(2), 489–501. http://doi.org/10.1093/toxsci/kfr124

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., … Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nature Immunology, 9(8), 847–56. http://doi.org/10.1038/ni.1631

Hristozov, D. R., Zabeo, A., Foran, C., Isigonis, P., Critto, A., Marcomini, A., & Linkov, I. (2014). A weight of evidence approach for hazard screening of engineered nanomaterials. Nanotoxicology, 8(1), 72–87. http://doi.org/10.3109/17435390.2012.750695

Ilinskaya, A., & Dobrovolskaia, M. (2013a). Nanoparticles and the blood coagulation system. Benefits of nanotechnology. Nanomedicine, 8(5), 773–84. http://doi.org/10.2217/nnm.13.48

Ilinskaya, A., & Dobrovolskaia, M. (2013b). Nanoparticles and the blood coagulation system. Part II: safety concerns. Nanomedicine (London, England), 8(6), 969–81. http://doi.org/10.2217/nnm.13.49

ISO. (1996). Particle size analysis -- Photon correlation spectroscopy (ISO/TC 24/SC 4 No. 13321:1996). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=21707

ISO. (2002). Surface chemical analysis -- High-resolution Auger electron spectrometers -- Calibration of energy scales for elemental and chemical-state analysis (ISO/TC 201/SC 7 No. 17974:2002). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=31659

ISO. (2003). ISO 18757:2003 - Fine ceramics (advanced ceramics, advanced technical ceramics) -- Determination of specific surface area of ceramic powders by gas adsorption using the BET method (Advanced ceramics No. ISO 18757:2003). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=31903

ISO. (2004). Microbeam analysis -- Scanning electron microscopy -- Guidelines for calibrating image magnification (ISO/TC 202/SC 4 No. 16700:2004). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=30420

ISO. (2005). Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 1: Mercury porosimetry (Particle size analysis. Sieving No. ISO 15901-1:2005). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=39385

ISO. (2006a). ISO 15901-2:2006 - Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 2: Analysis of mesopores and macropores by gas adsorption (Particle size analysis. Sieving No. ISO 15901-2:2006). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=39386&commid=47176

ISO. (2006b). Surface chemical analysis -- Auger electron spectroscopy -- Derivation of chemical information (ISO/TC 201/SC 7 No. 18394:2006). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=38675

ISO. (2007a). Determination of particle size distribution -- Single particle light interaction methods -- Part 2: Light scattering liquid-borne particle counter (ISO/TC 24/SC 4 No. 21501–2:2007). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=40275

ISO. (2007b). Determination of particle size distribution -- Single particle light interaction methods -- Part 3: Light extinction liquid-borne particle counter (ISO/TC 24/SC 4 No. 21501–3:2007). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=40276

ISO. (2007c). Determination of particle size distribution -- Single particle light interaction methods -- Part 4: Light scattering airborne particle counter for clean spaces (ISO/TC 24/SC 4 No. 21501–4:2007). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=40274

ISO. (2007d). ISO 15901-3:2007 - Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 3: Analysis of micropores by gas adsorption (Particle size analysis. Sieving No. ISO 15901-3:2007). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=40364

ISO. (2009a). Determination of particle size distribution -- Differential electrical mobility analysis for aerosol particles (ISO/TC 24/SC 4 No. 15900). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=39573

ISO. (2009b). Particle size analysis -- Laser diffraction methods (ISO/TC 24/SC 4 No. 13320–1:2009). Retrieved from http://www.iso.org/iso/home/store/catalogue_ics/catalogue_detail_ics.htm?csnumber=44929

ISO. (2010a). ISO/TS 10867:2010 - Nanotechnologies -- Characterization of single-wall carbon nanotubes using near infrared photoluminescence spectroscopy (Physics. Chemistry No. ISO/TS 10867:2010). Retrieved from http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46245

ISO. (2010b). ISO 9277:2010 - Determination of the specific surface area of solids by gas adsorption -- BET method (Particle size analysis. Sieving No. ISO 9277:2010). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=44941

ISO. (2010c). Surface chemical analysis -- X-ray photoelectron spectrometers -- Calibration of energy scales (ISO/TC 201/SC 7 No. 15472:2010). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=55796

ISO. (2011a). ISO/TS 10798:2011 - Nanotechnologies -- Charaterization of single-wall carbon nanotubes using scanning electron microscopy and energy dispersive X-ray spectrometry analysis (Physics. Chemistry No. ISO/TS 10798:2011). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=46128

ISO. (2011b). ISO/TS 10868:2011 - Nanotechnologies -- Characterization of single-wall carbon nanotubes using ultraviolet-visible-near infrared (UV-Vis-NIR) absorption spectroscopy (Physics. Chemistry No. ISO/TS 10868:2011). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=46247

ISO. (2011c). ISO 22309:2011 - Microbeam analysis -- Quantitative analysis using energy-dispersive spectrometry (EDS) for elements with an atomic number of 11 (Na) or above (Analytical Chemistry No. ISO 22309:2011). Retrieved from http://www.iso.org/iso/home/store/catalogue_ics/catalogue_detail_ics.htm?csnumber=57850

ISO. (2011d). Nanotechnologies -- Characterization of single-wall carbon nanotubes using thermogravimetric analysis (ISO/TC 229 No. ISO/TS 11308:2011). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=50357

ISO. (2011e). Nanotechnologies — Nanomaterial risk evaluation (No. ISO/TR 13121:2011(E)). Building (Vol. Technical).

ISO. (2012a). ISO/TR 10929:2012 - Nanotechnologies -- Characterization of multiwall carbon nanotube (MWCNT) samples (Physics. Chemistry No. ISO/TR 10929:2012). Retrieved from http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46424

ISO. (2012b). ISO/TS 10797:2012 - Nanotechnologies -- Characterization of single-wall carbon nanotubes using transmission electron microscopy (Physics. Chemistry No. ISO/TS 10797:2012).

Retrieved from http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46127

ISO. (2012c). ISO 13099-2:2012 - Colloidal systems -- Methods for zeta-potential determination -- Part 2: Optical methods (Particle size analysis. Sieving. No. ISO 13099-2:2012). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=52832

ISO. (2012d). ISO 9276-6:2008 - Representation of results of particle size analysis -- Part 6: Descriptive and quantitative representation of particle shape and morphology. Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=39389

ISO. (2013a). ISO/TR 13097:2013 - Guidelines for the characterization of dispersion stability (No. ISO/TR 13097:2013). Retrieved from http://www.iso.org/iso/catalogue_detail.htm?csnumber=52802

ISO. (2013b). ISO 13099-3:2014 - Colloidal systems -- Methods for zeta potential determination -- Part 3: Acoustic methods (Particle size analysis. Sieving No. ISO 13099-3:2014). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=52833&commid =47176

ISO. (2102). ISO 13099-1:2012 - Colloidal systems -- Methods for zeta-potential determination -- Part 1: Electroacoustic and electrokinetic phenomena (Particle size analysis. Sieving No. ISO 13099-1:2012). Retrieved from http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=52807&commid =47176

Jackson, P., Kling, K., Jensen, K. A., Clausen, P. A., Madsen, A. M., Wallin, H., & Vogel, U. (2015). Characterization of genotoxic response to 15 multiwalled carbon nanotubes with variable physicochemical properties including surface functionalizations in the FE1-Muta(TM) mouse lung epithelial cell line. Environmental and Molecular Mutagenesis, 56(2), 183–203. http://doi.org/10.1002/em.21922

Jacobsen, N. R., Wallin, H., De Jong, W., Oomen, A., Brandon, E., Krystek, P., … Czarny, B. (2013). Towards a method for detecting the potential genotoxicity of nanomaterials; Deliverable 7: Identification of target organs and biodistribution including ADME parameters. Retrieved from http://www.nanogenotox.eu/files/PDF/DELIVRABLES2/deliverable7__biodistribution.pdf

Jain, S., Thakare, V. S., Das, M., Godugu, C., Jain, A. K., Mathur, R., … Mishra, A. K. (2011). Toxicity of multiwalled carbon nanotubes with end defects critically depends on their functionalization density. Chemical Research in Toxicology, 24(11), 2028–2039. http://doi.org/10.1021/tx2003728

Jeliazkova, N., Chomenidis, C., Doganis, P., Fadeel, B., Grafström, R., Hardy, B., … Willighagen, E. (2015). The eNanoMapper database for nanomaterial safety information. Beilstein Journal of Nanotechnology, 6(1), 1609–1634. http://doi.org/10.3762/bjnano.6.165

Jensen, K. A., Kembouche, Y., & Nielsen, S. H. (2013). Nanogenotox deliverable 4.7: Hydrochemical reactivity, solubility, and biodurability of NANOGENOTOX nanomaterials. Copenhagen. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

Jensen, K. a, Saber, a T., Kristensen, H. V, Liguori, B., Jensen, a C. Ø., Koponen, I. K., & Wallin, H. (2014). NanoSafer vs . 1 . 1 Nanomaterial risk assessment using first order modeling. Retrieved from http://orbit.dtu.dk/en/publications/nanosafer-vs-11-nanomaterial-risk-assessment-using-first-order-modeling%2837472e3e-d4d0-4c8d-b7d7-5cadabc781c0%29.html

Jiang, J., Oberdörster, G., & Biswas, P. (2008). Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. Journal of Nanoparticle Research, 11(1), 77–89. http://doi.org/10.1007/s11051-008-9446-4

JIS. (2005). Method for particle size determination in metal catalysts by electron microscope (No. JIS H 7804:2005). Retrieved from http://infostore.saiglobal.com/store/details.aspx?ProductID=829829

JIS. (2007). Measurement of lattice parameters and crystallite sizes of carbon materials (No. 7651:2007).

Johnson, A. C., & Park, B. (2012). Predicting contamination by the fuel additive cerium oxide engineered nanoparticles within the United Kingdom and the associated risks. Environmental Toxicology and Chemistry, 31(11), 2582–2587. http://doi.org/10.1002/etc.1983

Johnson, P. R., & Elimelech, M. (1995). Dynamics of Colloid Deposition in Porous Media: Blocking Based on Random Sequential Adsorption. Langmuir, 11(3), 801–812. http://doi.org/10.1021/la00003a023

Johnston, H. J., Hutchison, G. R., Christensen, F. M., Peters, S., Hankin, S., Aschberger, K., & Stone, V. (2010). A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. Nanotoxicology, 4(June), 207–246. http://doi.org/10.3109/17435390903569639

Joris, F., Manshian, B. B., Peynshaert, K., De Smedt, S. C., Braeckmans, K., & Soenen, S. J. (2013). Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap. Chemical Society Reviews, 42(21), 8339–59. http://doi.org/10.1039/c3cs60145e

Jugan, M.-L., Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Sauvaigo, S., Douki, T., & Carriere, M. (2012). Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. Nanotoxicology, 6(5), 501–513. http://doi.org/10.3109/17435390.2011.587903

Kaempfer, A., La Spina, R., Gilliland, D., Valzacchi, S., Asturiol, D., Stone, V., & Kinsner-Ovaskainen, A. (2017). Silver nanoparticles and metallic silver interfere with the Griess reaction: reduction of the azo-dye formation via a competing Sandmeyer-like reaction. Chemical Research in Toxicology, acs.chemrestox.6b00280. http://doi.org/10.1021/acs.chemrestox.6b00280

Kagan, V. E., Tyurina, Y. Y., Tyurin, V. A., Konduru, N. V., Potapovich, A. I., Osipov, A. N., … Shvedova, A. A. (2006). Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: Role of iron. Toxicology Letters, 165(1), 88–100. http://doi.org/10.1016/j.toxlet.2006.02.001

Kakwere, H., Leal, M. P., Materia, M. E., Curcio, A., Guardia, P., Niculaes, D., … Pellegrino, T. (2015). Functionalization of strongly interacting magnetic nanocubes with (thermo)responsive coating and their application in hyperthermia and heat-triggered drug delivery. ACS Applied Materials & Interfaces, 7(19), 10132–45. http://doi.org/10.1021/am5088117

Kamath, P., Fernández, A., Giralt, F., & Rallo, R. (2015). Predicting cell association of surface-modified nanoparticles using protein corona structure - activity relationships (PCSAR). Current Topics in Medicinal Chemistry, 15(18), 175–182. http://doi.org/10.2174/1568026615666150506152808

Kansara, K., Patel, P., Shah, D., Shukla, R. K., Singh, S., Kumar, A., & Dhawan, A. (2015). TiO 2 nanoparticles induce DNA double strand breaks and cell cycle arrest in human alveolar cells. Environmental and Molecular Mutagenesis, 56(2), 204–217. http://doi.org/10.1002/em.21925

Kar, S., Gajewicz, A., Roy, K., Leszczynski, J., & Puzyn, T. (2016). Extrapolating between toxicity endpoints of metal oxide nanoparticles: Predicting toxicity to Escherichia coli and human keratinocyte cell line (HaCaT) with Nano-QTTR. Ecotoxicology and Environmental Safety, 126, 238–244. http://doi.org/10.1016/j.ecoenv.2015.12.033

Karlsson, H. L., Di Bucchianico, S., Collins, A. R., & Dusinska, M. (2015). Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environmental and Molecular Mutagenesis, 56(2), 82–96. http://doi.org/10.1002/em.21933

Keller, A. A., & Lazareva, A. (2014). Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. Environmental Science and Technology Letters, 1(1), 65–70. http://doi.org/10.1021/ez400106t

Keller, A. A., Mcferran, S., Lazareva, A., & Suh, S. (2013). Global life cycle releases of engineered nanomaterials. Journal of Nanoparticle Research, 15, 1692–1703. http://doi.org/10.1007/s11051-013-1692-4

Kermanizadeh, A. (2013). Engineered Nanomaterial Impact in the Liver following Exposure via an Intravenous Route–The Role of Polymorphonuclear Leukocytes and Gene Expression in the Organ. Journal of Nanomedicine & Nanotechnology, 4(157), 1–7. http://doi.org/10.4172/2157-7439.1000157

Kermanizadeh, A., Pojana, G., Gaiser, B. K., Birkedal, R., Bilanicova, D., Wallin, H., … Stone, V. (2012). In vitro assessment of engineered nanomaterials using C3A cells: Cytotoxicity, pro-inflammatory cytokines and function markers. Nanotoxicology, 5390(February 2016). http://doi.org/10.3109/17435390.2011.653416

Kermanizadeh, A., Vranic, S., Boland, S., Moreau, K., Baeza-Squiban, A., Gaiser, B. K., … Stone, V. (2013). An in vitro assessment of panel of engineered nanomaterials using a human renal cell line: cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity. BMC Nephrology, 14(1), 96. http://doi.org/10.1186/1471-2369-14-96

Kettiger, H., Schipanski, A., Wick, P., & Huwyler, J. (2013). Engineered nanomaterial uptake and tissue distribution: From cell to organism. International Journal of Nanomedicine. http://doi.org/10.2147/IJN.S49770

Kim, J. S., Lee, K., Lee, Y. H., Cho, H. S., Kim, K. H., Choi, K. H., … Yu, I. J. (2011). Aspect ratio has no effect on genotoxicity of multi-wall carbon nanotubes. Archives of Toxicology, 85(7), 775–786. http://doi.org/10.1007/s00204-010-0574-0

Klaine, S. J., Alvarez, P. J. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., … Lead, J. R. (2008). NANOMATERIALS IN THE ENVIRONMENT: BEHAVIOR, FATE, BIOAVAILABILITY, AND EFFECTS. Environmental Toxicology and Chemistry, 27(9), 1825. http://doi.org/10.1897/08-090.1

Kleandrova, V. V, Luan, F., González-Díaz, H., Ruso, J. M., Melo, A., Speck-Planche, A., & Cordeiro, M. N. D. S. (2014). Computational ecotoxicology: Simultaneous prediction of ecotoxic effects of nanoparticles under different experimental conditions. Environment International, 73, 288–294. http://doi.org/10.1016/j.envint.2014.08.009

Kleandrova, V. V, Luan, F., González-Díaz, H., Ruso, J. M., Melo, A., Speck-Planche, A., & Cordeiro, M. N. D. S. (2014). Computational ecotoxicology: simultaneous prediction of ecotoxic effects of nanoparticles under different experimental conditions. Environment International, 73, 288–94. http://doi.org/10.1016/j.envint.2014.08.009

Kleandrova, V. V, Luan, F., González-Díaz, H., Ruso, J. M., Speck-Planche, A., & Cordeiro, M. N. D. S. (2014). Computational tool for risk assessment of nanomaterials: Novel QSTR-perturbation model for simultaneous prediction of ecotoxicity and cytotoxicity of uncoated and coated nanoparticles under multiple experimental conditions. Environmental Science and Technology, 48(24), 14686–14694. http://doi.org/10.1021/es503861x

Kleandrova, V. V, Luan, F., González-Díaz, H., Ruso, J. M., Speck-Planche, A., & Cordeiro, M. N. D. S. (2014). Computational tool for risk assessment of nanomaterials: novel QSTR-perturbation model for simultaneous prediction of ecotoxicity and cytotoxicity of uncoated and coated nanoparticles under multiple experimental conditions. Environmental Science & Technology, 48(24), 14686–94. http://doi.org/10.1021/es503861x

Klebe, G., Abraham, U., & Mietzner, T. (1994). Molecular Similarity Indices in a Comparative Analysis (CoMSIA) of Drug Molecules to Correlate and Predict Their Biological Activity. Journal of Medicinal Chemistry, 37(24), 4130–4146. http://doi.org/10.1021/jm00050a010

Klimisch, H. J., Andreae, M., & Tillmann, U. (1997). A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regulatory Toxicology and Pharmacology, 25(1), 1–5. http://doi.org/10.1006/rtph.1996.1076

Knudsen, K. B., Northeved, H., Ek, P. K., Permin, A., Andresen, T. L., Larsen, S., … Lykkesfeldt, J. (2014). Differential toxicological response to positively and negatively charged nanoparticles in the rat brain. Nanotoxicology, 8(7), 764–74. http://doi.org/10.3109/17435390.2013.829589

Kreyling, W. G. (1992). Intracellular particle dissolution in alveolar macrophages. Environmental Health Perspectives, 97, 121–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1519559&tool=pmcentrez&rendertype =abstract

Kreyling, W. G., Semmler-Behnke, M., & Chaudhry, Q. (2010). A complementary definition of nanomaterial. Nano Today, 5(3), 165–168. http://doi.org/10.1016/j.nantod.2010.03.004

Kroll, A., Pillukat, M. H., Hahn, D., & Schnekenburger, J. (2009). Current in vitro methods in nanoparticle risk assessment: Limitations and challenges. European Journal of Pharmaceutics and Biopharmaceutics, 72(2), 370–377. http://doi.org/10.1016/j.ejpb.2008.08.009

Kroll, A., Pillukat, M. H., Hahn, D., & Schnekenburger, J. (2012). Interference of engineered nanoparticles with in vitro toxicity assays. Archives of Toxicology, 86(7), 1123–36. http://doi.org/10.1007/s00204-012-0837-z

Kuempel, E. D., Castranova, V., Geraci, C. L., & Schulte, P. a. (2012). Development of risk-based nanomaterial groups for occupational exposure control. Journal of Nanoparticle Research, 14(9), 1029. http://doi.org/10.1007/s11051-012-1029-8

Kumar, A., & Dhawan, A. (2013). Genotoxic and carcinogenic potential of engineered nanoparticles: an update. Archives of Toxicology, 87(11), 1883–1900. http://doi.org/10.1007/s00204-013-1128-z

L Browning, C., The, T., & Sr., M. D. M. and J. P. W. (2014). Titanium Dioxide Nanoparticles are not Cytotoxic or Clastogenic in Human Skin Cells. Journal of Environmental & Analytical Toxicology, 4(6). http://doi.org/10.4172/2161-0525.1000239

Lai, D. Y. (2012). Toward toxicity testing of nanomaterials in the 21st century: a paradigm for moving forward. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology, 4(1), 1–15. http://doi.org/10.1002/wnan.162

Landsiedel, R., Fabian, E., Ma-Hock, L., van Ravenzwaay, B., Wohlleben, W., Wiench, K., & Oesch, F. (2012). Toxico-/biokinetics of nanomaterials. Archives of Toxicology, 86(7), 1021–60. http://doi.org/10.1007/s00204-012-0858-7

Landsiedel, R., Ma-Hock, L., Kroll, A., Hahn, D., Schnekenburger, J., Wiench, K., & Wohlleben, W. (2010). Testing metal-oxide nanomaterials for human safety. Advanced Materials (Deerfield Beach, Fla.), 22(24), 2601–27. http://doi.org/10.1002/adma.200902658

Larsen, P. B., & Tyle, H. (2008). Nickel and Nickel Compounds: Report for the Danish Environmental Protection Agency. Regulation. Retrieved from http://eng.mst.dk/media/mst/67158/NickelBackground_EU_RAR_HH_March_2008_final_draft.pdf

Le, T. C., Mulet, X., Burden, F. R., & Winkler, D. A. (2013). Predicting the complex phase behavior of self-assembling drug delivery nanoparticles. Molecular Pharmaceutics, 10(4), 1368–1377. http://doi.org/10.1021/mp3006402

Le, T. C., Yin, H., Chen, R., Chen, Y., Zhao, L., Casey, P. S., … Winkler, D. A. (2016). An Experimental and Computational Approach to the Development of ZnO Nanoparticles that are Safe by Design. Small. CSIRO Manufacturing Bayview Avenue Clayton 3168 Australia. http://doi.org/10.1002/smll.201600597

Le, T., Epa, V. C., Burden, F. R., & Winkler, D. A. (2012). Quantitative structure-property relationship modeling of diverse materials properties. Chemical Reviews, 112(5), 2889–919. http://doi.org/10.1021/cr200066h

Lenz, W. (1988). A short history of thalidomide embryopathy. Teratology, 38(3), 203–215. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-0023789803&partnerID=tZOtx3y1

Li, D., Johanson, G., Emond, C., Carlander, U., Philbert, M., & Jolliet, O. (2014). Physiologically based pharmacokinetic modeling of polyethylene glycol-coated polyacrylamide nanoparticles in rats. Nanotoxicology, 8 Suppl 1, 128–37. http://doi.org/10.3109/17435390.2013.863406

Li, K., Zhao, X., K. Hammer, B., Du, S., & Chen, Y. (2013). Nanoparticles Inhibit DNA Replication by Binding to DNA: Modeling and Experimental Validation. ACS Nano, 7(11), 9664–9674. http://doi.org/10.1021/nn402472k

Li, X., Peng, Y., & Qu, X. (2006). Carbon nanotubes selective destabilization of duplex and triplex DNA and inducing B-A transition in solution. Nucleic Acids Research, 34(13), 3670–3676. http://doi.org/10.1093/nar/gkl513

Li, X., Peng, Y., Ren, J., & Qu, X. (2006). Carboxyl-modified single-walled carbon nanotubes selectively induce human telomeric i-motif formation. Proceedings of the National Academy of Sciences of the United States of America, 103(52), 19658–19663. http://doi.org/10.1073/pnas.0607245103

Li, Y., Wang, Y., Pennell, K. D., & Briola, L. M. A. (2008). Investigation of the transport and deposition of fullerene (C60) nanoparticles in quartz sands under varying flow conditions. Environmental Science and Technology, 42(19), 7174–7180. http://doi.org/10.1021/es801305y

Li, Y., Yang, C., Guo, X., Dang, Z., Li, X., & Zhang, Q. (2015). Effects of humic acids on the aggregation and sorption of nano-TiO2. Chemosphere, 119, 171–6. http://doi.org/10.1016/j.chemosphere.2014.05.002

Liaw, A., & Wiener, M. (2002). Classification and Regression by randomForest. R News, 2(3), 18–22.

Lidén, G. (2006). Dustiness testing of materials handled at workplaces. The Annals of Occupational Hygiene, 50(5), 437–9. http://doi.org/10.1093/annhyg/mel042

Liguori, B., Hansen, S. F., Baun, A., & Jensen, K. A. (2016). Control banding tools for occupational exposure assessment of nanomaterials — Ready for use in a regulatory context? NanoImpact, 2, 1–17. http://doi.org/10.1016/j.impact.2016.04.002

Lin, M. Y., Lindsay, H., Weitz, D. A., Ball, R., Klein, R., & Meakin, P. (1990). Universal reaction-limited colloid aggregation. Physical Review A, 41(4), 2005–2020. http://doi.org/10.1103/PhysRevA.41.2005

Lin, S., & Wiesner, M. R. (2012). Theoretical investigation on the interaction between a soft particle and a rigid surface. Chemical Engineering Journal, 191, 297–305. http://doi.org/10.1016/j.cej.2012.03.019

Linkov, I., Welle, P., Loney, D., Tkachuk, A., Canis, L., Kim, J. B., & Bridges, T. (2011). Use of multicriteria decision analysis to support weight of evidence evaluation. Risk Analysis : An Official Publication of the Society for Risk Analysis, 31(8), 1211–25. http://doi.org/10.1111/j.1539-6924.2011.01585.x

Liu, H. H., Bilal, M., Lazareva, A., Keller, A., & Cohen, Y. (2015). Simulation tool for assessing the release and environmental distribution of nanomaterials. Beilstein Journal of Nanotechnology, 6(1), 938–951. http://doi.org/10.3762/bjnano.6.97

Liu, H. H., & Cohen, Y. (2014). Multimedia environmental distribution of engineered nanomaterials. Environmental Science and Technology, 48(6), 3281–3292. http://doi.org/10.1021/es405132z

Liu, H. H., & Cohen, Y. (2014a). Multimedia environmental distribution of engineered nanomaterials. Environmental Science and Technology, 48, 3281–3292. http://doi.org/10.1021/es405132z

Liu, H. H., & Cohen, Y. (2014b). Multimedia environmental distribution of engineered nanomaterials. Environmental Science & Technology, 48(6), 3281–92. http://doi.org/10.1021/es405132z

Liu, H., Ma, L., Liu, J., Zhao, J., Yan, J., & Hong, F. (2010). Toxicity of nano-anatase TiO 2 to mice: Liver injury, oxidative stress. Toxicological & Environmental Chemistry, 92(1), 175–186. http://doi.org/10.1080/02772240902732530

Liu, H., Ma, L., Zhao, J., Liu, J., Yan, J., Ruan, J., & Hong, F. (2009). Biochemical toxicity of nano-anatase TiO2 particles in mice. Biological Trace Element Research, 129(1–3), 170–80. http://doi.org/10.1007/s12011-008-8285-6

Liu, H., Yao, X., Zhang, R., Liu, M., Hu, Z., & Fan, B. (2005). Accurate Quantitative Structure−Property Relationship Model To Predict the Solubility of C60 in Various Solvents Based on a Novel Approach Using a Least-Squares Support Vector Machine. The Journal of Physical Chemistry B, 109(43), 20565–20571. http://doi.org/10.1021/jp052223n

Liu, J., & Hurt, R. H. (2010). Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environmental Science and Technology, 44(6), 2169–2175. http://doi.org/10.1021/es9035557

Liu, R., Jiang, W., Walkey, C. D., Chan, W. C. W., & Cohen, Y. (2015). Prediction of nanoparticles-cell association based on corona proteins and physicochemical properties. Nanoscale, 7(21), 9664–9675. http://doi.org/10.1039/c5nr01537e

Liu, R., Liu, H. H., Ji, Z., Chang, C. H., Xia, T., Nel, A. E., & Cohen, Y. (2015). Evaluation of Toxicity Ranking for Metal Oxide Nanoparticles via an in Vitro Dosimetry Model. ACS Nano, 9(9), 9303–13. http://doi.org/10.1021/acsnano.5b04420

Liu, R., Rallo, R., Bilal, M., & Cohen, Y. (2015). Quantitative structure-activity relationships for cellular uptake of surface-modified nanoparticles. Combinatorial Chemistry and High Throughput Screening, 18(4), 365–375. http://doi.org/10.2174/1386207318666150306105525

Liu, R., Rallo, R., Bilal, M., & Cohen, Y. (2015). Quantitative structure-activity relationships for cellular uptake of surface-modified nanoparticles. Combinatorial Chemistry and High Throughput Screening, 18(4), 365–375. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-84931271982&partnerID=tZOtx3y1

Liu, R., Rallo, R., George, S., Ji, Z., Nair, S., Nel, A. E., & Cohen, Y. (2011). Classification NanoSAR development for cytotoxicity of metal oxide nanoparticles. Small, 7(8), 1118–1126. http://doi.org/10.1002/smll.201002366

Liu, R., Zhang, H. Y., Ji, Z. X., Rallo, R., Xia, T., Chang, C. H., … Cohen, Y. (2013a). Development of structure-activity relationship for metal oxide nanoparticles. Nanoscale, 5(12), 5644–53. http://doi.org/10.1039/c3nr01533e

Liu, R., Zhang, H. Y., Ji, Z. X., Rallo, R., Xia, T., Chang, C. H., … Cohen, Y. (2013b). Development of structure–activity relationship for metal oxide nanoparticles. Nanoscale, 5(12), 5644–5653. http://doi.org/10.1039/C3NR01533E

Liu, R., Zhang, H. Y., Ji, Z. X., Rallo, R., Xia, T., Chang, C. H., … Cohen, Y. (2013c). Development of structure–activity relationship for metal oxide nanoparticles. Nanoscale, 5(12), 5644–5653. http://doi.org/10.1039/C3NR01533E

Liu, X., O'Carroll, D. M., Petersen, E. J., Huang, Q., & Anderson, C. L. (2009). Mobility of multiwalled carbon nanotubes in porous media. Environmental Science & Technology, 43(21), 8153–8158. http://doi.org/10.1021/es901340d

Liu, X., Tang, K., Harper, S., Harper, B., Steevens, J. A., & Xu, R. (2013). Predictive modeling of nanomaterial exposure effects in biological systems. International Journal of Nanomedicine, 8 Suppl 1(Supplement 1 Nanoinformatics), 31–43. http://doi.org/10.2147/IJN.S40742

Liu, Y., Zhao, Y., Sun, B., & Chen, C. (2013). Understanding the toxicity of carbon nanotubes. Accounts of Chemical Research, 46(3), 702–713. http://doi.org/10.1021/ar300028m

Liu, Z., Dong, X., Song, L., Zhang, H., Liu, L., Zhu, D., … Leng, X. (2014). Carboxylation of multiwalled carbon nanotube enhanced its biocompatibility with L02 cells through decreased activation of mitochondrial apoptotic pathway. Journal of Biomedical Materials Research - Part A, 102(3), 665–673. http://doi.org/10.1002/jbm.a.34729

Lorenz, C., Von Goetz, N., Scheringer, M., Wormuth, M., & Hungerbühler, K. (2011). Potential exposure of German consumers to engineered nanoparticles in cosmetics and personal care products. Nanotoxicology, 5(1), 12–29. http://doi.org/10.3109/17435390.2010.484554

Louro, H., Tavares, A., Vital, N., Costa, P. M., Alverca, E., Zwart, E., … Silva, M. J. (2014). Integrated approach to the in vivo genotoxic effects of a titanium dioxide nanomaterial using LacZ plasmid-based transgenic mice. Environmental and Molecular Mutagenesis, 55(6), 500–509. http://doi.org/10.1002/em.21864

Love, S. A., Maurer-Jones, M. A., Thompson, J. W., Lin, Y. S., & Haynes, C. L. (2012). Assessing nanoparticle toxicity. Annu Rev Anal Chem (Palo Alto Calif), 5, 181–205. http://doi.org/10.1146/annurev-anchem-062011-143134

Lubinski, L., Urbaszek, P., Gajewicz, A., Cronin, M. T. D., Enoch, S. J., Madden, J. C., … Puzyn, T. (2013). Evaluation criteria for the quality of published experimental data on nanomaterials and their usefulness for QSAR modelling. Retrieved from http://www.tandfonline.com/doi/abs/10.1080/1062936X.2013.840679#.VMpMemOGd8E

Luoma, S. N. (2008). SILVER NANOTECHNOLOGIES AND THE ENVIRONMENT : OLD PROBLEMS OR NEW CHALLENGES ?, (September).

Luttrell, T., Halpegamage, S., Tao, J., Kramer, A., Sutter, E., & Batzill, M. (2014). Why is anatase a better photocatalyst than rutile? - Model studies on epitaxial TiO2 films. Scientific Reports, 4, 4043. Retrieved from http://dx.doi.org/10.1038/srep04043

Lynch, I. (2016). Compendium of Projects in the European NanoSafety Cluster, (June), 259.

Lynch, I., Feitshans, I. L., & Kendall, M. (2015). "Bio-nano interactions: new tools, insights and impacts": summary of the Royal Society discussion meeting. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 370(1661), 20140162-. http://doi.org/10.1098/rstb.2014.0162

Ma, L., Zhao, J., Wang, J., Liu, J., Duan, Y., Liu, H., … Hong, F. (2009). The Acute Liver Injury in Mice Caused by Nano-Anatase TiO2. Nanoscale Research Letters, 4(11), 1275–85. http://doi.org/10.1007/s11671-009-9393-8

Magdolenova, Z., Bilaničová, D., Pojana, G., Fjellsbø, L. M., Hudecova, A., Hasplova, K., … Dusinska, M. (2012). Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. Journal of Environmental Monitoring, 14(2), 455. http://doi.org/10.1039/c2em10746e

Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V., & Dusinska, M. (2014). Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology, 8(3), 233–278. http://doi.org/10.3109/17435390.2013.773464

Maggiora, G. M. (2006). On outliers and activity cliffs - Why QSAR often disappoints. Journal of Chemical Information and Modeling, 46(4), 1535. http://doi.org/10.1021/ci060117s

Mahapatra, I., Sun, T. Y., Clark, J. R. A., Dobson, P. J., Hungerbuehler, K., Owen, R., … Lead, J. (2015). Probabilistic modelling of prospective environmental concentrations of gold nanoparticles from medical applications as a basis for risk assessment. Journal of Nanobiotechnology, 13(1). http://doi.org/10.1186/s12951-015-0150-0

Mandelbrot, B. (1982). The Fractal Geometry of Nature. Holt, Henry & Company, Inc.

Mano, S. S., Kanehira, K., Sonezaki, S., & Taniguchi, A. (2012). Effect of Polyethylene Glycol Modification of TiO 2 Nanoparticles on Cytotoxicity and Gene Expressions in Human Cell Lines. International Journal of Molecular Sciences, 13, 3703–3717. http://doi.org/10.3390/ijms13033703

Marchese Robinson, R. L., Cronin, M. T. D., Richarz, A.-N., & Rallo, R. (2015). An ISA-TAB-Nano based data collection framework to support data-driven modelling of nanotoxicology. Beilstein Journal of Nanotechnology, 6(1), 1978–1999. http://doi.org/10.3762/bjnano.6.202

Marchese Robinson, R. L., Lynch, I., Peijnenburg, W., Rumble, J., Klaessig, F., Marquardt, C., … Harper, S. (2016). How should the completeness and quality of curated nanomaterial data be evaluated? Nanoscale. http://doi.org/10.1039/C5NR08944A

Markus, A. A., Parsons, J. R., Roex, E. W. M., de Voogt, P., & Laane, R. W. P. M. (2015). Modeling aggregation and sedimentation of nanoparticles in the aquatic environment. The Science of the Total Environment, 506–507, 323–9. http://doi.org/10.1016/j.scitotenv.2014.11.056

Martin, D., Maran, U., Sild, S., & Karelson, M. (2007). QSPR modeling of solubility of polyaromatic hydrocarbons and fullerene in 1-octanol and n-heptane. The Journal of Physical Chemistry. B, 111(33), 9853–7. http://doi.org/10.1021/jp071679x

Maruyama, K., Haniu, H., Saito, N., Matsuda, Y., Tsukahara, T., Kobayashi, S., … Kato, H. (2015). Endocytosis of Multiwalled Carbon Nanotubes in Bronchial Epithelial and Mesothelial Cells. BioMed Research International, 2015, 793186. http://doi.org/10.1155/2015/793186

Marvin, H. J. P., Bouzembrak, Y., Janssen, E. M., van der Zande, M., Murphy, F., Sheehan, B., … Bouwmeester, H. (2017). Application of Bayesian networks for hazard ranking of nanomaterials to support human health risk assessment. Nanotoxicology, 11(1), 123–133. http://doi.org/10.1080/17435390.2016.1278481

Mast, J., & De Temmerman, P.-J. (2016). Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids (NANoREG No. D2.10). Retrieved from http://www.nanoreg.eu/images/D2.10_Protocol_for_size-distribution_analysis_of_primary_NM_particles_in_air_powders_and_liquids_-approvedpublic_for_website_final09.11.pdf

Meesters, J. A. J., Koelmans, A. A., Quik, J. T. K., Hendriks, A. J., & Van De Meent, D. (2014). Multimedia modeling of engineered nanoparticles with simpleBox4nano: Model definition and evaluation. Environmental Science and Technology, 48(10), 5726–5736. http://doi.org/10.1021/es500548h

Meesters, J. A. J., Quik, J. T. K., Koelmans, A. A., Hendriks, A. J., & van de Meent, D. (2016). Multimedia environmental fate and speciation of engineered nanoparticles: a probabilistic modeling approach. Environmental Science-Nano, 3(4), 715–727. http://doi.org/10.1039/c6en00081a

Meesters, J., Koelmans, A., Quik, J., Hendriks, J., & van de Meent, D. (2014). Multimedia modeling of engineered nanoparticles with SimpleBox4nano: model definition and evaluation. Environmental Science & Technology, 48(10), 5726–36. http://doi.org/10.1021/es500548h

Meker, S., Margulis-Goshen, K., Weiss, E., Magdassi, S., & Tshuva, E. Y. (2012). High antitumor activity of highly resistant salan-titanium(IV) complexes in nanoparticles: An identified active species. Angewandte Chemie - International Edition, 51(42), 10515–10517. http://doi.org/10.1002/anie.201205973

Melagraki, G., & Afantitis, A. (2014). Enalos InSilicoNano platform: An online decision support tool for the design and virtual screening of nanoparticles. RSC Advances, 4(92), 50713–50725. http://doi.org/10.1039/c4ra07756c

Mercer, R. R., Hubbs, A. F., Scabilloni, J. F., Wang, L., Battelli, L. a, Friend, S., … Porter, D. W. (2011). Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes. Particle and Fibre Toxicology, 8(1), 21. http://doi.org/10.1186/1743-8977-8-21

Mercer, R. R., Scabilloni, J. F., Hubbs, A. F., Battelli, L. a, McKinney, W., Friend, S., … Porter, D. W. (2013). Distribution and fibrotic response following inhalation exposure to multi-walled carbon nanotubes. Particle and Fibre Toxicology, 10(1), 33. http://doi.org/10.1186/1743-8977-10-33

Metze, K. (2013). Fractal dimension of chromatin: potential molecular diagnostic applications for cancer prognosis. Expert Review of Molecular Diagnostics, 13(7), 719–35. http://doi.org/10.1586/14737159.2013.828889

Mikolajczyk, A., Gajewicz, A., Rasulev, B., Schaeublin, N., Maurer-Gardner, E., Hussain, S., … Puzyn, T. (2015). Zeta potential for metal oxide nanoparticles: A predictive model developed by a nano-quantitative structure-property relationship approach. Chemistry of Materials, 27(7), 2400–2407. http://doi.org/10.1021/cm504406a

Mitrano, D. M., Motellier, S., Clavaguera, S., & Nowack, B. (2015). Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. Environment International. http://doi.org/10.1016/j.envint.2015.01.013

Mitrano, D. M., Rimmele, E., Wichser, A., Erni, R., Height, M., & Nowack, B. (2014). Presence of Nanoparticles in Wash Water from Conventional Silver and Nano-silver Textiles. ACS Nano, 8(7), 7208–19. http://doi.org/10.1021/nn502228w

Money, E. S., Reckhow, K. H., & Wiesner, M. R. (2012). The use of Bayesian networks for nanoparticle risk forecasting: Model formulation and baseline evaluation. Science of the Total Environment, 426, 436–445. http://doi.org/10.1016/j.scitotenv.2012.03.064

Monopoli, M. P., Aberg, C., Salvati, A., & Dawson, K. A. (2012). Biomolecular coronas provide the biological identity of nanosized materials. Nature Nanotechnology, 7(12), 779–86. http://doi.org/10.1038/nnano.2012.207

Monopoli, M. P., Walczyk, D., Campbell, A., Elia, G., Lynch, I., Bombelli, F. B., & Dawson, K. a. (2011). Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles. Journal of the American Chemical Society, 133(8), 2525–34. http://doi.org/10.1021/ja107583h

Moreno, P. A., Vélez, P. E., Martínez, E., Garreta, L. E., Díaz, N., Amador, S., … García, F. (2011). The human genome: a multifractal analysis. BMC Genomics, 12(1), 506. http://doi.org/10.1186/1471-2164-12-506

Mortelmans, K., & Zeiger, E. (2000). The Ames Salmonella/microsome mutagenicity assay. Mutation Research, 455(1–2), 29–60.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 65(1–2), 55–63. http://doi.org/10.1016/0022-1759(83)90303-4

Mu, Q., Liu, W., Xing, Y., Zhou, H., Li, Z., Zhang, Y., … Yan, B. (2008). Protein binding by functionalized multiwalled carbon nanotubes is governed by the surface chemistry of both parties and the nanotube diameter. Journal of Physical Chemistry C, 112(9), 3300–3307. http://doi.org/10.1021/jp710541j

Mu, Y., Wu, F., Zhao, Q., Ji, R., Qie, Y., Zhou, Y., … Xing, B. (2016). Predicting toxic potencies of metal oxide nanoparticles by means of nano-QSARs. Nanotoxicology. State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing, China. http://doi.org/10.1080/17435390.2016.1202352

Mueller, N. C., Buha, J., Wang, J., Ulrich, A., & Nowack, B. (2013). Modeling the flows of engineered nanomaterials during waste handling. Environmental Science: Processes & Impacts, 15(1), 251. http://doi.org/10.1039/c2em30761h

Mueller, N. C., & Nowack, B. (2008). Exposure modeling of engineered nanoparticles in the environment. Environmental Science & Technology, 42(12), 4447–4453. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18605569

Mulware, S. J. (2012). Trace elements and carcinogenicity: a subject in review. 3 Biotech, 85–96. http://doi.org/10.1007/s13205-012-0072-6

Mura, S., Hillaireau, H., Nicolas, J., Le Droumaguet, B., Gueutin, C., Zanna, S., … Fattal, E. (2011). Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. International Journal of Nanomedicine, 6, 2591–2605. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-81255195201&partnerID=tZOtx3y1

Murphy, F. A., Poland, C. A., Duffin, R., & Donaldson, K. (2013). Length-dependent pleural inflammation and parietal pleural responses after deposition of carbon nanotubes in the pulmonary airspaces of mice. Retrieved from http://informahealthcare.com/doi/full/10.3109/17435390.2012.713527

Murray, A. R., Kisin, E. R., Tkach, A. V, Yanamala, N., Mercer, R. R., Young, S.-H., … Shvedova, A. a. (2012). Factoring-in agglomeration of carbon nanotubes and nanofibers for better prediction of their toxicity versus asbestos. Particle and Fibre Toxicology, 9(1), 10. http://doi.org/10.1186/1743-8977-9-10

Musee, N. (2011). Simulated environmental risk estimation of engineered nanomaterials: a case of cosmetics in Johannesburg City. Human & Experimental Toxicology, 30(9), 1181–95. http://doi.org/10.1177/0960327110391387

Mustad, A. P., Smeets, B., Jeliazkov, V., & Willighagen, E. L. (2014). Summary of the Spring 2014 NSC Database Survey. Retrieved from http://www.nanosafetycluster.eu/news/132/66/Summary-of-the-Spring-2014-NSC-Database-Survey.html

MWCNT REACH Dossier. (2016). REACH registration dossier: Multi-Walled Carbon Nanotubes (MWCNT), synthetic graphite in tubular shape (EC number: 936-414-1). Retrieved October 10, 2016, from http://echa.europa.eu/registration-dossier/-/registered-dossier/13454/7/2/2/?documentUUID=d042b6e7-1608-420a-b644-475a6450fe43

Nagai, H., Okazaki, Y., Chew, S., Misawa, N., Yamashita, Y., Akatsuka, S., … Toyokuni, S. (2011). Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. Pnas, 108(49), 1330–1338. http://doi.org/10.1073/pnas.1110013108

NanoDefine. (2013). FP7-NMP-2013-LARGE-7. Retrieved from www.nanodefine.eu

Nanogenotox. (2010). Safety evaluation of manufactured nanomaterials by characterisation of their potential genotoxic hazard. http://www.nanogenotox.eu/. EU Grant Agreement 2009 21 01.

Nanogenotox. (2012). Towards a method for detecting the potential genotoxicity of nanomaterials D4 . 2 : Transmission electron microscopic characterization of NANOGENOTOX nanomaterials Final report Key intrinsic physicochemical characteristics of NANOGENOTOX nanomaterials WP.

NanoGenoTox Joint Action. (2013). NANOGENOTOX Final report. Facilitating the safety evaluation of manufactured nanomaterials by characterising their potential genotoxic hazard. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

Nanogenotox WP6. (2013). Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

Nel, A. E., Parak, W. J., Chan, W. C. W., Xia, T., Hersam, M. C., Brinker, C. J., … Weiss, P. S. (2015). Where Are We Heading in Nanotechnology Environmental Health and Safety and Materials Characterization? ACS Nano, 9(6), 5627–5630. http://doi.org/10.1021/acsnano.5b03496

Nel, A., Xia, T., Mädler, L., & Li, N. (2006). Toxic potential of materials at the nanolevel. Science (New York, N.Y.), 311(5761), 622–7. http://doi.org/10.1126/science.1114397

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., & Zhang, H. (2013). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Accounts of Chemical Research, 46(3), 607–21. http://doi.org/10.1021/ar300022h

Neukum, C., Braun, A., & Azzam, R. (2014). Transport of engineered silver (Ag) nanoparticles through partially fractured sandstones. Journal of Contaminant Hydrology, 164, 181–192. http://doi.org/10.1016/j.jconhyd.2014.05.012

Nguyen, T., Pellegrin, B., Bernard, C., Rabb, S., Stuztman, P., Gorham, J. M., … Chin, J. W. (2012). Characterization of Surface Accumulation and Release of Nanosilica During Irradiation of Polymer Nanocomposites by Ultraviolet Light. Journal of Nanoscience and Nanotechnology, 12(8), 6202–6215. http://doi.org/10.1166/jnn.2012.6442

NIH. (2014). Nanomaterial Registry. Retrieved June 26, 2015, from https://www.healthdata.gov/dataset/nanomaterial-registry

NIOSH. (2013). Occupational Exposure to Carbon Nanotubes and Nanofibers (No. 2013–145). Current Intelligence Bulletin. Retrieved from http://www.cdc.gov/niosh/docs/2013-145/pdfs/2013-145.pdf

Nohynek, G. J., & Dufour, E. K. (2012). Nano-sized cosmetic formulations or solid nanoparticles in sunscreens: a risk to human health? Archives of Toxicology, 86(7), 1063–75. http://doi.org/10.1007/s00204-012-0831-5

Norppa, H., Siivola, K., Fessard, V., Tarantini, A., Apostolova, M., Jacobsen, N., … Silva, M. (2013). Nanogenotox deliverable 5 : In vitro testing strategy for nanomaterials including database Final report. Helsinki. Retrieved from http://www.nanogenotox.eu/index.php?option=com_content&view=article&id=136&Itemid=158

O'Brien, N., & Cummins, E. (2010). Nano-scale pollutants: Fate in irish surface and drinking water regulatory systems. Human and Ecological Risk Assessment, 16(4), 847–872. http://doi.org/10.1080/10807039.2010.501270

O'Brien, N., & Cummins, E. (2010). Ranking initial environmental and human health risk resulting from environmentally relevant nanomaterials. Journal of Environmental Science and Health. Part A, Toxic/hazardous Substances & Environmental Engineering, 45(8), 992–1007. http://doi.org/10.1080/10934521003772410

O'Brien, N. J., & Cummins, E. J. (2011). A risk assessment framework for assessing metallic nanomaterials of environmental concern: Aquatic exposure and behavior. Risk Analysis, 31(5), 706–726. http://doi.org/10.1111/j.1539-6924.2010.01540.x

Oberdörster, G., Castranova, V., Asgharian, B., & Sayre, P. (2015). Inhalation Exposure to Carbon Nanotubes (CNT) and Carbon Nanofibers (CNF): Methodology and Dosimetry. Journal of Toxicology and Environmental Health. Part B, Critical Reviews, 18(3–4), 121–212. http://doi.org/10.1080/10937404.2015.1051611

Oberdörster, G., & Cox, C. (1990). Carcinogenicity of cadmium in animals: What is the significance for man? Toxicological & Environmental Chemistry, 27(1–3), 181–195. http://doi.org/10.1080/02772249009357571

Oberdörster, G., Cox, C., & Baggs, R. (1987). Long term lung clearance and cellular retention of cadmium in rats and monkeys. Journal of Aerosol Science, 18(6), 745–748. http://doi.org/10.1016/0021-8502(87)90112-1

Oberdörster, G., Oberdörster, E., & Oberdörster, J. (2005). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives, 113(7), 823–39. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1257642&tool=pmcentrez&rendertype=abstract

OECD. (2004). The Report From The Expert Group On (Quantitative) Structure-Activity Relationships [(Q)SARs] On The Principles For The Validation of (Q)SARs (OECD Series on Testing And Assessmen No. ENV/JM/MONO(2004)24). Paris.

OECD. (2007a). Guidance document on the validation of (Quantitative) Structure-Activity Relationship [(Q) SAR] models (No. ENV/JM/MONO(2007)2). Paris. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2007)2

OECD. (2007b). Guidance Document on the Validation of (Quantitative) Structure-Activity Relationship [(Q)Sar] Models. OECD Environment Health and Safety Publications, Series on Testing and Assessment. (Vol. 69).

OECD. (2007c). Guidance on grouping of chemicals. Series on testing and assessment No. 80 (Series on testing and assessment No. ENV/JM/MONO(2007)28). Paris. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono%282007%2928

OECD. (2009a). Guidance manual for the testing of manufactured nanomaterials. OECD's sponsorship programme (No. ENV/JM/MONO(2009)20). Paris.

OECD. (2009b). Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials (Series on the Safety of Manufactured Nanomaterials). Paris. Retrieved from http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2009)21&doclanguage=en

OECD. (2010). Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; first revision (No. ENV/JM/MONO(2009)20/REV). Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282009%2920/rev&doclanguage=en

OECD. (2011). Report of the WPMN expert meeting on inhalation toxicity testing for nanomaterials (No. ENV/CHEM/NANO(2911)23/REV1).

OECD. (2012a). Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials (Series on the Safety of Manufactured Nanomaterials No. 36). Paris.

Retrieved   from
http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)40&
doclanguage=en

OECD. (2012b). Inhalation toxicity testing: expert meeting on potential revisions to OECD test guidelines and guidance document. Series on the Safety of Manufactured Nanomaterials No. 35 (Safety of Manufactured Nanomaterials No. ENV/JM/MONO(2012)14). Paris.

OECD. (2012c). The Adverse Outcome Pathway for skin sensitisation initiated by covalent binding to proteins. Part 2: Use of the AOP to develop chemical categories and integrated assessment and testing approaches. Series on testing and assessment No. 168 (Series on testing and assessment No. ENV/JM/MONO(2012)10/PART2). Paris.

OECD. (2012d). The Adverse Outcome Pathway for skin sensitisation initiated by covalent binding to proteins Part 1: Scientific evidence. Series on testing and assessment No. 168 (Series on testing and assessment No. ENV/JM/MONO(2012)10/PART1).

OECD. (2014a). Draft Report Of The OECD Expert Meeting on Genotoxicity of Manufactured Nanomaterials.

OECD. (2014b). Ecotoxicology and environmental fate of manufactured nanomaterials: Test guidelines. Expert meeting report. Series on the safety of manufactured nanomaterials No. 40 (No. ENV/JM/MONO(2014)1).   Paris.   Retrieved   from
http://www.oecd.org/officialdocuments/displaydocument/?cote=ENV/JM/MONO(2014)1&doclangu
age=en

OECD. (2014c). Ecotoxicology and Environmental Fate of Manufactured Nanomaterials:Test Guidelines   Expert   Meeting   Report.   Paris.   Retrieved   from
http://www.oecd.org/env/ehs/nanosafety/publicationsintheseriesonthesafetyofmanufacturednano
materials.htm

OECD. (2014d). Genotoxicity of manufactured nanomaterials: Report of the OECD expert meeting. Series on the safety of manufactured nanomaterials No. 43 (No. ENV/JM/WRPR(2014)51).

OECD. (2014e). Guidance on grouping of chemicals. Second edition. Series on testing and assessment No. 194 (Series on Testing & Assessment No. ENV/JM/MONO(2014)4). Paris. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282014%
294&doclanguage=en

OECD. (2014f). New guidance document on the integrated approach on an integrated approach on testing and assessment (IATA) for skin corrosion and irritation. Series on Testing and Assessment No. 203 (Series on Testing and Assessment No. ENV/JM/MONO(2014)19). Paris. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)19&
doclanguage=en

OECD. (2014g). Report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines. Series on the safety of manufactured nanomaterials No. 41 (Series on the Safety of Manufactured Nanomaterials No. ENV/JM/MONO(2014)15).   Paris.   Retrieved   from
http://www.oecd.org/officialdocuments/displaydocument/?cote=env/jm/mono(2014)15&doclangua
ge=en

OECD. (2015). Titanium dioxide dossier (NM100-NM105) (Working Party on Manufactured Nanomaterials No.   ENV/CHEM/NANO(2015)21/Part   1   to   5).   Paris.   Retrieved   from
http://www.oecd.org/chemicalsafety/nanosafety/titanium-dioxide-nm100-nm105-manufactured-
nanomaterial.htm

OECD. (2016a). Approaches to develop or use concepts of Grouping, Equivalence abd Read-Across based on Physical-Chemical properties (GERA-PC) of nanomaterials for their human health abd ecosystem hazard assessment in regulatory regimes: analysis of a survey (Series on the Safety of Manufactured Nanomaterials No. 64). Paris.

OECD. (2016b). Guidance Document for the Use of Adverse Outcome Pathways in Developing Integrated Approaches to Testing and Assessment (IATA). ENV/JM/MONO(2016)67.

OECD. (2016c). Guidance Document on The Reporting of Defined Approaches to be used within Integrated Approaches to Testing and Assessment. ENV/JM/MONO(2016)28.

OECD. (2016d). Toxicokinetic of manufactured nanomaterials: report from the OECD expert meeting (Series on the Safety of Manufactured Nanomaterials No. 72) (Vol. 1). paris. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282016%2924&doclanguage=en

OECD. (2017). Alternative testing strategies in risk assessment of manufactured nanomaterials: current state of knowledge and research needs to advance their use (Series on the Safety of Manufactured Nanomaterials No. 80). Paris.

OECD WPMN. (2016). Multiwalled Carbon Nanotubes (Mwcnt): Summary of the Dossier, ENV/JM/MON, 1–48. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2016)20&docLanguage=En

Oksel, C., Ma, C. Y., Liu, J. J., Wilkins, T., & Wang, X. Z. (2015). (Q) SAR modelling of nanomaterial toxicity : A critical review. Particuology, (21), 1–19.

Oksel, C., Ma, C. Y., Liu, J. J., Wilkins, T., & Wang, X. Z. (2015). (Q)SAR modelling of nanomaterial toxicity: A critical review. Particuology, 21, 1–19. http://doi.org/10.1016/j.partic.2014.12.001

Oksel, C., Winkler, D. A., Ma, C. Y., Wilkins, T., & Wang, X. Z. (2016). Accurate and interpretable nanoSAR models from genetic programming-based decision tree construction approaches. Nanotoxicology, 10(7), 1001–1012. http://doi.org/10.3109/17435390.2016.1161857

Oksel, C., Winkler, D. A., Ma, C. Y., Wilkins, T., & Wang, X. Z. (2016). Accurate and interpretable nanoSAR models from genetic programming-based decision tree construction approaches. Nanotoxicology.

Oomen, A., Bleeker, E., Bos, P., Broekhuizen, F. van, Gottardo, S., Groenewold, M., … Landsiedel, R. (2015, October 26). Grouping and Read-Across Approaches for Risk Assessment of Nanomaterials. International Journal of Environmental Research and Public Health. Multidisciplinary Digital Publishing Institute. http://doi.org/10.3390/ijerph121013415

Oomen, A. G., Bos, P. M. J., Fernandes, T. F., Hund-Rinke, K., Boraschi, D., Byrne, H. J., … Landsiedel, R. (2014). Concern-driven integrated approaches to nanomaterial testing and assessment--report of the NanoSafety Cluster Working Group 10. Nanotoxicology, 8(3), 334–48. http://doi.org/10.3109/17435390.2013.802387

Osmond-McLeod, M. J., Oytam, Y., Kirby, J. K., Gomez-Fernandez, L., Baxter, B., & McCall, M. J. (2014). Dermal absorption and short-term biological impact in hairless mice from sunscreens containing zinc oxide nano- or larger particles. Nanotoxicology, 8 Suppl 1, 72–84. http://doi.org/10.3109/17435390.2013.855832

Paik, S. Y., Zalk, D. M., & Swuste, P. (2008). Application of a pilot control banding tool for risk level assessment and control of nanoparticle exposures. Annals of Occupational Hygiene, 52(6), 419–428. http://doi.org/10.1093/annhyg/men041

Pan, Y., Li, T., Cheng, J., Telesca, D., Zink, J. I., & Jiang, J. (2016). Nano-QSAR modeling for predicting the cytotoxicity of metal oxide nanoparticles using novel descriptors. RSC Advances, 6(31), 25766–25775. http://doi.org/10.1039/c6ra01298a

Pantano, A., M. Parks, D., & Boyce, M. C. (2004). Mechanics of deformation of single- and multi-wall carbon nanotubes. Journal of the Mechanics and Physics of Solids, 52(4), 789–821. http://doi.org/10.1016/j.jmps.2003.08.004

Papa, E., Doucet, J. P., & Doucet-Panaye, A. (2015). Linear and non-linear modelling of the cytotoxicity of TiO2 and ZnO nanoparticles by empirical descriptors. SAR and QSAR in Environmental Research, 1–19. http://doi.org/10.1080/1062936X.2015.1080186

Papa, E., Doucet, J. P., Sangion, A., & Doucet-Panaye, A. (2016). Investigation of the influence of protein corona composition on gold nanoparticle bioactivity using machine learning approaches. SAR and QSAR in Environmental Research. QSAR Research Unit in Environmental Chemistry and Ecotoxicology, DiSTA, University of Insubria, Varese, Italy. http://doi.org/10.1080/1062936X.2016.1197310

Park, B., Donaldson, K., Duffin, R., Tran, L., Kelly, F., Mudway, I., … Martin, P. (2008). Hazard and risk assessment of a nanoparticulate cerium oxide-based diesel fuel additive - a case study. Inhalation Toxicology, 20(6), 547–566. http://doi.org/10.1080/08958370801915309

Park, E.-J., Yi, J., Chung, K.-H., Ryu, D.-Y., Choi, J., & Park, K. (2008). Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicology Letters, 180(3), 222–9. http://doi.org/10.1016/j.toxlet.2008.06.869

Pashley, R., & Karaman, M. (2005). Applied Colloid and Surface Chemistry. John Wiley & Sons. Retrieved from https://books.google.com/books?id=FGyIJ1Z5Tr4C&pgis=1

Patlewicz, G., Ball, N., Booth, E. D., Hulzebos, E., Zvinavashe, E., & Hennes, C. (2013). Use of category approaches, read-across and (Q)SAR: general considerations. Regulatory Toxicology and Pharmacology : RTP, 67(1), 1–12. http://doi.org/10.1016/j.yrtph.2013.06.002

Patlewicz, G., Roberts, D. W., Aptula, A., Blackburn, K., & Hubesch, B. (2013). Workshop: use of "read-across" for chemical safety assessment under REACH. Regulatory Toxicology and Pharmacology, 65(2), 226–8. http://doi.org/10.1016/j.yrtph.2012.12.004

Pauluhn, J. (2009). Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: Toxic effects are determined by density of agglomerate structures, not fibrillar structures. Toxicological Sciences, 113(1), 226–242. http://doi.org/10.1093/toxsci/kfp247

Peijnenburg, W. J. G. M., Baalousha, M., Chen, J., Chaudry, Q., Von Der Kammer, F., Kuhlbusch, T. A. J., … Koelmans, A. A. (2015). A Review of the Properties and Processes Determining the Fate of Engineered Nanomaterials in the Aquatic Environment. Critical Reviews in Environmental Science and Technology, 45(19), 2084–2134. http://doi.org/10.1080/10643389.2015.1010430

Peijnenburg, W., Praetorius, A., Scott-Fordsmand, J., & Cornelis, G. (2016). Fate assessment of engineered nanoparticles in solids dominated media ??? Current insights and the way forward. Environmental Pollution, 218, 1365–1369. http://doi.org/10.1016/j.envpol.2015.11.043

Petersen, E. J., Zhang, L., Mattison, N. T., O'Carroll, D. M., Whelton, A. J., Uddin, N., … Chen, K. L. (2011). Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. Environmental Science and Technology. http://doi.org/10.1021/es201579y

Petosa, A. R., Jaisi, D. P. E. B. P., Quevedo, I. R., Elimelech, M., Tufenkji, N., & Haven, N. (2010). Aggregation and deposition of engineered nanomaterials in aquatic environments: role of physicochemical interactions. Environmental Science & Technology, 44(17), 6532–49. http://doi.org/10.1021/es100598h

Petosa, A. R., Jaisi, D. P., Quevedo, I. R., Elimelech, M., & Tufenkji, N. (2010). Aggregation and deposition of engineered nanomaterials in aquatic environments: role of physicochemical interactions. Environmental Science & Technology, 44(17), 6532–49. http://doi.org/10.1021/es100598h

Petrova, T., Rasulev, B. F., Toropov, A. A., Leszczynska, D., & Leszczynski, J. (2011). Improved model for fullerene C60 solubility in organic solvents based on quantum-chemical and topological descriptors. Journal of Nanoparticle Research, 13(8), 3235–3247. http://doi.org/10.1007/s11051-011-0238-x

Piccapietra, F., Allué, C. G., Sigg, L., & Behra, R. (2012). Intracellular silver accumulation in Chlamydomonas reinhardtii upon exposure to carbonate coated silver nanoparticles and silver nitrate. Environmental Science and Technology, 46(13), 7390–7397. http://doi.org/10.1021/es300734m

Pippa, N., Dokoumetzidis, A., Demetzos, C., & Macheras, P. (2013). On the ubiquitous presence of fractals and fractal concepts in pharmaceutical sciences: a review. International Journal of Pharmaceutics, 456(2), 340–52. http://doi.org/10.1016/j.ijpharm.2013.08.087

Poater, A., Gallegos Saliner, A., Solà, M., Cavallo, L., & Worth, A. P. (2010). Computational methods to predict the reactivity of nanoparticles through structure-property relationships. Expert Opinion on Drug Delivery, 7(3), 295–305. http://doi.org/10.1517/17425240903508756

Poater, A., Saliner, A. G., Sol, M., Cavallo, L., & Worth, A. P. (2010). Computational methods to predict the reactivity of nanoparticles through structure-property relationships. Expert Opinion on Drug Delivery, 7(3), 295–305. http://doi.org/10.1517/17425240903508756

Poulsen, S. S., Jackson, P., Kling, K., Knudsen, K. B., Skaug, V., Kyjovska, Z. O., … Vogel, U. (2016). Multi-walled carbon nanotube physicochemical properties predict pulmonary inflammation and genotoxicity. Nanotoxicology, 5390(August), 1–13. http://doi.org/10.1080/17435390.2016.1202351

Poulsen, S. S., Saber, A. T., Williams, A., Andersen, O., Købler, C., Atluri, R., … Vogel, U. (2015). MWCNTs of different physicochemical properties cause similar inflammatory responses, but differences in transcriptional and histological markers of fibrosis in mouse lungs. Toxicology and Applied Pharmacology, 284(1), 16–32. http://doi.org/10.1016/j.taap.2014.12.011

Powell, J. J., Faria, N., Thomas-McKay, E., & Pele, L. C. (2010). Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. Journal of Autoimmunity, 34(3), J226-33. http://doi.org/10.1016/j.jaut.2009.11.006

Praetorius, A., Arvidsson, R., Molander, S., & Scheringer, M. (2013). Facing complexity through informed simplifications: A research agenda for aquatic exposure assessment of nanoparticles. Environmental Sciences: Processes and Impacts, 15(1), 161–168. http://doi.org/10.1039/c2em30677h

Praetorius, A., Gundlach-Graham, A., Goldberg, E., Fabienke, W., Navratilova, J., Gondikas, A., … von der Kammer, F. (2017). Single-particle multi-element fingerprinting (spMEF) using inductively-coupled plasma time-of-flight mass spectrometry (ICP-TOFMS) to identify engineered nanoparticles against the elevated natural background in soils. Environ. Sci.: Nano, 4, 307–314. http://doi.org/10.1039/C6EN00455E

Praetorius, A., Labille, J., Scheringer, M., Thill, A., Hungerbühler, K., & Bottero, J.-Y. (2014). Heteroaggregation of titanium dioxide nanoparticles with model natural colloids under environmentally relevant conditions. Environmental Science and Technology, 48(18), 10690–10698. http://doi.org/10.1021/es501655v

Praetorius, A., Scheringer, M., & Hungerbu, K. (2012). Development of Environmental Fate Models for Engineered Nanoparticles ꞏ A Case Study of TiO 2 Nanoparticles in the Rhine River.

Praetorius, A., Scheringer, M., & Hungerbühler, K. (2012). Development of environmental fate models for engineered nanoparticles-a case study of TiO2 nanoparticles in the Rhine River. Environmental Science & Technology, 46(12), 6705–13. http://doi.org/10.1021/es204530n

Prasad, R. Y., Simmons, S. O., Killius, M. G., Zucker, R. M., Kligerman, A. D., Blackman, C. F., … DeMarini, D. M. (2014). Cellular interactions and biological responses to titanium dioxide nanoparticles in HepG2 and BEAS-2B cells: Role of cell culture media. Environmental and Molecular Mutagenesis, 55(4), 336–342. http://doi.org/10.1002/em.21848

Prasad, R. Y., Wallace, K., Daniel, K. M., Tennant, A. H., Zucker, R. M., Strickland, J., … Demarini, D. M. (2013). Effect of treatment media of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle. ACS Nano, 7(3), 1929–1942. http://doi.org/10.1021/nn302280n

Puzyn, T., Gajewicz, A., Leszczynska, D., & Leszczynski, J. (2010). Nanomaterials - the next great challenge for QSAR modelers. In T. Puzyn, J. Leszczynski, & M. T. D. Cronin (Eds.), Recent advances in QSAR studies (pp. 383–403). New York: Springer.

Puzyn, T., Leszczynska, D., & Leszczynski, J. (2009). Toward the development of "Nano-QSARs": Advances and challenges. Small, 5(22), 2494–2509. http://doi.org/10.1002/smll.200900179

Puzyn, T., Leszczynska, D., & Leszczynski, J. (2009). Toward the development of "nano-QSARs": advances and challenges. Small (Weinheim an Der Bergstrasse, Germany), 5(22), 2494–509. http://doi.org/10.1002/smll.200900179

Puzyn, T., Rasulev, B., Gajewicz, A., Hu, X., Dasari, T. P., Michalkova, A., … Leszczynski, J. (2011). Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature Nanotechnology, 6(3), 175–178. http://doi.org/10.1038/nnano.2011.10

Puzyn, T., Rasulev, B., Gajewicz, A., Hu, X., Dasari, T. P., Michalkova, A., … Leszczynski, J. (2011a). Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature Nanotechnology, 6(3), 175–8. http://doi.org/10.1038/nnano.2011.10

Puzyn, T., Rasulev, B., Gajewicz, A., Hu, X., Dasari, T. P., Michalkova, A., … Leszczynski, J. (2011b). Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature Nanotechnology, 6(3), 175–8. http://doi.org/10.1038/nnano.2011.10

Quik, J. T. K., de Klein, J. J. M., & Koelmans, A. A. (2015). Spatially explicit fate modelling of nanomaterials in natural waters. Water Research, 80, 200–8. http://doi.org/10.1016/j.watres.2015.05.025

Quik, J. T. K., Stuart, M. C., Wouterse, M., Peijnenburg, W., Hendriks, A. J., & van de Meent, D. (2012). Natural colloids are the dominant factor in the sedimentation of nanoparticles. Environmental Toxicology and Chemistry / SETAC, 31(5), 1019–22. http://doi.org/10.1002/etc.1783

Quik, J. T. K., Velzeboer, I., Wouterse, M., Koelmans, A. A., & van de Meent, D. (2014). Heteroaggregation and sedimentation rates for nanomaterials in natural waters. Water Research, 48, 269–279. http://doi.org/10.1016/j.watres.2013.09.036

Quik, J. T. K., Vonk, J. A., Hansen, S. F., Baun, A., & Van De Meent, D. (2011). How to assess exposure of aquatic organisms to manufactured nanoparticles? Environment International, 37(6), 1068–1077. http://doi.org/10.1016/j.envint.2011.01.015

Rajagopalan, R., & Tien, C. (1976). Trajectory analysis of deep-bed filtration with the sphere-in-cell porous media model. AIChE Journal, 22(3), 523–533. http://doi.org/10.1002/aic.690220316

Rallo, R., France, B., Liu, R., Nair, S., George, S., Damoiseaux, R., … Cohen, Y. (2011). Self-organizing map analysis of toxicity-related cell signaling pathways for metal and metal oxide nanoparticles. Environmental Science & Technology, 45(4), 1695–702. http://doi.org/10.1021/es103606x

Randic, M. (1975). Characterization of molecular branching. Journal of the American Chemical Society, 97(23), 6609–6615. http://doi.org/10.1021/ja00856a001

Rasmussen, K., González, M., Kearns, P., Sintes, J. R., Rossi, F., & Sayre, P. (2016). Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines. Regulatory Toxicology and Pharmacology, 74, 147–160. http://doi.org/10.1016/j.yrtph.2015.11.004

Rasmussen, K., Mast, J., & Temmerman, P.-J. (2014). Multi-walled Carbon Nanotubes, NM-400, NM-401, NM-402, NM-403: Characterisation and Physico-Chemical Properties. (JRC Science and Policy Reports). Luxembourg: Publications Office of the European Union. Retrieved from http://publications.jrc.ec.europa.eu/repository/bitstream/JRC91205/mwcnt-online.pdf

Rasmussen, K., Mast, J., Temmerman, P. De, Verleysen, E., Waegeneers, N., Steen, F. Van, … Gibson, N. (2014). Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico- Chemical Properties. Luxembourg. http://doi.org/10.2788/79554

Rauscher, H., Roebben, G., Amenta, V., Boix Sanfeliu, A., Calzolai, L., Emons, H., … Commission, E. (2014). Towards a review of the EC Recommendation for a definition of the term "nanomaterial"; Part 1: Compilation of information concerning the experience with the definition . (H. Rauscher & G. Roebben, Eds.). European Commission Joint Research Centre.

Raychoudhury, T., Tufenkji, N., & Ghoshal, S. (2012). Aggregation and deposition kinetics of carboxymethyl cellulose-modified zero-valent iron nanoparticles in porous media. Water Research, 46(6), 1735–1744. http://doi.org/10.1016/j.watres.2011.12.045

RCC. (2013a). Nanotechnology Initiative Work Element 2 , Priority Setting : Development of a Joint Nanomaterials Classification Scheme (Nanotechnology Initiative No. Final report). Retrieved from http://nanoportal.gc.ca/666215CB-929F-4F75-B78C-4D9DD0F1093B/FINAL DRAFT RCC Nanotechnology Initiative Work Element 2.pdf

RCC. (2013b). Nanotechnology Initiative Work Element 3 : Risk Assessment / Risk Management (Nanotechnology Initiative No. Final report). Retrieved from http://www.nanoportal.gc.ca/9073BB1A-4560-4D8E-B53E-DFBE216676E2/FINAL DRAFT RCC Nanotechnology Initiative Work Element 3.pdf

Rice, Z., Cady, N. C., Bergkvist, M., Lobert, P. E., Bourgeois, D., Pampin, R., … Bard, A. J. (2009). Terminal phosphate group influence on DNA - TiO2 nanoparticle interactions. MRS Proceedings, 1236, 1236-NaN-15. http://doi.org/10.1557/PROC-1236-SS05-15

Richarz, A.-N., Avramopoulos, A., Benfenati, E., Gajewicz, A., Golbamaki Bakhtyari, N., Leonis, G., … Puzyn, T. (2017). Compilation of Data and Modelling of Nanoparticle Interactions and Toxicity in the NanoPUZZLES Project. In L. Tran, M. A. Bañares, & R. Rallo (Eds.), Modelling the Toxicity of Nanoparticles (pp. 303–324). Cham: Springer International Publishing. http://doi.org/10.1007/978-3-319-47754-1_10

Richarz, A.-N., Madden, J. C., Marchese Robinson, R. L., Lubiński, Ł., Mokshina, E., Urbaszek, P., … Cronin, M. T. D. (2014). Development of computational models for the prediction of the toxicity of nanomaterials. Perspectives in Science. http://doi.org/10.1016/j.pisc.2014.11.015

Riediker, M., Ostiguy, C., Triolet, J., Troisfontaine, P., Vernez, D., Bourdel, G., … Cad??ne, A. (2010). Development of a control banding tool for nanomaterials (Control Banding No. 2008-NaN-407). Expert Committee (CES) on Physical Agents (Vol. 2008-NaN-4). Retrieved from https://www.anses.fr/en/content/anses-proposes-innovative-approach-prevention-occupational-risks-nanomaterials

Rispoli, F., Angelov, A., Badia, D., Kumar, A., Seal, S., & Shah, V. (2010). Understanding the toxicity of aggregated zero valent copper nanoparticles against Escherichia coli. Journal of Hazardous Materials, 180(1–3), 212–216. http://doi.org/10.1016/j.jhazmat.2010.04.016

Rittinghausen, S., Bellmann, B., Creutzenberg, O., Ernst, H., Kolling, A., Mangelsdorf, I., … Ziemann, C. (2013). Evaluation of immunohistochemical markers to detect the genotoxic mode of action of fine and ultrafine dusts in rat lungs. Toxicology, 303, 177–186. http://doi.org/10.1016/j.tox.2012.11.007

RIVM, JRC, & ECHA. (2016). Usage of (eco)toxicological data for bridging data gaps between and grouping of nanoforms of the same substance. Elements to consider. Retrieved from https://echa.europa.eu/documents/10162/13630/eco_toxicological_for_bridging_grouping_nanoforms_en.pdf

Rivolo, P. (2012). Encyclopedia of Nanotechnology. (B. Bhushan, Ed.). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-90-481-9751-4

Rivolo, P., Rose, J., Auffan, M., Proux, O., Niviere, V., & Bottero, J. Y. (2012). Encyclopedia of Nanotechnology. (B. Bhushan, Ed.). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-90-481-9751-4

Robinson, R. L. M., Cronin, M. T. D., Richarz, A.-N., & Rallo, R. (2015). An ISA-TAB-Nano based data collection framework to support data-driven modelling of nanotoxicology. Beilstein Journal of Nanotechnology, 6, 1978–1999. http://doi.org/10.3762/bjnano.6.202

Roebben, G., Rauscher, H., Amenta, V., Aschberger, K., Boix Sanfeliu, A., Calzolai, L., … Commission, E. (2014). Towards a review of the EC Recommendation for a definition of the term "nanomaterial"; Part 2: Assessment of collected information concerning the experience with the definition. (G. Roebben & H. Rauscher, Eds.). European Commission Joint Research Centre.

Rofouei, M. K., Salahinejad, M., & Ghasemi, J. B. (2014a). An alignment independent 3D-QSAR modeling of dispersibility of single-walled carbon nanotubes in different organic solvents. Fullerenes Nanotubes and Carbon Nanostructures, 22(7), 605–617. http://doi.org/10.1080/1536383X.2012.702157

Rofouei, M. K., Salahinejad, M., & Ghasemi, J. B. (2014b). An alignment independent 3D-QSAR modeling of dispersibility of single-walled carbon nanotubes in different organic solvents. Fullerenes Nanotubes and Carbon Nanostructures, 22(7), 605–617. http://doi.org/10.1080/1536383X.2012.702157

Roller, M. (2011). In vitro genotoxicity data of nanomaterials compared to carcinogenic potency of inorganic substances after inhalational exposure. Mutation Research, 727(3), 72–85. http://doi.org/10.1016/j.mrrev.2011.03.002

Romoser, A. A. (2012). CYTOTOXICOLOGICAL RESPONSE TO ENGINEERED NANOMATERIALS: A PATHWAY-DRIVEN PROCESS. Statewide Agricultural Land Use Baseline 2015. http://doi.org/10.1017/CBO9781107415324.004

Royce, S. G., Mukherjee, D., Cai, T., Xu, S. S., Alexander, J. A., Mi, Z., … Georgopoulos, P. G. (2014). Modeling population exposures to silver nanoparticles present in consumer products. Journal of Nanoparticle Research, 16(11). http://doi.org/10.1007/s11051-014-2724-4

Rushton, E. K., Jiang, J., Leonard, S. S., Eberly, S., Castranova, V., Biswas, P., … Oberdörster, G. (2010). Concept of Assessing Nanoparticle Hazards Considering Nanoparticle Dosemetric and Chemical/Biological Response Metrics. Journal of Toxicology and Environmental Health, Part A, 73(5). http://doi.org/10.1080/15287390903489422.Concept

Sager, T. M., Wolfarth, M. W., Andrew, M., Hubbs, A., Friend, S., Chen, T., … Holian, A. (2014). Effect of multi-walled carbon nanotube surface modification on bioactivity in the C57BL/6 mouse model. Nanotoxicology, 8(3), 317–27. http://doi.org/10.3109/17435390.2013.779757

Sakharova, N. A., Pereira, A. F. G., Antunes, J. M., & Fernandes, J. V. (2016). Numerical simulation study of the elastic properties of single-walled carbon nanotubes containing vacancy defects. Composites Part B: Engineering, 89, 155–168. http://doi.org/10.1016/j.compositesb.2015.11.029

Salahinejad, M., & Zolfonoun, E. (2013). QSAR studies of the dispersion of SWNTs in different organic solvents. Journal of Nanoparticle Research, 15(11). http://doi.org/10.1007/s11051-013-2028-0

Saliner, A. G., Burello, E., & Worth, A. (2008). Review of computational approaches for predicting the physicochemical and biological properties of nanoparticles BT - JRC Sci Tech Reports EUR.

Sani-Kast, N., Scheringer, M., Slomberg, D., Labille, J., Praetorius, A., Ollivier, P., & Hungerbühler, K. (2015). Addressing the complexity of water chemistry in environmental fate modeling for engineered nanoparticles. The Science of the Total Environment, 535, 150–9. http://doi.org/10.1016/j.scitotenv.2014.12.025

Sargent, L. M., Hubbs, A. F., Young, S. H., Kashon, M. L., Dinu, C. Z., Salisbury, J. L., … Reynolds, S. H. (2012). Single-walled carbon nanotube-induced mitotic disruption. Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 745(1–2), 28–37. http://doi.org/10.1016/j.mrgentox.2011.11.017

Sargent, L., Shvedova, A., Hubbs, A., Salisbury, J., Benkovic, S., Kashon, M., … Reynolds, S. (2009). Induction of aneuploidy by single-walled carbon nanotubes. Environ Mol Mutagen., 50(8), 708–717. http://doi.org/10.1002/em.20529

Savolainen, K., Backman, U., Brouwer, D., Fadeel, B., Fernandes, T., Kuhlbusch, T., … Pylkkänen, L. (2013). Nanosafety in Europe 2015-2025 : Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations Nanosafety in Europe Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations. Helsinki: EDITA. Retrieved from www.ttl.fi/en/publications/electronic_publications/pages/default.aspx

Sayes, C., & Ivanov, I. (2010). Comparative Study of Predictive Computational Models for Nanoparticle-Induced Cytotoxicity. Risk Analysis, 30(11), 1723–1734. http://doi.org/10.1111/j.1539-6924.2010.01438.x

Sayes, C., & Ivanov, I. (2010). Comparative study of predictive computational models for nanoparticle-induced cytotoxicity. Risk Analysis, 30(11), 1723–34. http://doi.org/10.1111/j.1539-6924.2010.01438.x

SCCS. (2012). Guidance on the safety assessment of nanomaterials in cosmetics. Brussels: Scientific Committee on Consumer Safety.

SCCS. (2013a). Opinion on Titanium Dioxide (nano form) (No. COLIPA n. S75). Brussels: Scientific Committee on Consumer Safety. Retrieved from http://www.nononsensecosmethic.org/wp-content/uploads/2013/06/sccs-TiO2nano.pdf

SCCS. (2013b). Opinion SCCS/1489/12 on Titanium Dioxide (nano form); COLIPA S75. Brussels: Scientific Committee on Consumer Safety.

SCCS. (2014). Memorandum on Relevance, Adequacy and Quality of Data in Safety Dossiers on Nanomaterials (No. SCCS/1524/13). Retrieved from https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_142.pdf

SCCS. (2015). Notes of guidance for the testing of cosmetic ingredients and their safety evaluation (No. SCCS/1564/15). Retrieved from http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf

SCENIHR. (2009). Risk Assessment of Products of Nanotechnologies. Brussels: Scientific Committee on Emerging and Newly Identified Health Risks. Retrieved from http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf

SCENIHR. (2010). Risks Scientific Basis for the Definition of the Term " nanomaterial ." Brussels. Retrieved from http://ec.europa.eu/health/archive/ph_risk/committees/04_scenihr/docs/scenihr_o_012.pdf

Schins, R. P. F. (2002). Mechanisms of genotoxicity of particles and fibers. Inhalation Toxicology, 14(1), 57–78. http://doi.org/10.1080/089583701753338631

Schins, R. P. F., & Knaapen, A. M. (2007). Genotoxicity of poorly soluble particles. Inhalation Toxicology, 19 Suppl 1(781062662), 189–98. http://doi.org/10.1080/08958370701496202

Schinwald, A., Murphy, F. A., Prina-Mello, A., Poland, C. A., Byrne, F., Movia, D., … Donaldson, K. (2012). The threshold length for fiber-induced acute pleural inflammation: Shedding light on the early events in asbestos-induced mesothelioma. Toxicological Sciences, 128(2), 461–470. http://doi.org/10.1093/toxsci/kfs171

Schröder, K., Pohlenz-Michel, C., Simetska, N., Voss, J. U., Escher, S., & Mangersdorf, I. (2014). Carcinogenicity and Mutagenicity of Nanoparticles – Assessment of Current Knowledge as Basis for Regulation. Hannover. Retrieved from http://www.umweltbundesamt.de/sites/default/files/medien/378/publikationen/texte_50_2014_ca rcinogenicity_and_mutagenicity_of_nanoparticles_1.pdf

Schultz, C., Powell, K., Crossley, A., Jurkschat, K., Kille, P., Morgan, A. J., … Spurgeon, D. J. (2014). Analytical approaches to support current understanding of exposure, uptake and distributions of engineered nanoparticles by aquatic and terrestrial organisms. Ecotoxicology (London, England), 24(2), 239–61. http://doi.org/10.1007/s10646-014-1387-3

Schultz, T. W., Amcoff, P., Berggren, E., Gautier, F., Klaric, M., Knight, D. J., … Cronin, M. T. D. (2015). A Strategy for Structuring and Reporting a Read-Across Prediction of Toxicity. Regulatory Toxicology and Pharmacology. http://doi.org/10.1016/j.yrtph.2015.05.016

Scientific Committee on Consumer Safety. (2015). Safety Silica , Hydrated Silica , and Silica Surface Modified with Alkyl Silylates (nano form). Retrieved from http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_175.pdf

Sellers, K., Deleebeek, N., Messiaen, M., Jackson, M., Bleeker, E., Sijm, D., & Broekhuizen, F. (2015). Grouping nanomaterials A strategy towards grouping and read-across (RIVM report No. 2015–61). Retrieved from http://www.nanowerk.com/nanotechnology-news/newsid=40311.php

Shao, C.-Y., Chen, S.-Z., Su, B.-H., Tseng, Y. J., Esposito, E. X., & Hopfinger, A. J. (2013). Dependence of QSAR models on the selection of trial descriptor sets: a demonstration using nanotoxicity endpoints of decorated nanotubes. Journal of Chemical Information and Modeling, 53(1), 142–58. http://doi.org/10.1021/ci3005308

Shaw, S. Y., Westly, E. C., Pittet, M. J., Subramanian, A., Schreiber, S. L., & Weissleder, R. (2008). Perturbational profiling of nanomaterial biologic activity. Proceedings of the National Academy of Sciences of the United States of America, 105(21), 7387–7392. http://doi.org/10.1073/pnas.0802878105

Shinohara, N., Gamo, M., & Nakanishi, J. (2009). Risk Assessment of Manufactured Nanomaterials - Fullerene (C 60). October.

Shinohara, N., Matsumoto, K., Endoh, S., Maru, J., & Nakanishi, J. (2009). In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. Toxicology Letters, 191(2–3), 289–96. http://doi.org/10.1016/j.toxlet.2009.09.012

Siegrist, K. J., Reynolds, S. H., Kashon, M. L., Lowry, D. T., Dong, C., Hubbs, A. F., … Sargent, L. M. (2014). Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses. Particle and Fibre Toxicology, 11(1), 6. http://doi.org/10.1186/1743-8977-11-6

Silva, T., Pokhrel, L. R., Dubey, B., Tolaymat, T. M., Maier, K. J., & Liu, X. (2014). Particle size, surface charge and concentration dependent ecotoxicity of three organo-coated silver nanoparticles: Comparison between general linear model-predicted and observed toxicity. Science of the Total Environment, 468–469, 968–976. http://doi.org/10.1016/j.scitotenv.2013.09.006

Simkó, M., Tischler, S., & Mattsson, M. O. (2015). Pooling and analysis of published in vitro data: A proof of concept study for the grouping of nanoparticles. International Journal of Molecular Sciences, 16(11), 26211–26236. http://doi.org/10.3390/ijms161125954

Sivaraman, N., Srinivasan, T. G., Vasudeva Rao, P. R., & Natarajan, R. (2001). QSPR Modeling for Solubility of Fullerene (C60) in Organic Solvents. Journal of Chemical Information and Computer Sciences, 41(4), 1067–1074. http://doi.org/10.1021/ci010003a

Sizochenko, N., Kuz'min, V., Ognichenko, L., & Leszczynski, J. (2016). Introduction of simplex-informational descriptors for QSPR analysis of fullerene derivatives. Journal of Mathematical Chemistry, 54(3), 698–706. http://doi.org/10.1007/s10910-015-0581-8

Sizochenko, N., & Leszczynski, J. (2016). Review of current and emerging approaches for quantitative nanostructure-activity relationship modeling – the case of inorganic nanoparticles. Journal of Nanotoxicology and Nanomedicine, 1(1), 1–16. http://doi.org/10.4018/JNN.2016010101

Sizochenko, N., Rasulev, B., Gajewicz, A., Kuz'Min, V., Puzyn, T., & Leszczynski, J. (2014). From basic physics to mechanisms of toxicity: The "liquid drop" approach applied to develop predictive classification models for toxicity of metal oxide nanoparticles. Nanoscale, 6(22), 13986–13993. http://doi.org/10.1039/c4nr03487b

Sizochenko, N., Rasulev, B., Gajewicz, A., Mokshyna, E., Kuz'min, V. E., Leszczynski, J., & Puzyn, T. (2015). Causal inference methods to assist in mechanistic interpretation of classification nano-SAR models. RSC Advances, 5(95), 77739–77745. http://doi.org/10.1039/c5ra11399g

Smoluchowski, M. V. (1917). Versuch einer mathematischen Theorie der Koagulationskinetik. Zeitschrift Für Physikalische Chemie, 92, 129–168.

Soliman, M. M., Attia, H. F., Hussein, M. M., Mohamed, E. H., & Ismail, T. A. (2013). Protective effect of N-Acetylcystiene aganst titanium dioxide nanoparticles modulate immune responses in male albino rats. American Journal of Immunology, 9(4), 148–158. http://doi.org/10.3844/ajisp.2013.148.158

Stefaniak, A., Hoover, M., Day, G., Ekechukwu, A., Whitney, G., Brink, C., & Scripsick, R. (2005). Characteristics of Beryllium Oxide and Beryllium Metal Powders for Use as Reference Materials. Journal of ASTM International, 2(10), 15. http://doi.org/10.1520/JAI13174

Stern, S. T., & McNeil, S. E. (2008). Nanotechnology safety concerns revisited. Toxicological Sciences : An Official Journal of the Society of Toxicology, 101(1), 4–21. http://doi.org/10.1093/toxsci/kfm169

Stoccoro, A., Di Bucchianico, S., Uboldi, C., Coppedè, F., Ponti, J., Placidi, C., … Migliore, L. (2016). A panel of in vitro tests to evaluate genotoxic and morphological neoplastic transformation potential on Balb/3T3 cells by pristine and remediated titania and zirconia nanoparticles. Mutagenesis. http://doi.org/10.1093/mutage/gew015

Stone, A. V., Önlü, S., Bergamaschi, E., Carlander, D., Costa, A., Engelmann, W., … Wohlleben, W. (2017). Research priorities relevant to development or updating of nano-relevant regulations and guidelines. Retrieved from https://www.nanosafetycluster.eu/uploads/files/pdf/RRR_Final_version_090317.pdf

Stone, U. K. V., Balharry, D., Fernandes, T., Johnston, H., Munro, L., & Hartl, M. (2013). RESEARCH PRIORITISATION TO DELIVER AN INTELLIGENT TESTING STRATEGY FOR THE HUMAN AND ENVIRONMENTAL SAFETY OF NANOMATERIALS.

Stone, U. K. V., Balharry, D., Fernandes, T., Johnston, H., Munro, L., Hartl, M., & V. Stone, S. Pozzi-Mucelli, L. Tran, K. Aschberger, S. Sabella, U.B. Vogel, C. Poland, D. Balharry, T. Fernandes, S. Gottardo, S. Hankin, M. Hartl, N. Hartmann, D. Hristozov, K. Hund-Rinke, H. Johnston, A. Marcomini, O. Panzer, D. Roncato, A.T. Saber, H. W. and J. J. S.-F. (2013). RESEARCH PRIORITISATION TO DELIVER AN INTELLIGENT TESTING STRATEGY FOR THE HUMAN AND ENVIRONMENTAL SAFETY OF NANOMATERIALS AS SE SM. Retrieved from www.its-nano.eu

Stone, V., Johnston, H., & Schins, R. P. F. (2009). Development of in vitro systems for nanotoxicology: methodological considerations. Critical Reviews in Toxicology, 39(7), 613–26. http://doi.org/10.1080/10408440903120975

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., … Scott-Fordsmand, J. J. (2014). ITS-NANO--prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Particle and Fibre Toxicology, 11, 9. http://doi.org/10.1186/1743-8977-11-9

Sun, T. Y., Bornhöft, N. A., Hungerbühler, K., & Nowack, B. (2016). Dynamic Probabilistic Modeling of Environmental Emissions of Engineered Nanomaterials. Environmental Science and Technology, 50(9), 4701–4711. http://doi.org/10.1021/acs.est.5b05828

Sun, T. Y., Conroy, G., Donner, E., Hungerbühler, K., Lombi, E., & Nowack, B. (2015). Probabilistic modelling of engineered nanomaterial emissions to the environment: A spatio-temporal approach. Environmental Science: Nano, 2(4), 340–351. http://doi.org/10.1039/c5en00004a

Sun, T. Y., Gottschalk, F., Hungerbühler, K., & Nowack, B. (2014). Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. Environmental Pollution, 185, 69–76. http://doi.org/10.1016/j.envpol.2013.10.004

Suzuki, T., Miura, N., Hojo, R., Yanagiba, Y., Suda, M., Hasegawa, T., … Wang, R.-S. (2016). Genotoxity assessment of intravenously injected titanium dioxide nanoparticles in gpt delta transgenic mice. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 802, 30–37. http://doi.org/10.1016/j.mrgentox.2016.03.007

Sweeney, S., Grandolfo, D., Ruenraroengsak, P., & Tetley, T. D. (2015). Functional consequences for primary human alveolar macrophages following treatment with long, but not short, multiwalled carbon nanotubes. International Journal of Nanomedicine, 10, 3115–3129. http://doi.org/10.2147/IJN.S77867

Sycheva, L. P., Zhurkov, V. S., Iurchenko, V. V, Daugel-Dauge, N. O., Kovalenko, M. A., Krivtsova, E. K., & Durnev, A. D. (2011). Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo. Mutation Research, 726(1), 8–14. http://doi.org/10.1016/j.mrgentox.2011.07.010

Taft, W. (1956). Steric Effects in Organic Chemistry. (M. S. Newman, Ed.). New York: John Wiley & Sons Inc.

Taghavy, A., Mittelman, A., Wang, Y., Pennell, K. D., & Abriola, L. M. (2013). Mathematical modeling of the transport and dissolution of citrate-stabilized silver nanoparticles in porous media. Environmental Science and Technology, 47(15), 8499–8507. http://doi.org/10.1021/es400692r

Taghavy, A., Pennell, K. D., & Abriola, L. M. (2015). Modeling coupled nanoparticle aggregation and transport in porous media: A Lagrangian approach. Journal of Contaminant Hydrology, 172, 48–60. http://doi.org/10.1016/j.jconhyd.2014.10.012

Tantra, R., Oksel, C., Puzyn, T., Wang, J., Robinson, K. N., Wang, X. Z., … Wilkins, T. (2014). Nano(Q)SAR: Challenges, pitfalls and perspectives. Nanotoxicology, 1–7. http://doi.org/10.3109/17435390.2014.952698

Tantra, R., Oksel, C., Puzyn, T., Wang, J., Robinson, K. N., Wang, X. Z., … Wilkins, T. (2015). Nano(Q)SAR: Challenges, pitfalls and perspectives. Nanotoxicology, 9(5), 636–642. http://doi.org/10.3109/17435390.2014.952698

Tavares, A. M., Louro, H., Antunes, S., Quarré, S., Simar, S., De Temmerman, P.-J., … Silva, M. J. (2014). Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. Toxicology in Vitro, 28(1), 60–69. http://doi.org/10.1016/j.tiv.2013.06.009

Teeguarden, J. G., Hinderliter, P. M., Orr, G., Thrall, B. D., & Pounds, J. G. (2007). Particokinetics In Vitro: Dosimetry Considerations for In Vitro Nanoparticle Toxicology Assessments. Toxicological Sciences, 97(2), 614–614. http://doi.org/10.1093/toxsci/kfm097

Tervonen, K., Waissi, G., Petersen, E. J., Akkanen, J., & Kukkonen, J. V. K. (2010). Analysis of fullerene-c60 and kinetic measurements for its accumulation and depuration in Daphnia magna. Environmental Toxicology and Chemistry, 29(5), 1072–1078. http://doi.org/10.1002/etc.124

Thomas, D. G., Chikkagoudar, S., Chappell, A. R., & Baker, N. A. (2012). Annotating the structure and components of a nanoparticle formulation using computable string expressions. Proceedings. IEEE International Conference on Bioinformatics and Biomedicine, 2012, 889–894. http://doi.org/10.1109/BIBMW.2012.6470259

Tiede, K., Hanssen, S. F., Westerhoff, P., Fern, G. J., Hankin, S. M., Aitken, R. J., … Boxall, A. B. A. (2016). How important is drinking water exposure for the risks of engineered nanoparticles to consumers? Nanotoxicology, 10(1), 102–110. http://doi.org/10.3109/17435390.2015.1022888

Todeschini, R., & Consonni Viviana. (2009). Molecular Descriptors for Chemoinformatics. (R. Mannhold, H. Kubinyi, & G. Folkers, Eds.) (2nd ed.). Wiley.

Topliss, J. G., & Edwards, R. P. (1979). Chance factors in studies of quantitative structure-activity relationships. Journal of Medicinal Chemistry, 22(10), 1238–1244. http://doi.org/10.1021/jm00196a017

Toropov, A. A., Leszczynska, D., & Leszczynski, J. (2007). Predicting water solubility and octanol water partition coefficient for carbon nanotubes based on the chiral vector. Computational Biology and Chemistry, 31(2), 127–8. http://doi.org/10.1016/j.compbiolchem.2007.02.002

Toropov, A. A., & Leszczynski, J. (2006). A new approach to the characterization of nanomaterials: Predicting Young's modulus by correlation weighting of nanomaterials codes. Chemical Physics Letters, 433(1–3), 125–129. http://doi.org/10.1016/j.cplett.2006.11.010

Toropov, A. A., Rallo, R., & Toropova, A. P. (2015). Use of Quasi-SMILES and monte carlo optimization to develop quantitative feature property/activity relationships (QFPR/QFAR) for nanomaterials. Current Topics in Medicinal Chemistry, 15(18), 1837–1844. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-84934783593&partnerID=tZOtx3y1

Toropov, A. A., Rasulev, B. F., Leszczynska, D., & Leszczynski, J. (2008). Multiplicative SMILES-based optimal descriptors: QSPR modeling of fullerene C60 solubility in organic solvents. Chemical Physics Letters, 457(4–6), 332–336. http://doi.org/10.1016/j.cplett.2008.04.013

Toropov, A. A., & Toropova, A. P. (2015a). Quasi-QSAR for mutagenic potential of multi-walled carbon-nanotubes. Chemosphere, 124(1), 40–46. http://doi.org/10.1016/j.chemosphere.2014.10.067

Toropov, A. A., & Toropova, A. P. (2015b). Quasi-SMILES and nano-QFAR: United model for mutagenicity of fullerene and MWCNT under different conditions. Chemosphere, 139, 18–22. http://doi.org/10.1016/j.chemosphere.2015.05.042

Toropov, A. A., Toropova, A. P., Benfenati, E., Gini, G., Puzyn, T., Leszczynska, D., & Leszczynski, J. (2012). Novel application of the CORAL software to model cytotoxicity of metal oxide nanoparticles to bacteria Escherichia coli. Chemosphere, 89(9), 1098–1102. http://doi.org/10.1016/j.chemosphere.2012.05.077

Toropov, A. A., Toropova, A. P., Benfenati, E., Leszczynska, D., & Leszczynski, J. (2009). Additive InChI-based optimal descriptors: QSPR modeling of fullerene C 60 solubility in organic solvents. Journal of Mathematical Chemistry, 46(4), 1232–1251. http://doi.org/10.1007/s10910-008-9514-0

Toropov, A. A., Toropova, A. P., Puzyn, T., Benfenati, E., Gini, G., Leszczynska, D., & Leszczynski, J. (2013). QSAR as a random event: Modeling of nanoparticles uptake in PaCa2 cancer cells. Chemosphere, 92(1), 31–37. http://doi.org/10.1016/j.chemosphere.2013.03.012

Toropova, A. P., & Toropov, A. A. (2013). Optimal descriptor as a translator of eclectic information into the prediction of membrane damage by means of various TiO2 nanoparticles. Chemosphere, 93(10), 2650–2655. http://doi.org/10.1016/j.chemosphere.2013.09.089

Toropova, A. P., Toropov, A. A., Manganelli, S., Leone, C., Baderna, D., Benfenati, E., & Fanelli, R. (2016). Quasi-SMILES as a tool to utilize eclectic data for predicting the behavior of nanomaterials. NanoImpact, 1, 60–64. http://doi.org/10.1016/j.impact.2016.04.003

Tosco, T., & Sethi, R. (2009). MNM1D: A numerical code for colloid transport in porous media: Implementation and validation. American Journal of Environmental Sciences, 5(4), 516–524. http://doi.org/10.3844/ajessp.2009.516.524

Tournebize, J., Sapin-Minet, A., Bartosz, G., Leroy, P., & Boudier, A. (2013). Pitfalls of assays devoted to evaluation of oxidative stress induced by inorganic nanoparticles. Talanta. http://doi.org/10.1016/j.talanta.2013.07.077

Tran, C. L., Buchanan, D., Cullen, R. T., Searl, A., & Jones, A. D. (2000). Inhalation of poorly soluble particles. II Influence of particle surface area on inflammation and clearence. Inhalation Toxicology, 12, 1113–1126.

Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., & Schiestl, R. H. (2009). Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Research, 69(22), 8784–9. http://doi.org/10.1158/0008-5472.CAN-09-2496

Tschopp, J., & Schroder, K. (2010). NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nature Reviews. Immunology, 10(3), 210–5. http://doi.org/10.1038/nri2725

Tufenkji, N., & Elimelech, M. (2004). Correlation Equation for Predicting Single-Collector Efficiency in Physicochemical Filtration in Saturated Porous Media. Environmental Science & Technology, 38(2), 529–536.

UBA, BfR, & BAuA. (2011). Nanomaterials and REACH Background Paper on the Position of German Competent Authorities. Retrieved from http://www.reach-clp-biozid-helpdesk.de/de/Downloads/Hintergrundpapier Nano und REACH engl. Version.pdf?__blob=publicationFile

Uboldi, C., Urbán, P., Gilliland, D., Bajak, E., Valsami-Jones, E., Ponti, J., & Rossi, F. (2016). Role of the crystalline form of titanium dioxide nanoparticles: Rutile, and not anatase, induces toxic effects in Balb/3T3 mouse fibroblasts. Toxicology in Vitro : An International Journal Published in Association with BIBRA, 31, 137–45. http://doi.org/10.1016/j.tiv.2015.11.005

Union, C. of the E., & Council of the European Union. (1991). Council Directive of 15 July 1991 concerning the placing of plant protection products on the market (91/414/EEC). Official Journal of the European Union, L230(L 230), 290.

US-EPA, & EPA. (2013). Comprehensive Environmental Assessment Applied to: Multiwalled Carbon Nanotube Flame-Retardant Coatings in Upholstery Textiles - A Case Study Presenting Priority Research Gaps for Future Risk Assessments. EPA/600/R-12/043F (Vol. EPA/600/R-). Washington DC: U.S. Environmental Protection Agency. Retrieved from http://cfpub.epa.gov/ncea/nano/recordisplay.cfm?deid=253010

US EPA. (2014). U.S. Environmental Protection Agency (USEPA). 2014. Risk Assessment Forum White Paper: Probabilistic Risk Assessment Methods and Case Studies. EPA/100/R‐09/001A. Washington, D.C.: Risk Assessment Forum, Office of the Science Advisor, USEPA.

Utembe, W., Potgieter, K., Stefaniak, A. B., & Gulumian, M. (2015). Dissolution and biodurability: Important parameters needed for risk assessment of nanomaterials. Particle and Fibre Toxicology, 12, 11. http://doi.org/10.1186/s12989-015-0088-2

Vales, G., Rubio, L., & Marcos, R. (2014). Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells. Nanotoxicology, 9(5), 568–578. http://doi.org/10.3109/17435390.2014.957252

Van Berlo, D., Clift, M., Albrecht, C., & Schins, R. (2012). Carbon nanotubes: An insight into the mechanisms of their potential genotoxicity. Swiss Medical Weekly. http://doi.org/10.4414/smw.2012.13698

van der Zande, M., Vandebriel, R. J., Groot, M. J., Kramer, E., Herrera Rivera, Z. E., Rasmussen, K., … Bouwmeester, H. (2014). Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Particle and Fibre Toxicology, 11(1), 8. http://doi.org/10.1186/1743-8977-11-8

Van Duuren-Stuurman, B., Vink, S. R., Verbist, K. J. M., Heussen, H. G. A., Brouwer, D. H., Kroese, D. E. D., … Fransman, W. (2012). Stoffenmanager Nano Version 1.0: A Web-Based Tool for Risk Prioritization of Airborne Manufactured Nano Objects. Annals of Occupational Hygiene, 56, 1–17. http://doi.org/10.1093/annhyg/mer113

van Kesteren, P. C. E., Cubadda, F., Bouwmeester, H., van Eijkeren, J. C. H., Dekkers, S., de Jong, W. H., & Oomen, A. G. (2014). Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology, 0(0), 1–10. http://doi.org/10.3109/17435390.2014.940408

Vandebriel, R. J., & De Jong, W. H. (2012). A review of mammalian toxicity of ZnO nanoparticles. Nanotechnology, Science and Applications, 5, 61–71. http://doi.org/10.2147/NSA.S23932

Vietti, G., Lison, D., & van den Brule, S. (2016). Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). Particle and Fibre Toxicology, 13(1), 11. http://doi.org/10.1186/s12989-016-0123-y

Vilanova, O., Franzese, G., & Barnabei, M. (2014). BUBBLES User-friendly Bundle for Bio-nano Large-scale Efficient Simulations. Retrieved from https://github.com/bubbles-suite/BUBBLES/wiki

Vilaseca, P., Dawson, K. A., & Franzese, G. (2013). Understanding and modulating the competitive surface-adsorption of proteins through coarse-grained molecular dynamics simulations. Soft Matter, 9(29), 6978. http://doi.org/10.1039/c3sm50220a

Vroman, L., & Adams, A. L. (1969). Findings with the recording ellipsometer suggesting rapid exchange of specific plasma proteins at liquid/solid interfaces. Surface Science, 16, 438–446. http://doi.org/10.1016/0039-6028(69)90037-5

Walkey, C. D., Olsen, J. B., Song, F., Liu, R., Guo, H., Olsen, D. W. H., … Chan, W. C. W. (2014). Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. ACS Nano, 8(3), 2439–2455. http://doi.org/10.1021/nn406018q

Wang, F., Yu, L., Monopoli, M. P., Sandin, P., Mahon, E., Salvati, A., & Dawson, K. A. (2013). The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine : Nanotechnology, Biology, and Medicine, 9(8), 1159–68. http://doi.org/10.1016/j.nano.2013.04.010

Wang, X., Xia, T., Addo Ntim, S., Ji, Z., Lin, S., Meng, H., … Nel, A. E. (2011). Dispersal state of multiwalled carbon nanotubes elicits profibrogenic cellular responses that correlate with fibrogenesis biomarkers and fibrosis in the murine lung. ACS Nano, 5(12), 9772–9787. http://doi.org/10.1021/nn2033055

Wang, Y., Kalinina, A., Sun, T., & Nowack, B. (2016). Probabilistic modeling of the flows and environmental risks of nano-silica. Science of the Total Environment, 545–546, 67–76. http://doi.org/10.1016/j.scitotenv.2015.12.100

Wang, Y., Li, Y., Fortner, J. D., Hughes, J. B., Abriola, L. M., & Pennell, K. D. (2008). Transport and retention of nanoscale C60 aggregates in water-saturated porous media. Environmental Science & Technology, 42(10), 3588–3594.

Weed, D. L. (2005). Weight of evidence: a review of concept and methods. Risk Analysis : An Official Publication of the Society for Risk Analysis, 25(6), 1545–57. http://doi.org/10.1111/j.1539-6924.2005.00699.x

Wei, Y.-T., & Wu, S.-C. (2010). Development of a trajectory model for predicting attachment of submicrometer particles in porous media: Stabilized NZVI as a case study. Environmental Science and Technology, 44(23), 8996–9002. http://doi.org/10.1021/es102191b

Weissleder, R., Kelly, K., Sun, E. Y., Shtatland, T., & Josephson, L. (2005). Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. Nature Biotechnology, 23(11), 1418–23. http://doi.org/10.1038/nbt1159

Wen DAI, Xue-Ying SHAN, G.-Y. He. and H.-Q. C. (2015). PREDICTION FOR CELLULAR UPTAKE OF MANUFACTURED NANOPARTICLES TO PANCREATIC CANCER CELLS. Revue Roumaine de Chimie, 60(4), 367–370.

WHO. (2012). Nanotechnology and human health : Scientific evidence and risk governance. Bonn Germany.

WHO. (2014). GUIDANCE DOCUMENT ON EVALUATING AND EXPRESSING UNCERTAINTY IN HAZARD CHARACTERIZATION. Retrieved from http://www.who.int/ipcs/methods/harmonization/uncertainty_in_hazard_characterization.pdf

Wigger, H., Hackmann, S., Zimmermann, Köser, J., Thöming, J., & von Gleich, A. (2015). Influences of use activities and waste management on environmental releases of engineered nanomaterials. Science of the Total Environment, 535, 160–171. http://doi.org/10.1016/j.scitotenv.2015.02.042

Winkler, D. A., Burden, F. R., Yan, B., Weissleder, R., Tassa, C., Shaw, S., & Epa, V. C. (2014). Modelling and predicting the biological effects of nanomaterials. SAR and QSAR in Environmental Research, 25(2), 161–72. http://doi.org/10.1080/1062936X.2013.874367

Winkler, D. A., Mombelli, E., Pietroiusti, A., Tran, L., Worth, A., Fadeel, B., & McCall, M. J. (2013). Applying quantitative structure-activity relationship approaches to nanotoxicology: Current status and future potential. Toxicology, 313(1), 15–23. http://doi.org/10.1016/j.tox.2012.11.005

Winter, M., Beer, H.-D., Hornung, V., Krämer, U., Schins, R. P. F., & Förster, I. (2011). Activation of the inflammasome by amorphous silica and TiO2 nanoparticles in murine dendritic cells. Nanotoxicology, 5(3), 326–40. http://doi.org/10.3109/17435390.2010.506957

Wirnitzer, U., Herbold, B., Voetz, M., & Ragot, J. (2009). Studies on the in vitro genotoxicity of baytubes®, agglomerates of engineered multi-walled carbon-nanotubes (MWCNT). Toxicology Letters, 186(3), 160–165. http://doi.org/10.1016/j.toxlet.2008.11.024

Wohlleben, W. (2012). Validity range of centrifuges for the regulation of nanomaterials: from classification to as-tested coronas. Journal of Nanoparticle Research : An Interdisciplinary Forum for Nanoscale Science and Technology, 14(12), 1300. http://doi.org/10.1007/s11051-012-1300-z

Wohlleben, W., Ma-Hock, L., Boyko, V., Cox, G., Egenolf, H., Freiberger, H., … Landsiedel, R. (2013). Nanospecific guidance in REACH: A comparative physical-chemical characterization of 15 materials with methodical correlations. Journal of Ceramic Science and Technology, 4(August), 93–104. http://doi.org/10.4416/JCST2012-00045

Wolfgang Koch, W. S. (2008). A SIMPLE PULMONARY RETENTION MODEL ACCOUNTING FOR DISSOLUTION AND MACROPHAGE-MEDIATED REMOVAL OF DEPOSITED POLYDISPERSE PARTICLES. Retrieved from http://informahealthcare.com/doi/abs/10.1080/089583701300001069

Wolfram, J., Yang, Y., Shen, J., Moten, A., Chen, C., Shen, H., … Zhao, Y. (2014). The nano-plasma interface: Implications of the protein corona. Colloids and Surfaces. B, Biointerfaces. http://doi.org/10.1016/j.colsurfb.2014.02.035

Wu, S., Blackburn, K., Amburgey, J., Jaworska, J., & Federle, T. (2010). A framework for using structural, reactivity, metabolic and physicochemical similarity to evaluate the suitability of analogs for SAR-based toxicological assessments. Regulatory Toxicology and Pharmacology : RTP, 56(1), 67–81. http://doi.org/10.1016/j.yrtph.2009.09.006

Xia, X.-R., Monteiro-Riviere, N. a, & Riviere, J. E. (2010). An index for characterization of nanomaterials in biological systems. Nature Nanotechnology, 5(9), 671–5. http://doi.org/10.1038/nnano.2010.164

Xia, X. R., Monteiro-Riviere, N. A., Mathur, S., Song, X., Xiao, L., Oldenberg, S. J., … Riviere, J. E. (2011). Mapping the surface adsorption forces of nanomaterials in biological systems. ACS Nano, 5(11), 9074–9081. http://doi.org/10.1021/nn203303c

Yang, S.-T., Liu, Y., Wang, Y.-W., & Cao, A. (2013). Biosafety and bioapplication of nanomaterials by designing protein-nanoparticle interactions. Small (Weinheim an Der Bergstrasse, Germany), 9(9–10), 1635–53. http://doi.org/10.1002/smll.201201492

Yang, W., Peters, J. I., & Williams, R. O. (2008). Inhaled nanoparticles--a current review. International Journal of Pharmaceutics, 356(1–2), 239–47. http://doi.org/10.1016/j.ijpharm.2008.02.011

Yao, K.-M., Habibian, M. T., & O'Melia, C. R. (1971). Water and waste water filtration. Concepts and applications. Environmental Science & Technology, 5(11), 1105–1112.

Yilmaz, H., Rasulev, B., & Leszczynski, J. (2015). Modeling the dispersibility of single walled carbon nanotubes in organic solvents by quantitative structure-activity relationship approach. Nanomaterials, 5(2), 778–791. http://doi.org/10.3390/nano5020778

Ying, J., Zhang, T., & Tang, M. (2015). Metal oxide nanomaterial QNAR models: Available structural descriptors and understanding of toxicity mechanisms. Nanomaterials, 5(4), 1620–1637. http://doi.org/10.3390/nano5041620

Yousefinejad, S., Honarasa, F., Abbasitabar, F., & Arianezhad, Z. (2013). New LSER model based on solvent empirical parameters for the prediction and description of the solubility of buckminsterfullerene in various solvents. Journal of Solution Chemistry, 42(8), 1620–1632. http://doi.org/10.1007/s10953-013-0062-2

Zalk, D. M., Paik, S. Y., & Swuste, P. (2009). Evaluating the Control Banding Nanotool: a qualitative risk assessment method for controlling nanoparticle exposures. Journal of Nanoparticle Research, 11(7), 1685–1704. http://doi.org/10.1007/s11051-009-9678-y

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., … Nel, A. E. (2012). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano, 6(5), 4349–4368. http://doi.org/10.1021/nn3010087

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., … Nel, A. E. (2012a). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano, 6(5), 4349–68. http://doi.org/10.1021/nn3010087

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., … Nel, A. E. (2012b). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano, 6(5), 4349–68. http://doi.org/10.1021/nn3010087

Zhang, W., Crittenden, J., Li, K., & Chen, Y. (2012). Attachment efficiency of nanoparticle aggregation in aqueous dispersions: modeling and experimental validation. Environmental Science & Technology, 46(13), 7054–62. http://doi.org/10.1021/es203623z

Zhang, W., Rittmann, B., & Chen, Y. (2011). Size effects on adsorption of hematite nanoparticles on E. coli cells. Environmental Science & Technology, 45(6), 2172–8. http://doi.org/10.1021/es103376y

Zhang, W., Yao, Y., Sullivan, N., & Chen, Y. (2011). Modeling the primary size effects of citrate-coated silver nanoparticles on their ion release kinetics. Environmental Science and Technology, 45(10), 4422–4428. http://doi.org/10.1021/es104205a

Zhao, C.-M., & Wang, W.-X. (2010). Biokinetic uptake and efflux of silver nanoparticles in Daphnia magna. Environmental Science and Technology, 44(19), 7699–7704. http://doi.org/10.1021/es101484s

Zhou, H., Mu, Q., Gao, N., Liu, A., Xing, Y., Gao, S., … Yan, B. (2008). A nano-combinatorial library strategy for the discovery of nanotubes with reduced protein-binding, cytotoxicity, and immune response. Nano Letters, 8(3), 859–65. http://doi.org/10.1021/nl0730155

Zhou, Z., Son, J., Harper, B., Zhou, Z., & Harper, S. (2015a). Influence of surface chemical properties on the toxicity of engineered zinc oxide nanoparticles to embryonic zebrafish. Beilstein Journal of Nanotechnology, 6(1), 1568–1579. http://doi.org/10.3762/bjnano.6.160

Zhou, Z., Son, J., Harper, B., Zhou, Z., & Harper, S. (2015b). Influence of surface chemical properties on the toxicity of engineered zinc oxide nanoparticles to embryonic zebrafish. Beilstein Journal of Nanotechnology, 6(1), 1568–1579. http://doi.org/10.3762/bjnano.6.160

Ziemann, C., Rittinghausen, S., Ernst, H., Kolling, A., Mangelsdorf, I., & Creutzenberg, O. (2011). Genotoxic Mode of Action of Fine and Ultrafine Dusts in Lungs, 107. Retrieved from http://www.baua.de/de/Publikationen/Fachbeitraege/F2135.pdf?__blob=publicationFile&v=2

3.5.2 PBK, PBD and dosimetry models

Anjilvel S, Asgharian B. A multiple-path model of particle deposition in the rat lung. Fundam Appl Toxicol. 1995;28(1):41-50. http://www.ncbi.nlm.nih.gov/pubmed/8566482.

Bachler G, Von Goetz N, Hungerbuhler K. Using physiologically based pharmacokinetic (PBPK) modeling for dietary risk assessment of titanium dioxide (TiO$_2$) nanoparticles. Nanotoxicology. 2015;9(3):373-380. doi:10.3109/17435390.2014.940404.

Bachler G, von Goetz N, Hungerbühler K. A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. Int J Nanomedicine. 2013;8:3365-3382. doi:10.2147/IJN.S46624.

Cohen J, Deloid G, Pyrgiotakis G, Demokritou P. Interactions of engineered nanomaterials in physiological media and implications for in vitro dosimetry. Nanotoxicology. 2013;7(4):417-431. doi:10.3109/17435390.2012.666576.

Cohen, J. M., Teeguarden, J. G., & Demokritou, P. (2014). An integrated approach for the in vitro dosimetry of engineered nanomaterials. http://doi.org/10.1186/1743-8977-11-20

Cohen JM, DeLoid GM, Demokritou P. A critical review of in vitro dosimetry for engineered nanomaterials. Nanomedicine. 2015;10(19):3015-3032. doi:10.2217/nnm.15.129.

Deloid, G., Cohen, J. M., Darrah, T., Derk, R., Wang, L., Pyrgiotakis, G., … Demokritou, P. (2014). Estimating the effective density of engineered nanomaterials for in vitro dosimetry. Nat. Commun. 5:3514. http://doi.org/10.1038/ncomms4514.

Demin VA, Gmoshinsky I V, Demin VF, et al. Modeling interorgan distribution and bioaccumulation of engineered nanoparticles (using the example of silver nanoparticles). Nanotechnologies Russ. 2015;10(3-4):288-296. doi:10.1134/S1995078015020081.

EMA/CHMP/458101/2016 July 2016 Committee for Medicinal Products for Human Use (CHMP) Guideline on the qualification and reporting of physiologically based pharmacokinetic (PBPK) modelling and simulation.

Fallon MS, Varshney M, Dennis DM, Chauhan A. A physiologically-based pharmacokinetic model of drug detoxification by nanoparticles. J Pharmacokinet Pharmacodyn. 2004;31(5):381-400. doi:10.1007/s10928-004-8322-9.

Fan, W., Liu, L., Peng, R., & Wang, W.-X. (2016). High bioconcentration of titanium dioxide nanoparticles in Daphnia magna determined by kinetic approach. Science of the Total Environment. Department of Environmental Science and Engineering, School of Space and Environment, Beihang University, Beijing 100191, PR China. http://doi.org/10.1016/j.scitotenv.2016.06.197

Garcia GJ, Kimbell JS. Deposition of inhaled nanoparticles in the rat nasal passages: dose to the olfactory region. Inhal Toxicol. 2009;21(14):1165-1175.

Henry FS, Tsuda A. Onset of alveolar recirculation in the developing lungs and its consequence on nanoparticle deposition in the pulmonary acinus. J Appl Physiol. 2016;120(1):38-54. doi:10.1152/japplphysiol.01161.2014.

Hinderliter PM, Minard KR, Orr G, et al. ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. Part Fibre Toxicol. 2010;7(1):36. doi:10.1186/1743-8977-7-36.

Kolanjiyil A V, Kleinstreuer C. Nanoparticle mass transfer from lung airways to systemic regions--Part I: Whole-lung aerosol dynamics. J Biomech Eng. 2013;135(12):121003. doi:10.1115/1.4025332.

Li D, Johanson G, Emond C, Carlander U, Philbert M, Jolliet O. Physiologically based pharmacokinetic modeling of polyethylene glycol-coated polyacrylamide nanoparticles in rats. Nanotoxicology.

2014;8(SUPPL. 1):128-137. doi:10.3109/17435390.2013.863406.

Li M, Reineke J. Mathematical modelling of nanoparticle biodistribution: Extrapolation among intravenous, oral and pulmonary administration routes. Int J Nano Biomater. 2011;3(3):222-238. doi:10.1504/IJNBM.2011.042131.

Lin Z, Monteiro-Riviere NA, Riviere JE. A physiologically based pharmacokinetic model for polyethylene glycol-coated gold nanoparticles of different sizes in adult mice. Nanotoxicology. 2016;10(2):162-172. doi:10.3109/17435390.2015.1027314.

Liang X, Wang H, Grice JE, et al. Physiologically Based Pharmacokinetic Model for Long-Circulating Inorganic Nanoparticles. Nano Lett. 2016;16(2):939-945. doi:10.1021/acs.nanolett.5b03854.

Maher MA, Naha PC, Mukherjee SP, Byrne HJ. Numerical simulations of in vitro nanoparticle toxicity - the case of poly(amido amine) dendrimers. Toxicol Vitr. 2014;28(8):1449-1460. doi:10.1016/j.tiv.2014.07.014.

Mahnama A, Ghorbaniasl G, Allaei SM V, Nourbakhsh A. Semi-analytical solution for the in-vitro sedimentation, diffusion and dosimetry model: Surveying the impact of the Peclet number. Colloids Surfaces B Biointerfaces. 2014;122:324-331. doi:10.1016/j.colsurfb.2014.06.063.

Mukherjee D, Botelho D, Gow AJ, Zhang J, Georgopoulos PG. Computational multiscale toxicodynamic modeling of silver and carbon nanoparticle effects on mouse lung function. PLoS One. 2013;8(12). doi:10.1371/journal.pone.0080917.

Mukherjee D, Leo BF, Royce SG, et al. Modeling physicochemical interactions affecting in vitro cellular dosimetry of engineered nanomaterials: application to nanosilver. J Nanoparticle Res. 2014;16(10). doi:10.1007/s11051-014-2616-7. Mukherjee et al., 2014 (a)

Mukherjee D, Royce SG, Sarkar S, et al. Modeling in vitro cellular responses to silver nanoparticles. J Toxicol. 2014; doi:10.1155/2014/852890. Mukherjee et al., 2014 (b)

Mukherjee D, Porter A, Ryan M, et al. Modeling in vivo interactions of engineered nanoparticles in the pulmonary alveolar lining fluid. Nanomaterials. 2015;5(3):1223-1249. doi:10.3390/nano5031223.

Péry ARR, Brochot C, Hoet PHM, Nemmar A, Bois FY. Development of a physiologically based kinetic model for 99m-Technetium-labelled carbon nanoparticles inhaled by humans Human PBPK model for carbon nanoparticles. Inhal Toxicol. 2009;21(13):1099-1107. doi:10.3109/08958370902748542.

Sahneh FD, Scoglio CM, Monteiro-Riviere NA, Riviere JE. Predicting the impact of biocorona formation kinetics on interspecies extrapolations of nanoparticle biodistribution modeling. Nanomedicine. 2015;10(1):25-33. doi:10.2217/nnm.14.60.

Shelley ML, Wagner AJ, Hussain SM, Bleckmann C. Modeling the in vivo case with in vitro nanotoxicity data. Int J Toxicol. 2008;27(5):359-367. doi:10.1080/10915810802503487.

Schroeter JD, Asgharian B, Price OT, McClellan GE. Computational fluid dynamics simulations of inhaled nano- and microparticle deposition in the rhesus monkey nasal passages. Inhal Toxicol. 2013;25(12):691-701. doi:10.3109/08958378.2013.835889.

Su D, Ma R, Salloum M, Zhu L. Multi-scale study of nanoparticle transport and deposition in tissues during an injection process. Med Biol Eng Comput. 2010;48(9):853-863. doi:10.1007/s11517-010-0615-0.

Teeguarden, J. G., Mikheev, V. B., Minard, K. R., Forsythe, W. C., Wang, W., Sharma, G., … Thrall, B. D. (2014). Comparative iron oxide nanoparticle cellular dosimetry and response in mice by the inhalation and liquid cell culture exposure routes, 1–18. http://doi.org/10.1186/s12989-014-0046-4.

Tien N-C, Jen C-P. Analytical model for nanotoxic assessment of a human respiratory system. J Wuhan Univ Technol Mater Sci Ed. 2010;25(6):903-908.

van Kesteren PCE, Cubadda F, Bouwmeester H, et al. Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology. 2015;9(4):442-452. doi:10.3109/17435390.2014.940408.

Zhang Z, Kleinstreuer C. Computational analysis of airflow and nanoparticle deposition in a combined nasal–oral–tracheobronchial airway model. J Aerosol Sci. 2011;42(3):174-194. doi:10.1016/j.jaerosci.2011.01.001.

4 Case studies on grouping and read-across between nanoforms

In this chapter we report two case studies on the application of read-across between nanoforms of the same substance. The aims of this exercise were to:

- evaluate the applicability of the workflow for grouping and read-across proposed in the draft REACH guidance update

- illustrate how computational methods can be used in the formulation of a grouping hypothesis

- illustrate how a grouping and read-across argument should be documented

- identify the different sources of uncertainty associated with filling data gaps by grouping and read-across

- evaluate the extent to which ECHA's Read-across Assessment Framework (RAAF) captures the different sources of uncertainty for nanoforms

- identify data gaps related to the selected case studies on grouping and read-across

This work was carried out to explore the practical process of grouping and read-across between nanoforms, with a view to sharing the lessons learned about the overall process. Thus, the conclusions obtained for specific substances should not be regarded as recommendations for regulatory action.

4.1 Selection of case studies

Two case studies of different composition (nano-TiO_2 and carbon nanotubes (CNTs)) were identified for the application of the workflow for grouping and read-across proposed by the ECHA's draft REACH guidance document (ECHA, 2017b). The two manufactured nanomaterials (NMs) were selected mainly due to 1) the availability of a significant amount of data on toxicity and physicochemical characterisation (Creutzenberg, 2013; MWCNT REACH Dossier, 2016; NIOSH, 2013; OECD, 2015; OECD WPMN, 2016; SCCS, 2013a; Sellers et al., 2015; US-EPA & EPA, 2013)[38], 2) their industrial relevance as they are produced in high volumes (more than 10000 t for TiO_2 and more than 1000 t for CNTs per year) (EC, 2012a) and 3) in-house experience on the behaviour of these specific nanomaterials from EU-funded projects (ENPRA, NanoMILE, NanoTEST and DEROCA).

It is noted that both NMs are the subject of on-going policy discussions. An IARC monograph[39] on the carcinogenicity of CNTs is in preparation and the proposal of classifying TiO_2 as carcinogen 1B by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, 2016) is subject to debate as the industry has firmly opposed to

---

[38] REACH registration dossiers for TiO_2 were screened online but information on the analogues of interest in our study were not identified.

[39] IARC Monograph Volume 111, Fluoro-edenite, silicon carbide fibres and whiskers, and single-walled and multi-walled carbon nanotubes. Under preparation.
The IARC Monograph Volume 93, Carbon Black, Titanium Dioxide, and Talc contains mostly old studies form TiO_2 and the nanoform is not assessed separately from the bulk form.

such classification by making more than 500 comments to the proposal (European Industry, 2016). As mentioned above, the case studies in this chapter are intended to evaluate, illustrate and inform the overall process of grouping and read-across between nanoforms.

4.1.1 The structure of the dataset

In order to survey the landscape of the available information on the identified NMs, a dataset was built containing two sets of information. Two clearly differentiated blocks of information were considered:

a)   the physicochemical characterisation, fundamental behaviour and reactivity of the identified NMs

b)   toxicological data of relevant REACH endpoints (e.g. genotoxicity, acute toxicity, skin sensitisation, carcinogenicity, etc.).

The choice of properties to capture in the database was informed by the templates proposed by Schultz et al. (T. W. Schultz et al., 2015), which were developed to assist in assessing and reporting similarity assumptions in the context of chemistry, toxicokinetics and toxicodynamics, and thereby supporting read-across. Since the case studies presented here correspond to NMs, the templates were adapted by taking into account physicochemical parameters specific to NMs. These are identified in the REACH guidance update for NMs (ECHA, 2017b) as key physicochemical parameters following a proposal by ITS-Nano (U. K. V. Stone, Balharry, Fernandes, Johnston, Munro, Hartl, et al., 2013). Other physicochemical parameters were taken into account, as reported thoroughly in Table 1.1 of Chapter 1. These parameters are either held under the OECD harmonised templates or considered relevant in the scientific literature.

The following physicochemical characteristics were collected in our dataset:

1. **What they are**: Name, JRC Nanomaterials Repository number, Chemical composition, Impurities, Crystal type, Crystal size, Surface coating, Porosity, Basic morphology, Primary particle diameter, Average particle diameter, Average length (TEM), Aspect ratio, Particle size distribution, Pour density (weighing), Specific surface area, Volume specific surface area

2. **Where they go:** Agglomeration, Hamaker constant, Dustiness, $logK_{OW}$, Hydrophobicity, Solubility(ies), Dispersability, Stability of the dispersion, (Bio)persistence, Redox potential, Zeta potential, Isoelectric point, Abiotic transformation, Toxicokinetics, Metabolic products, Steric hindrance

3. **What they do**: Conduction band, Radical formation potential, Catalytic activity, Photocatalytic activity, Hydrolysis, Protein binding, Electrophilicity/Nucleophilicity (electrophilicity index), Physical hazards (flammability, autoflammability and explosiveness), Dissociation constant

Regarding toxicological endpoints, the following information was collected:

1. Acute toxicity

2. Irritation, corrosion, sensitisation: Skin irritation, Eye irritation, Skin sensitisation

3. Genotoxicity: Mutagenicity, Genotoxicity *in vitro* and *in vivo*

4. Repeated dose toxicity: 28 days, 90 days, other duration, oral, instillation, inhalation

5. Other endpoints (e.g. dermal/percutaneous absorption, immunotoxicity, epidemiological studies).

Biological activity such as the induction of reactive oxygen species (ROS) is a relevant initiating event in nanotoxicology and was initially considered for its inclusion in the dataset. However, since the objective of the WPMN OECD dossiers was to test the applicability of existing OECD test guidelines to NMs, and there is no guidance on biological activity, test conditions are not harmonised and available results in the literature were so variable (Barillet et al., 2010; Jugan et al., 2012) that it was finally decided to neglect this information. The development of a test guideline to measure ROS generation would support the classification of nanomaterials as biologically active or inactive, which would be of great importance in read-across.

All the listed properties were searched for during data collection, but some were not used in developing the case studies because of lack of information or low data reliability.

4.1.2   Selection of the endpoint to read-across

To compile the Excel spreadsheets (the dataset) for the selected case studies, information was scrutinised from the following sources:

- Data available from public OECD dossiers (OECD, 2015; OECD WPMN, 2016)

- REACH registration dossiers as of March 2016

- IARC dossier on MWCNTs

- report on nano-TiO_2 proposal for classification from ANSES (ANSES, 2016),

- in-house data (cytotoxicity data from the Nanomile project),

- data from the Scientific Committee on Consumer Safety (for nano-TiO_2) (SCCS, 2013a)

- JRC Repository (K Rasmussen, Mast, & Temmerman, 2014; Kirsten Rasmussen et al., 2014)

- Nanogenotox FP7 project (Nanogenotox, 2012)

Figure 4.1 below shows the number of tests collected for each toxicological endpoint for the different forms of nano-TiO_2 and MWCNT. "Other toxicological tests" include dermal/percutaneous absorption, immunotoxicity, cytotoxicity and epidemiological studies, if available. Considering the number of test results recorded, the endpoint with most

information was genotoxicity as it included ~30% of the available toxicological tests for nano-TiO$_2$ and ~60% for MWCNTs (repeated dose toxicity tests include different routes of exposure and different test durations). Accordingly, genotoxicity was selected as the endpoint to read-across in both case studies as it was not only the toxicological endpoint with most tests available, but these tests were also covering the largest set of nanoforms (e.g. the maximum number of nano-TiO2 tested in a repeated dose toxicity study was three, whereas 6 nanoforms were tested in genotoxicity studies).

The data available on genotoxicity consisted mainly of tests carried out within the Nanogenotox Joint Action (NanoGenoTox Joint Action, 2013) and covered *in vitro* and *in vivo* comet and micronucleus assays. Results from bacterial mutagenicity test (Bacterial reverse mutation assay; Ames test) were not included in the count, as this test is not considered applicable to NMs in its current form (Clift et al., 2012; OECD, 2014d).

A) nano-TiO$_2$ B) MWCNTs
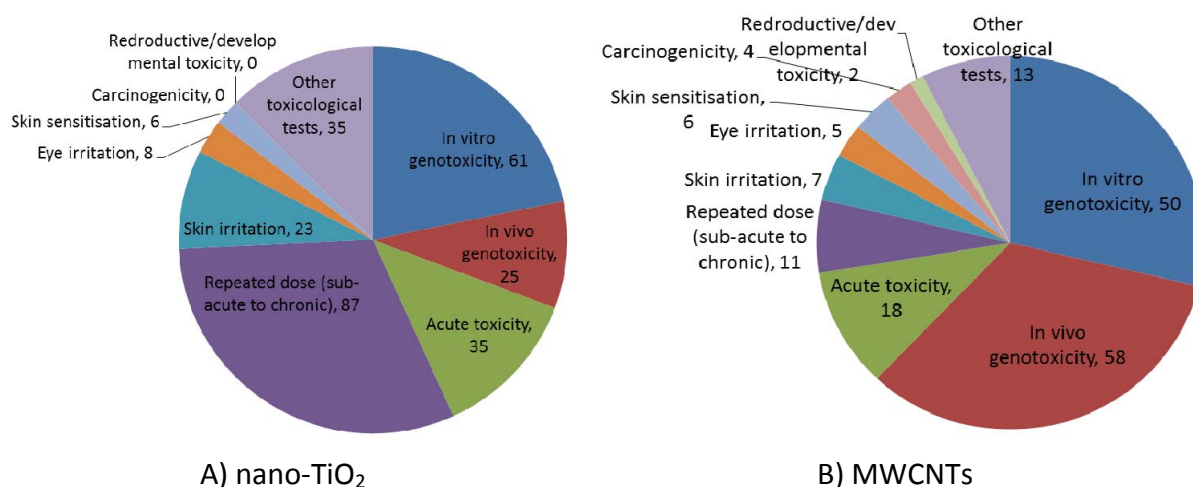
Figure 4.1 Number of toxicological tests identified for A) the nano-TiO$_2$ case study and B) for the MWCNT case study

Mutagenicity testing is required by the REACH regulation for all substances manufactured or imported in the EU above 1 tonne (per year per manufacturer/importer). At the lowest tonnage level (REACH Annex VII) an *in vitro* gene mutation study in bacteria is required. At higher tonnage levels both *in vitro* (in mammalian cells) and *in vivo* genotoxicity and mutagenicity tests may be required.

Table 4.1 shows the mutagenicity tests that are accepted or required by the REACH regulation with increasing tonnage levels. The applicability of those tests to NMs is also addressed as some of the in vitro tests are applicable, but none of the in vitro tests would be because toxikokinetic investigations are needed to determine if a NM reaches the target tissue (Kirsten Rasmussen et al., 2016). The adaptation for the *in vitro* micronucleus test would involve the addition of cytochalasin B as a post treatment to allow the cell to be exposed solely to the tested NMs.

Table 4.1 List of *in vitro* and *in vivo* mutagenicity and genotoxicity tests with their corresponding guidance documents and applicability to NMs

REACH recommended mutagenicity tests[*]	EU or OECD test guideline (TG)	Applicability of OECD TGs to NMs
In vitro tests		
Bacterial reverse mutation assay	EU: B.13/14 OECD TG 471	Not applicable
In vitro mammalian cell gene mutation test – hprt test	EU: B.17 OECD TG 476	Applicable
In vitro mammalian cell gene mutation test – Mouse lymphoma assay	EU: B.17 OECD TG 476	Applicable
In vitro mammalian chromosome aberration test	EU: B.10 OECD TG 473	Applicable after modification
In vitro micronucleus test	EU: B.49 OECD TG 487	Applicable after modification (modification for NMs is not included in the TG)
In vivo tests		
In vivo mammalian bone marrow chromosome aberration test	EU: B.11 OECD TG 475	Not applicable[1]
In vivo mammalian erythrocyte micronucleus test	EU: B.12 OECD TG 474	Not applicable[1]
Unscheduled DNA synthesis (UDS) test with mammalian liver *cells in vivo*	EU: B.39 OECDTG 486	Not applicable[1]
Transgenic rodent (TGR) somatic and germ cell gene mutation assays	EU: B.58 OECD TG 488	Not applicable[1]
In vivo alkaline single-cell gel electrophoresis assay for DNA strand breaks (comet assay)	EU: none OECD TG 489	Not applicable[1]

[*] according to ECHA guideline (ECHA, 2015a)

[1] If there is evidence that the test substance(s), or its metabolite(s) will not reach the target tissue, it may not be appropriate to use this test.

In general it is observed that the availability of applicable tests guidelines for NMs is relatively limited. Furthermore, as reported in more detail under the case studies below, the data available were scarce and not always of good quality. Nano-TiO$_2$ repeated dose toxicity studies include sub-acute to chronic toxicity studies and account for 34% of the results but the collected results do not cover the whole set of nanoforms that are considered in this

study but only 3 nanoforms. The genotoxicity studies are the most populated endpoint for both nano-TiO$_2$ and MWCNTs.

The main purpose of these case studies is to determine the genotoxic hazard potential of the target substance via read-across and document the process. The case studies will identify groups of substances (category) that can be used to determine the in vitro comet assay results of the target substances. One key step will be the determination of the physicochemical properties that can be used to define the groups and similarities between analogues that will form the categories. Ideally, a relationship between physicochemical properties and genotoxic potential will be established. Such information could facilitate data gap filling for those target analogues that are sufficiently similar and could also be used for prioritisation of testing (to fill data gaps for other analogues not investigated in this case study). However, the latter is not within the scope of this work.

Objective of this case study is also to consider the possibility of to read-across non-nanoforms: in the particular case of TiO$_2$, an analogue is considered a non-nanoform in the OECD Dossiers (NM-100).

The case studies will follow the workflow structure proposed by ECHA for grouping and read-across of nanomaterials (ECHA, 2017b) and will show how some chemoinformatic techniques (hierarchical clustering, principal component analysis, random forest variable selection) can be used to substantiate the grouping hypothesis.

Since the two case studies are based on the genotoxic potential of NMs, the description of the modes of action that can lead to genotoxicity of NMs are explained next.

4.1.3   Possible modes of action of genotoxicity

Two principle modes of genotoxic action have been reported for particles, known as primary and secondary genotoxicity and these are also reported specifically for MWCNT (Van Berlo, Clift, Albrecht, & Schins, 2012) and nano-TiO$_2$ (Golbamaki et al., 2015). Primary genotoxicity is defined as genetic damage elicited by particles in the absence of inflammation (Schins & Knaapen, 2007). Hence, it may be principally indicated from *in vitro* genotoxicity/mutagenicity studies or *in vivo* studies performed with particle concentrations that do not elicit significant (pulmonary) inflammation.

Direct primary genotoxicity can result from direct physical interaction between nanoparticles and the genomic DNA (Golbamaki et al., 2015; K. Li, Zhao, K. Hammer, Du, & Chen, 2013; Magdolenova et al., 2014; L. M. Sargent et al., 2012; Siegrist et al., 2014), whereas indirect primary genotoxicity may be the consequence of increased ROS formation upon interaction with other cellular components (e.g. mitochondria, cell membrane) or from depletion of intracellular antioxidants (Di Giorgio et al., 2011; Ken Donaldson, Poland, & Schins, 2010; Schins & Knaapen, 2007) (Table 4.2). The presence as impurities or in the composition of the NMs of reactive transition metals may also contribute to oxidative DNA damage induction.

Table 4.2 Pathways of particle-mediated ROS generation and their involvement in processes of primary and secondary genotoxicity (from Schins and Knaapen 2007)

Genotoxicity	Process/mechanism
Primary (direct or indirect)	Intrinsic ROS generation from particles. • Surface associated free radicals or oxidative groups (e.g., SiO· and SiO·$_2$ on crystalline silica) • ROS generation by particles in aqueous suspension (e.g., Haber–Weiss reactions by available metals, semiquinone radical redox cycling of biotransformed PAH.
Primary (indirect)	ROS generation upon interaction of particle with cellular components. • Damage to mitochondria/interaction with the electron transport chain • Activation of NAD(P)H-like enzyme systems. • Disturbance of endogenous antioxidant defences
Secondary	Generation of ROS and reactive nitrogen species (RNS) during particle-elicited inflammation • Phagocytes: NADPH oxidase, nitric oxide synthase, myeloperoxidase

Oxidative stress in cells may be formed directly via physicochemical reactivity (e.g. via active residual metal catalysts including Fenton chemistry) or indirectly via the activation of enzymatic pathways leading to release of oxidative species.

Oxidative DNA damage based on the formation of intracellular ROS production is the best described and discussed mechanism following exposure to nanoparticles (Golbamaki et al., 2015; Schins, 2002; Tournebize, Sapin-Minet, Bartosz, Leroy, & Boudier, 2013).

High levels of ROS can be deleterious not only to DNA but to all classes of cell components: lipids, proteins, nucleic acids and other macromolecules (Tournebize et al., 2013). Reactive species are also formed under physiological conditions, reacting with cellular components, leading to the activation of intracellular signalling pathways, nuclear transcription factors, inducing gene expression and cell responses such as repair, adaptation or transformation. This process is described as redox signalling. Apart from ROS mediated genotoxicity, other genotoxic mechanisms have been reported, e.g. the disturbance of membrane stability (Cveticanin et al., 2010) by the negative charge of some materials (e.g. transition metals) or the inhibition of various DNA repair machineries (Van Berlo et al., 2012).

Secondary genotoxicity implies a pathway of genetic damage resulting from an oxidative DNA attack by ROS and reactive nitrogen species (RNS) and possible other mediators. The aspect of secondary genotoxicity originates from observations in which various poorly soluble particles (e.g. MWCNT, carbon black, TiO$_2$) were found tumorigenic in rat lungs after chronic high exposures. The process is associated with overload and persistent inflammation and seems not to be related to their chemical composition (Borm, Schins, & Albrecht, 2004; Greim et al., 2001). Genotoxicity in lung epithelial cells has been found to have a high correlation with cell proliferation and tumour frequencies, indicating that it may be a secondary effect following cell proliferation (Rittinghausen et al., 2013). These toxic reactive species can be formed through phagocytic oxidative burst as a consequence of frustrated

phagocytosis of inhaled fibres. Frustrated phagocytosis was observed for rigid MWCNT longer than 20 µm (Ken Donaldson, Poland, & Wargo, 2009). Particle-elicited inflammation from recruited and activated phagocytes (macrophages, neutrophils) is considered to involve a threshold, meaning that it is dependent on an exposure concentration triggering inflammation and overwhelming antioxidant and DNA damage repair capacities in the lung.

Although secondary genotoxicity seems to be the most common mechanism for NMs like MWCNTs or asbestos, several *in vitro* studies in which nanoparticles including MWCNTs induced genotoxicity in the absence of inflammatory cells (NanoGenoTox Joint Action, 2013) indicate that primary genotoxicity cannot be ruled out per se. However, the predictive value of in vitro studies carried out with dispersed NMs in cultured cells to identify genotoxic NMs *in vivo* that could eventually be carcinogenic is presently unclear (NanoGenoTox Joint Action, 2013).

A distinction between primary and secondary genotoxicity seems relevant as currently available literature data merely indicate that the tumourigenesis of poorly soluble particles involves a mechanism of secondary genotoxicity (Ken Donaldson, Poland, et al., 2010; Schins & Knaapen, 2007). However, the (1) causality between pulmonary inflammation and genotoxicity has not yet been established, and (2) effects of inflammation on fundamental DNA damage responses that orchestrate mutagenesis and carcinogenic outcome, that is, cell cycle arrest, DNA repair, proliferation, and apoptosis, are currently poorly understood (Schins & Knaapen, 2007).

4.2   Workflow for grouping and read-across

A review on available read-across frameworks and case studies was presented in chapter 2. In this project, the workflow recently presented in the draft appendix to the REACH guidance (ECHA, 2017b) is considered as a reference for reporting the read-across information. The framework is shown in Figure 4.2. In the case studies sections in this chapter each step of the framework is developed in a paragraph reporting the same title.
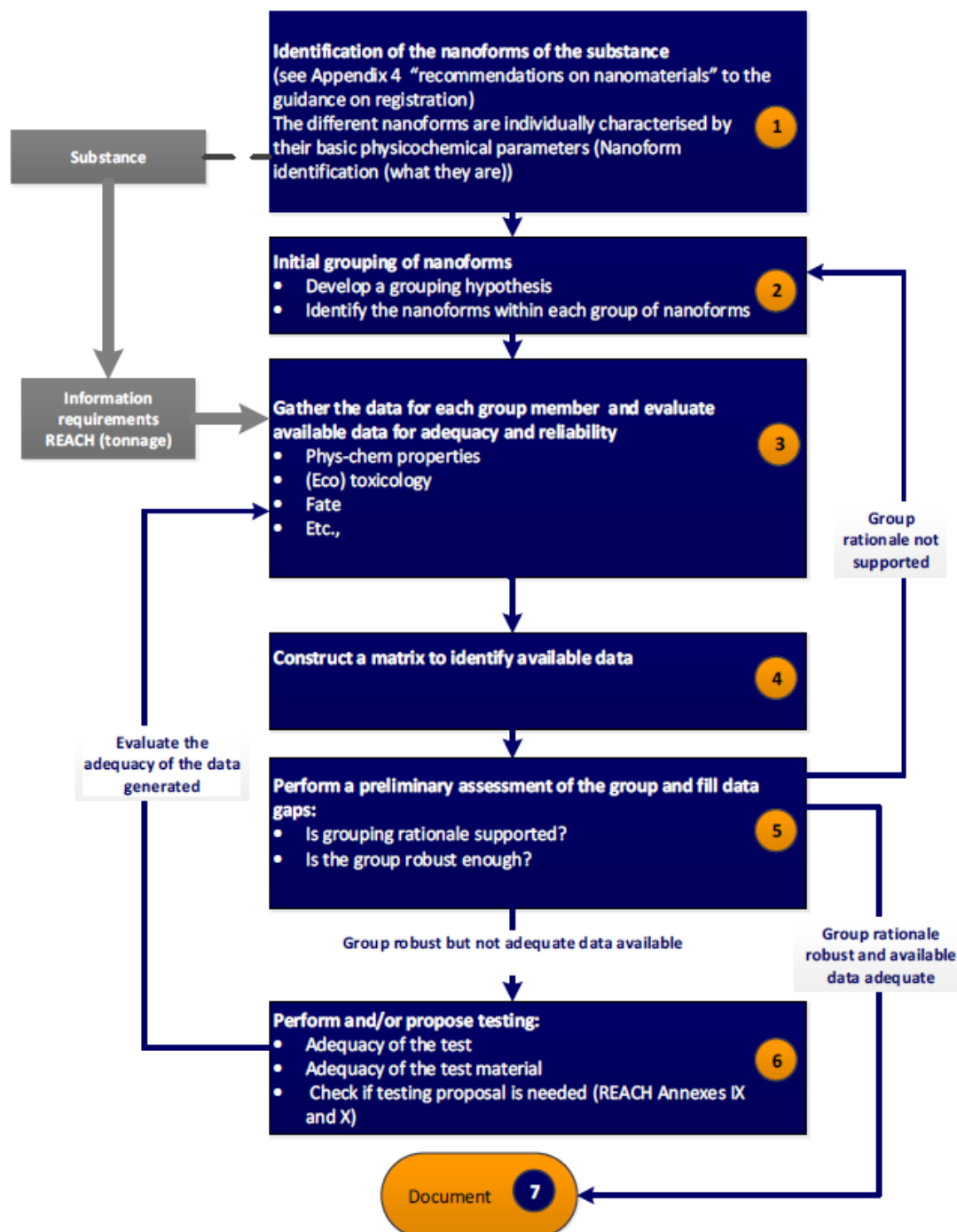

Figure 4.2 Read-across workflow followed in the Nanocomput case studies (ECHA, 2017b)

Generally, read-across is an iterative process that starts defining a target substance with a property that needs to be determined. In the current case this will be genotoxicity of nano-TiO$_2$ and MWCNTs. The information for these case studies is obtained from data that is already available for a set of identified analogues.

In the case of NMs the definition of analogues is not straightforward as for e.g. organic chemicals, because the influence of the different properties of NMs (e.g. size, coating, composition, or solubility) is not yet well understood. Step 1 of the framework is to define the nanoforms of the substance, i.e. "what they are". In order to infer the property of interest, there is a need of a hypothesis that will substantiate the interpolation of data. In the case of NMs there is not a well established and unique mechanism of mutagenicity. It could be hypothesised, for instance, that NMs reactive to DNA would be genotoxic. This hypothesis would include primary and secondary mechanisms and would imply that particles reach the target organ. The hypothesis definition takes place in step 2 of the framework and is followed by the definition of 2 groups (categories), reactive vs non-reactive. Step 3 consists of the gathering of data for each member of the group that will be used to assess the similarity within the groups and the grouping hypothesis. The disposition of these data in matrix form (step 4), will facilitate this grouping assessment, which will take place at step 5. In case the group rationale is not supported by the underlying data, a new grouping hypothesis will need to be formulated. On the contrary, if the group is robust but data is missing, new data will need to be generated and a testing strategy will be defined in step 6. If the group is robust and the data adequate, the prediction (read-across) can be accepted.

4.3   Methods used to investigate similarity between nanoforms

Given that there is not an agreement in the literature about the mechanism that can lead to genotoxicity of nano-TiO2 and MWCNTs, e.g. chemical reactivity, ROS generation, agglomeration and sedimentation, a set of chemoinformatic tools have been used to determine the (physicochemical) properties that differentiate the analogues, their similarity and that may drive genotoxicity. Thus, for each case study, the grouping hypothesis was based on the similarity between the physicochemical properties reported in the dataset. The formulation of similarity rules was supported by the application of the following data mining methods:

1. Hierarchical clustering was applied to identify possible clusters or groups of analogues in the dataset (similar NMs)

2. Principal component analysis was applied to have an indication of what are the physicochemical properties that differentiate the NMs and that can help in justifying the clustering, which is important in defining the grouping hypothesis

3. Random forest variable selection was applied to verify which are the most relevant properties in predicting the assay results. For this methodology the toxicological information needs to be taken into consideration as the aim was to classify the relevance of each variable (physicochemical property) in predicting the result (toxicological assay). In this chapter the toxicological endpoint was genotoxicity but the same technique could be used for any other endpoint. The technique can be applied to any endpoint, but the result will be exclusive to each endpoint.

The three data mining methods are introduced next.

4.3.1 Hierarchical clustering

Hierarchical clustering is an unsupervised technique that supports the definition of categories of data, in the present case studies groups of TiO_2 and CNTs nanoforms. In the divisive type of clustering the algorithm recursively divides the initial population, i.e. the source analogues, into two groups depending on their relative distances (similarity) until no more divisions are possible. The distances correspond to the Euclidean distances between two points in a space and are calculated using all the properties of each analogue. There are different ways of calculating the distances, which can determine the shape of the clusters. The most usual ones are the "complete", "single", and "average". The complete distance is determined from the members of the two groups that have the largest Euclidean distance. The single distance corresponds to the distance of the members of the two groups that are closest, and the average distance corresponds to the average of distances of all members of each group with all members of the other group. For this exercise it was considered that the average distance was the most adequate measure as the different members of the two groups would be equally represented.

Before clustering the NMs, the dataset had to be modified in order to remove those properties that were not useful to differentiate NMs. The usual procedure in these cases is the removal of properties with low variability/invariant values and the removal of properties with redundant information, i.e. with values similar to other properties across the NMs (highly correlated properties). Since the present two case studies corresponded to different forms of TiO_2 and CNTs, respectively; chemical composition was not one of the properties considered as it was constant in each case study. In an hypothetical case in which different substances were used as analogues (e.g. read-across between different metal oxides), properties accounting for the differences in chemical composition as could be charge of the metallic atom, molecular weight, number of electrons, bond order, etc. could be included in the list of physicochemical properties that are used to determine the distance between analogues and that ultimately define the clusters.

4.3.2 Principal component analysis

Principal component analysis is another unsupervised common technique that can be used to determine similar substances (clusters) from their variables (properties) and to find the properties that differentiate them the most, i.e. show which properties are important and which properties are redundant. The PCA uses vectors of data, i.e. properties for each NM, and transforms them via a linear combination into other vectors (principal components, PCs) that are orthogonal to each other. PCs are formed from a linear combination of the initial set of properties and are usually named in decreasing order of variance. In general the first two or three PC account for more than 80% of the total variance across NMs and can be used to describe the whole dataset with just two or three vectors.

4.3.3 Random forest for variable selection

The two methods mentioned above are called "unsupervised" because they only make use of the properties of substances to determine their "similarity" and identify the properties that differentiate them. Random forest is a classification algorithm that uses the properties

of substances to classify them into categories. The algorithm uses predefined categories of the substances, e.g. genotoxic vs non-genotoxic, to determine the usefulness of the (physicochemical) properties to predict the categories. This type of algorithms is usually used to select a subset of variables from a large pool of variables and are called variable selection algorithms.

One of the most commonly used methods for variable selection is implemented in the package "randomForest" of the R software(Liaw & Wiener, 2002), which is open source and freely accessible software (https://cran.r-project.org/). The algorithm determines the importance of the variables by building a random forest model and computing the mean decrease in accuracy that each variable causes when left out of the pool of variables when constructing the random forest. A random forest classifier operates by constructing a multitude of decision trees and outputting the class (category) that is the mode of the classes. Random forests correct the tendency for individual trees to overfit their training set. The variables that cause the highest accuracy decrease are considered the most important ones. In general the mean decrease of the Gini index (Gini, 1912) is used instead of the mean decrease of accuracy to determine variable importance. The Gini index is a measure of how each variable contributes to the homogeneity of the nodes and leaves in the resulting random forest and ranges from 0 (homogeneous) to 1 (heterogeneous). Each time a particular variable is used to split a node, the Gini indexes of the child nodes are calculated and compared to that of the original node. The variables that yield nodes with higher purity (homogeneity) have a higher decrease in Gini index, and therefore are considered more important.

4.4   The nano-TiO2 case study: predicting the in vitro comet assay result

In this chapter a set of 6 TiO$_2$ nanoforms will be used as source substances (analogues) to predict via read-across the genotoxic potential of two target TiO$_2$ nanoforms. The genotoxic potential is defined by corresponding in vitro comet assay results and is predicted using the category approach. The case study follows the workflow proposed by ECHA for grouping and read-across of NMs (ECHA, 2017b).

4.4.1   Identification and characterisation of the nanoforms of the substance

In this case study, the result of the comet Assay for TiO$_2$ Rutile (R) nano (Sigma 637262) and TiO$_2$ Anatase (A) nano (Sigma 637254) will be the data gaps to be filled by read-across. The first step in the read-across workflow (see Figure 4.5Figure 4.) is the identification and appropriate characterisation of the nanoforms of the substance (ECHA, 2017b).

Identification of target NMs
According to ECHA draft recommendations for the definition of nanoforms, the requirements to register a nanomaterial are:

- Particle size (in one or more dimensions)

- Particle shape, e.g. spheroidal-like, high aspect ratio (≥5:1, nanotubes, nanorods), two-dimensional (flakes or platelets), other (mixtures of particles with different shapes)

- Surface chemistry (chemical identity)

The information for the target NMs used in the present case study was extracted from Guichard et al. (2012) and presented in Table 4.3.

Table 4.3 Physicochemical properties of the NMs used as target substances in the case study. Data obtained from (Guichard et al., 2012).

Properties	TiO_2 R nano	TiO_2 A nano
Crystal type	Rutile	Anatase
Total non-TiO_2 content (including coating and impurities) (% w/w)	13	0.50
Surface chemistry (as declared by manufacturer)	SiO_2 (<5%) Na_2SO_4 SO_4^{-2}	uncoated
Surface coating (% w/w)	11	0
Primary particle diameter (nm)	10 nm diameter 62 nm length	14
Shape	Rod	Sphere
Specific surface area (m^2/g)	149	177

According to the physicochemical properties, the target materials consist of a nanopowder with particles of rutile and anatase TiO_2 respectively, specific surface area of 149 and 177 m^2/g, different levels of non-TiO_2 content (one is uncoated and has 99.5% w/w purity, the coated one has 87% w/w purity). The producer indicates that TiO_2 R nano may contain up to 5% w/w of SiO_2 as surface coating. This NM has been tested for cytotoxicity and genotoxicity in Syrian hamster embryo cells by Guichard et al. (2012), and the reporting of results includes some physicochemical analysis that will be used to determine analogues, and the test results will be used to va\lidate the read-across prediction.

The analysis of the physicochemical properties of the target substances shows that the measured ones are slightly different from those reported by the manufacturer. For instance, (Guichard et al., 2012) found for TiO_2 R nano 11% w/w of impurities corresponding mainly to SiO_2 (manufacturer declared up to 0.5%), the measured particle size corresponded to a rod of 62±10 x 24±2 nm (manufacturer declared 40x10nm[40]), and the surface area to 177m^2/g (manufacturer declared 50m^2/g). For the purpose of this study it is assumed that the substance used by (Guichard et al., 2012) in their experiments really corresponds to coated TiO_2 manufactured by Sigma. It is not clear though where is the limit to consider that two substances are the same.

---

[40] According to details provided in the NM Aldrich catalogue http://www.sigmaaldrich.com/catalog/product/aldrich/637262?lang=it&region=IT

Identification of source analogues

To identify analogues of the target NM, the initial sources for collecting information on physicochemical properties and on toxicological endpoints were the SCCS report and the OECD WPMN dossier on TiO$_2$ nanoforms nano-(OECD, 2015; SCCS, 2013a). The information obtained from these sources was used to construct a table with 53 TiO$_2$ nanoforms. Unfortunately, most of these analogues could not be identified because the reported data (mainly in the SCCS report) had been anonymised, and the information reported did not include systematic physicochemical characterisation that could be used to identify them.

We therefore decided to consider only the nanoforms that were identified properly by means of fundamental parameters like solubility, hydrophobicity, zeta potential, and dispersability, which are among the ones that need to be taken into account in read-across of NMs nano-(ECHA, 2017b). This led to a dataset with 6 TiO$_2$ nanoforms whose data were mainly obtained from the OECD dossier (from the version published online in March 2016). The 6 nanoforms differ in their size (from 7 to 117 nm), coating (two of them are declared coated by the manufacturer and the others are declared without a coating), crystal type (anatase or rutile) and hydrophobicity (hydrophobic or hydrophilic). For an overview of physicochemical characterisation of these TiO$_2$ nanoforms, see Rasmussen et al. (2014b). As shown in Table 4.5, all collected nanoforms had several toxicological data available including comet assay results. The 6 analogues and the physicochemical properties relevant for their identification ("what they are") are shown in Table 4.4.

One of the difficulties in identifying NMs is the presence of coating and impurities. Impurities are defined as "An unintended constituent present in a substance as manufactured" (ECHA, 2012a), while surface coating consists in the surface chemistry purposely added to the NM. The measurement of the elements present on the surface of the NM does not distinguish between the two, and hence "Total non-TiO$_2$ content including coating and impurities (% w/w)" is reported as the measure for the total elements detected other than the core material. Thus, this measure includes also the coating, which is separately declared by the supplier and is also reported separately in our dataset and in Table 4.4. as "Surface chemistry (as declared by manufacturer)" and "Surface coating (%)" indicating the quantity of coating with respect to total weight of the NM. In the present case, NM-103 and NM-104 are declared coated and in fact the amount of total non-TiO$_2$ content is >10% w/w. NM-101 has quite a high content of impurities if compared to the uncoated NM-100, NM-102 and NM-105.

Table 4.4.shows one of the problems of NMs which is data variability. For instance, some of the properties like crystallite size were obtained averaging different values provided by different laboratories (see Appendix VII and IX for further information). In the particular case of NM-100, 117nm is the average of 141, 61, 168, and 100nm. The way in which the data is summarized depends on the distribution of the values. If the distribution is normal, the values can be averaged but if the distribution is not normal and there are extremes, then the median is a better option. However, in these cases we do not have enough data to determine which is the right value. In this case, we have values obtained by different laboratories and some of these values correspond to triplicate measures with very low SD. Luckily, the mean and median were very similar and the statistical method used did not compromise the value. Similarly, the particle size diameter was obtained from two different measures with triplicates, one of 70 ± 20nm and another one of 116.9 ± 36.9nm. The final results indicate that the crystallite size is very similar to the particle size diameter (117 vs 93

nm), which would imply that the NMs are formed by a single crystal and this may not be 100% true in some cases. NM-100 is a dry-milled NM and the variability in the measured values of its particle diameter and crystallite size just indicates that it does not correspond to a mono-dispersed substance but that it contains particle sizes ranging from 61-168nm. This will not represent a major problem in this case study as the endpoint of interest is in vitro genotoxicity, in which the primary size of the NMs may not dramatically affect the exposure. However, this could have big implications in in vivo endpoints in which the size of the particles may significantly affect the biodistribution of particles throughout the body and organs (e.g. lungs). The fact that the particle size diameter and crystallite size are very similar or that the latter is even larger is unexpected as it would indicate that the NMs are composed of just one crystal. Another factor that can help explaining this issue is the fact that the particle size diameter was measured with TEM and the crystallite size by XRD, which are two different methods whose comparison might be difficult. In this case, the latter would be less accurate as the authors of the Nanogenotox report acknowledged having some problems fitting the data to some of the equations that are used to derive the crystallite size from the diffraction values.

Table 4.4 Physicochemical properties of the source analogues obtained from the OECD dossiers (downloaded March 2016)

Property	NM100	NM101	NM102	NM103	NM104	NM105
Crystal type	Anatase	Anatase	Anatase	Rutile	Rutile	83% anatase 17% rutile
Other info	Dry-milled	Semiconductor catalyst used in photocatalytic process	photocatalytic	hydrophobic	hydrophilic	-
Total non-TiO$_2$ content including coating and impurities (% w/w)	1.5	9	5	11	11	0.11
Surface chemistry (as declared by manufacturer)	uncoated	uncoated	uncoated	Al$_2$O$_3$ and SiO$_2$	Al$_2$O$_3$ and SiO$_2$	uncoated
Surface coating (% w/w)	0	0	0	8	8	0
Primary particle diameter (TEM) (nm)	93 ± 23	5 ± 1	22 ± 10	24 ± 2	24 ± 2	20 ± 3
Crystallite size (XRD) (nm)[*]	117 ± 40	7 ± 2	24 ± 5	24 ± 4	25 ± 4	22 ± 5
Particle Size Distribution (Z-average) (nm)[β]	210 ± 10	278	440 ± 37	135 ± 25	145 ± 35	177 ± 39
Shape	Spheroidal	Spheroidal	Spheroidal	Spheroidal	Spheroidal	Spheroidal
Aspect ratio	1.53	1.53	1.53	1.7	1.53	1.36
Specific surface area (m^2/g)[£]	9	242 ± 73	77 ± 10	54 ± 4	54 ± 2	47 ± 0.5

[*] values averaged from different instruments and principles (Peak fit, TOPAS, Fullprof, Scherrer eq., TOPAS, IB, TOPAS FWHM)
[β] values averaged from ICP-MS and DLS experiments.
[£] values averaged from SAXS/USAXS and BET

These analogues are quite data rich because they have been investigated in several FP7 projects and the WPMN testing program. The information available for each NM was

restructured starting from the OECD dossiers to build a complete dataset including both physicochemical information and toxicological information (genotoxicity) for NM-100, NM-101, NM-102, NM-103, NM-104, NM-105.

A summary of the genotoxicity studies that were collected from the cited sources is reported in Table 4.5. The table shows that there are *in vitro* comet assay results for all the identified source NMs and they exhibit both positive and negative results. This justifies the selection of *in vitro* comet assay as the endpoint to read-across. The results in the table report the positive tests over the total tests available. All the genotoxicity tests performed in the Nanogenotox WP6 were taken into consideration for the purpose of this case study. The testing protocol included a post-treatment of cythochalasin B that was added 6 h after the start of the treatment for all cell lines except CaCO2 where cytochalasin B was added after 24 h, so it can be assumed that this protocol applied the adaptation of the OECD TG 473 as recommended in Rasmussen et al. (2016). However, only the *in vitro* comet assay was performed for the 6 source analogues, hence this endpoint was selected for the read-across case study, although no OECD TG is available.

Table 4.5 Availability of genotoxicity tests for the source NMs in our dataset (number of positives over the total number of tests performed). Data obtained from the Nanogenotox Joint Action (Nanogenotox WP6, 2013; Norppa et al., 2013).

Assay		NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
in vivo	comet assays	-	1/5	2/12	1/12	2/12	3/12
in vitro	comet assays	2/2	0/2	4/6	0/6	0/6	3/6
in vivo	micronucleus	-	0/3	0/5	0/5	0/5	0/5
in vitro	micronucleus	-	-	1/7	2/7	2/7	1/7

4.4.2   Initial grouping of nanoforms

The second step of the ECHA guidance for grouping of nanoforms corresponds to the proposal of an initial grouping of the identified materials (ECHA, 2017b).

Table 4.5 reports the genotoxicity tests available in the Nanogenotox project for the source analogues. Our hypothesis will be based on the fact that the data in Table 4.5 show that NM-100, NM-102 and NM-105 have a higher tendency to giving positive results in the in vitro comet assay, whereas NM-101, NM-103 and NM-104 tend to give negative results.

As mentioned in paragraph 4.1.2, and as reported in the literature (Golbamaki et al., 2015; Magdolenova et al., 2014), there are more experimental data on the *in vitro* comet assay for testing the DNA damage caused by NMs, compared to other tests. The *in vitro* comet assay detects DNA strand breaks at the level of single cells. In our case study, we aim at identifying NMs physicochemical properties that may affect DNA damage, to be able to read-across test results to our target NMs.

Regarding the mechanism(s) of genotoxic action of NMs, Magdolenova et al. (2014) and Golbamaki et al. (2015) reviewed the mechanisms of genotoxicity of NMs and of the subset of metal oxides (including nano-TiO$_2$), respectively. They report that DNA damage caused by nano-TiO2 may be classified as direct primary genotoxicity, indirect primary damage, and secondary genotoxicity. Direct genotoxicity assumes that DNA and NM are in contact. An

example of direct genotoxicity by TiO_2 was found to involve the reaction of terminal DNA phosphate groups that influence the binding of DNA to nano-TiO_2 (Rice et al., 2009). Indirect primary genotoxicity may be elicited by interaction of NMs with nuclear proteins (involved in replication, transcription, and repair), disturbance of cell cycle checkpoint functions, ROS arising from the NM surface, release of toxic metal ions from the NM surface, ROS produced by cell components, and inhibition of antioxidant defence (Jugan et al., 2012). Finally, secondary genotoxicity may be elicited by ROS production in inflammatory cells via an inflammation signalling pathway (Romoser, 2012), i.e. macrophages or neutrophils (activated phagocytes) generate ROS while trying to digest the NMs. This can cause an inflammatory reaction that may subsequently cause oxidative DNA damage (Trouiller et al., 2009).

Although most experimental studies provide evidence for a mechanism of action for indirect primary genotoxicity via ROS (Golbamaki et al., 2015), several studies report that a clear correlation between the level of ROS production and DNA damage was not supported by their findings (Barillet et al. 2010, Golbamaki et al. 2015, Li et al 2013). For example, Li et al. (2013) investigated the ability of a set of NMs (including nano-TiO_2, plus metal and metal oxide NMs and quantum dots) to inhibit DNA replication by binding to DNA: they concluded that ROS generation as an important cause of genotoxicity was not supported by their experiments as the amount of generated ROS did not explain the effect of NMs on DNA binding reported in their study. They suggested instead that direct binding activity of NMs to DNA was the likely genotoxicity mechanism.

Development of grouping hypothesis
Our dataset includes 6 NMs with different properties: different primary and crystallite sizes, different crystalline types and surface characteristics (some are coated and some uncoated), as reported in Table 4.4. From combining the *in vitro* comet assay results reported in Table 4.5 and the physicochemical characteristics identifying the nanoforms shown in Table 4.4 we can formulate our grouping hypothesis:

Nano-TiO_2 in its uncoated form has the potential to damage DNA, but this can be masked by the presence of coating or by the large amounts of impurities on the surface of the NM.

This hypothesis is supported by the random forest analysis reported in paragraph 4.4.5. The variable importance plot in Figure 4.8 shows that the Total non-TiO2 content, which includes coating and impurities, and organic matter, which accounts mostly for coating, are the most valuable properties to predict *in vitro* comet assay results.

In fact, it can be readily seen in the dataset of analogues that the coated NMs turn out negative in the comet assay while the ones without coating and organic impurities turn out positive. This can be explained if a direct interaction mechanism of genotoxicity or an indirect primary genotoxicity are considered (Magdolenova et al., 2014). The conduction band of TiO_2 falls in the range of biological redox potentials (Burello & Worth, 2011a), meaning that TiO_2 with or without the presence of UV light can generate reactive species that react with cell constituents such as DNA. In both direct and indirect primary genotoxicity, physical interaction of the NM with DNA (direct) or another cellular component (e.g. enzyme mediated a redox reaction) that generates ROS (indirect) is necessary for the DNA damage to occur. The NM coating acts as a physical barrier that can prevent this contact between the Ti and O atoms of TiO_2 and DNA or other cellular components.

Therefore, coated nano-TiO$_2$ will not turn out positive in the comet assay as there will be no physical interaction between the Ti / O atoms and DNA / cellular components.

The way in which the coating can prevent DNA damage is not entirely clear, in fact several works show contradictory results and explanations for the *in vitro* genotoxicity of TiO$_2$ with coating playing a main role. For instance, it was shown (Falck et al., 2009; Mano, Kanehira, Sonezaki, & Taniguchi, 2012) that the addition of PEG coating to nano-TiO$_2$ increased the dispersion of NMs which resulted in lower cytotoxicity and genotoxicity. Magdolenova et al. (2012) showed that the degree of dispersion of TiO$_2$ NMs had an influence on the DNA damage in three cell lines. Agglomerates of less than 200 nm had no effect on genotoxicity while larger ones showed positive results. These results could be due to larger agglomerates precipitate and deposit on the cells increasing the actual exposure to the NM or even covering them completely and suffocating them. Another consideration is the effect that the use of media with proteins (e.g. BSA, FBS) can have on the results. If the NMs are surrounded by proteins, they are more dispersed and also less toxic as the "reactive" part is encapsulated ("hidden") behind the protein corona. Another aspect that cannot be ignored when analysing the in vitro results of TiO$_2$ is its photocatalytic activity, which can be even triggered by a simple fluorescent tube (Karlsson, Di Bucchianico, Collins, & Dusinska, 2015). Thus, it is obvious that the mechanism of genotoxicity of TiO$_2$ is not well defined and that there might be more than one that could even take place simultaneously. Probably the truth is a combination of all factors that have as common source the presence of coating either by preventing aggregation of NMs, deposition, and therefore reducing exposure, or by preventing physical contact with DNA and/or other cell components after uptake. However, what is relevant in this case is that the majority of studies agree with the hypothesis presented here which is the fact that coated nano-TiO$_2$ show fewer positive results in the comet assay than the uncoated ones, therefore it can be fairly concluded that the presence of coating reduces the genotoxic effects of nano-TiO$_2$. It is important to keep in mind that the present coatings are mainly not "charged" as could be coatings with reactive or non-neutral groups such as terminal –COOH or –NH$_2$, in which cases the grouping hypothesis might change.

Identification of the nanoforms within each group

From Table 4.6 it can be seen that a trend can be identified only for the *in vitro* comet results, where the test has positive results (the group of positives) for NM-100, NM-102 and NM-105. The group of materials that are not causing DNA damage (the group of negatives) is NM-101, NM-103 and NM-104. This grouping is schematically presented in Table 4.6.

Table 4.6 Assignment of the analogues to the two groups identified upon DNA damage potential measured with the *in vitro* comet assay, depending on the characteristics of the NMs.

	Naked particle	Particle with high non-TiO$_2$ content (coating + impurities)
Source NMs	NM-100, NM-102, NM-105	NM-103, NM-101, NM-104
DNA damage	+	-

4.4.3 For each group member, gather and evaluate data adequacy and reliability

The third step of the framework is aimed at defining the purpose of the grouping (endpoint(s)), and at developing the hypothesis and the scientific basis for a robust justification of the read-across (ECHA, 2017b).

The dataset of source analogues was adapted to other properties relevant for grouping as provided in Appendix 1 of the ECHA guidance (ECHA, 2017b) and following Table 1.1. Table 4.7 shows the complete dataset for the identified source analogues. The table was analysed to identify a set of properties that can be used to define (structurally) similar NMs as well as to identify those physicochemical properties that can help in predicting by read-across the results of an *in vitro* comet assay. The dataset reported here contains only data that were used in the analyses. The full dataset is reported and described in Appendix X.

Available information was collected considering the list of physicochemical properties reported in Section 4.1.1. A detailed analysis on physicochemical properties is reported in Appendix VIII.

Physicochemical parameters

The total non-TiO_2 content of the source analogues varies from 0.11% to 11%, where the highest values are justified by the presence of coating. NM-103 and NM-104 contain 6% of Al_2O_3 6 and 2% of organic functionalisation (silanes and dimethicone for NM-103 making it hydrophobic; tetramethyl silicate; glycerol; silanes; hexadecanoic acid, methyl ester, octadecaonic acid for NM-104 making it hydrophilic). NM-101 with 9% of non-TiO2 content of organic composition (silane, Hexadecanoic acid, methyl ester, octadecaonic acid) could be considered to have coating although this nanoform was not declared coated by the manufacturer (Birkedal et al., 2012). This difference is reflected in Table 4.7, where the presence of surface coating is represented by its %w/w and where the Total non-TiO2 content accounts for the amount of matter that is not TiO_2, thus including coating and impurities.

Since there was abundant information on the particle size distribution in biological media submitted in the OECD dossiers, this was also considered in the reported dataset.

The dependence of the NM properties on the environment (fate) is addressed in the dataset by including physicochemical properties measured in different media. For example, particle size distribution, zeta potential, polydisperisity index are measured in milli-Q water, in Dulbecco's modified eagle medium, and in foetal bovine serum, whereas solubility and redox potential are measured in Gamble's solution (representing a lung fluid) and Caco2 medium (representing the intestinal environment).

The numerical values in the table are deducted from the data analysis presented in Appendix VIII; particle size distribution is determined in MilliQ water and biological media. Biological media applied in the studies reported in the table are Dulbecco's modified eagle medium with and without L-glutamine, fetal bovine serum and phosphate-buffered saline. Dispersions were either untreated or underwent 1 minute probe sonication or 20 minutes ultrasound bath sonication. Inputs on solubility and biodurability were deducted by elemental analysis of the particle-free tested media (K. A. Jensen et al., 2013).

Table 4.7. Physicochemical properties of the target analogues. MQ: milli-Q water; 1 min: sonication time, as direct probe; 20 min: sonication time, ultrasound batch mode; DMEM: Dulbecco's modified eagle medium; PBS: phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 0.02 M PO4) FBS: fetal bovine serum; PdI: polydispersity index; BSA: bovine serum albumin.

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
In vitro comet assay	1	0	1	0	0	1
Total non-TiO$_2$ content including coating and impurities (% w/w)	1.5	9	5	11	11	0.11
Impurity(% w/w Fe)	0.49	0	0.07	0.06	0	0.06
Impurity(% w/w Si)	0.28	0.29	0.08	0.68	0.018	0.07
Impurity(% w/w K)	0.25	0	0.001	0.001	0.001	0
Impurity(% w/w P)	0.21	0.27	0.001	0	0	0
Impurity – coating (% w/w Al)	0.09	0.09	0.05	3.4	3.2	0.04
Impurity(% w/w Cr)	0.03	0	0	0	0	0
Impurity(% w/w Zr)	0.005	0.01	0.005	0.001	0.001	0
Impurity(% w/w Ca)	0.001	0	0.005	0.005	0.01	0
Impurity(% w/w Na)	0.001	0.1	0.001	0.01	0	0.001
Impurity(% w/w S)	0	0.22	0.001	0.01	0.01	0.26
Impurity(% w/w Mg)	0	0	0	0.001	0.001	0
Crystal type (Anatase)	1	1	1	0	0	0.84
Crystal type (Rutile)	0	0	0	1	1	0.16
Crystal type (Cubic)	0	0	0	0	0	0
Crystallite size (mean)	117.81	7.69	23.93	24.32	24.71	22.44
Primary particle diameter (mean)	93.45	5.25	22.00	24.00	24.50	20.13
Aspect ratio	1.53	1.53	1.53	1.70	1.53	1.36
Specific surface area (m^2/g)	9.23	316.07	77.86	53.98	54.33	47.00
Shape (elongated=1, spherical=0)	0	0	0	1	0	1
Surface coating (% w/w)	0	0	0	8	8	0
Organic matter (% w/w)	0	8	0	2	2	0
IsoelectricPoint(Mean)	7.02	5.5	6	8.3	8.5	6.8
IsoelectricPoint(Min)	6.86	5.3	6	8.2	8.2	6.6
IsoelectricPoint(Max)	7.18	5.7	6	8.5	8.8	6.9
Density	3.84	3.99	3.84	4.015	4.09	4.052
Mean of total pore volume (ml/g)	0.0324	0.319	0.2996	0.2616	0.1935	0.1937
Micro surface area (m2/g)	0	13.625	1.108	0	0	0
Micropore volume (ml/g)	0	0.00179	0.00034	0	0	0
Specific surface area (mean)	9.23	242.785	77.864	53.984	54.331	47
Dustiness-Respirable(mg/kg)	1500	5600	9200	19000	6400	11000
Biodurability 24h 0.05% BSA (Ti content) (µg/l)	5.2	0	0	0	0	0
Biodurability 24h Gambles solution (Ti content) (µg/l)	0	0	3388	0	0	0
Biodurability 24h Caco2 (Ti content) (µg/l)	796	3414	1741	222	3386	2724
Biodurability 24h 0.05% BSA (Al content) (µg/l)	0	175	0	198	137	0

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
In vitro comet assay	1	0	1	0	0	1
Biodurability 24h Gambles solution (Al content) (µg/l)	0	177	0	868	413	0
Biodurability 24h Caco2 (Al content) (µg/l)	24	252	0	182	413	0
Biodurability 24h 0.05% BSA (Si content) (mg/l)	0	0	0	0.9	0	0
Redox Caco2 medium $^{\Omega}$	1	-1	-1	1	-1	-1
Redox Gamble's solution $^{\Omega}$	1	0	-1	1	-1	-1
Redox BSA $^{\Omega}$	0	0	0	0	0	0
Particle Size Distribution (Z-average) (nm)	210	278	439.8	135.11	144.47	176.78
Particle Size Distribution in PBS, untreated (nm)	2289	1229	1579	1397	1600	3342
Particle Size Distribution in MQ Water, Mode 1,1min sonication (nm)	259.3	719.5	703	2649	207.7	352.6
Particle Size Distribution in DMEM + L-glutamate, Mode 1, 20min sonication (nm)	1059	1974	2001	2916	3207	1956
Zeta Potential in MQ Water,1min sonication (mV)	-24.5	-27.2	-27.1	39.1	-23.4	-23.8
Zeta Potential in DMEM + 10%FBS, Mode 1, 1min sonication (mV)	78.4	0.13	-10.5	-12.4	-9.38	-9.92
Zeta Potential in PBS, 20min sonication (mV)	-20.2	-21.7	-18.5	-20.9	-20.3	-33.2
Zeta Potential in DMEM + 5% FBS, 20min sonication (mV)	-10.4	-11.3	-9.47	-13.7	-9.38	-11.9
Polidispersability Index in DMEM + 1% FBS - 1min sonication	0.207	0.232	0.243	0.243	0.194	0.177
Polidispersability Index in MQ water - 1min sonication	0.205	0.274	0.248	0.393	0.236	0.211
Polidispersability Index in DMEM + Lglutamine - 20min sonication	0.515	0.247	0.227	0.264	0.209	0.341
Polidispersability Index (PdI)	0.303	0.323	0.427	0.292	0.227	0.245

$^{\Omega}$ values obtained from Nanogenotox 4.7 determined by measuring the content of O_2. Oxidising properties (1), neutral (0), reducing (-1)

Toxicological information

ANSES (2016) performed a literature search on available genotoxicity case studies published in the period 2010-2015, reporting also registration dossiers collected by ECHA. Studies identified by ANSES that were missing in our dataset were then included and a further literature search was carried out to include other available studies (Appendix XI) on the six source analogues, and a reliability assessment of the studies was made according to the criteria identified by ANSES and reported here.

In vitro studies were considered reliable if:

1. The NMs are characterized (at least size, crystallinity and coating) and a description of the dispersed materials are provided (particle size distribution, zeta potential, polydispersity index)

2. The NM uptake is observed and/or cytotoxicity is tested

3. Positive and negative controls are considered, and replicates are included.

The *in vivo* studies are considered reliable if conditions 1 and 3 above are applied; negative results are taken into account only when it has been proven that the nanoparticles have reached the organ investigated (condition 2). In *in vitro* studies, this could be confirmed with data on uptake or if cytotoxicity was detected.

In our data collection we reported micronucleus, comet and chromosomal aberration tests because the current OECD test guidelines fort these tests are considered applicable to NMs. Although some efforts are being done to evaluate the *in vitro* comet assay (Azqueta & Dusinska, 2015; Golbamaki et al., 2015), an OECD test guideline is not available. This endpoint was considered anyway in our data collection because of larger availability of studies. Previous reviews on genotoxicity tests applied to NMs claimed that the comet and micronucleus assays are the most commonly used tests in the field (Golbamaki et al., 2015), and our search results confirm this.

Details on the literature search for genotoxicity studies is reported in Appendix XI. Table 4.8 shows the results of the tests reported in the collected literature; the studies taken into consideration include the tests reported previously in Table 4.5. The results are shown as the number of positives out of the total studies. The numbers refer to reliable studies, identified according to the criteria identified by ANSES.

Table 4.8 Total number of reliable genotoxicity studies for nano-TiO$_2$ found in the literature and reported in Appendix XI. The comet *in vitro* assay is the most performed assay and it gives mostly positive results for NM-100, NM-102 and NM-105.

N of positives / total N studies	*in vitro*			*in vivo*		
	Micronucleus assay	comet	Chromosomal aberration	Micronucleus assay	comet	Chromosomal aberration
NM-100	-	2/2	-	-	-	-
NM-101	-	2/6	-	0/3	1/5	-
NM-102	3/10	5/8	-	0/6	2/13	0/2
NM-103	3/8	0/6	-	0/5	1/12	-
NM-104	3/8	0/6	-	0/5	2/12	-
NM-105	4/18	10/14	0/1	2/9	4/15	-

4.4.4 *Construct a matrix to identify available data*

The fourth step of the framework proposed in the ECHA draft guidance on grouping for read-across is dedicated to building a matrix for reported the data collected and evaluated in the previous step (ECHA, 2017b).

The target NMs identified in Table 4.3 are allocated to the two identified groups according to the presence or absence of coating. Table 4.9 reports graphically our read-across, and includes the physicochemical properties of the source and target NMs ("what they are"). For the analogues also information on fundamental behaviour ("where they go") and reactivity ("what they do") is reported.

As mentioned earlier, our target NMs have actually been tested, so we can validate our conclusion by means of the *in vitro* comet assay results (Guichard et al., 2012). A detailed analysis is reported in Section 4.3.5.

Table 4.9 Grouping hypothesis and read-across of comet assay results. TiO$_2$ R and TiO$_2$ A are the two target nanomaterials. According to the grouping hypothesis based on the presence or absence of the coating, the two targets NMs are assigned to the positive and negative group, respectively.

	Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105	TiO$_2$ R	TiO$_2$ A
	In vitro comet assay	+	-	+	-	-	+	?	?
What they are	Total non-TiO2 content including coating and impurities (% w/w)	1.5	9	5	11	11	0.11	13	0.5
	Surface coating (%)	0	0	0	8	8	0	11	0
	Organic matter (%)	0	8	0	2	2	0	9	0
	Crystal type (Anatase)	1	1	1	0	0	0.84	0	1
	Crystal type (Rutile)	0	0	0	1	1	0.16	1	0
	Crystal type (Cubic)	0	0	0	0	0	0	0	0
	Crystallite size (mean) (nm)	117.81	7.69	23.93	24.32	24.71	22.44		
	Shape (rod=1, spherical=0)	0	0	0	1	0	1	1	0
	Aspect ratio	1.53	1.53	1.53	1.7	1.53	1.36	6.2	1
	Primary particle diameter (mean) (nm)	93.45	5.25	22.00	24.00	24.50	20.13	62x10	14
	Specific surface area (m^2/g)	9.23	316.07	77.87	53.98	54.33	47	149	177
Where they go	Isoelectric Point (Mean) (pH)	NA	5.5	6	8.3	8.5	6.8		
	Density (g/mL)	3.84	3.99	3.84	4.02	4.09	4.05		
	Mean of total pore volume (mL/g)	0.032	0.319	0.300	0.262	0.194	0.194		
	Micro surface area (m^2/g)	0	13.625	1.108	0	0	0		
	Micropore volume (mL/g)	0	0.00179	0.00034	0	0	0		
	Dustiness-Respirable(mg/kg)	1500	5600	9200	19000	6400	11000		
	Biodurability 24h 0.05% BSA (Ti content) (µg/l)	5.2	0	0	0	0	0		
	Biodurability 24h Gambles solution (Ti content) (µg/l)	0	0	3388	0	0	0		
	Biodurability 24h Caco2 (Ti content) (µg/l)	796	3414	1741	222	3386	2724		
What they do	Redox Caco2 medium [Ω]	1	-1	-1	1	-1	-1		
	Redox Gamble's solution [Ω]	1	0	-1	1	-1	-1		
	Redox BSA [Ω]	0	0	0	0	0	0		

[Ω] values obtained from Nanogenotox 4.7 determined by measuring the content of O$_2$. Oxidising properties (1), neutral (0), reducing (-1)

4.4.5 Perform a preliminary assessment of the group and fill data gaps

The fifth step of the ECHA draft appendix to the ECHA guidance on grouping for read-across (ECHA, 2017b) aims at combining all the gathered information into an overall assessment.

The identification of the structural similarities[41] in the analogues dataset is a way to assess the strength of the stated grouping hypothesis. This was done through the application of hierarchical clustering, principal component analysis and was supported by decision tree analysis, as introduced in paragraph 4.3.

Assessment of the grouping hypothesis

Hierarchical clustering

The initial dataset included 6 analogues with approximately 147 properties for each of them (Table A8.1 in Appendix X). Four properties were discarded because of low variability (Crystal type cubic, Redox BSA, Biodurability after 24h in Caco2 ((Si content) and Biodurability after 24h in Gamble's solution (Si content)). If a correlation filter was applied to such a small dataset, the correlation would be derived from just 6 values and this could lead to the filtering of properties that are not really related. For instance, the correlation between "Organic matter" and "Micro surface area" is 0.93. Such a correlation is not meaningful because the former property only contains values for three NMs, and the latter for two. Thus, a correlation filter was not applied in order to avoid such problems. The dataset contained a number of DLS measures for the NMs in 6 different solvents (MQ water, PBS, DMEM + 1% FBS, DMEM + 5% FBS, DMEM + 10% FBS, and DMEM + L-glutamate) and 3 different treatments (untreated, 1min tip sonication, 20min bath sonication). This made a highly biased dataset as it contained 62 properties related to DLS particle size distribution, 21 to Zeta potential, and 20 to polydispersability index. In order to reduce the weight of such measures and obtain a more balance dataset, these properties were reduced to 4 DLS measures, 4 Zeta Potential and 4 PdI. A hierarchical clustering of the transposed dataset was used to determine clusters of similar properties (e.g. particle size distribution in DMEM + 5% FBS and 1min sonication with particle size distribution in DMEM + 10% FBS and 1min sonication). This allowed the determination of groups of similar properties which could be reduced to a single property and yield a more balanced dataset. Four groups were considered for each type of property, i.e. particle size distribution, Zeta Potential, and PdI, and one property was kept for each set of related properties and the others were discarded.

The hierarchical clustering of the resulting dataset, which contained 50 variables, is presented in Figure 4.3 and shows that NM-103 and NM-104 form a very solid group (p<0.01). The other 4 NMs form another group as they are clustered together with high significance (AU value). It is worth mentioning that the clusters obtained here must be only considered from an exploratory point of view and in a weight of evidence context. This information alone cannot be used to define clusters of NMs but must be complemented with other techniques and rationales (e.g. PCA, variable selection, mechanistic information) to be used in read-across.

---

[41] In this context with structural similarity we intend the similarity in the properties under the nanoforms identification, fundamental behaviour and reactivity
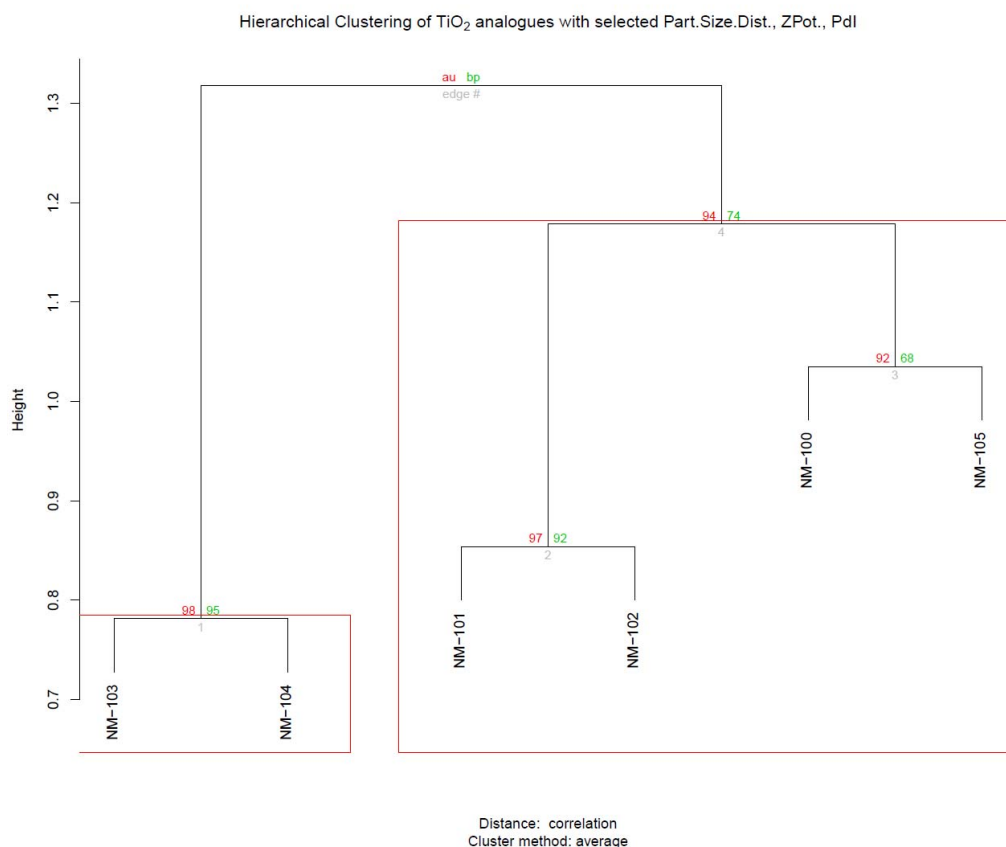
Figure 4.3. Hierarchical clustering of the TiO$_2$ analogues. The numbers in red correspond to the "Approximately Unbiased" (AU) p-value that is computed by multiscale bootstrap resampling, and the ones in green to "Bootstrap Probability" p-value (BP), which is computed by normal bootstrap resampling. The height in the Y-axis indicates the distance between clusters computed as average linkage. AU p-value will be used for the interpretation as it is usually a better approximation to the real p-value.

Principal component analysis (PCA)

While the hierarchical clustering indicates similar NMs by taking into account all physicochemical properties and forming subsequent groups of 2 substances, the PCA is a dimensionality reduction technique that shows the properties that account for the maximum variance between individuals, NMs in this case. The PCA also uses all properties to determine each of the principal components (PC) but are weighted in such a way that a minimum number of properties can be used to explain the differences between the different NMs.

The principal component analysis of the same dataset used for the hierarchical clustering of the analogues shows a similar picture (Figure 4.4) to the one obtained in the hierarchical clustering. The different NMs are placed in the plot by using the PC1 and PC2 scores and the loadings of each property with respect to PC1 and PC2 are indicated as arrows. NMs that appear close to each other indicate similarity in the space defined by PC1 and PC2. Long and light blue arrows indicate high contribution of that specific property to one of the PCs. The closer the arrow is to an axis, i.e. to a PC, the higher the contribution it has to that PC. It is

269

necessary to remember that the PCs are simplifications of the whole picture and that the fact that NMs appear close to each other only indicates that these NMs are similar to each other in that reduced representation of reality given by 2 variables, i.e. PC1 vs PC2. PC1 and PC2 typically account for a rather large variance (>50%) and indicate what are the variables that differentiate the NMs. The fact that these variables be related with the endpoint of interest cannot be assured and is not the purpose of PCA or other unsupervised techniques.

In Figure 4.4, NM-103 and NM-104 appear close to each other at the positive side of PC2. The arrows show that these positions are mainly driven by the properties related to impurities of Al (Biodurability 24h in Gambles solution (Al content)), Mg, by the crystal type rutile, and % of surface coating. NM-100 appears at the top part of the plot mainly driven by Particle primary diameter and Crystallite size, which matches the fact that NM-100 is the biggest NM of the series (considered as bulk material). For the same reason, NM-101 appears at the bottom of the plot as it is the smallest NM, and NM-102 and NM-105 appear next to each other on the negative side of PC1, mainly driven by Crystal type anatase and by not having surface coating.
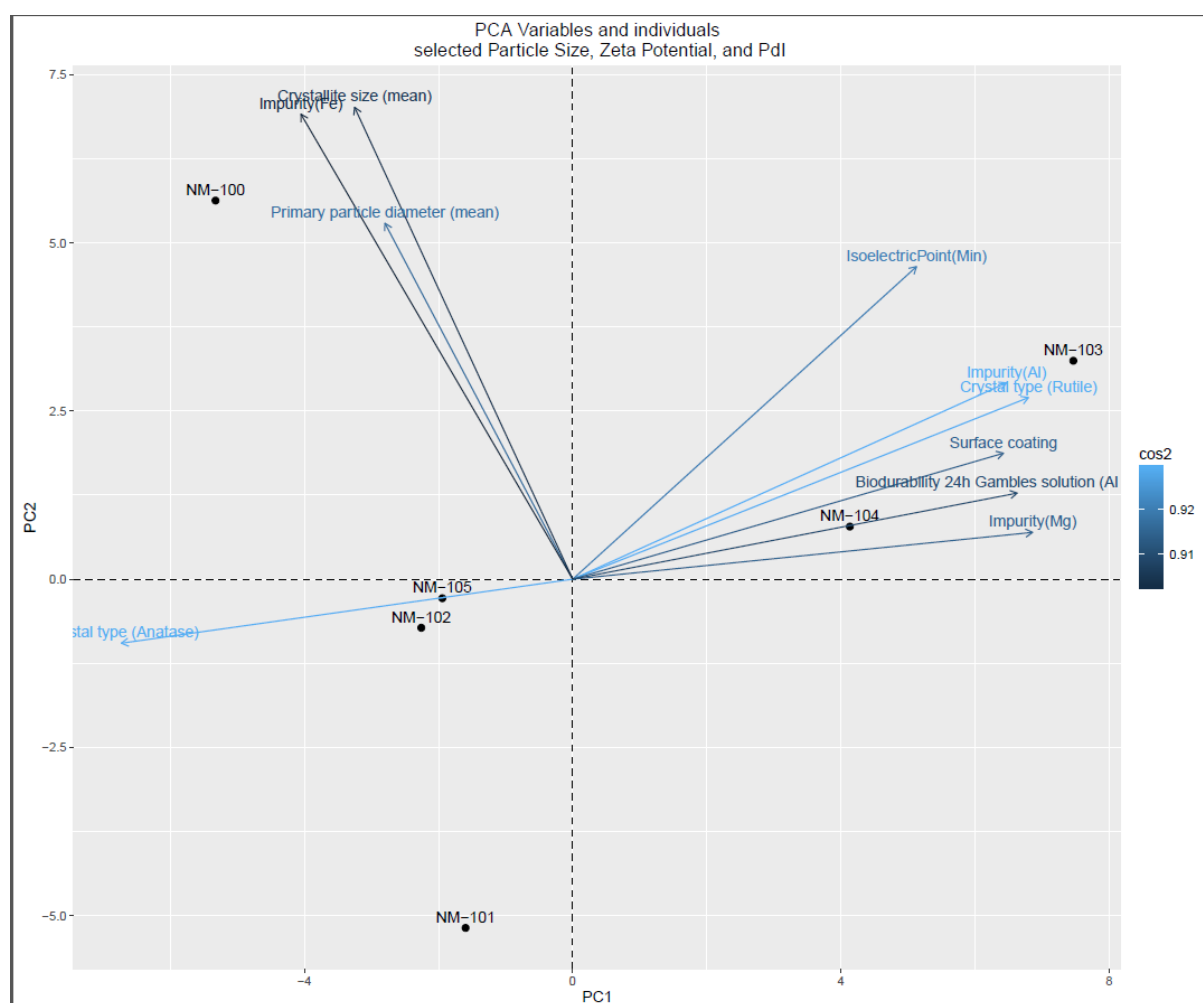

Figure 4.4. Principal component analysis of the dataset of 6 TiO$_2$ analogues. The position of the analogues (individuals) on the space of PC1 vs PC2 are indicated as black dots. Arrows

correspond to the 10 variables with higher contribution to the PCs. The colours are defined by the squared loadings (cos2) and indicate their contributions to the PCs.

The squared loadings of the two first principal components are given in Table 4.10 and show that the properties with the higher contributions to PC1 are the Biodurability 24h Gambles solution (Al content) and Impurity (Al), which are similar properties; crystal type (anatase and rutile), and % of surface coating and Mg impurity. For PC2 the main contributors are the specific surface area, total pore volume, primary particle diameter, crystallite size, and Fe impurities.

Table 4.10. Squared loadings of PC1 and PC2 of the PCA of the source analogues.

Property	PC1 loadings^2	Property	PC2 loadings^2
Biodurability 24h Gambles solution (Al content)	0.90	Specific surface area (mean)	0.77
Impurity(Al)	0.89	Mean of total pore volume (ml/g)	0.74
Crystal type (Rutile)	0.89	Primary particle diameter (mean)	0.73
Crystal type (Anatase)	0.89	Crystallite size (mean)	0.67
Surface coating	0.87	Micropore volume (ml/g)	0.63
Impurity(Mg)	0.87	Impurity (Fe)	0.63

It can be deduced from the PCA that the differences between NMs are mainly due to the presence of impurities and coating (Al content mainly provides from coating), crystal type (anatase vs rutile), particle size (including surface area), and pore volume. The fact that crystal type variables appear so high in the list is due to they correspond to the percentage of crystal of that type, and since most of the particles are either 100% anatase or 100% rutile, the differences between the NMs is extreme. Primary particle diameter is also one of the main differences between NMs as the biggest one is 115 nm and the smallest is 5 nm. Biodurability 24h Gambles solution (Al content) and Impurity(Al) are very similar properties as the former one corresponds to the quantity of Al dissolved in media after 24h, and the second one correspond to the quantity of Al found after calcination of the NMs. The former, is also part of coating as NM-103 and NM-104 have around 6% of Al_2O_3 coating and therefore a large amount of Al. Surface coating, which includes not only Al_2O_3 but also organic matter like sylanes or glycol is also one of the main contributors to PC1. The loadings also show that other properties like Zeta Potential, PdI, or particle size distribution have less influence.

Random forest as variable selection to predict the comet assay

Hierarchical clustering and PCA are unsupervised techniques as they only make use of the physicochemical properties of substance to determine clusters of similar substances and the properties that differentiate the most those substances. The random forest variable selection algorithm, instead, is a supervised technique as it uses the physicochemical

properties to predict a given outcome, in this case positive or negative results in comet assays, and provides a relative importance of the variables for the prediction.

The variable importance plot of the source analogues (Figure 4.5) clearly shows that the most important variables to predict the comet assay results for the 6 analogues are the Total non-TiO2 content and the amount of organic matter. As mentioned above, Total non-TiO2 content corresponds to the sum of the impurities and coating found on the analogues, and this distinction had to be made because it is impossible to determine from the data whether NM-101 was coated because, according to the manufacturer, NM-101 has no coating. However, the tests performed in the Nanogenotox Joint Action (see Deliverable 4.3 Table 2-1, and page 57) showed that there was a significant mass loss at 200 °C that was identified by GC-MS analysis as hexa/octadecanoic acid and others. These impurities accounted for around 9% of the total weight and were considered as coating by Nanogenotox. They were not considered coating in this work because NM-101 is stated to be uncoated by the manufacturer, and according to REACH, it can only be considered coating those materials that were intentionally added. Therefore, the 9% of organic matter of NM-101 has been considered as "impurities" in this report. In addition, the level of stability or binding of these "impurities" to the particles is unknown. It could be that they were just adsorbed on the particles and would dissociate from the particle when put in contact with the media. The properties that follow in the list correspond to Al impurities (Biodurability 24h), which are also related to the presence of coating as they are measured as the content of Al in solution after 24h.

**Variable importance of the target analogues
to predict Comet assay results**

Organic matter
Total non-TiO2 content
Biodurability 24h Caco2 (Al content)
Biodurability 24h Gambles solution (Al content)
Biodurability 24h 0.05% BSA (Al content)
Impurity(Fe)
Impurity(Al)
Polidispersability Index MQ water - 1min sonication
Part.Size.Dist(Size (d.nm) DMEM + Lglutamate Mode #1 - 20min sonication)
IsoelectricPoint(Mean)
Part.Size.Dist(Z-Avergae (d.nm) PBS - untreated)
Zeta Potential (mV) MQ Water - 1min sonication
Impurity(Na)
Impurity(Si)
Polidispersability Index DMEM + Lglutamine - 20min sonication
Crystal type (Rutile)
Impurity(S)
Part.Size.Dist(Size (d.nm) MQ Water Mode #1 - 1min sonication)
Surface coating
Part.Size.Dist(Zaverage)
IsoelectricPoint(Min)
Impurity(Mg)
IsoelectricPoint(Max)
Specific surface area (mean)
Biodurability 24h Caco2 (Ti content)
Zeta Potential (mV) PBS - 20min sonication
Dustiness-Respirable(mg/kg)
Density
Crystal type (Anatase)
Zeta Potential (mV) DMEM + 5% FBS - 20min sonication
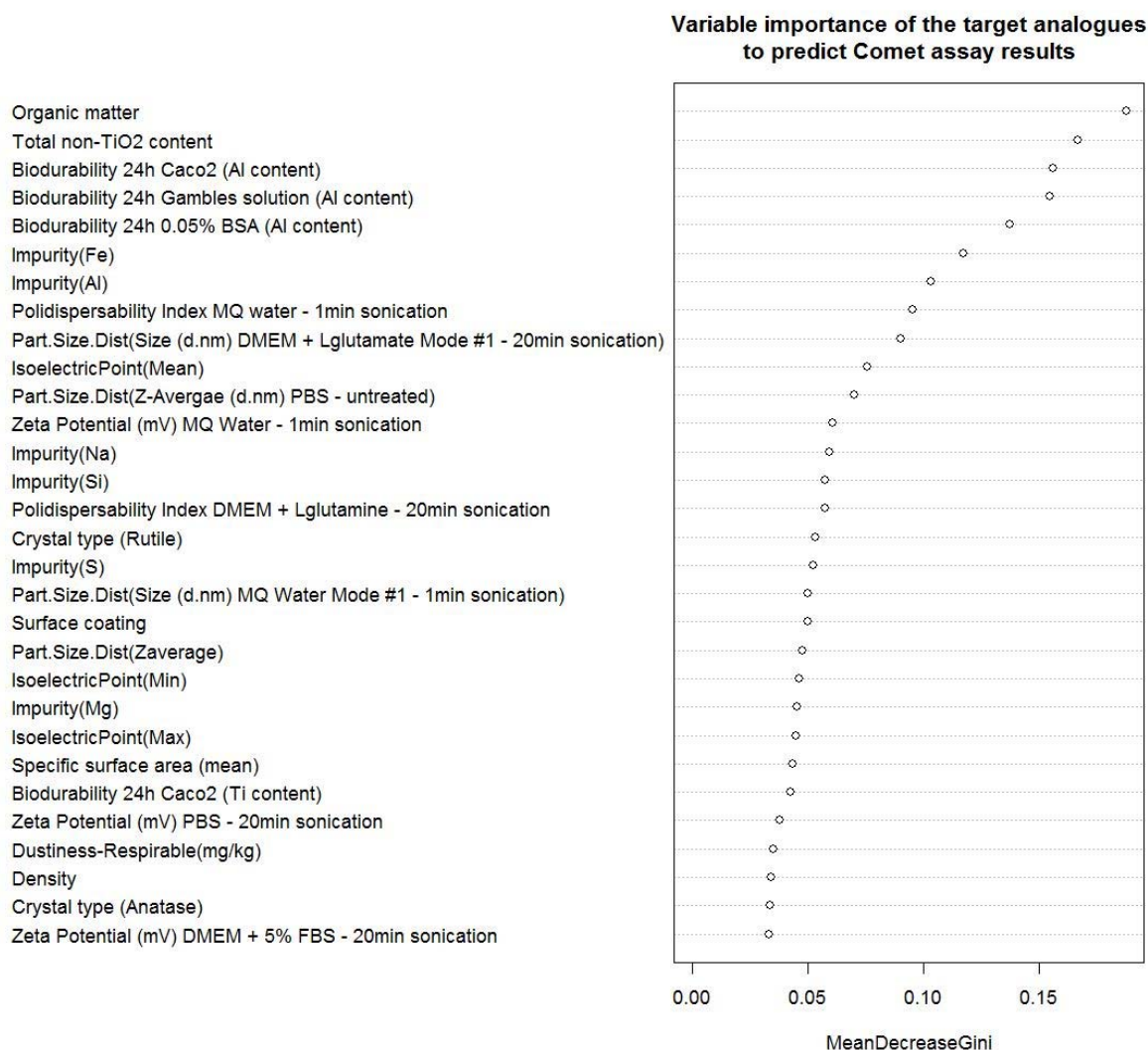
0.00   0.05   0.10   0.15

MeanDecreaseGini

Figure 4.5. Relative importance of variables in terms of their predictivity of the comet assay. Variable importance expressed as mean decrease of the Gini index of the source nanoforms.

Conclusion

Hierarchical clustering and PCA source nanoforms show that two groups of NMs can be clearly defined from their physicochemical properties. NM-103 and NM-104 (negative in the comet assay) form a very strong group (p<0.01). Actually, they are very similar NMs of rutile type with a size of ~24nm, high content of impurities like Al and surface coating. The coating in fact contains Al_2O_3, which explains that Al impurities content was found as a relevant property. Another group is the one formed by the other NMs, more precisely NM-102 and NM-105 appear next to each other in the PCA (both are positive in the comet assay) as both correspond to uncoated anatase TiO2 (100% and 84%) with ~23nm and low amount of impurities. NM-100 does not cluster together with any of the other NMs in the PCA. The reason for this is because it corresponds to a relatively large "NM" (>117nm), which makes it significantly different from the rest. For instance in the PCA, PC2 has a strong component of particle size and, therefore these property sets NM-100 at the higher part of the plot.

273

However, if only the crystal type and coating are considered, NM-100 groups perfectly with NM-102 and NM-105 as it is uncoated, and 100% anatase. In addition, such a classification matches the toxicological profile of these NMs as they all turn out positive in the comet assay.

NM-101 is more difficult to classify because it is the smallest of all NMs with a diameter of 5nm (lower part of PCA), it is also declared as uncoated by the producer but the elemental analysis showed the presence of around 9% of organic matter or impurities. Moreover NM-101 is of type anatase but negative in the comet assay.

If NM-101 is not considered, it can be clearly stated that uncoated anatase NMs are the ones positive in the comet assay. This in fact is in line with the higher photoreactivity of anatase with respect to rutile (Luttrell et al., 2014). Although comet assays are carried in lab facilities were very little UV light should be present to differentially photoactivate anatase, it was recently shown that anatase samples exposed to fluorescent tube lab light caused statistically significant higher amounts of DNA breaks (Karlsson et al., 2015) to BEAS-2B cells. However, NM-101 is also of anatase type but turns out negative in the comet assay breaking the abovementioned relation between anatase and positive comet assays. NM-101 contains a large amount of organic impurities (9%), which is of similar composition to the coating of NM-103 and NM-104. Whether these organic impurities (organic matter in Table 4.) are considered coating or not – in order to be considered coating the substance must be intentionally added – it is clear that there is a correlation between the NMs that have coating and/or organic impurities and the result of the comet assay. Moreover, this correlation is supported by the results of the random forest variable selection which shows that the two variables with the highest discriminant power to predict comet assay are Total non-TiO$_2$ and Organic matter. Following our hypothesis, uncoated rutile would also be predicted as possibly genotoxic. However, it would be desirable to dispose of data for this type of nanoform in order to be able to have a prediction with less uncertainty.

Filling data gaps by read-across

In the previous paragraph the grouping hypothesis for the nano-TiO$_2$ analogues was supported by the results obtained from hierarchical clustering, PCA and random forest variable selection algorithms.

The two target NMs were identified in Table 4.3, including the coating of the two nanoforms. According to the physicochemical properties of the identified target NMs, we can assume they are included in the same variable space as the source NMs: primary particle size, shape, total non-TiO2 content, organic matter, crystal type, and specific surface area are included in the range defined by the source NMs. Because of the lack of some physicochemical data for the target NMs, it was not possible to include them in the PCA analysis or in the clustering exercise. However, it is possible to assign the two target NMs to a class according to some of their characteristics. Since the presence of coating or high amount of non-TiO2 content on the surface of nano-TiO$_2$ appears to prevent NM to cause DNA damage detected by the *in vitro* comet assay, it is possible to group TiO$_2$ R nano with the analogues –NM-103 and NM-104 and possibly NM-101, giving negative results, and TiO$_2$ A nano with NM-100, -102 and -105, which cause DNA damage.

As shown in Table 4.9 and Table 4.11, the two target NMs have different characteristics with respect to coating or non-TiO2 content. TiO_2 R has a coating, and thus it is predicted to have a negative outcome in the *in vitro* comet assay. TiO_2 A, instead, has a relatively low level of impurities and no coating, and thus our prediction is that it gives positive result in the *in vitro* comet assay. The prediction is reported in Table 4.11. This outcome is confirmed by the *in vitro* comet assay carried out by Guichard et al. (2012) which shows that TiO_2 A is genotoxic and TiO_2 R is not.

Table 4.11 Read-across matrix showing the prediction for the identified target NMs.

	Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105	TiO$_2$ R	TiO$_2$ A
	In vitro comet assay	+	-	+	-	-	+	-	+
What they are	Total non-TiO2 content including coating and impurities (% w/w)	1.5	9	5	11	11	0.11	13	0.5
	Surface coating (%)	0	0	0	8	8	0	11	0
	Organic matter (%)	0	8	0	2	2	0	2	0
	Crystal type (Anatase)	1	1	1	0	0	0.84	0	1
	Crystal type (Rutile)	0	0	0	1	1	0.16	1	0
	Crystal type (Cubic)	0	0	0	0	0	0	0	0
	Crystallite size (mean) (nm)	117.81	7.69	23.93	24.32	24.71	22.44		
	Shape (rod=1, spherical=0)	0	0	0	1	0	1	1	0
	Aspect ratio	1.53	1.53	1.53	1.7	1.53	1.36	6.2	1
	Primary particle diameter (mean) (nm)	93.45	5.25	22.00	24.00	24.50	20.13	62x10	14
	Specific surface area (m^2/g)	9.23	316.07	77.86	53.98	54.33	47	149	177
Where they go	Isoelectric Point (Mean) (pH)	NA	5.5	6	8.3	8.5	6.8		
	Density (g/mL)	3.84	3.99	3.84	4.02	4.09	4.05		
	Mean of total pore volume (mL/g)	0.032	0.319	0.300	0.262	0.194	0.194		
	Micro surface area (m2/g)	0	13.625	1.108	0	0	0		
	Micropore volume (mL/g)	0	0.00179	0.00034	0	0	0		
	Dustiness-Respirable(mg/kg)	1500	5600	9200	19000	6400	11000		
	Biodurability 24h 0.05% BSA (Ti content) (µg/l)	5.2	0	0	0	0	0		
	Biodurability 24h Gambles solution (Ti content) (µg/l)	0	0	3388	0	0	0		
	Biodurability 24h Caco2 (Ti content) (µg/l)	796	3414	1741	222	3386	2724		
What they do	Redox Caco2 medium $^\Omega$	1	-1	-1	1	-1	-1		
	Redox Gamble's solution $^\Omega$	1	0	-1	1	-1	-1		
	Redox BSA $^\Omega$	0	0	0	0	0	0		

$^\Omega$ values obtained from Nanogenotox 4.7 determined by measuring the content of O$_2$. Oxidising properties (1), neutral (0), reducing (-1)

276

4.4.6 Perform and/or propose testing

The sixth step of ECHA draft guidance (ECHA, 2017b) asks for identifying testing, where the data collected to support the grouping hypothesis is not considered reliable or sufficient. This case study was intended to show how to document read-across following the workflow proposed in the recommendation for grouping of NMs. For this purpose, genotoxicity by means of comet assay was selected as it allowed the definition of two groups of NMs, positive in the comet assay and negative in the comet assay. The adequacy of using in vitro comet assay for NMs has been debated in the literature (Azqueta & Dusinska, 2015; Magdolenova et al., 2014) and if compared with other methods it shows some tendency to provide more positive results (Armand et al., 2016; Guichard et al., 2012; Jugan et al., 2012; Kansara et al., 2015; L Browning, The, & Sr., 2014; Prasad et al., 2014; Prasad, Wallace, Daniel, Tennant, Zucker, Strickland, Dreher, Kligerman, Blackman, & DeMarini, 2013; Stoccoro et al., 2016; Tavares et al., 2014b; Vales, Rubio, & Marcos, 2014). Therefore, the available data for nano-TiO$_2$ does not seem to confirm genotoxicity and the overall conclusion should be limited to the result of the in vitro comet assay and not on the genotoxic potential of TiO$_2$ nanoforms. Extension of the dataset with reliable *in vivo* genotoxicity could be a possible next step. In *in vivo* studies, toxicokinetic data show that after intravenous administration, liver, spleen and lungs may be considered as target organs as there is evidence that nano-TiO$_2$ reaches these organs and tend to accumulate there. This evidence is supported by the Heringa et al. (2016), who identify a potential human health risk for liver, spleen, ovaries and testes.

Since biological reactivity is an important property in supporting similarity, it would be interesting to have a test guideline to fulfil to this requirement, as at the moment there are a few data available and there is no standardised experimental approach to investigate this property.

4.5 The carbon nanotube case study: predicting genotoxicity

Genotoxicity was also selected as the toxicological endpoint to read-across, as most data for the analogues were available for this endpoint (Figure 4.). Under the Nanogenotox Joint Action *in vitro* and *in vivo* comet assays and chromosomal damage (micronucleus) tests were carried out for some of the analogues (NanoGenoTox Joint Action, 2013). 10 analogues were selected based on their data from *in vitro* and *in vivo* comet assays as performed by the Danish National Research Centre for the Working Environment (NRCWE) (Poulsen et al., 2016). The *in vitro* comet assay detects DNA strand breaks as well as oxidative damage to DNA at the level of single cells. The chromosomal aberration assay or the micronucleus test assess chromosomal damage, i.e. when part(s) of a chromosome is/are deleted, added or rearranged (clastogenic effects), or aneuploidy (numerical modifications of chromosomes).

In our case study, we aim at identifying MWCNTs PC properties that may affect DNA damage, to be able to read-across to target MWCNTs.

4.5.1 Identification and characterisation of the nanoforms of the substance

Carbon nanotubes are graphene sheets rolled into a cylinder (Figure 4.6). Depending on the number of cylinders arranged in concentric layers single walled (SWCNT) and multiple walled carbon nanotubes (MWCNT) can be distinguished. CNTs are considered a nanoform without a corresponding bulk form.
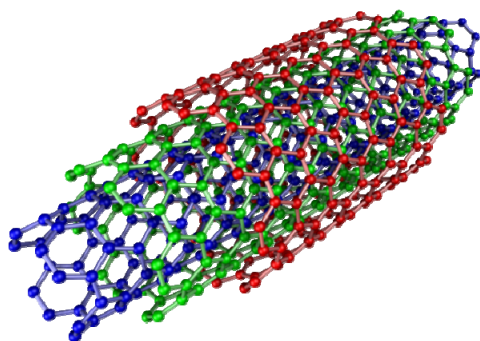

Figure 4.6. A multi-walled armchair carbon nanotube, rendered in POVRay" by Eric Wieser, published on 27 December 2010 on Wikimedia Commons at https://commons.wikimedia.org/wiki/File:Multi-walled_Carbon_Nanotube.png under the Creative Commons Attribution-ShareAlike 3.0 Unported (CC BY-SA 3.0) licence.
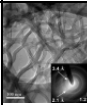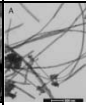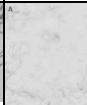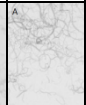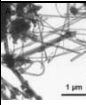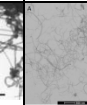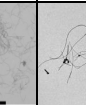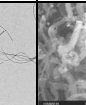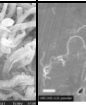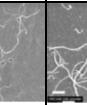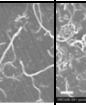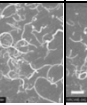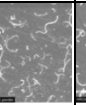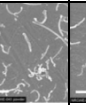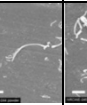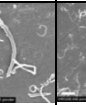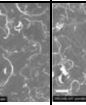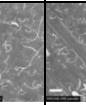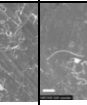
The physicochemical properties of MWCNT can vary, depending on the production process and consequent modifications. Catalytic metals are required in the manufacturing process and may remain as impurities in the CNTs. The most important differences in physicochemical properties that may have been reported to have an impact on toxicological outcomes are: Length, diameter, impurities/catalysts, surface reactivity, surface modification, flexibility/rigidity and agglomeration state (Allegri et al. 2016; Braakhuis et al. 2014; Hamilton et al. 2013; Jackson et al. 2015).

Other relevant parameters such as chemical composition, biopersistence and (in)solubility apply to all (non-functionalised) analogues as MWCNT consist of elemental carbon which is not soluble in any (physiological) media tested and recalcitrant to biodegradation.

19 analogues (Table 4.12) were selected for this case study based on the richness and quality of the physicochemical and toxicological data. Information on these MWCNT was retrieved from the i) OECD WPMN Testing Programme; ii) FP7 Nanogenotox Joint Action, iii) REACH dossier iv) IARC dossier v) peer reviewed literature, of which the publication by Jackson et al. 2015 (Jackson et al., 2015) and Poulsen et al 2016 (Poulsen et al., 2016) was given special attention (see below). The analogues are arranged by increasing number (NM-400, NM-401, NM-402, NM-403 and NM-404) in the JRC repository, then the 2 Mitsui types, Nikkiso and Hanwha and finally the NRCWE analogues tested by Jackson et al. (Jackson et al., 2015). Analogues 1-8 are synthesised by chemical vapour deposition (CVD) and are not functionalised. Analogues 11-12, 14-15 and 17-19 are surface modified (Table 4.12). The order of the first 8 analogues was not informed by any physicochemical or other property; the other analogues are grouped by size and surface modification as suggested by (Jackson et al., 2015). Carbon black and Crocidolite (asbestos) were added as reference materials.

The information available for each NM was restructured starting from the above mentioned sources to build a complete dataset including both physicochemical and toxicological information with focus on genotoxicity, as this was the endpoint with the most abundant information.

Table 4.12 Analogues selected for the case study

Analogue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Ref 1	Ref 2
Name	NM-400	NM-401	NM-402	NM-403	Mitsui NRCWE-006	Mitsui NRCWE-007	Nikkiso	Hanwha CM-100	NRCWE-26	NRCWE-040	NRCWE-041	NRCWE-042	NRCWE-043	NRCWE-044	NRCWE-045	NRCWE-046	NRCWE-047	NRCWE-048	NRCWE-049	Printex 90	Crocidolite
Production process	CVD	CVD	CVD	CVD	CVD	CVD	CVD	CVD													
TEM image																					
Main source of information	JRC Rep; OECD, Nanogenotox, REACH dossier	JRC Rep; Jackson 2015	JRC Rep; OECD, Nanogenotox; Jackson 2015,	JRC Rep; OECD, Nanogenotox, REACH dossier; Jackson 2015	OECD, Nanogenotox; Jackson 2015	Nanogenotox	OECD SP	OECD SP	Jackson et al 2015; Poulsen et al 2016												

280

Identification of target analogues

Defining similarity between NM analogues is challenging as it is not only based on similarity of the chemical structure/composition, but is also influenced by physicochemical properties. 19 MWCNT were selected as analogues for which data for relevant physicochemical and most toxicological endpoints was available (Table 4.13). Physicochemical data was not reported consistently for the analogues and it was found to be very heterogeneous. For example the description of crystalline phase is very dissimilar; diameter and length are reported as average values or ranges, impurities are reported as absolute values or percentages and information on rigidity or tangle can be identified only from TEM pictures. This makes it difficult to compare the analogues as with regard to their similarity of physicochemical properties.

The data matrix was filled with available data to determine the source substances, i.e. those with data on genotoxicity, and the target analogues, i.e. those with data gaps. In this case study we follow a category approach, thus source and target analogues may change depending on the endpoint of interest and the availability of data for these endpoints (Section 4.5.4 and Table 4.17).

Table 4.13 Identification of the 19 analogues and 2 reference materials: Overview the physicochemical properties

Analogue	1	2	3	4	5	6	7	8	Ref 1	Ref 2
Name	NM-400	NM-401	NM-402	NM-403	Mitsui NRCWE-006	Mitsui NRCWE-007	Nikkiso	Hanwha CM-100	Printex 90	Crocidolite
Chemical composition (carbon purity)	89.91% - 99%	99.19% (	>92 - 99.19%	90% - >95%	> 99%		>98%	95%	>99%	
Impurities (% elements)	> 0.01%: Al, Fe, Na, S; 0.005-0.01%: Co, 0.0001-0.005%: Ca, K	> 0.01%: Al. Fe. S;	<5% (OECD) >0.0% 0.005-0.01 %: not detected; 0.001.0.005%: Ag	<5% (OECD) <3wt% Mn Mg, Al, Na, Ni, Fe; >0.01%: Al, Co, Mg, Mn, Ca	Fe: 0.3 - 1.06% Cr 14 ppm, Bismuth: 6ppm, Ni 4 ppm	Na: 0.05%, Al: 0.00086%, Cr: 0.0149%, Fe: 0.048%, Ni: 0.4843	Li 0.00005%; Al 0.0080%; Ca 0.0176%; Fe 0.0053-0.36%; Cd 0.0016 Ga 0.0176% Cr 0.0014%, Bi 0.00006%, Ni 0.00004%	Fe: 5%	FeO_3 0.006%; NiO 0.0003; Cl 0.03, CuO 0.0005%, SO_3 0.66%, ZnO 0.001%;	FeO_3 7.23%; MgO 0.19; MnO 0.03,
Surface coating	pristine	pristine	pristine	pristine					-	-
COOH (mmol/g)		0.02	0.14	0.09	0.04					
Primary particle diameter (nm)	5-35 (mean 9)	11-90	6-69 internal: 4.8; external: 11.7	10-16 D50: 11 nm	40-90	15	48 -63 nm	10-15	90% <460	9-14
Average length (TEM) (nm)	100-10.000; mean 1500	1300 - 5000	100-1300	400 ->10000	1000-19000	368, 10000-50000	940 -1100	From < 20000 to >20000 µm	90% <4500	ND
Aspect ratio	79±50	66±46	125±66	42±29	63.7 - 100	24.1				
Mass median diameter (µm)	85; D10: 31.6; D50: 85 D90: 228		0.416 - 2.56	0.400 - 3	0.07--1.6		0.45	0.543.-8.421		

Analogue	1	2	3	4	5	6	7	8	Ref 1	Ref 2
Name	NM-400	NM-401	NM-402	NM-403	Mitsui NRCWE-006	Mitsui NRCWE-007	Nikkiso	Hanwha CM-100	Printex 90	Crocidolite
Pour density (weighing) (g/cm3)	0.06 - 0.08	0.11	0.02 - 0.09	0.16			3.8			
Specific surface area (m^2/g) (BET)	250-300	226.4	24-300	189-300	23-28		69	224.9	5.24	182
Respirable Dustiness	< 420 mg/kg	< 4200 mg/kg	< 1700 mg/kg	< 4900 mg/kg	2.4%		0.061 mg/m^3			
Solubility in water	< 2 mg/L (20°C, pH 7.5 - 9.2) (practically insoluble)			< 2 mg/L (20°C, pH 7.5 - 9.2) (practically insoluble)				oxidised MWCNT was completely dispersed in deionised water		
(Bio)persistence (time) (Bio)degradation			not readily biodegradable	not readily biodegradable	not readily biodegradable		not readily biodegradable			
Zeta potential (mV)	not relevant for MWCNT	not relevant for MWCNT	not relevant for MWCNT	not relevant for MWCNT			ca -14.7 mV +/-0.9 (DME-FBS 10%)	-45 < -10		

Analogue	9	10	11	12	13	14	15	16	17	18	19
Name	NRCWE-26	NRCWE-040	NRCWE-041	NRCWE-042	NRCWE-043	NRCWE-044	NRCWE-045	NRCWE-046	NRCWE-047	NRCWE-048	NRCWE-049
Chemical composition (% carbon purity)	84.4	98.6	99.2	99.2	98.5	98.6	96.3	98.7	98.7	98.8	98.8
Impurities (% elements)	Al_2O_3 14.97; CaO 0.01; Cl 0.01, CoO 0.11, Cr2O3 0.0007, FeO3 0.29; NiO 0.0008, ZnO 0.002;	CaO 0.05; Cl 0.05, CoO 0.001, Cr2O3 0.02, CuO 0.0013, FeO3 0.2; La2O3 0.32, MgO 0.01, MnO 0.002, NiO 0.56, P_2O_5 0.15, SiO_2 0.02, SO_3 0.01, ZnO 0.001;	CaO 0.13; Cl 0.02, CoO 0.001, Cr_2O_3 0.02, FeO_3 0.13; K_2O 0.003, La_2O_3 0.03, MgO 0.02, MnO 0.001, NiO 0.31, P_2O_5 0.20, SiO_2 0.01, SO_3 0.01, ZnO 0.001;	CaO 0.25; Cl 0.02, Cr_2O_3 0.02, CuO 0.008, CuO_2 0.007, FeO_3 0.08; K_2O 0.005, La_2O_3 0.02, MgO 0.03, MnO 0.001, NiO 0.21, P_2O_5 0.14, SiO_2 0.1, SO_3 0.01, ZnO 0.001;	CaO 0.04; CoO 0.001, Cr_2O_3 0.02, FeO_3 0.008; K_2O 0.001, La_2O_3 0.02, MgO 0.01, NiO 1.2, P_2O_5 0.15, SiO_2 0.006, SO_3 0.04, ZnO 0.001;	CaO 0.08; Cl 0.01, CoO 0.002 CuO 0.0024, FeO_3 0.004; K_2O 0.003, La_2O_3 0.01, MgO 0.02, NiO 1.04, P_2O_5 0.14, SiO_2 0.01, SO_3 0.03, ZnO 0.001;	Al_2O_3 0.52; BaO 0.06, CaO 0.08; Cl 0.02, CoO 0.250, Cr_2O_3 0.02, CuO 0.0038, FeO_3 1.17; K_2O 0.003, La_2O_3 0.01, MgO 0.02, MnO 0.002 NiO 1.34, P_2O_5 0.16, SiO_2 0.02, SO_3 0.06, ZnO 0.001;	Al_2O_3 0.29; CaO 0.03; Cl 0.01, CoO 0.25, Cr_2O_3, 0.02, CuO 0.0015, FeO_3 0.008; MgO 0.22, MnO 0.3, NiO 0.0045, P_2O_5 0.14, SiO_2 0.007, ZnO 0.001;	Al_2O_3 0.27; CaO 0.03; Cl 0.02, CoO 0.25 Cr_2O_3, 0.001, CuO 0.0006, FeO_3 0.007; MgO 0.22, MnO 0.3, NiO 0.0043, P_2O_5 0.15, SiO_2 0.02, SO_3 0.01, ZnO 0.001;	Al_2O_3 0.26; CaO 0.02; CoO 0.24 Cr_2O_3, 0.001, FeO_3 0.007; MgO 0.19, MnO 0.28, NiO 0.0037, P_2O_5 0.14, SiO_2 0.007, ZnO 0.001;	Al_2O_3 0.26; CaO 0.03; CoO 0.25, Cr_2O_3, 0.001, CuO 0.0004, FeO_3 0.004; MgO 0.19, MnO 0.29, NiO 0.0038, P_2O_5 0.15, SiO_2 0.008, SO_3 0.01, ZnO 0.001;
Surface coating	pristine	pristine	OH	COOH	pristine	OH	COOH	pristine	OH	COOH	NH_2

284

Analogue	9	10	11	12	13	14	15	16	17	18	19
Name	NRCWE-26	NRCWE-040	NRCWE-041	NRCWE-042	NRCWE-043	NRCWE-044	NRCWE-045	NRCWE-046	NRCWE-047	NRCWE-048	NRCWE-049
COOH (mmol/g)	0.4	0.18	0.84	2.04	0.09	0.11	0.31	0.32	0.13	0.29	0.16
Average length (TEM) (nm ± SD)	1500	518.9 (±598)	1005 (±2948)	723.2 (±971.9)	771.3 (±3471)	1330 (±2454)	1553 (±2954)	717.2 (±1214)	532.5 (±591.9)	1604 (±5609)	731.1 (±1473)
Aspect ratio											
Pour density (weighing) (g/cm3)											
Specific surface area (m²/g) (BET)	245	150	152	141	82	74	119	223	216	185	199
Respirable Dustiness											
Solubility in water											
(Bio)persistence (time) (Bio)degradation											
Zeta potential (mV)											

4.5.2 *Initial grouping of nanoforms*

In the literature grouping approaches for different types of nano-objects have been proposed, based on the presumption that there are common modes of action for several types of nanomaterials (see also previous chapter on nano-TiO$_2$). Some grouping approaches (Arts et al., 2015, 2016; Schröder et al., 2014) have proposed 4 different groups including Granular Biopersistent Particles (GBP) with no or little intrinsic chemical toxicity, (nano)particles with specific toxicity, soluble (nano)particles without significant toxicity and Nanotubes (Nanofibres). This case study focuses on nanotubes and the evaluation of MWCNTs as part of this group. All MWCNTs were initially gathered in one group. The possibility to form subgroups based on physicochemical properties (e.g. length, diameter, content of impurities) was also considered but no other groups than those defined by Jackson et al. could be identified.

For the selection of analogues it was decided to focus only on MWCNT to keep i) the number of analogues and ii) influencing properties manageable. The most important physicochemical properties for the analogues are summarised in Table 4.13. Those properties for which no data for any of the analogues was found or which were considered not applicable/relevant for MWCNTs (e.g. Crystal size, Log K$_{ow}$, Hamaker constant, hydrophobicity) were removed from the list. More MWCNT analogues from different producers have been found in literature but were not included in the case study due to lack of consistent data (e.g. uncertainties about identity, physicochemical characteristics). It is anticipated that the grouping could apply also to other MWCNTs that fit the category as defined within this case study and which are reasonably presumed to follow the same toxicokinetics and mode of action. Although the present case study is limited to the analysis of multiple walled carbon nanotubes, it is likely that the category can be expanded to include other CNTs (e.g. single walled carbon nanotubes) or even other carbon based nanomaterials such as fullerenes or carbon black, on a case by case base, depending on their similarities in physicochemical, translocation and toxicological properties. Carbon black as spherical carbon based material and crocidolite as high aspect ratio material have been included as reference material to assess the potential impact of the chemical and morphological similarity respectively.

Develop a grouping hypothesis (rationale for similarity)
Multiwalled carbon nanotubes consisting of >90% carbon within a size range of 5–90 nm diameter and 0.1 –20 μm length have the potential to induce ROS production and (chronic) lung inflammation following inhalation. This can potentially lead to genotoxicity (DNA strand breaks and/or increased mutation frequency) as secondary effect and increased risk of tumour formation (Schins and Knaapen, 2007).

The mutagenic activity of CNTs can be influenced by its size, morphology, rigidity, stiffness (the property of a solid body to resist deformation), surface coating and nature of impurities.

For none of the analogues a proposal for classification (according to CLP) for mutagenicity (and carcinogenicity) has been identified.

One of the analogues, Mitsui NRCWE-006 or MWNT-7, was suggested by IARC (International Agency for Research on Cancer) to be classified as possibly carcinogenic to humans (Group 2B) based on the evidence of observed mesothelioma following intraperitoneal (or intrascrotal) injection in rats. All other MWCNTs and SWCNTs were categorised in group 3 as

not classifiable to their carcinogenicity to humans (Grosse et al., 2014). Assuming carcinogenicity would be a consequence of genotoxicity, this could suggest two subgroups based on long (MWNT-7like) and short MWCNTs (see discussion below under 4.5.3.1).

Hereunder we assess if subgrouping based on size or other physicochemical properties is feasible or recommended.

Identification of the nanoforms within each group (attempts to create subgroups)

Physicochemical Properties (what they are)
Based on common starting material used during their synthesis, all category members are considered structurally/chemically similar: all MWCNT are synthetic graphite in tubular shape, with a purity (carbon content) ≥90%.

One of the problems to determine similarity of CNT is the fact that there are no "classical" structural alerts (fragments) for MWCNTs and molecular properties are not applicable, as MWCNT do not have a uniquely defined molecular structure like regular chemicals. Though some physicochemical properties such as morphology, size (long fibre-like shape; high aspect ratio), content of oxidising impurities, and rigidity could be comparable to structural alerts as they have been suggested to be predictive for MWCNT toxicity in some studies (Allegri et al., 2016; Braakhuis, Oomen, & Cassee, 2015; Jackson et al., 2015; Poulsen et al., 2016). Biopersistence, length and rigidity have been proposed to be determinants of MWCNT *in vivo* toxicity, in analogy with the toxicity of asbestos and other inorganic fibres (Ken Donaldson et al., 2006; Murphy, Poland, Duffin, & Donaldson, 2013). All these properties were investigated to be used to subgroup MWCNTs by using chemoinformatic techniques like hierarchical clustering and principal component analysis, which will be presented in section 4.5.5.

Physicochemical properties that were found for most of the analogues were presented in Table 4.. The properties that are considered necessary by ECHA (ECHA, 2017b) to identify nanoforms were individually analysed to be used to subgroup MWCNTs. The analysis and rationalisation are presented below. Raman spectroscopy which would yield information about the purity, defects and tube alignment, and which assists in the distinction of MWCNTs with respect to other carbon allotropes was only available for the analogues 1-3 (NM-400, NM-401 and NM-402) (K Rasmussen et al., 2014).

Chemical composition, content of impurities (residual metals)
All MWCNT analogues consist of > 90% pure carbon. MWCNTs possess intrinsic ROS-scavenging properties but they are also capable of generating intracellular ROS upon interaction with cellular components, and can cause antioxidant depletion (Van Berlo et al., 2012). Catalysts on CNT surface could be released and may have an impact on toxicity (Aldieri et al., 2013; Kagan et al., 2006; Y. Liu, Zhao, Sun, & Chen, 2013). Fenton-reactive metals content (Van Berlo et al., 2012) used as catalysts during synthesis of MWCNTs may contribute to oxidative stress and thereby promoting MWCNT-induced toxicity. Fe-rich (SW)CNTs (26% w/w) were found to be significantly cytotoxic and genotoxic in murine alveolar macrophages and induced a severe oxidative stress, compared to Fe-free (SW)CNTs (0.23% w/w) (Aldieri et al., 2013; Vietti, Lison, & van den Brule, 2016). Iron has also been identified as contributing to asbestos toxicity, which has been described to be consequence

287

of frustrated phagocytosis and multiple cellular processes (Boyles et al., 2015). The MWCNT analogues in this case study have very low levels of iron impurities; in general the amount of iron impurities of MWCNT are much lower than those of SWCNT which can reach up to 30% w/w. The contribution of other metals (aluminium, nickel, cobalt (see Table 4.)) to MWCNT toxicity have been suggested to be rather low (Boyles et al., 2015) although some of these metals are classified as carcinogens (Ni) or possible carcinogens (Co) (Mulware, 2012). The highest amount of impurities was reported for analogue 9, which contained 15% of Al_2O_3 (Jackson et al., 2015). Analogue 8, contained 5% Fe and analogue 4 <3% (Mn, Mg, Na, Ni, Fe) (OECD WPMN, 2016). Not only the amount but also bioavailability is a key determinant of metal impurities. In addition, a potential contamination with endotoxin (lipopolysaccharide, LPS) is also suggested to have an impact and therefore needs to be carefully tested (Esch, Han, Foarde, & Ensor, 2010; Jackson et al., 2015). Analogues 1-4 and 9-18 were tested for endotoxins and showed very low amounts of endotoxins (Jackson et al., 2015).

Besides catalytic metals, other types of impurities that might cause pulmonary toxicity (Ken Donaldson et al., 2006) can be present. For instance, support material such as aluminates, silicates, and magnesium oxide and residual organics like amorphous particles, or micro-structured particles such as graphite sheets, which might arrange into carbon nanofibers or spheres. Nevertheless, there is little toxicity information available that correlates them to adverse pulmonary effects and the presence of such substances is hardly found to be reported.

Morphology (size and rigidity)

Long MWCNTs are usually considered to be more hazardous than short ones in *in vivo* tests(Ken Donaldson, Duffin, Murphy, & Poland, 2012; Grosse et al., 2014; Murphy et al., 2013; Sweeney, Grandolfo, Ruenraroengsak, & Tetley, 2015). Long MWCNTs were shown to induce frustrated phagocytosis and affect alveolar macrophage function, including cell death, ROS generation and release of pro-inflammatory cytokines (reviewed e.g. by (Johnston et al., 2010; Vietti et al., 2016). Long and rigid fibres were shown to have problems to be incorporated into phagosomes, leading to the release of harmful oxygen radicals and hydrolytic enzymes which can cause chronic inflammation (Ken Donaldson, Murphy, Duffin, & Poland, 2010). The length and rigidity of MWCNT may also influence the translocation and clearance from respiratory airways making the smaller and more flexible tubes get more easily into deeper compartments of the lungs like alveoli.

Such results may suggest a possible sub-categorisation into long and short MWCNTs that would be relevant in predicting *in vivo* effects. Different thresholds have been suggested, but there is no agreement on a cut-off value. For this case study we suggest a threshold of 5 μm in accordance with the WHO fibre definition[42]. A similar threshold of 4 μm for pathogenicity of fibres to the pleura has also been suggested by (Schinwald et al., 2012). 4

---

[42] The World Health Organization (WHO) characterised the properties of bio persistent fibers. This refers to inorganic fiber dusts (except asbestos fibers) with a length > 5 microns, a diameter < 3 microns and a length-to-diameter ratio of > 3:1.

μm is also the median aerodynamic diameter of particles that fall within the respirable size range[43].

The groups based on this threshold are presented in Table 4.. Due to the varying numbers for MWCNT length reported in different reports, not all MWCNT types could be clearly assigned. Analogues 2, 3 and 4 are usually considered as short tubes with a length of ≤ 1 μm, however the Nanogenotox reports also stated a length from up to or above 10 μm.

Lengths greater than 15-20 μm can be important as they are associated with frustrated phagocytosis (Boyles et al., 2015; Ken Donaldson, Murphy, et al., 2010) due to the relatively small size of macrophages (10 μm) compared to the length of the fibre. Applying this threshold would separate analogue 8 with a length of ~ 20 μm from the other analogues. Numbers for length presented in (Poulsen et al., 2016) suggest that analogues 10-19 are short; based on data in (Jackson et al. 2015; from manufacturer) analogues 10-12 could be considered as very long (10-50 μm) and 13-15 as probably long (10-20 μm). Preference was given to the values determined by SEM image analysis.

Diameter and rigidity of MWCNT have also been reported as critical factors in mesothelial injury and carcinogenesis (Allegri et al., 2016; Fenoglio et al., 2012; Nagai et al., 2011). The outer diameter of MWCNT is directly related to surface curvature which influences protein adsorption and the formation of a corona (Gu et al., 2015). Thin MWCNTs (~10-50 nm) showed mesothelial cell membrane piercing and in general higher cytotoxicity, inflammogenicity and mesotheliomagenicity compared to thicker ones (diameter >40-150 nm) or tangled MWCNTs (Allegri et al., 2016; Fenoglio et al., 2012; Nagai et al., 2011). Larger diameter however has been suggested in some studies to be a significant predictor of genotoxicity in BAL and lung tissue (Jackson et al., 2015; Poulsen et al., 2016) while no significant difference between short/thin and longer/thicker MWCNTs with regard to pulmonary inflammatory response was found by (Poulsen et al., 2015).

A categorisation of MWCNT based on their diameters is difficult as no cut-off values have been suggested; moreover, the diameters reported for MWCNT show a big variability making it difficult to assign them to a thin or thick group. All analogues can probably be considered thin with a diameter <150nm (Table 4.15).

Straight/rigid MWCNT have been described to have higher potential for inflammogenicity and mesotheliomagenicity than tangled ones. They were also described to induce DNA damage *in vitro* and DNA damage and micronuclei in mouse lungs while tangled MWCNTs showed only slight increase in DNA damage *in vitro* (Catalán et al., 2015).

Information on rigidity is only described in few sources. If MWCNTs are tangled or straight can be estimated from scanning electron microscope (SEM) images if they are published. Analogue 1, 3, 4 and 6 have been described as tangled or highly bended, while analogues 2 and 4 as straight-wall MWCNTs (Catalán et al., 2015; de Temmerman et al., 2012). The TEM image of analogue 8 suggests tangled MWCNTs, while analogue 7 tubes seem rather straight (see images in Table 4.12 and overview in Table 4.16).

---

[43] European Committee for Standardisation 1993. Workplace atmospheres – Size fraction definitions for measurement of airborne particles. CEN Standard EN481, Brussels; http://www.inhaledparticles.org.uk/files/2013/08/G-Fern-Presentation-Session-.pdf

Results from raman spectroscopy show that analogue 2 had a spectroscopic profile with a high G-band (G for Graphite, sp2 hybridised bonds) intensity and low D-band (D for diamond or defect; sp3-hybridised carbon-carbon bond) (K Rasmussen et al., 2014) suggesting it has a more graphitic structure than analogues 1 and 3. It was also found to have the largest tube diameters and analogue 1 and 3 the smallest tube diameters among the similar MWCNT characterized by TEM. This is interesting as for MWCNT the intensity of the D-band increases compared to the G-band with increasing number walls. It was suggested to analyse further the influence of graphite particle impurities (K Rasmussen et al., 2014). No such information was found for the other analogues to allow a comparison.

Table 4.14 Attempts to subgroup MWCNT analogues based on length

	Short MWCNT (< 5 μm)	Probably short (< 5 μm)	Not assignable	Long MWCNT (≥ 5 μm	Very Long MWCNT (≥ 20 μm
Analogue		1, 2, 3, 4, 10-19	6	5	8

Table 4.15 Attempts to subgroup MWCNT analogues based on diameter

	Thin MWCNT (≤50 nm)	Probably thin (≤150nm)	Not assignable	Thick MWCNT (≥150 nm)
Analogue	1, 2, 4, 6, 8, 10-19	3, 5, 7		

Table 4.16 Attempts to subgroup MWCNT analogues based on rigidity

	Tangled	Not assignable/probably straight	straight
Analogue	1, 3, 4, 6, 8, 10-19	7	2, 5

Aspect ratio
MWCNT by definition have a high aspect ratio and are considered HARNs; aspect ratio was reported only for analogue 5 (63->100) and 6 (24). Aspect ratio as such has been described as not affecting genotoxicity of MWCNT (Kim et al., 2011).

Surface area
The surface area concept may apply to the fibrous structure of MWCNT; however also the length and diameter may be determinants of their biological/toxicological effects, based on the three-dimensional (3D) paradigm that is well known from asbestos and glass fiber toxicology; it is considered unlikely to be applicable for highly tangled/agglomerated CNT (Günter Oberdörster, Castranova, Asgharian, & Sayre, 2015) As most MWCNT agglomerates do not usually consist of straight, well-aligned parallel tubes, theoretical calculations or BET measurements may not provide direct information on the surface area which becomes available to the biological environment and may trigger a reaction. Such information may be more relevant to estimate the biologically effective dose (BED) (Ken Donaldson et al., 2013), however as it is system-dependent it will not be generally available. Functional groups attached to the sidewalls can also influence the surface area values (Allegri et al., 2016).

The fibrotic potency of SWCNT > MWCNT ≫ CNF was observed to correlate with their increasing specific surface areas (Mercer et al., 2011; Murray et al., 2012), though.

Surface chemistry

Functionalisation of MWCNT with hydroxyl or carboxyl groups can increase their dispersibility. It may alter surface charge, stability, functionality and reactivity and is often used to reduce MWCNT toxicity (Allegri et al., 2016). So far diverging results have been shown on its effects *in vitro* (Kakwere et al., 2015; Z. Liu et al., 2014) and *in vivo* (Jain et al., 2011; Sager et al., 2014). Surface chemistry influences the absorption of macromolecules to CNT, depending on available specific surface area, solution, pH, pKa value, and ionic strength in the solution (Cho, Huang, & Schwab, 2011). Sub-categorisation based on surface modification has been assessed for analogues 11-19 based on their surface coating, but no specific trends were identified (Jackson et al., 2015; Poulsen et al., 2016).

Surface charge was not reported for any of the analogues. For some it was stated that it was not measurable as they were agglomerated (W. Wohlleben et al., 2013). Zeta potential was only reported for two analogues and was found to be negative. Analogue 7 was -14.7mV and analogue 8 -45>-10. Zeta potential was considered not relevant for the other analogues (OECD WPMN, 2016).

Agglomeration and Dispersion

MWCNT are rarely present as isolated fibres but rather as self-assembled, intertwined, and coil-like structures (aggregates or agglomerates) depending on the various types of adhesion forces acting between tube surfaces (Pauluhn, 2009).

Agglomeration has an impact on the deposition of MWCNTs within the airways of the respiratory tract and assemblages of MWCNT may trigger pulmonary overload-related cascades of events at lower mass-based exposure levels than the high density poorly soluble particles (Pauluhn, 2009). Functionalised MWCNT were shown to form larger agglomerates than pristine counterparts when suspended in cell culture medium which is probably influenced by the different interaction with proteins (Allegri et al., 2016; Hamilton, Wu, Mitra, Shaw, & Holian, 2013). CNTs taken up as agglomerates have been shown to be less easily degraded by macrophages and could thus represent a higher risk of long-term toxicity (X. Wang et al., 2011).

Agglomeration behaviour was assessed from variation in the average particle size for the NRCWE analogues in different concentrations in exposure media (Poulsen et al., 2016).

As no single physicochemical parameter seems suitable to be predictive of toxicity, it is generally recommended to consider a number of property combinations in order to assess the overall hazard of a single material type (Arts et al., 2016; A. G. Oomen et al., 2014). It should also be considered that primary physicochemical descriptors of nanomaterials may not be the most appropriate to predict their toxicological behaviour, in part as many of these are "context" dependent, i.e. are affected by the surrounding matrix (pH, ionic strength, biomolecules or macromolecules etc.) or the route of exposure. Many NM properties are interdependent such that changing one property may inadvertently result in changing several others, e.g. changing NM shape/length may cause surface defects or change the surface chemistry. NM can be transformed by ageing and throughout their lifecycle (Mitrano

et al., 2015) what will inevitably affect surface chemistry, and thus should be considered when grouping CNTs and NMs in general.

Transformation

MWCNT consist of elemental carbon which is recalcitrant to biodegradation, though some biodegradation of CNTs by naturally occurring enzymes or organelles has been observed (Petersen et al., 2011). Nevertheless, data of various category members consistently show across *in vivo*, *in vitro,* and *in chemico* methods that MWCNTs do not undergo metabolism and are persistent (OECD WPMN, 2016). It can reasonable be assumed/predicted that untested category members (analogues 1, 2, 6, 8 for biodegradation/biopersistence) behave in the same way. The bioavailability of CNTs is generally considered low and CNTs are not considered to be transformed in the human body (Binderup et al., 2013; Jacobsen et al., 2013; MWCNT REACH Dossier, 2016). Catalysts on CNT surface could be released and may have an impact on toxicity (Aldieri et al., 2013; Y. Liu et al., 2013) due to their oxidising properties caused mainly by metals such as iron, aluminium, nickel, or cobalt (see Table 4.). However, the concentration of these metals on the surface of the MWCNTs is usually so low that its impact can generally be considered negligible; at least for the analogues considered in this case study.

Translocation (Physiological and cellular) (where they go)

The translocation determines the target organ/site where possible genotoxic actions could take place.

The deposition efficiency following inhalation in the parts pharynx, bronchi and alveoli differs and depends on the size of MWCNT which is determined by their agglomeration state (Günter Oberdörster et al., 2015). Agglomeration state and size have also been described to affect the clearance rate and the translocation from deposition sites (lungs of mice) to pleural sites, to local lymph nodes, and to secondary organs (Braakhuis et al., 2014; Mercer et al., 2013).

Uptake strategies in cells are dependent on the cell and the NM property. From the described pathways, phagocytosis (immunogenic > 0.5 µm) seems to be the most relevant for MWCNT (Kettiger, Schipanski, Wick, & Huwyler, 2013), whereas also Clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis have been described to play a role for uptake in bronchial epithelial and mesothelial cells (Maruyama et al., 2015).

The adsorption of proteins and biomolecules to NM surfaces can influence physicochemical properties (e.g. size and surface charge) and thus shape the biological identity and influence the interactions at the nano-bio interface (Aggarwal et al., 2009). However, due to continuous protein association and dissociation the composition of the corona varies over time (Cedervall et al., 2007). The binding of proteins to MWCNT is dependent on the surface chemistry. Negatively charged MWCNTs enhanced binding for some proteins but not others. However, in the case of CNTs it was also suggested that besides electrostatic properties also the stereochemical nature of both nanotubes (3-dimensional arrangement of carbon atoms) and proteins will determine the nanotube/protein binding (Cai et al., 2013; Q. Mu et al., 2008). Proteins were suggested to require a suitable surface curvature for binding as (functionalised) MWNTs with a larger diameter (~40 nm) generally exhibited stronger protein binding compared to those with a smaller diameter (~10 nm) (Q. Mu et al., 2008)

Toxicity (Genotoxicity) (what they do)

As described above, different mechanisms for possible genotoxicity of nanoparticles and CNTs have been proposed. Assuming that MWCNT genotoxicity is a secondary effect following inflammation, all factors influencing inflammation may be also relevant for the onset of genotoxicity.

Inhalation is considered the critical route of exposure route for MWCNT as no absorption via the oral or dermal route is expected (Binderup et al., 2013; OECD WPMN, 2016).

Following pulmonary exposure of mice or rats MWCNTs were observed to induce inflammation, granulomas and interstitial fibrosis. The mechanisms involved with adverse pulmonary responses of MWCNT are not completely understood, as well as the contribution of biopersistence of catalytic metals, fiber dimensions, and surface functionalisation.

Following inhalation, MWCNT due to their morphology could be too long to be engulfed and removed by macrophages and due to their biopersistence have very long retention times in the lungs (Ken Donaldson et al., 2006; Pauluhn, 2009). MWCNT are suspected to show similar toxicity to asbestos fibres and due to their needle-like structure may penetrate biological membranes, thereby inflicting mechanical damage (Kettiger et al., 2013). Specifically long MWCNTs have shown to be more cytotoxic and more potent in inducing pro-inflammatory and pro-fibrotic immune responses (Boyles et al., 2015; Ken Donaldson et al., 2012). The most evident response to long CNTs is frustrated phagocytosis, respiratory (oxidative) burst and pro-inflammatory and pro-fibrotic reactions (Johnston et al., 2010).

Micronucleus formation has been observed in different cell types following exposure to MWCNTs (see Table 2 in Van Berlo et al. 2012; NanoGenoTox Joint Action 2013). Micronucleus formation can result froth either a high level of chromosome damage or mitotic spindle disruption. SWCNTs have been described to interact with the mitotic spindle apparatus, including mitotic tubulin and chromatin, which could explain aneugenic effects as observed in several studies (L. Sargent et al., 2009). The similarity of CNTs and microtubules may facilitate interaction with the centrosome and mitotic spindle (Cortez & Machado-Santelli, 2008; L. Sargent et al., 2009). (SW)CNTs have been described to bind to DNA at G-C rich regions in the chromosomes including telomeric DNA (X. Li, Peng, & Qu, 2006; X. Li, Peng, Ren, & Qu, 2006). DNA intercalation and telomeric binding can induce chromosome breakage suggesting that interaction of the CNT with DNA may also be a source of chromosome damage.

4.5.3 For each group member, gather and evaluate data adequacy and reliability

The dataset of source analogues (Table 4.13) was analysed to identify a set of properties that can be used to define (structurally) similar NMs as well as to identify those PC properties that can help in predicting by read-across the results of genotoxicity. The dataset reported here contains only data that were used in the analyses. The full dataset is reported and described in Appendix X.

Physicochemical parameters

In order to properly study all CNTs it is necessary to have the same type of data. If not possible, a subset with the most consistent data can be used. This is the case for the NRCWE analogues (Jackson et al., 2015; Poulsen et al., 2016), which were used for the hierarchical clustering and PCA. The data found in (Jackson et al., 2015) was pre-treated in order to be able to use it for clustering and for PCA. For instance, the values that were given as ranges,

e.g. Diameter 67 nm (24-138nm), were transformed into 3 different properties corresponding to minimum diameter (24nm), maximum diameter (138nm), and average diameter (67nm). The contents of impurities that were not declared were considered to be 0%. Subsequently, all properties were scaled. In the case of content of impurities, viability, and proliferation; the maximum and minimum were set to 100 and 0%, respectively. Otherwise, small differences in viability (94% vs 97%) would have been over-represented after scaling. Invariant properties and those highly correlated (>0.90) were removed from the dataset that was finally used for the clustering and PCA exercise.

Toxicological information

Genotoxicity data from studies carried out with the MWCNT analogues was collected. Based on the availability of data, the following tests were considered for the grouping hypothesis assessment: *in vitro* studies in mammalian cells for gene mutation such as chromosome aberration (micro nucleus) and DNA damage (Comet assay), and *in vivo* studies such as chromosome aberration (micro nucleus) and DNA damage (Comet assay) if they were of reliability 1 or 2 (Klimisch et al., 1997).

Data were considered reliable if:

1. The NMs were characterized (size, coating or trade name or repository number provided to allow identification of the analogue) and the description of the dispersed materials should ideally be provided (particle size distribution, zeta potential, polydispersity index)

2. NM uptake was observed and/or cytotoxicity was tested

3. Positive and negative controls were considered, and replicates were included.

The *in vivo* studies were considered reliable if conditions 1 and 3 above were fulfilled; negative results should be taken into account only when it has been proven that the nanoparticles have reached the organ investigated. This could be confirmed with data on uptake or if cytotoxicity was detected.

Results from the bacterial mutagenicity test (Bacterial reverse mutation assay; Ames test) were collected but not taken into consideration in the evaluation because this test was concluded to not be applicable to NMs (OECD, 2014a) as they may not penetrate the cell wall and therefore potentially lead to false negative results. (Clift et al., 2012).

Most of the assessed genotoxicity studies of analogues 1-8 were carried out as part of the Nanogenotox Joint Action (Nanogenotox, 2010). WP5 of this project investigated the in vitro genotoxicity of MNs (Norppa et al., 2013). Various human cell lines of different origin were used for the comet and the micronucleus assays: pulmonary (bronchial epithelial BEAS 2B and 16 HBE; adenocarcinomic human alveolar basal epithelial A549), intestinal (epithelial colorectal adenocarcinoma Caco2, primarily undifferentiated cells used) and epidermal (NHEK). In addition, the comet assay was also performed in a 3-dimensional human reconstructed full thickness skin model. The micronucleus assay was also performed in human primary lymphocytes, a cell system widely used in genotoxicity testing of soluble chemicals.

The alkaline comet assay, a simple and sensitive method for the detection of DNA strand breaks (single- and double-strand breaks), alkali-labile sites, and excision repair sites, was used in all the human cell systems applied in the 1st part of WP5. In some laboratories, a

modified comet assay based on the use of FpG (formamidopyrimidine-DNA-glycoslyase), which improves the detection of oxidative DNA damage, was voluntarily used as a supplementary assay. In addition, all NMs were also tested in the mouse lymphoma assay, performed in mouse lymphoma L5178Y TK+/- cells. This technique is able to detect a wide spectrum of mutations, including gene mutations.

Additional genotoxicity test results were found in the OECD testing programme (OECD WPMN, 2016) and in peer reviewed literature, where we also found information on the analogues 7 and 8. We carefully checked for multiple reporting of the same studies in these sources.

In the Nanogenotox Joint Action data from selected *in vivo* genotoxicity tests (work package 6) was generated with the aim to assess the correlation between *in vivo* and *in vitro* results also taking into account the kinetic results (Nanogenotox WP6, 2013). 3 complementary tests were performed on rodents: comet assay, micronucleus assay (chromosome and genome mutations) and mutation Lac Z assay to measure gene mutations. The oral route was tested for analogues 1-3, whereas the endotracheal route for analogues 1-3 and 5.

For analogues 9-19 systematic *in vitro* and *in vivo* comet assays were carried out.

4.5.4   Construct a matrix to identify available data

All collected information on the analogues on physicochemical and toxicological properties was inserted into an Excel data sheet (see supplementary information S4). From these data sheets the availability of genotoxicity studies was determined and data gaps identified (see Table 4.17 and discussion below). Positive results out of the total number of identified tests are presented in Table 4.17.

Table 4.17 Total number of reliable genotoxicity studies for MWCNT found in the literature and reported in Supplementary material S4. *In vitro* and *in vivo* genotoxicity studies are presented as number of positive results/total number of tests identified. Data gaps are highlighted in red.

Analogue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Ref 1	Ref 2
Name	NM-400	NM-401	NM-402	NM-403	Mitsui MWNT-7	Mitsui NRC WE-007	Nikkiso	Hanwha CM-100	NRC WE-26	NRC WE-040	NRC WE-041	NRC WE-042	NRC WE-043	NRC WE-044	NRC WE-045	NRC WE-046	NRC WE-047	NRC WE-048	NRC WE-049	Printex 90	Crocidolite
in vitro gene mutation in mammalian cells	0/1	0/1	0/2	0/2	0/2	0/1															
in vitro chromosome aberration - micro nucleus	3/7	1/5	3/6	3/8	4/8	3/5	0/1	0/1													
in vitro DNA damage (Comet assay)	0/4	0/4	0/4	0/5	0/4	0/4			0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		
In vivo DNA damage - COMET assay	0/2	0/1	1/3		0/2		0/1	2/2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
In vivo micronucleus frequency and chromosomal aberrations	1/6	0/4	0/5		0/5		0/1	0/1													

The majority (59 out of 77) of the *in vitro* genotoxicity (Table 4.17) tests were found negative. The highest rate of positive *in vitro* tests (3 out of 10, for details refer to Annex X) was found in analogue 6, the lowest (2 out of 11) in analogue 2. All identified *in vitro* gene mutation studies in mouse lymphoma L5178Y TK+/- cells and V79, Chinese hamster lung fibroblast for analogues 1-6 were negative. Likewise, all *in vitro* comet assays in four different cell lines were negative. No gene mutation and chromosome aberration tests were found for analogues 7-8.

From the *in vitro* chromosome aberration (micronucleus) tests, some analogues showed in almost half of the tests increased numbers of micronuclei at concentrations starting from 10 – 30 μg/ml. Based on these results analogue 6 could be interpreted as positive, whereas analogues 1, 3, 4 and 5 may be considered borderlines. Positive results were mainly observed in BEAS-2B cells, human pulmonary epithelial cell A549 and undifferentiated human epithelial colorectal adenocarcinoma Caco2 cells. In 16 HBE cells all analogues tested were negative.

The authors concluded that although dose-dependent effects could be seen in many experiments, the genotoxicity of the MNs studied was usually relatively low; in such a situation, experimental variation may determine if the result will turn out positive or negative. They also considered that it was presently unclear how much of this variation represented true differences among the cell systems and how much could be explained by experimental variation, e.g. in MN dispersion, agglomerate size in the cell culture, MN sedimentation on the cells, and thereby cellular uptake and intracellular dose (agglomerates of different size and shape may have differential effects on cells), and variation in scoring.

In a second phase of the Nanogenotox Joint Action, a round robin study (an inter-laboratory test performed independently in 12 different laboratories) was carried out to assess the reproducibility of the genotoxicity tests. In this study (see Table 4.18), analogue 3 showed relatively reproducible results for the comet assay in Caco2 cells, and for the micronucleus assay in BEAS-2B cells. Both tests turned out mostly negative, 1/4 and 1/6, respectively. The comet assay in BEAS-2B cells showed low reproducibility (3/6) while micronucleus in Caco2 cells showed high reproducibility with positive results (3/4).

The authors concluded that in the case of the tested NMs the usual practice applied in validating short-term assays for genotoxic carcinogens cannot be followed. In particular it is unclear how important are genotoxic events in the carcinogenesis of the NMs that have been shown to be carcinogenic (Norppa et al., 2013).

Table 4.18 Results of *in vitro* round-robin test (Nanogenotox; Norppa et al. 2013)

Partner	MWCNT analogue 3	
	Comet	CBMN
Caco-2 cells		
ANSES	-	+
NRCWE	+	+
BfR	-	+
IPL	-	
RIVM		
INRS	-	(+)
BEAS 2B cells		
IMB-BAS	+	+
FIOH	-	-
NIOM	+	-
UAB	-	-
IPH	-	-
INSA	+	-

+: positive, -: negative; (+): equivocal; grey box: no data;

From the *in vivo* genotoxicity tests carried out (Table 4.17) the majority (30 out of 34) of the *in vivo* tests were negative. The *in vivo* micronucleus tests of the Nanogenotox Joint Action following oral and endotracheal route were all negative, whereas in the comet assay analogue 1 lead to an equivocal result based on positive findings in kidney following intratracheal instillation. Positive *in vivo* results were found in other studies published in peer reviewed papers whose reliability was doubtful (see comments in the annexed Excel sheet).

We conclude from these results, that the tested MWCNT analogues are most probably not genotoxic *in vivo*.

The NRCWE analogues 9-19 and 2-5 were tested in *in vitro* and *in vivo* comet assay (Jackson et al., 2015; Poulsen et al., 2016). These studies had the aim to find physicochemical properties that are predictive of pulmonary inflammation and genotoxicity. Levels of DNA strand breaks (% DNA in tail and tail length) for concentrations ≤ 200 µg/ml were tested in *in vitro* comet assays. All analogues of this case study except the carboxylated MWCNT NRCWE042 were found negative at all tested concentrations. NRCWE042 showed a statistically significant increase of % DNA in the tail with respect to control for the highest concentration. Other NRCWE analogues showed a decrease in tail length at the highest concentrations.

In the *in vivo* follow up study three different doses (6, 18 and 54 µg) were administered via intratracheal instillation in mice. DNA strand breaks in lung tissue and neutrophils in BALF were measured to assess genotoxicity and lung inflammation, respectively. After day 1, a statistically significant increase in % of DNA strand breaks were detected for analogue 14 (NRCWE-044) at the lowest concentration (6 µg/mouse). After 28 days the same MWCNT showed statistically significant increases of % of DNA strand breaks for the two highest concentrations (18 and 54µg/mouse), and NRCWE-045 for the highest concentration. After

90 days no significant increase in % of DNA strand breaks compared to the control were observed for any of the MWCNT.

After 1 day the total number of neutrophils in BALF was statistically significantly increased at 18 and 54 µg/mouse for all MWCNTs and for three of them also at 6 µg/mouse. 28 days after exposure only the highest dose groups had increased numbers of neutrophils in BALF, whereas at the lower concentrations most analogues reached normal level. At 92 days only group 3 (NRCWE 46-49, analogues 16-19) MWCNT had statistically significant increased neutrophil counts, all other MWCNTs had "normal" neutrophil counts. These data suggest that lung inflammation is the predominant effect following exposure to high concentrations of MWCNTs and that MWCNT are not genotoxic.

These results support our conclusion that the assessed MWCNTs including the analogues of this case study are (probably) not genotoxic and this conclusion may be extrapolated to other MWCNTs within the assessed ranges of size, content of impurities and surface area. Surface modification as tested in these studies did not have an impact on the results.

4.5.5   Perform a preliminary assessment of the group and fill data gaps

A preliminary assessment of the grouping hypothesis was carried out by identifying the structural similarities between the identified nanoforms. Two unsupervised chemoinformatic techniques were used, i.e. hierarchical clustering and principal component analysis.

The MWCNTs for which *in vitro* and *in vivo* studies of sufficient reliability (1, 2) were available were used for this assessment. This in in line with the good read-across practice (GRAP) which among others recommends that the source data must be adequate to meet the REACH information (Ball et al., 2016). To allow comparison of the different analogues we did not consider the different information requirements according to REACH Annexes and took into account all possible *in vitro* and *in vivo* tests required at the highest tonnage level. So the data gaps in this case study are only data gaps for the exercise with the option to be filled by read-across and do not represent real data gaps in the context of a REACH registration as according to the tonnage level these (e.g. *in vivo*) test may not be required or only a chromosome aberration or DNA damage study may be necessary.

Target substances were those analogues for which data gaps for one of the 5 study types as described above were identified (highlighted in red in Table 4.17).

When deciding on the read-across approach according to the RAAF (ECHA, 2015b) the category approaches Scenario 4 and 6 were considered (see Section 4.6.1). This is justifiable as no biotransformation is taking place. Although there are variations in physicochemical parameters (length, rigidity/straightness, impurities) between the analogues (Scenario 4 RAAF), they do not seem to play a relevant role for the endpoint genotoxicity and therefore similar strength of effect(s) for the target substances can be predicted (Scenario 6 RAAF).

299

Hierarchical clustering

Due to the availability of data, this part only covers analogues 9-19. Analogues 1-8 will be included in case additional physicochemical data becomes available.

The data used for the hierarchical clustering is that published by (Jackson et al., 2015) and includes several properties like purity, length, diameter, amount of impurities, cell viability, agglomeration state, etc. The list of properties used is detailed in Table 4.19.

Table 4.19. Physicochemical parameters and used for the assessment of the grouping hypothesis via hierarchical clustering and PCA

Property	Explanation
Purity (%)	Purity
LengthMin(µm)	Minimum length
LengthMax(µm)	Maximum length
LengthAverage(µm)	Average length
DiameterMin(nm)	Minimum diameter
DiameterMax(nm)	Maximum diameter
DiameterAverage(nm)	Average diameter
BET(m^2/g)	Surface area
Impurities (Al_2O_3, BaO, Fe, CuO, Cl, etc.)	Amount of impurities (%)
Endotoxin (EU/ml)	Amount of endotoxine
CEA:C,H,N,O (wt%)	Combustion elemental analysis of different elements
-OH,-COOH, -NH_2 (mmol/g)	Amount of surface coating
Zave at 12.5 and 200 µg/ml	Aggregation/agglomeration
PdI at 12.5 and 200 µg/ml	Polidispersibility Index
ROS	Reactive oxygen species determined by Dichlorofluorescin (DCFH) oxidation assay
Viability at different concentrations (0-200 µg/ml)	Cell viability
Cell proliferation at different concentrations (0-200 µg/ml)	Cell proliferation

The result of the hierarchical clustering is shown in Figure 4.7. Three strong groups (p<0.05) can be identified and are shown in red squares. Cluster c (right hand side) includes the analogues 13-15 NRCWE 043-045 which are the MWCNTs classified as group II by Jackson et al. These MWCNT are the ones with the largest diameter (50-80nm). Cluster b includes NM-401 and NRCWE-006, which are named (reference) Materials by Jackson et al. and which are MWCNTs of different sizes but very similar BET (18 and 26 m^2/g, respectively) with similar viabilities and proliferation values. Cluster a is the biggest of the clusters and contains Jackson et al. Group III MWCNTs (analogues 16-19) and some reference materials (NM-402, NM-403) as well as the Standard MWCNT. Group III MWCNTs correspond to short CNTs (1-10µm) with intermediate diameters (11-15.5nm). However, some of the MWCNTs of the reference materials group have similar sizes to Group III CNTs and this is probably the reason why they clustered together.

On the left hand side of Figure 4.7 there are analogues 10-12 NRCWE 040-042. They do not show significant similarity between them as (p>0.15). These MWCNT correspond to Group I defined by Jackson et al., the long MWCNTs (10-50µm). The fact that they are different from all other MWCNT can make them a group of their own. Similarly, Carbon black was not clustered with any MWCNT as it is quite different from the MWCNTs and was also missing some of the data, which the algorithm assigned as 0.
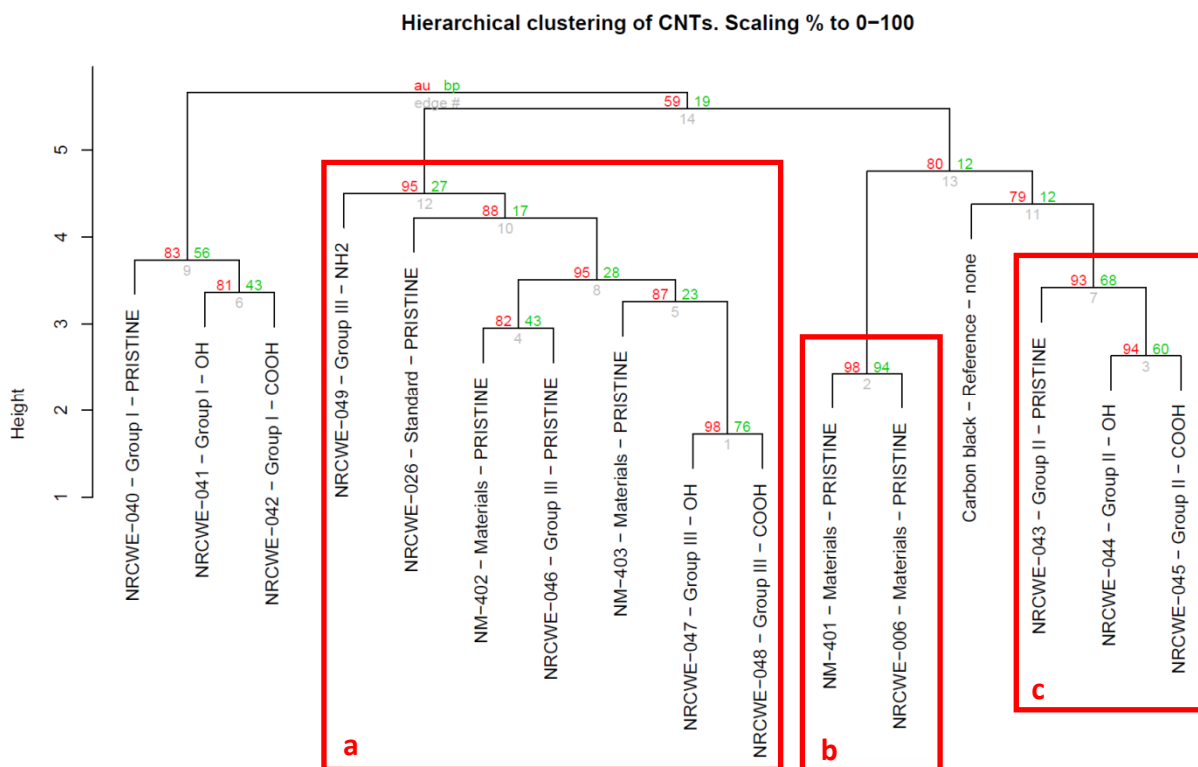
Figure 4.7. Hierarchical clustering of the Jackson et al. dataset. Distances between CNTs correspond to Euclidean distance and the clusters were generated from the "average" distance of the members of each group. Main clusters are indicated in red boxes.

Summing up, hierarchical clustering shows that the clusters are mainly driven by the size of the CNTs and by proliferation and viabilities although to a lower extent. At the same time it seems that the groups are not determined by the impurities and functionalization of the CNTs. Interestingly, the clusters formed correspond to some extent to those defined by Jackson et al., which were mainly defined by size. A way to determine the physicochemical parameters that account for the variability between CNTs, therefore that could explain eventual differences in toxicological behaviour, is through a PCA.

Principal component analysis (PCA)
PCA is dimensionality reduction technique that uses linear combinations of the original variables (see   Table 4.19) to define new variables (principal components) that are orthogonal to each other and that can describe a large amount of the total variance of the system (~60-70%) with just two variables, principal component 1 and principal component 2. This technique provides information about the variables that distinguish the individuals (MWCNTs) the most. The PCA of the same dataset that was used for the hierarchical clustering is shown in Figure 4.8.

It is interesting to observe in the PCA that Group I, Group II, and Group III CNTs as defined by Jackson et al. appear at different parts of the representation and that they cluster in different groups. Additionally, it can be observed that the MWCNTs that belong to the Materials group are spread between Group II and Group III. Actually, clusters b and c of Figure 4.7 appear in the same region of the PCA at the bottom left part. Regarding the variables that compose PC1 and PC2, the minimum length, maximum length and BET, are

the ones that contribute the most to the principal components, that is the ones that differentiate them the most.

Regarding the analysis of the positions of the MWCNTs in the PCA plot (Group III falls at the positive side of PC1 while Group II falls at the negative side of PC1), the fact that BET and ROS are parallel to PC1 explains this disposition. Group III CNTs, which are the short CNTs, have statistically significant larger BET (average ~ 205m²/g) than Group II CNTs, which are the thick CNTs (average ~ 92m²/g), p<0.001. NM-402 and NRCWE-026, which appear in the same cluster with Group III CNTs, also have very high BETs, 226 and 245m²/g, respectively. Therefore, BET clearly drives PC1 and the position of the MWCNT with respect to the X-axis of the plot.
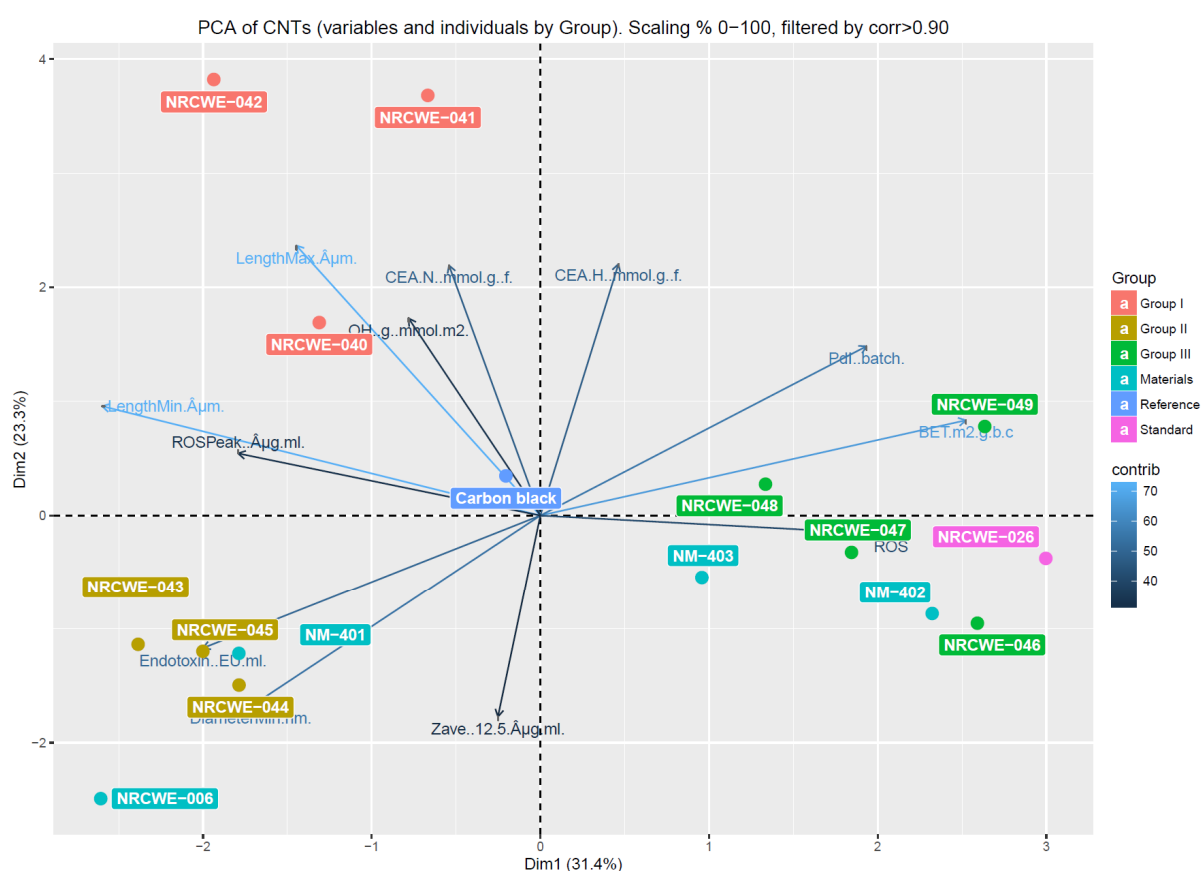
Figure 4.8. Principal component analysis of the MWCNTs dataset. The MWCNTs are colour-coded according to the Groups defined by Jackson et al., and the variables are colour-coded by their contributions to PC1 and PC2. The variance explained by each PC is indicated in the axis title.

Group I CNTs appear at the positive part of PCA2, which according to the loadings (see Table 4.19) is mainly driven by maximum length and combustion elemental analysis of H and N, CEA.H..mmol.g..f and CEA.N..mmol.g..f., respectively. In fact, Group I corresponds to long CNTs, which are 50μm long while Group II CNTs are 20μm long. The combustion analysis also shows a statistically significant difference between CEA.H..mmol.g..f of Group I and II (p<0.004). The difference for CEA.N..mmol.g..f. is not statistically significant because the value for NRCWE-040 is very low, but the values of NRCWE-041 and NRCWE-042 are statistically significant from those of Group III (p<0.005).

Thus, the PCA shows that the CNTs of Group I, II, and III can be distinguished by the minimum and maximum length, BET, ROS, and combustion elemental analysis of H and N. The PCA also shows that NM-401 and NRCWE-006 cluster with Group II CNTs, and that NM-403, NM-402, and NRCWE-026 cluster with Group III CNTs. The variables that contribute the most to PC1 and PC2 (loadings) are shown in Table 4.19 and confirm the analysis above.

Table 4.20. PC1 and PC2 loadings of the corresponding PCA carried out for the CNTs dataset.

Variable	PC1 loadings^2	Variable	PC2 loadings^2
Minimum Length (μm)	16.14	Maximum Length (μm)	18.07
BET (m^2/g)	15.22	CEA-H (mmol/g)	15.67
ROS	10.31	CEA-N (mmol/g)	15.5
Endotoxin (EU/ml)	9.57	Zave at 12.5 (μg/ml)	10.13
PdI	8.95	OH mmol/m^2	9.64
ROS peak (μg/ml)	7.68	Minimum diameter (nm)	9.14
Minimum Diameter (nm)	7.06	PdI	7.1
PdI at 12.5 μg/ml	5.51	Endotoxin (EU/ml)	4.42
Maximum Length (μm)	5.01	Minimum Length (μm)	2.96

In order to show whether the functionalization could explain the physicochemical differences between CNTs, the same PCA shown above was repeated and colour-coded by type of functionalization in Figure 4.9. However, it is clear in this figure that pristine, COOH, and OH functionalised CNTs do not form any group, and that therefore, the physicochemical differences of the CNTs cannot be explained by their functionalization. In fact, Jackson et al. already noted that the amount of functionalization of the CNTs was rather small, what confirms this observation. It may be necessary to define what amount of surface functionalization is needed in order to consider a CNT functionalised.
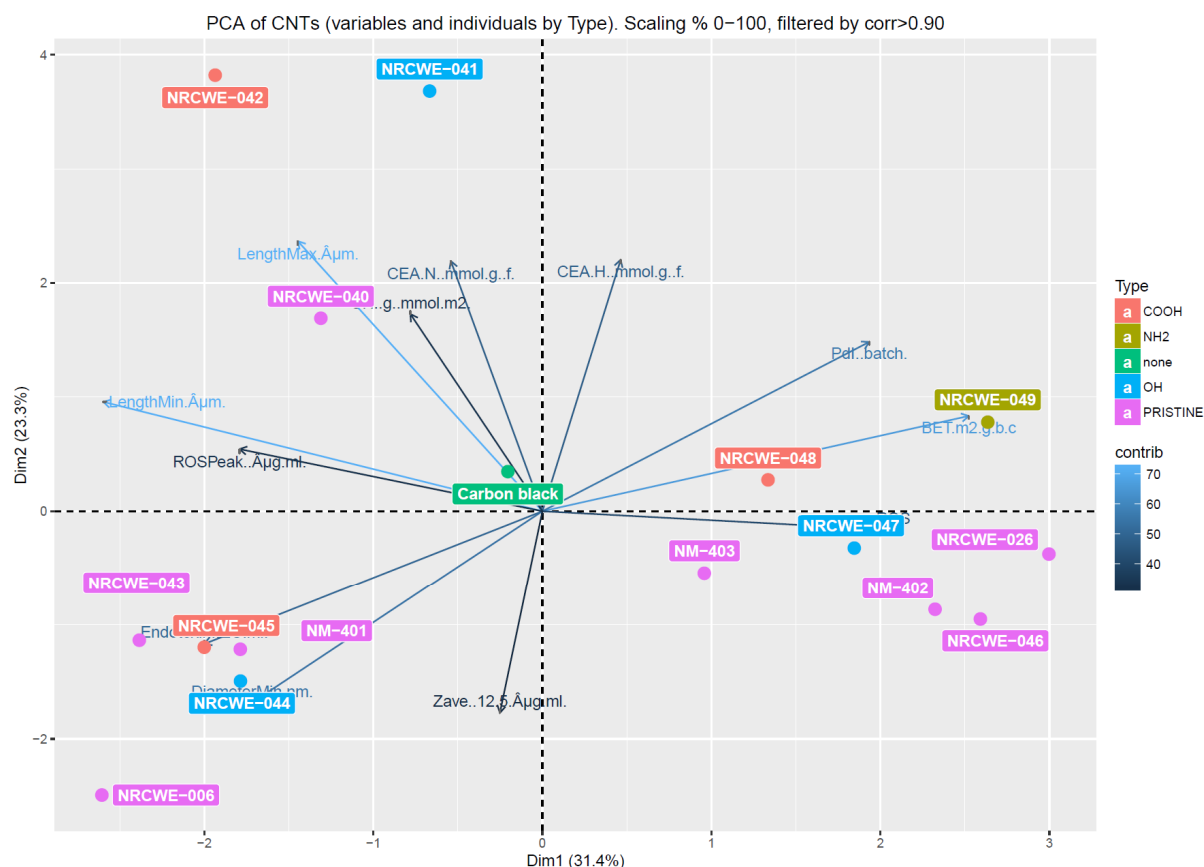
Figure 4.9. Principal component analysis of the CNTs dataset. The individuals CNT are colour-coded according to the type of functionalization reported by Jackson et al., and the variables are colour-coded by their contributions to PC1 and PC2. The variance explained by each PC is indicated in the axis title.

In order to analyse the similarity of the CNTs present in Jackson et al. with the targets, the amount of variables used for the initial PCA were reduced. Only the main contributors to PC1 and PC2 were selected to carry out another PCA, in this case using only LengthMin, LengthMax, BET, CEA H, and CEA N. Ideally, this PCA would also contain the targets but since this kind of data was not available they could not be added. Either way, the plot is presented here to show that the reduction of variables shows a very similar picture to the previous PCA and that it seems that the physicochemical differences between the Jackson et al. MWCNTs can be explained in terms of these 5 variables. The resulting PCA is shown in Figure 4.10 which is obviously different from the one in Figure 4.9 but the three main clusters groups seen in Figure 4.9 remain almost invariable.
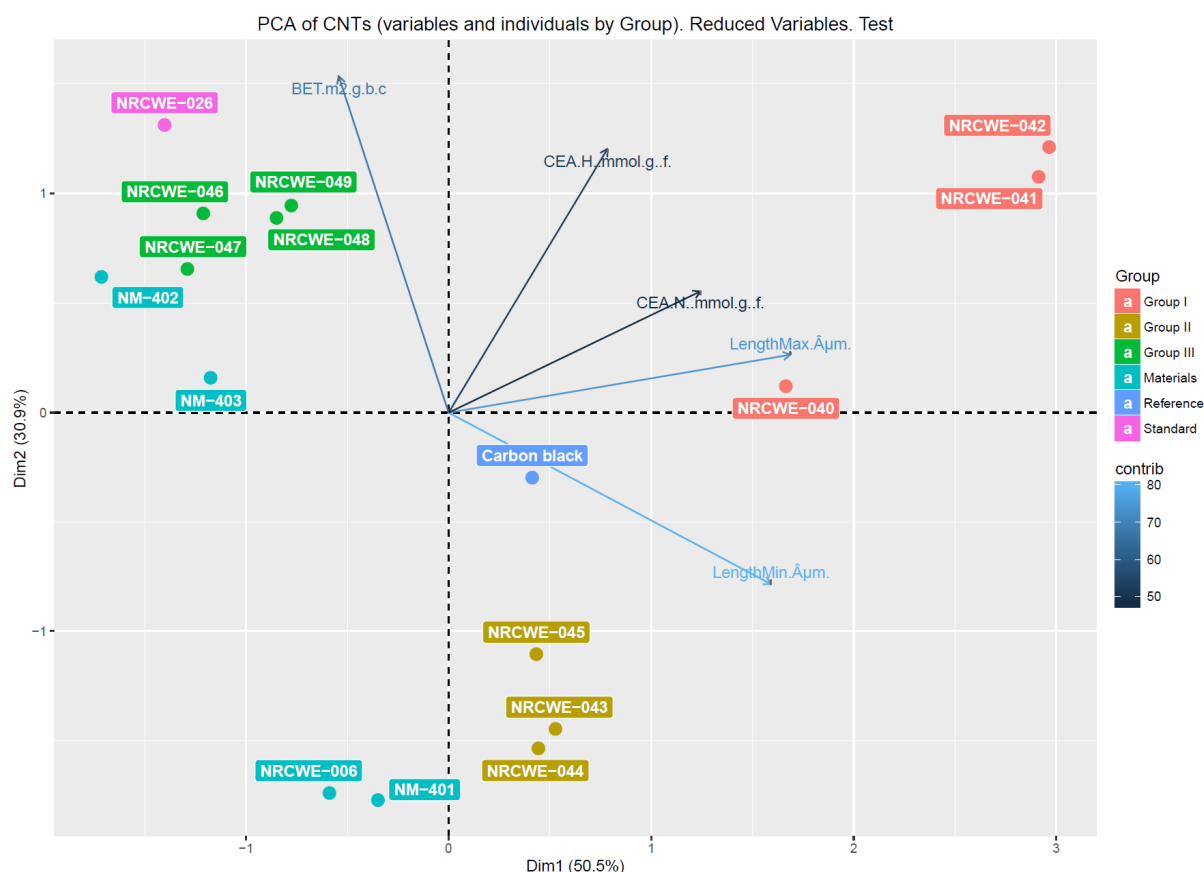
Figure 4.10. PCA of the CNTs with a reduced set of variables. The variables used were LengthMax, LengthMin, BET, CEA H, CEA N. The MWCNTs are colour-coded according to the Groups defined by Jackson et al., and the variables are colour-coded by their contributions to PC1 and PC2. The variance explained by each PC is indicated in the axis title.

Conclusions of hierarchical clustering and PCA

The hierarchical clustering and PCAs shown above have served to determine the clusters or groups of similar CNTs as well as the physicochemical properties that determine their similarity and that drive the grouping. However, we are interested in reading across the *in vitro* comet assay. In order to determine the physicochemical properties that can be related to genotoxic properties of CNTs, *in vitro* comet assay results for the CNTs used in the PCAs above were collected from (Jackson et al., 2015). The comet assay results were to be used to assign the CNTs to different categories, in this case genotoxicant or non-genotoxicant. Subsequently, a random forest algorithm was to be used to determine the physicochemical properties that are more discriminating in predicting these categories.

Unfortunately (in this case study), the results obtained by Jackson et al. showed that all CNTs at all concentrations were not different from the vehicle control. Only NRCWE-042 showed a statistically significant increase in % of DNA strand breaks at 200µg/ml compared to the control. Taking into account all these results, it does not seem reasonable to consider any of the CNTs as genotoxic. Since none of the source CNTs can be considered positive, the random forest algorithm cannot be used as there is need of having at least two categories. Otherwise, all variables will be equally important to predict negative genotoxicity.

The results obtained by Jackson et al. for % of DNA strand breaks and tail length for the different CNTs at the tested concentrations (0-200 µg/ml) are shown in Figure 4.11A and Figure 4.11B, respectively. Figure 4.11A only shows statistically significant differences with respect to vehicle control for NRCWE-042 (200µg/ml) and H_2O_2 (60µg/ml), which is the positive control. Figure 4.11B shows a statistically significant decrease in tail length for the highest concentrations of NRCWE-042, NRCWE-046, NRCWE-047, and NRCWE-049. Although it could seem that these variations indicate genotoxicity, it was shown by the authors that these variations were mainly caused by the reduced cell proliferation that occurred for these CNTs at high concentrations.
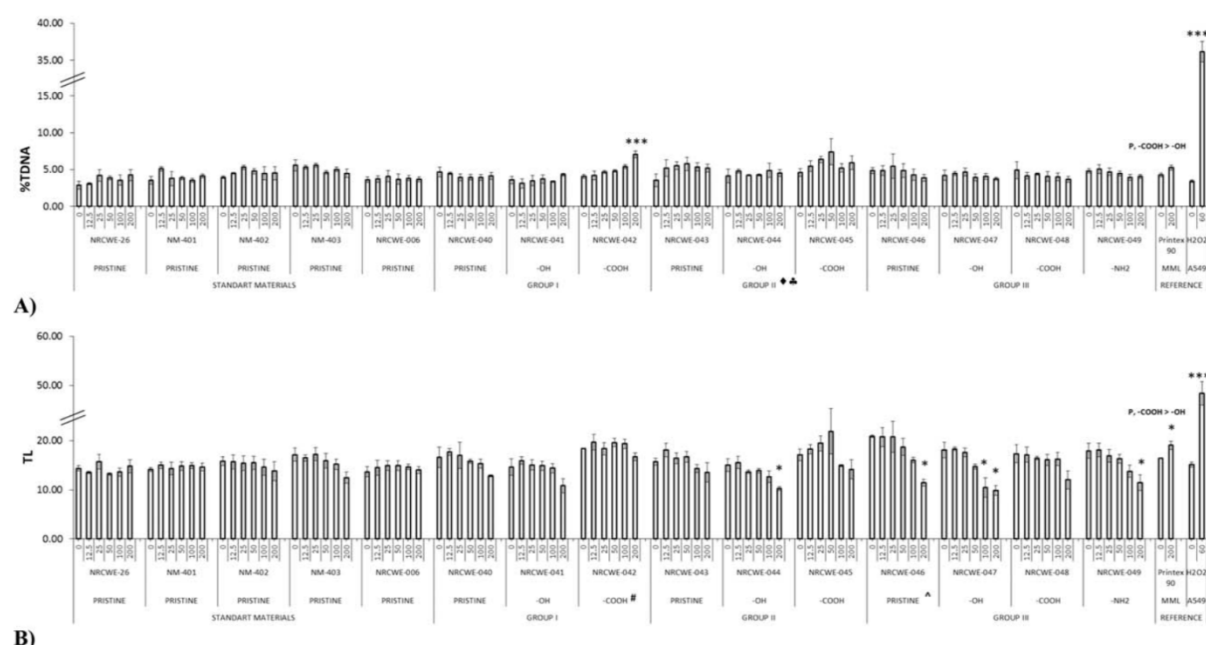
Figure 4.11. *In vitro* **comet assay results for different CNTs and at different concentrations as reported in Jackson et al. 2015. A) corresponds to % of DNA strand breaks. B) corresponds to tail length. Carbon black (Printex 90) and H_2O_2 were used as negative and positive control, respectively.**

The authors observed that the content of Fe_2O_3 and NiO clustered with % of strand breaks in a PCA and suggested that the content of Fe and Ni could be related to genotoxic effects, but no statistical significance with respect to controls was observed. In fact, the CNTs with higher content of Fe_2O_3 and NiO are NM-402 and NRCWE-045 for Fe_2O_3, and NRCWE-042, NRCWE-043, and NRCWE-044 for NiO. Of these CNTs, only NRCWE-044 at the highest concentration shows a decrease in tail length significantly different from the control. The rest do not show differences with the control in % DNA strand breaks or tail length. Therefore, the present results do not clearly show a causal effect between Fe and Ni content and genotoxic effects.

The analysis that we performed on the data provided by Jackson et al., however, shows that NRCWE-042, the CNT that shows significant % of DNA strand breaks at the highest concentration, has the highest content of OH or COOH, 4.09 and 2.04 mmol/g, respectively. In spite of the high content, the corresponding surface concentration of OH and COOH is not different from the other CNTs. However, the total content of OH and COOH in the samples of NRCWE-042 is 10 times higher than the average of the rest of CNTs. This of course, should

not affect toxicity unless some of the OH or COOH detached or reacted with biological entities. Having a loose functionalization would explain the dose response shape of NRCWE-042 and possibly the positive result for mutagenicity at the highest dose.

Overall, judging by the data found in Jackson et al. and the other analogues (see Table 4.17), it seems that CNTs turn out negative in the *in vitro* comet assay and that they should not be considered genotoxic on such basis.

Figure 4.12. DNA strand breaks in the lung tissue after exposure to MWCNT and reference materials at day 1(A), 28 (B), and 92(C).

This observation can be extrapolated to the *in vivo* situation as the same group published a follow-up article on the genotoxicity of CNTs *in vivo* (Poulsen et al., 2016).

The authors analysed the genotoxic effects of the CNTs (comet assay) in rats after instillation (Figure 4.12). They measu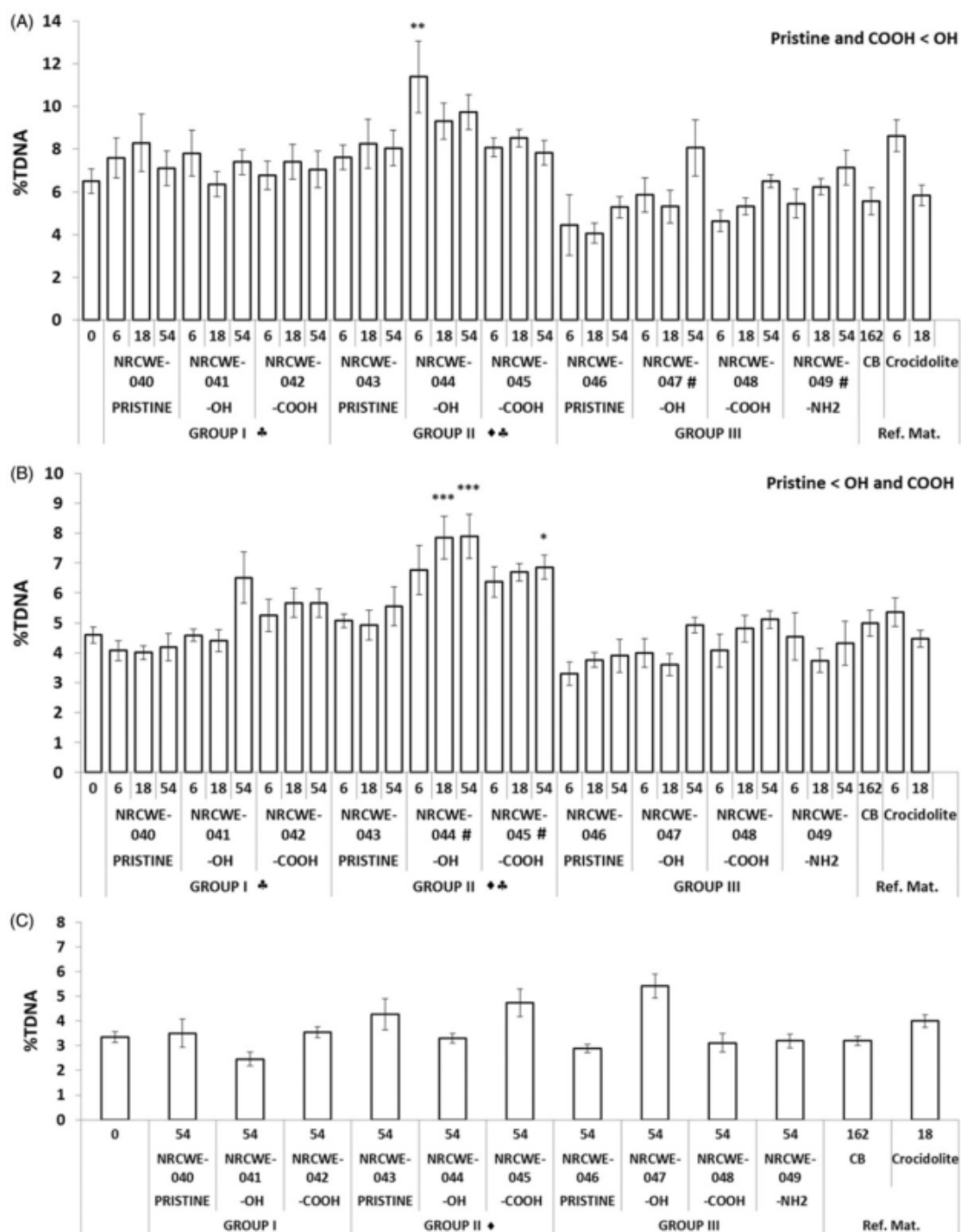red the % of DNA strand breaks in lung tissue and BAL fluid at day 1, 28, and 92. Of all CNTs and doses tested, only NRCWE-044 showed positive results for 6µg/ml at day 1, and 18 and 54 µg/ml at day 28. All other CNTs and doses tested at day 1, 28, and 92 were not significantly different from the control. These results point out that CNTs are in general not genotoxic, and that in the rare cases in which they turn out positive (e.g. positive at day 1 for NRCWE-044 at 6µg/ml but not at 18 or 54 µg/ml), there is full recovery after 92 days.

The results in the BAL fluid were similar, with the only difference that from NRCWE-040 to NRCWE-045 were found positive at day 1 for at least one test concentration. Nevertheless, all CNTs were found negative for all concentrations after 28 and 92 days, showing again a recovery process.

Taking into account the results obtained by Jackson et al. 2015 and Poulsen et al. 2016, it is reasonable to conclude that CNTs are most probably negative in the comet assay, both *in vitro* and *in vivo*, up to 200µg/ml *in vitro* and 54µg/ml *in vivo*.

Poulsen et al. showed, however, that inflammation behaviour is different as all CNTs showed statistically significant increase in neutrophils count in BAL fluid at day 1 and 28; and Group III CNTs at day 92 too. This result shows that CNTs can cause lung inflammation and that it can still be present 92 days after exposure. This seems to be a common mechanism for fibres, which may induce carcinogenicity via long-term inflammation.

Fill data gaps by reading across
Data gaps (as highlighted in red in Table 4.17) exist for analogues 4, 6, 7 and 8 for some endpoints and for the analogues 9-19 for all endpoints except the comet assay *in vitro* and *in vivo*. Considering read-across endpoint by endpoint, the *in vitro* gene mutation in mammalian cells and *in vitro* DNA damage may be predicted to be negative for analogue 7 and 8 as all tests with the other analogues were negative. For analogue 4 and 6 no *in vivo* data was located. Both analogues had some positive results in the *in vitro* micronucleus tests, but the other tests were all negative. As the other analogues tested under more reliable conditions (see discussion above) were negative, it may be predicted that also these analogues would not be genotoxic *in vivo*. From their physicochemical properties, their differences to be considered are: analogue 7 seems to be straight/rigid, similar to analogue 5 and analogue 8 has been described as very long MWCNT which also had the highest Fe content. Analogues 4 and 6 do not show properties that would make them suspicious to have a higher propensity for genotoxicity/lung inflammation.

4.5.6 Perform and/or propose testing

In this case study we have made an appraisal to fill identified data gaps. In this case no further testing of the assessed analogues seems necessary. Based on the available data, we do not suggest to extrapolate this conclusion to other MWCNT, whose size is beyond the tested ones (i.e. longer or larger diameter), which are surface modified or which have a higher content of oxidising impurities.

In general to increase the knowledge and, considering the low reproducibility of some *in vitro* tests, it is suggested to adapt the test protocols to increase the reliability of test results.

Further, the predictability of *in vitro* genotoxicity of NMs for *in vivo* genotoxicity and carcinogenicity needs to be better understood.

4.5.7 Summary and conclusion

Previous research has shown that physicochemical properties can influence translocation and toxicity of MWCNT (Braakhuis et al., 2014; Ken Donaldson et al., 2012). Although length and rigidity/straightness are cited in the literature as important properties in influencing lung deposition and thereby the onset, location and/or severity of the pulmonary response (e.g. genotoxicity/carcinogenicity) (Braakhuis et al., 2014; Ken Donaldson et al., 2012), experimental measures of "rigidity" are usually not reported. In fact, it is complex to characterise and measure such mechanical property at the nanometer scale (Pantano, M. Parks, & Boyce, 2004), and in our attempt to group MWCNTs depending on this parameter, rigidity was defined based on TEM images. As reported in the literature, Young's modulus is a measure of rigidity (Pantano et al., 2004; Sakharova, Pereira, Antunes, & Fernandes, 2016) and this physical characteristic could be required and used to quantitatively classify MWCNTs in terms of rigidity. A QSPR for Predicting Young's modulus for metal-based NMs is available in the Nanocomput model inventory (supporting material S1, Toropov and Leszczynski 2006). Considering the relevance of this property, further research activities could aim at developing predictive models for (MW)CNTs rigidity.

Concerning genotoxicity no major differences between the analogues assessed in this case study were observed that could be attributed to length or rigidity/straightness.

In vitro gene mutation tests in mammalian cells and comet assays were negative for the analogues. Some analogues showed a higher rate of positive results in the *in vitro* micronucleus assay. There were fewer *in vivo* genotoxicity tests available but the majority did not point to a genotoxic effect for MWCNT. Genotoxicity and the generation of reactive oxygen species are frequently investigated in *in vitro* studies with respect to their general predictive power for *in vivo* situations. However as they neglect defence mechanisms their predictivity for *in vivo* situations may be limited (Ken Donaldson, Poland, et al., 2010). In addition, recent analyses have shown, that carcinogenicity is not predicted very well for particles (Roller, 2011). It is thus also difficult to establish a clear correlation between MWCNT properties with *in vivo* results (Roller, 2011; Ziemann et al., 2011).

Size and rigidity may however determine fate and kinetics of MWCNT and thus bioavailability and target organs.

In vitro tests ROS generation has been shown to correlate well with inflammation *in vivo* (increase of neutrophils after intratracheal instillation) (Rushton et al., 2010), provided that particles are compared based on particle surface and the steepest slope of the dose response curve as dose metric. This could be relevant if genotoxicity arises as secondary effect.

Our results suggest that the investigated MWCNT are not genotoxic. Genotoxicity thus does not seem to be (the sole) responsible for the initiation of carcinogenicity following MWCNT inhalation. It is therefore not possible to determine physicochemical properties that are responsible for the presence or absence of a genotoxic effect. MWCNT physicochemical properties have shown to have an impact on the translocation, clearance and persistence of MWCNTs in the body and thus on the onset and persistence of inflammation and release of reactive oxygen species. This cannot be tested in an *in vitro* test.

4.6   Uncertainty analysis

The approach of chemical grouping and read-across includes uncertainties. There are various areas that contribute to the overall uncertainty: uncertainty associated with the data, assumptions, and predictions used to justify similarity and analogue suitability between the group members; and toxicological uncertainty with the read-across prediction of hazard derived (evaluated based on the number and suitability of analogues contributing data, source study quality, likelihood of effect and potency concordance between target and source chemical).

For the present case studies we have used the ECHA Read-Across Assessment Framework (RAAF) (ECHA, 2017a) as a systematic guidance to identify and summarise the different sources of uncertainty associated with filling data gaps by grouping and read-across, with a view also to evaluate the applicability of the RAAF for nanomaterials and to find possible issues that need to be taken into account particularly for nanomaterials.

4.6.1   Uncertainty assessment using the Read-Across Assessment Framework

The Read-Across Assessment Framework has been developed by ECHA to provide guidance for a structured analysis of read-across submissions and justifications (ECHA, 2017a). Six possible read-across scenarios are considered, depending on the number of substances (analogue or category approach), the effect caused by common or different substances for source(s) and target(s) or, for a category, whether the predicted property is following a regular pattern (trend) or not changing across source structures. Sets of Assessment Elements (AEs) per scenario describe 'crucial scientific aspects to judge validity and reliability of read-across' (ECHA, 2015b). For each AE multiple considerations are listed, which should be addressed in the justification of the read-across argumentation. There are general (common) AEs and scenario-specific AEs. Assessment Options (AOs) reflect the conclusions on adequacy and scientific robustness. They are defined as scores as from 1 to 5 according to whether the information provided is not acceptable (1), not acceptable in its current form (2), acceptable with just sufficient (3), medium (4) or high (5) confidence.

The ECHA RAAF was used to assess the uncertainties in the read-across for the two nanomaterial case studies. Moreover, the case study evaluation was used to identify

nanomaterial particularities considered in the read-across exercise. Thus this exercise also evaluates the applicability of the RAAF for the assessment of read-across for nanomaterials and might point out possible respective adaptions or extensions of the RAAF AEs for this specific case of read-across.

Generally the reliability, relevance, consistency and completeness of data and argumentation are evaluated.

From the six RAAF scenarios, Scenario 6 does describe the nanomaterial case studies best. Both the TiO_2 and MWCNT case studies use category approaches (reading-across from a group of substances to a target), different compounds (in this case nanoforms) are considered in the category which have the same type of effect, and there are no variations in effect (e.g. Comet assay result either positive or negative). Although there are variations in the strengths of observed different effects for CNT depending on e.g. length and impurities, they do not influence the considered binary genotoxicity endpoints (yes/no).

In Table 4.21, the AEs for RAAF Scenario 6 are listed. They cover the general overarching topics:

- similarity hypothesis, substance(s) considered, and available data
- toxicant(s) the organism is exposed to
- mechanism of toxicity.

AEs C.1-C.6 are common AEs for all scenarios considering a category approach; AEs 6.1-6.5 are scenario-specific. The terms used correspond to the terminology used in the RAAF, i.e. applicable to conventional organic substances. Nanospecific adaptions of the terms will also be discussed in the following.

Table 4.21. RAAF Assessment Elements for Scenario 6 (category approach, different compounds with same effect, no variation in effect), summarising the crucial points of a read-across argumentation that should be justified (see ECHA 2017a).

	RAAF Assessment Elements (Scenario 6)	
C.1	Substance characterisation including impurity profile	**Similarity hypothesis,** **Substance(s) considered,** **Available data**
C.2	Structural similarity and category hypothesis Allowed/non-allowed differences (category)	
C.3	Link of structural similarities and structural differences with the proposed regular pattern	
C.4	Consistency of effects in the data matrix	
C.5	Reliability and adequacy of the source study(ies) Data quality	
C.6	Bias that influences the prediction Other possibilities excluded?	
6.1	Compounds the test organism is exposed to Different compounds with same effect	**Toxicant the organism is exposed to**
6.4	Exposure to other compounds than to those linked to the prediction Other compounds present or formed, e.g. metabolites...? Impurities? Systemic availability of the compounds?	
6.5	Occurrence of other effects than covered by the hypothesis and justification	
6.2	Common underlying mechanism, qualitative aspects Mode of action hypothesis (link to structure); What is the biological target? Same for source/target compounds?	**Mechanism of toxicity**
6.3	Common underlying mechanism, quantitative aspects Similar exposure to source/target compounds?	

4.6.2 The TiO₂ case study

The RAAF AE elements (Scenario 6) are used to highlight uncertainties in the grouping and read-across argumentations. The evaluation and nanospecific considerations are summarised in Table 4.22.

Substance characterisation (C.1)

In the first step of the read-across process the identification of the nanoforms is considered, i.e. "what they are". The identification includes particle size, particle shape and surface chemistry. The characterisation should also include impurities present, which are defined as "unintended constituent present in a substance as manufactured" (ECHA 2012); on the contrary, NMs contain surface coating purposely added.

The core chemical composition is TiO_2, the crystal type and size are reported. Impurity information is given by the provider only as a generic percentage of purity. Additional measurements are available from different Nanogenotox deliverables; however, in some cases there were some inconsistencies between different tests.

For the considered target substances, analysis of the physicochemical properties (as reported in (Guichard et al., 2012)) showed that the measured properties were slightly different from those reported by the manufacturer.

Of the physicochemical properties considered during data collection, some were disregarded because of lack of information or low data reliability. Appendix VIII reports the analysis of the variability of the physiochemical property measurements, particle size distribution, Zeta potential being dependent for example from the dispersion medium used, different sonication methods. As a consequence of the huge data variability in particle size distribution (and consequently on Zeta potential), this measurement is not reliable and could in principle be excluded from the grouping exercise.

Overall there is uncertainty associated with the nanoform identification and physicochemical characterisation, which is subject to high variability in measurements (different experimental conditions, result ranges; see Appendix VIII).

Structural similarity and category hypothesis (C.2)

The criterion of structural similarity considered in the RAAF for conventional organic substances needs to be extended for nanoforms to their properties regarding identification, fundamental behaviour and reactivity.

In order to investigate criteria for similarity of the nanoforms considered and form a category hypothesis, cheminfomatics methods have been used to determine the (physicochemical) properties that differentiate the analogues, their similarity and that may drive genotoxicity. The properties differentiating the two groups were surface coating, Al coating and crystallite type.

The nanoform NM-101 was not declared coated by the manufacturer (Birkedal et al., 2012). However it contains 9% organic impurities. Therefore it is considered coated for the purpose of this case study and part of the respective group of nanoforms.

Link between structural (physicochemical) similarity and predicted property (C.3)
For NMs, the similarity cannot be based on molecular structure as for conventional chemicals. Moreover, in general very little is known about the mechanisms of toxic action of NMs. Therefore it can be challenging to develop the grouping hypothesis of the link between similar properties and the predicted property/toxicity, in this case the outcome of the *in vitro* comet assay.

The link between physicochemical properties of the analogues and the predicted property, i.e. genotoxicity, has been investigated with cheminformatics methods. The hypothesis is that Nano-TiO$_2$ in its uncoated form has the potential to damage DNA, but this can be masked by the presence of coating or by large amounts of impurities on the surface of the NM (total non-TiO$_2$ content).

Consistency of effects in the data matrix (C.4), Reliability and adequacy of the source studies (C.5)
Data were collected following a reliability assessment based on the ANSES criteria (see ANSES 2016).

The data for micronucleus, comet and chromosomal aberration assays were included in the data collection, because the current OECD test guidelines fort these tests are considered applicable to NMs and thus sufficiently reliable. For the *in vitro* comet assay however, an OECD test guideline is not available. This endpoint was considered nevertheless in the data collection because of larger availability of studies. Previous reviews on genotoxicity tests applied to NMs claimed that the comet and micronucleus assays are the most commonly used tests in the field (Golbamaki et al. 2015).

Generally, the assessment of quality, reliability and relevance to human health endpoints of measured toxicity data as well as their interpretation is difficult, partly due to the uncertainty in applying existing testing protocols to nanomaterials. The applicability of current OECD test guidelines to NMs is still under discussion and artefacts affecting the results of toxicity assessment of NMs have been reported (see Marchese Robinson et al. 2016).

Bias in selection of category members (C.6)
Because of the scarcity of the data available (as full datasets covering all properties considered) or insufficient quality, the set of analogues considered in this case study was limited. Only the nanoforms that were completely identified by means of fundamental parameters like solubility, hydrophobicity (currently not well defined for nanomaterials), zeta potential, dispersability were considered. This led to a dataset with 6 TiO$_2$ nanoforms, differing in their primary particle size (from 7 to 117 nm), coating (two of them are declared coated by the manufacturer and the others are declared without a coating), crystal type (anatase and rutile) and hydrophobicity (materials functionalised to be hydrophobic or hydrophilic). Thus, our hypothesis is based on a small dataset and considers a single *in vitro* endpoint (Comet assay). Other *in vitro* tests available for TiO$_2$ nanoforms do not confirm genotoxicity, so an overall conclusion on genotoxic potential cannot be made with certainty.

Compound the test organism is exposed to (6.1)
TiO$_2$ nanoforms without coating are considered to cause the adverse effect. The presence of coating and/or a high amount of organic impurities is hypothesised to mask the potential

DNA damage. The definition of the toxicant is based on evaluation of the physicochemical properties. Some uncertainty remains about the property driving the genotoxicity.

Exposure to other compounds (6.4)
Exposure to impurities might influence the observed genotoxic effects. Another factor to take into consideration is the presence of proteins in the medium. If the NMs are surrounded by proteins, they are more dispersed and also less toxic since the "reactive" part is "hidden" behind the protein corona.

Common underlying mechanism, qualitative and quantitative aspects (6.2, 6.3)
The mechanisms of primary and secondary genotoxicity are not fully understood.

Generally, there is some uncertainty related to the mechanism: The majority of studies supported the hypothesis that the genotoxic effect of TiO_2 is masked by the presence of coating (i.e. total non-TiO_2 content including impurities). However, the way in which the coating can prevent DNA damage is not entirely clear. The mechanism of genotoxicity of TiO_2 is not well defined and still discussed in the literature, it is also possible that several effects take place at the same time. There is general uncertainty about the mechanism of action for indirect primary genotoxicity via ROS (Golbamaki et al., 2015), a clear correlation between the level of ROS production and DNA damage was not supported in several studies. Therefore measured information on ROS formation (bioactivity) would be useful for supporting the hypothesis in the case study.

Exposure to other compounds (6.4)
The presence of reactive transition metals as impurities or in the NM composition may also contribute to oxidative DNA damage induction.

Occurrence of other effects (6.5)
There might be more than one mechanism responsible for genotoxicity of TiO_2; and possibly a combination of several factors are responsible for masking the DNA damage. They may have as common source the presence of coating / high amount of impurities either by preventing aggregation of NMs, or by preventing physical contact with DNA and/or other cell components.

There is also an indication for the fact that the degree of agglomeration of TiO_2 nanoforms, which depends on the presence of coating, may have an influence on the DNA damage.

A direct interaction mechanism of genotoxicity or an indirect primary genotoxicity are considered (Magdolenova et al. 2014). Furthermore, the conduction band of TiO_2 falls in the range of biological redox potentials (Burello and Worth 2011), meaning that TiO_2 with or without the presence of UV light can generate reactive species that react with cell constituents such as DNA. However, the band gap is not the only predictor of reactivity, but also for example the geometry of the crystal, the nature of surface defects.

Table 4.22 Evaluation of the uncertainties of the TiO$_2$ case study according to the ECHA RAAF Scenario 6.

	RAAF Assessment Element (Scenario 6)	Uncertainties in the TiO$_2$ case study	Nanospecific issues
C.1	Substance characterisation	• Measured physicochemical characteristics of the NMs vary: measurement uncertainty. Is there an influence on other properties of the nanomaterials? • Impurity information not always available or inconsistent	• Physicochemical characterisation of NMs: high variability of measurements (influence of different experimental conditions)
C.2	Structural similarity and category hypothesis	• NM-101 is not declared as coated, but has % of impurities corresponding to a coating. Thus it was considered coated.	• For NMs, the similarity cannot be based on chemical (e.g. molecular) structure as for conventional chemicals, but should consider physical form and key physicochemical properties
C.3	Link of structural similarities and structural differences with the proposed property	• Little is known about the mechanisms of toxic action, making it challenging to link similarity to the property (genotoxicity) considered	
C.4	Consistency of effects in the data matrix	• Uncertainty in applying existing testing protocols to nanomaterials and thus uncertainty in assessment of quality, reliability and relevance to human health endpoints of measured toxicity data	• Artefacts affecting the results of toxicity assessment of NMs are discussed in the literature
C.5	Reliability and adequacy of the source study(ies)		
C.6	Bias that influences the prediction	• Selection of analogues based only on data-availability	
6.1	Compounds the test organism is exposed to		• For conventional chemicals, either the parent molecule of (bio)transformation products are the indirect/direct toxicants; for NMs the considerations extend to coating, released metals etc

	RAAF Assessment Element (Scenario 6)	Uncertainties in the TiO$_2$ case study	Nanospecific issues
6.2	Common underlying mechanism, qualitative aspects	• The mechanism of genotoxicity of TiO$_2$ is not well defined. It is also possible that several effects take place at the same time.	
6.3	Common underlying mechanism, quantitative aspects		
6.5	Occurrence of other effects than covered by the hypothesis and justification		
6.4	Exposure to other compounds than to those linked to the prediction	• For example the presence of reactive transition metals may also contribute to oxidative DNA damage induction	

4.6.3   The CNT case study

The RAAF AE elements (Scenario 6) are used to highlight uncertainties in the grouping and read-across argumentations. The evaluation and nanospecific considerations are summarised in Table 4.23.

Substance characterisation (C.1)
The MWCNT are considered structurally/chemically similar based on the same starting material for their synthesis. However, the purity of the MWCNT varies between 90 and 99%, and thus may influence similarity.

The same material from the same producer and identified by the trade name may still be subject to variations, such as batch to batch variability, surface modification (though this is usually reported) or ageing. Impurity information is given by the provider as a generic percentage of purity. Furthermore, measurements are available from different Nanogenotox deliverables; however, in some cases there were inconsistencies between different tests. Measured physicochemical characteristics measured by the customer may vary from those delivered by the manufacturer, sometimes because they are dependent on the sample preparation and test conditions.

Thus there is uncertainty associated with the nanoform identification and physicochemical characterisation, which is subject to high variability in measurements (different experimental conditions, result ranges).

Structural similarity and category hypothesis (C.2), Link between structural (physicochemical) similarity and predicted property (C.3)
For NM, the similarity cannot be based on molecular structure as for conventional chemicals. Therefore physicochemical and NM characterisation parameters were analysed to identify possible categories and link the parameters to genotoxicity.

Subgrouping of the MWCNT can be done according to different parameters, e.g. length or diameter, which influence different toxic behaviour, resulting in different subcategories. However, there is uncertainty for example in the lengths reported for MWCNT, therefore not all MWCNT types could be clearly assigned to respective subcategories. Subcategorisation based on diameter is also difficult since there is no clear cut-off value and the values for the same analogue are reported sometimes for huge ranges. No major differences concerning genotoxicity between the analogues assessed in this case study were observed that could be attributed to length or diameter. Size for example may however determine fate and kinetics of MWCNT and thus bioavailability and target organs. Overall no single physicochemical parameter seems suitable to predict toxicity, and some may be interdependent.

As a more in depth-analysis, the dataset of physicochemical parameters was analysed by hierarchical clustering and principal component analysis in order to identify the properties possibly linked to genotoxicity. Generally there is a large variation in several of the physicochemical properties reported, i.e. only available as ranges. Therefore the data had to

be pre-treated for the analysis, for example values given as ranges such as for the diameter were considered as minimum, average and maximum diameter. Moreover, all properties were scaled.

Consistency of effects in the data matrix (C.4), Reliability and adequacy of the source studyies (C.5)

The case study analogues have been selected according to the quality of the available physicochemical and toxicological data, according to defined reliability criteria (see 0). However the data for physicochemical characterisation possess a high variability. To increase the knowledge and considering the low reproducibility of some *in vitro* tests it could be suggested to adapt the test protocols to increase the reliability of test results.

Generally, it has to be noted that not all test protocols are suitable for nanomaterials, including artefacts affecting the results of toxicity assessment of NMs (see (Marchese Robinson et al., 2016)). For this reason Ames test results were not considered in this case study: the test has a limited applicability to NMs as they may not penetrate the cell wall and therefore potentially lead to false negative results (Clift et al., 2012). The predictability of *in vitro* genotoxicity of NMs for *in vivo* genotoxicity and carcinogenicity needs to be better understood.

Bias in selection of category members (C.6)

The case study was focused on MWCNT to keep the number of analogues and influencing properties manageable. The MWCNT analogues were selected based on data availability for all relevant physicochemical and most toxicological endpoints, leading to a dataset of 19 NMs. There are many more MWCNT types described in the literature but because lack of consistent data leading to uncertainty about the identity and physicochemical characteristics they were not included in this case study. Generally, it is assumed that other MWCNTs would also fit into the category considered as defined within this case study, if they can be reasonably presumed to follow the same toxicokinetics and mode of action, and the category could be extended to other CNT types, e.g. SWCNTs, or even fullerenes or carbon black, on a case by case base, depending on their similarities in physicochemical, translocation and toxicological properties (see 4.5.2).

Compound the test organism is exposed to (6.1)

The toxicants are the considered MWCNT, high aspect ratio nanomaterials consisting of >90 carbon which are not biodegradable (see Table 4.12).

Common underlying mechanism, qualitative and quantitative aspects (6.2, 6.3)

There is currently no agreement in the literature on the mode of action, nor on the genotoxic potential of nanoparticles as the overall results seem to indicate that some tests are more prone to give positive results than others.

Ring trials carried out as part of the Nanogenotox Joint Action showed that for some tests there was low reproducibility. Remarkably, the comet assay showed better reproducibility in Caco-2 cells, while micronucleus test was better in BEAS 2B cells (see Table 4.18).

Genotoxicity and the generation of reactive oxygen species are frequently investigated in *in vitro* studies with respect to their general predictive power for *in vivo* situations. However as

they neglect defence mechanisms, their predictivity for *in vivo* situations may be limited (Ken Donaldson, Poland, et al., 2010). In addition, recent analyses have shown that carcinogenicity is not predicted very well for particles (Roller, 2011). It is thus also difficult to establish a clear correlation between MWCNT properties with *in vivo* results (Roller, 2011; Ziemann et al., 2011).

There is also some uncertainty related to the adverse effects, due to different types of effects that can be caused by properties related to the particle, nanosize or inorganic nature of the NM or a combination thereof. Test conditions and selection of tested concentrations may contribute to a big variability.

Exposure to other compounds (6.4)
As discussed above, impurities might *de facto* constitute differences between MWCNT which then may influence the toxicity. For example, catalytic metals from the production process and remaining as impurities on the CNT surface may be released and increase toxicity, e.g. contribute to oxidative stress. This has been mainly described for SWCNTs which may have up to 30% of iron; MWCNT have much lower concentration of catalytic metals.

Table 4.23. Evaluation of the uncertainties of the CNT case study according to the ECHA RAAF Scenario 6.

	RAAF Assessment Element (Scenario 6)	Uncertainties in the CNT case study	Nanospecific issues
C.1	Substance characterisation	• Uncertainty related to the nanoform identification • Measured physicochemical characteristics of the NMs vary: measurement uncertainty. Is there an influence on other properties of the nanomaterials?	• Physicochemical characterisation of NMs: high variability of measurements (influence of different experimental conditions)
C.2	Structural similarity and category hypothesis	• No single physicochemical parameter seems suitable to predict toxicity, and some may be interdependent	• For NMs, the similarity cannot be based on chemical (e.g. molecular) structure as for conventional chemicals, but should consider physical form and key physicochemical properties
C.3	Link of structural similarities and structural differences with the proposed property		
C.4	Consistency of effects in the data matrix	• Uncertainty in applying existing testing protocols to nanomaterials and thus uncertainty in assessment of quality, reliability and relevance to human health endpoints of measured toxicity data	• Artefacts affecting the results of toxicity assessment of NMs are discussed in the literature
C.5	Reliability and adequacy of the source study(ies)		
C.6	Bias that influences the prediction	• Selection of analogues based only on data-availability; many MWCNT types not considered	
6.1	Compounds the test organism is exposed to		• For conventional chemicals, either the parent molecule of (bio)transformation products are the indirect/direct toxicants; for NMs the considerations extend to coating, released metals etc

	RAAF Assessment Element (Scenario 6)	Uncertainties in the CNT case study	Nanospecific issues
6.2	Common underlying mechanism, qualitative aspects	• The mechanism of genotoxicity of CNT is not well defined • It is also possible that several effects take place at the same time	
6.3	Common underlying mechanism, quantitative aspects		
6.5	Occurrence of other effects than covered by the hypothesis and justification		
6.4	Exposure to other compounds than to those linked to the prediction	• Impurities might constitute differences between MWCNTs which then may influence the toxicity	

4.6.4 Summary of uncertainties in the case studies

The major uncertainties encountered in the nanomaterial case studies were related to:

- Complexity of nanostructures: similarity, category boundaries and members

- Identification of nanomaterials

- Quality and inconsistency/reproducibility of study data; missing protocols or uncertainty in the applicability of protocols to nanomaterials, both for

- Material characterisation

- Toxicity assays

- Physicochemical properties driving the toxicity

- Limited datasets

- Read-across of negative effects

- Possible combination of physicochemical properties affecting toxicity

- Relevant test systems (*in vitro* mechanistic studies).

Nanospecificities to be considered in the RAAF

The following nanospecific issues have been identified related to a read-across for nanomaterials:

- Similarity based on chemical structure to be replaced with appropriate and relevant other parameter(s), i.e. consider consider physical form and key physicochemical properties

- However, for soluble nanoparticles the similarity in chemical structure can be applied; the solubility bringing back the NM into a classical substance case.

- Definition of the "identical or different compounds" in the RAAF should be adapted to nanoforms and take factors such as coating, size/length into consideration

- Definition of the compound the organism is exposed to and leading to an adverse effect:
  In the case of nanomaterials it is not only a distinction between parent compounds and (bio)transformation products such as metabolites, but for example needs to consider either the nanomaterial as such, or impurities/coating, released metals.

- Are there specific nano-toxicity mechanisms or kinetics of exposure which have to be taken into consideration?

The question of similarity is a specific fundamental issue. The RAAF, as related to the REACH legislation, is anchored on similarity of chemical structures.

For nanomaterials, the first step of defining a group of similar analogues, i.e. nanoforms, has to be approached differently and similarity has to be defined based on the essential and relevant properties of the NMs. There is not a generally accepted way of defining similarity between NMs. It has been reported that the importance of physicochemical data for NM are dependent on the type of NMs (Sellers et al., 2015). Composition can be crucial for some applications and it could allow grouping (e.g. CNTs), however in other occasions coatings or solubility can be more important as could be the case for NMs that leach ions (e.g. Ag, ZnO). In addition, there is lack of publicly available data for NMs what makes it difficult to determine if two NMs have similar properties. But most importantly there is lack of standardised methods that guarantee that the same kind of data (e.g. size distribution) are generated in the same conditions and are comparable, starting with differences in the methods for the sample preparation. Currently, some data for NMs can be found in publications but closer inspection shows that the same type of data was usually measured with different protocols (solvents, sonication times, sonication types, etc.) and by different research groups. These variations in protocol raise the question of whether data generated in different conditions are comparable. All this results in smaller datasets and data gaps, taking also into account that there are very few *in silico* tools that can be used to calculate properties for specific NMs based on similarity between nanoforms for the purpose of prediction of toxicity.

Thus, for nanomaterials the RAAF Assessment Elements based on chemical structure would need to be adapted appropriately.

Furthermore, the variability of the measurement data of the NM characterisation hampers linking the characteristics to the observed effects. In the literature, appropriate definition of the requirements for characterisation is repeatedly called for (e.g. Fadeel, Fornara, Toprak, & Bhattacharya, 2015; Marchese Robinson et al., 2016; A. E. Nel et al., 2015).

In general, however, the case studies have shown that the RAAF framework is applicable to nanomaterials as well as to conventional chemicals.

4.7 Conclusions

In this chapter the ECHA draft framework for grouping and read-across of NMs has been applied for the first time to two NM case studies. These case studies have been documented by providing mechanistic interpretation of the available data, where possible, and according to the state of the art in the field. However, it has also to be acknowledged that our dataset for application of read-across to NMs was quite limited due to the data availability. Although TiO_2 nanoforms and MWCNTs are well studied NMs, it was not possible to identify a more suitable/relevant endpoint for carrying out our read-across case studies, and also the set of analogues was limited.

In this paragraph, the conclusions and lessons learnt while carrying out the read-across case studies are summarised.

__Applicability of the ECHA workflow__

- The workflow proposed in the draft ECHA guidance for grouping and read-across of NMs (ECHA, 2017b) was applied to our case studies, and as reported in this chapter, it allows to present all the information collected and used to build and test the grouping hypothesis. Figure 4.13 shows a graphic where some pragmatic suggestions on how to deal with each step of the workflow are summarised. Such specifications would guide the applicant through the workflow, and are formulated according to our experience in grouping of NMs for read-across.

- The approach followed in presenting the case studies was based on the pattern identified in the toxicological and physicochemical tests that are presented in step 2; then, chemoinformatic tools are applied in step 5 to assess the grouping hypothesis. Figure 4.16 reports some suggestions on the computational methods that could be suitable for this purpose. It would be possible also to apply computational methods in step 2 of the ECHA original workflow to define the grouping hypothesis that would then be assessed in step 5 by looking for toxicological tests to confirm it (the literature review on genotoxicity studies).

- The narrative supported by the framework proposed by ECHA is complex and tends to cause repetition in reporting the case, because it assumes the hypothesis (stated in step 2, Figure 4.13) is built a priori from the data gathered for the identified analogues (steps 3 and 4). From the examples examined in this chapter it is evident that the hypothesis is a result of data evaluation and treatment, but this step comes after in the workflow. The read-across framework proposed by RIVM et al. (2016) goes in this direction as the whole dataset is introduced earlier in the process (step 2), together with the initial grouping. Our suggestion in this respect would be to anticipate the data gathering process in step 2, so that the grouping hypothesis would take into account all the available information. This is also presented on the right-hand side of Figure 4.13.

- The ECHA workflow is not specific to read-across between nanoforms only, but can also be used, in principle, for read-across from the bulk form to the nanoform. This depends on how similar the analogues are to one another, which has to be judged on a case by case basis. Our suggestion would be to make this clearer in the nomenclature applied to the workflow, for instance by referring to "different physical forms of a substance" instead of using the term "nanoform".
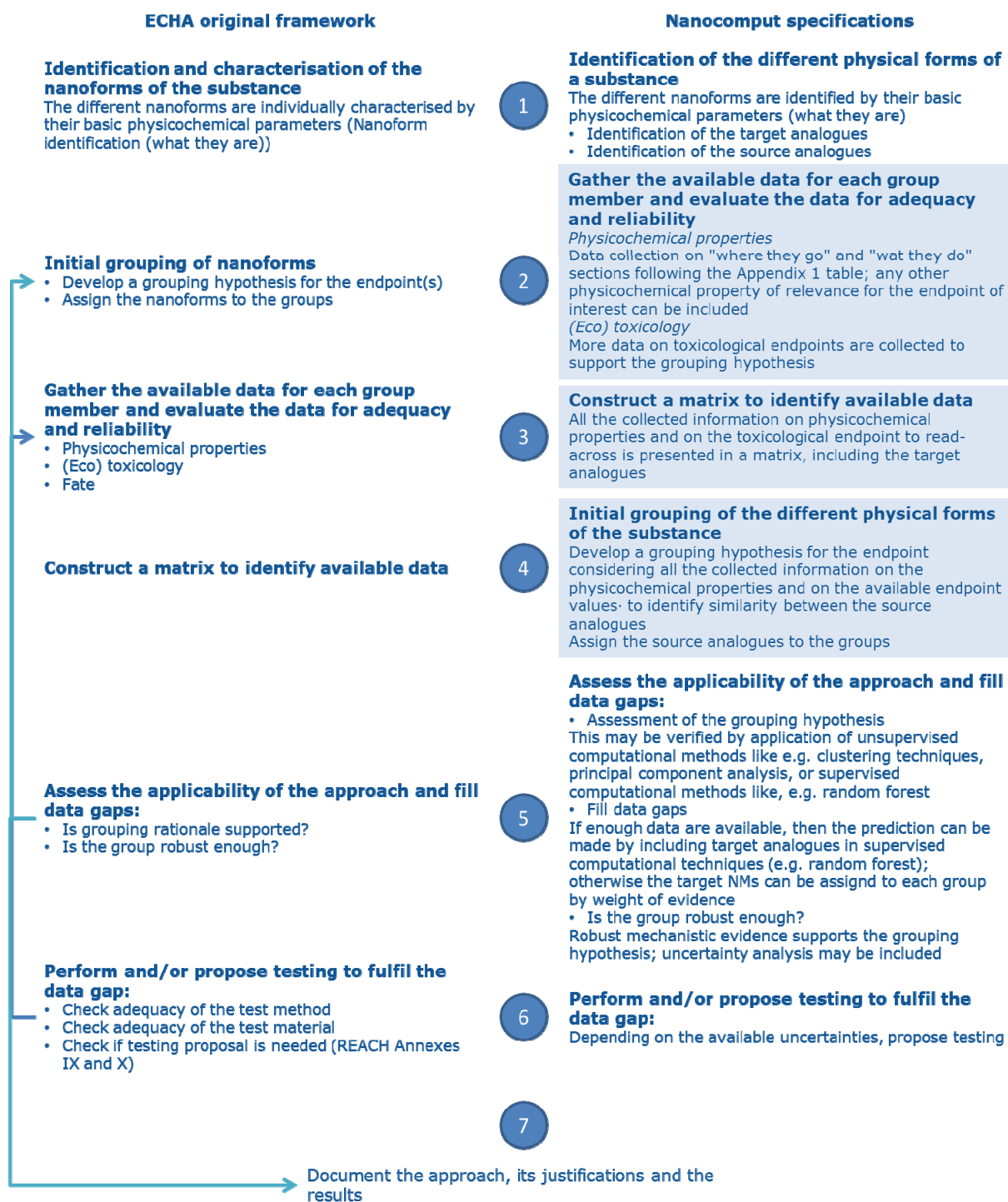
Identification and characterisation of the nanoforms of the substance
The different nanoforms are individually characterised by their basic physicochemical parameters (Nanoform identification (what they are))

Identification of the different physical forms of a substance
The different nanoforms are identified by their basic physicochemical parameters (what they are)
- Identification of the target analogues
- Identification of the source analogues

(1)

Gather the available data for each group member and evaluate the data for adequacy and reliability
Physicochemical properties
Data collection on "where they go" and "wat they do" sections following the Appendix 1 table; any other physicochemical property of relevance for the endpoint of interest can be included
(Eco) toxicology
More data on toxicological endpoints are collected to support the grouping hypothesis

(2)

Initial grouping of nanoforms
- Develop a grouping hypothesis for the endpoint(s)
- Assign the nanoforms to the groups

Construct a matrix to identify available data
All the collected information on physicochemical properties and on the toxicological endpoint to read-across is presented in a matrix, including the target analogues

(3)

Gather the available data for each group member and evaluate the data for adequacy and reliability
- Physicochemical properties
- (Eco) toxicology
- Fate

Initial grouping of the different physical forms of the substance
Develop a grouping hypothesis for the endpoint considering all the collected information on the physicochemical properties and on the available endpoint values· to identify similarity between the source analogues
Assign the source analogues to the groups

(4)

Construct a matrix to identify available data

Assess the applicability of the approach and fill data gaps:
- Assessment of the grouping hypothesis
This may be verified by application of unsupervised computational methods like e.g. clustering techniques, principal component analysis, or supervised computational methods like, e.g. random forest
- Fill data gaps
If enough data are available, then the prediction can be made by including target analogues in supervised computational techniques (e.g. random forest); otherwise the target NMs can be assignd to each group by weight of evidence
- Is the group robust enough?
Robust mechanistic evidence supports the grouping hypothesis; uncertainty analysis may be included

(5)

Assess the applicability of the approach and fill data gaps:
- Is grouping rationale supported?
- Is the group robust enough?

Perform and/or propose testing to fulfil the data gap:
- Check adequacy of the test method
- Check adequacy of the test material
- Check if testing proposal is needed (REACH Annexes IX and X)

Perform and/or propose testing to fulfil the data gap:
Depending on the available uncertainties, propose testing

(6)

(7)

Document the approach, its justifications and the results

Figure 4.13. ECHA framework for grouping and read-across of NM properties. The ECHA proposed framework is presented on the left side, while on the right side suggestions on possible specifications to help applicants based on the lessons learnt in Nanocomput are presented. According to the case studies that were carried out, the step "Gather the available data for each group member and evaluate the data for adequacy and reliability" should occur earlier in the process, before the "Initial grouping of nanoforms". Construction of a matrix could come as a result of the data gathering, instead of consisting of a separate step.

Considerations on the read-across approach applied to NMs

- Analogue vs category approaches. The analogue approach (one-to-one read-across) is implicitly covered in the category approach (many-to-one read-across). Multivariate approaches like PCA and cluster analysis are best suited to the category approach, where multiple analogues are available, and where relative pairwise similarities are meaningful.

- Possibility to read across more than one endpoint. In principle this is possible, provided that the underlying rationale for grouping is valid for the different endpoints. This could be the case when the mode of action is based on the same fundamental properties, e.g. skin sensitisation and mutagenicity being dependent on particle reactivity. In practice, though, the group will be endpoint-specific due to the multiplicity of underlying modes of action.

- In the hypothesis formulation stage, the starting point for grouping analogues is flexible and subject to expert judgement, depending on scientific (mechanistic knowledge) and practical (data availability) considerations. The initial choice of descriptors follows from this. For example, if the initial category hypothesis is that the toxicity of all metal oxides depends on their reactivity, and that reactivity is dependent on the atoms present, then metal composition could be a useful descriptor. If however the broad category of metal oxides is subcategorised to a specific subgroup (e.g. iron oxides), composition itself is unlikely to provide a means of discriminating between members of the group. Instead, additional descriptors would be required, e.g valence and/or band gap energy. This is consistent with the approach for categorisation and subcategorisation of organic substances in the OECD QSAR Toolbox.

Relevance of computational methods in grouping for read-across

- It has been shown how unsupervised techniques like hierarchical clustering and PCA can support the grouping hypothesis by identifying the differences between nanoforms and by supporting the weight of evidence in the read-across hypothesis.

- Supervised and unsupervised techniques were applied in the case studies presented in this chapter. Unsupervised techniques are more useful early in the process for hypothesis formulation, to identify which properties are more relevant for grouping and to define the similarity between analogues on the basis of these properties. Supervised techniques are more useful later in the process, for model building when there is already evidence of property-activity relationships. In our case study, a supervised variable selection algorithm was used to determine the most valuable properties in predicting genotoxicity, to support the grouping hypothesis. Irrespective of the techniques used, they should be considered as feeding information into an overall weight of evidence, rather than being conclusive themselves.

Uncertainty in grouping NMs for read-across

- Uncertainty associated to the read-across exercises has been taken into account; uncertainty is related to the identification of the (non-)nanoforms, experimental variability associated with the physicochemical and toxicological information and to the lacking measurement protocols, and, finally, to the lack of knowledge on the mechanisms of genotoxic action of NMs.

- We have developed a table of uncertainties that relate back to the ECHA RAAF. This indicates clearly additional considerations that need to be considered: e.g.

  o Applicability of *in vitro* test methods

  o Problem of particles precipitating onto cell surface and causing false positives due to membrane damage / "suffocation"

- A RAAF scenario was applied to our case studies for uncertainty analysis; therefore, the RAAF is applicable to NMs. A key aspect that is missing and would need to be more articulated for its application to NMs, is the concept of similarity, as in the RAAF it is limited to chemical similarity, and for a more consistent application to NMs, other principles for similarity shall be included.

4.8 References

Aggarwal, P., J. B. Hall, C. B. McLeland, M. a Dobrovolskaia, and S. E. McNeil. (2009). Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. Advanced drug delivery reviews 61:428–37.

Aldieri, E., I. Fenoglio, F. Cesano, E. Gazzano, G. Gulino, D. Scarano, A. Attanasio, G. Mazzucco, D. Ghigo, and B. Fubini. (2013). The role of iron impurities in the toxic effects exerted by short multiwalled carbon nanotubes (MWCNT) in murine alveolar macrophages. J Toxicol Environ Health A 76:1056–1071.

Allegri, M., D. K. Perivoliotis, M. G. Bianchi, M. Chiu, A. Pagliaro, M. A. Koklioti, A. F. A. Trompeta, E. Bergamaschi, O. Bussolati, and C. A. Charitidis. (2016). Toxicity determinants of multi-walled carbon nanotubes: The relationship between functionalization and agglomeration. Toxicology Reports 3:230–243.

ANSES. (2016). CLH report Proposal for Harmonised Classification and Labelling Substance Name : Titanium dioxide.

Arts, J. H. E., M. Hadi, M.-A. Irfan, A. M. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, T. Petry, U. G. Sauer, D. Warheit, K. Wiench, W. Wohlleben, and R. Landsiedel. (2015). A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regulatory toxicology and pharmacology 71:S1–S27.

Arts, J. H. E., M. Hadi, M.-A. Irfan, A. M. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, T. Petry, U. G. Sauer, D. Warheit, K. Wiench, W. Wohlleben, and R. Landsiedel. (2016). Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regulatory toxicology and pharmacology 76:234–261.

Azqueta, A., and M. Dusinska. (2015). The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetics 6:239.

Ball, N., M. T. D. Cronin, J. Shen, M. D. Adenuga, K. Blackburn, E. D. Booth, M. Bouhifd, E. Donley, L. Egnash, J. J. Freeman, C. Hastings, D. R. Juberg, A. Kleensang, N. Kleinstreuer, E. D. Kroese, T. Luechtefeld, A. Maertens, S. Marty, J. M. Naciff, J. Palmer, D. Pamies, M. Penman, A.-N. Richarz, D. P. Russo, S. B. Stuard, G. Patlewicz, B. van Ravenzwaay, S. Wu, H. Zhu, and T. Hartung. (2016). Toward Good Read-Across Practice (GRAP) guidance. ALTEX.

Barillet, S., A. Simon-Deckers, N. Herlin-Boime, M. Mayne-L'Hermite, C. Reynaud, D. Cassio, B. Gouget, and M. Carrière. (2010). Toxicological consequences of TiO2, SiC nanoparticles and multi-walled carbon nanotubes exposure in several mammalian cell types: an in vitro study. Journal of Nanoparticle Research 12:61–73.

Van Berlo, D., M. Clift, C. Albrecht, and R. Schins. (2012). Carbon nanotubes: An insight into the mechanisms of their potential genotoxicity.

Binderup, M.-L., L. Bredsdorff, V. M. Beltoft, A. Mortensen, K. Löschner, E. H. Larsen, and F. D. Eriksen. (2013). Systemic Absorption of Nanomaterials by Oral Exposure; Part of the "Better control of nano" initiative 2012-2015.

Birkedal, R., B. Shivachev, L. Dimova, O. Petrov, R. Nikolova, J. Mast, P. de Temmerman, N. Waegeneers, L. Delfosse, F. Van Steen, J. C. Pizzolon, L. De Temmerman, V. Kofoed-sørensen, P. A. Clausen, A. Parks, P. Ferguson, C. Motzkus, and K. A. Jensen. (2012). Nanogenotox deliverable 43: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials.

Borm, P. J. A., R. P. F. Schins, and C. Albrecht. (2004). Inhaled particles and lung cancer, part B: Paradigms and risk assessment.

Boyles, M. S. P., L. Young, D. M. Brown, L. MacCalman, H. Cowie, A. Moisala, F. Smail, P. J. W. Smith, L. Proudfoot, A. H. Windle, and V. Stone. (2015). Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. Toxicology in Vitro 29:1513–1528.

Braakhuis, H. M., A. G. Oomen, and F. R. Cassee. (2015). Grouping nanomaterials to predict their potential to induce pulmonary inflammation. Toxicology and applied pharmacology.

Braakhuis, H. M., M. V. D. Z. Park, I. Gosens, W. H. De Jong, and F. R. Cassee. (2014). Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. Particle and fibre toxicology 11:18.

Burello, E., and A. P. Worth. (2011). A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5:228–35.

Cai, X., R. Ramalingam, H. S. Wong, J. Cheng, P. Ajuh, S. H. Cheng, and Y. W. Lam. (2013). Characterization of carbon nanotube protein corona by using quantitative proteomics. Nanomedicine: Nanotechnology, Biology, and Medicine 9:583–593.

Catalán, J., K. M. Siivola, P. Nymark, H. K. Lindberg, S. Suhonen, H. Järventaus, A. J. Koivisto, C. Moreno, E. Vanhala, H. Wolff, K. I. Kling, K. A. Jensen, K. M. Savolainen, and H. Norppa. (2015). In vitro and in vivo genotoxic effects of straight versus tangled multi-walled carbon nanotubes. Nanotoxicology 5390:1–47.

Cedervall, T., I. Lynch, S. Lindman, T. Berggård, E. Thulin, H. Nilsson, K. A. Dawson, and S. Linse. (2007). Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proceedings of the National Academy of Sciences of the United States of America 104:2050–5.

Cho, H. H., H. Huang, and K. Schwab. (2011). Effects of solution chemistry on the adsorption of ibuprofen and triclosan onto carbon nanotubes. Langmuir 27:12960–12967.

Clift, M. J. D., D. O. Raemy, C. Endes, Z. Ali, A. D. Lehmann, C. Brandenberger, A. Petri-Fink, P. Wick, W. J. Parak, P. Gehr, R. P. F. Schins, and B. Rothen-Rutishauser. (2012). Can the Ames test provide an insight into nano-object mutagenicity? Investigating the interaction between nano-objects and bacteria. Nanotoxicology 7:1–13.

Cortez, B. A., and G. M. Machado-Santelli. (2008). Chrysotile effects on human lung cell carcinoma in culture: 3-D reconstruction and DNA quantification by image analysis. BMC Cancer 8:181.

Creutzenberg, O. (2013). Toxic Effects of Various Modifications of a Nanoparticle Following Inhalation.

Cveticanin, J., G. Joksic, A. Leskovac, S. Petrovic, A. V Sobot, and O. Neskovic. (2010). Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells. Nanotechnology 21:15102.

Donaldson, K., R. Aitken, L. Tran, V. Stone, R. Duffin, G. Forrest, and A. Alexander. (2006). Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety.

Donaldson, K., R. Duffin, F. A. Murphy, and C. A. Poland. (2012). The Toxicology of Carbon Nanotubes. Pages (K. Donaldson, C. Poland, R. Duffin, and J. Bonner, Eds.) The Toxicology of Carbon Nanotubes. Cambridge University Press, Cambridge.

Donaldson, K., F. a Murphy, R. Duffin, and C. a Poland. (2010a). Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Particle and fibre toxicology 7:5.

Donaldson, K., C. A. Poland, and E. Wargo. (2009). Nanotoxicology: new insights into nanotubes. Nature Nanotechnology 4:708–710.

Donaldson, K., C. a Poland, and R. P. F. Schins. (2010b). Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies. Nanotoxicology 4:414–20.

Donaldson, K., A. Schinwald, F. Murphy, W.-S. Cho, R. Duffin, L. Tran, and C. Poland. (2013). The biologically effective dose in inhalation nanotoxicology. Accounts of chemical research 46:723–32.

EC. (2012). Commission Staff Working Paper: Types and uses of nanomaterials, indcluding safety aspects. Pages European Commission.

ECHA. (2012). Guidance for identification and naming of substances under REACH and CLP.

ECHA. (2015a). Guidance on information requirements and chemical safety assessment Chapter R 5 : Adaptation of information requirements. Version 4. ECHA, Helsinki.

ECHA. (2016). Appendix 4 : Recommendations for nanomaterials applicable to the Guidance on Registration Draft (Public) Version 10.

ECHA. (2017a). Read-Across Assessment Framework (RAAF). ECHA-17-R-01-EN

ECHA. (2017b). Appendix R 6-1 : Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping.

Esch, R. K., L. Han, K. K. Foarde, and D. S. Ensor. (2010). Endotoxin contamination of engineered nanomaterials. Nanotoxicology 4:73–83.

European Industry. (2016). COMMENTS AND RESPONSE TO COMMENTS ON CLH ON TITANIUM DIOXIDE: PROPOSAL AND JUSTIFICATION Comments.

Fadeel, B., A. Fornara, M. S. Toprak, and K. Bhattacharya. (2015). Keeping it real: The importance of material characterization in nanotoxicology. Biochemical and Biophysical Research Communications 468:498–503.

Falck, G., H. Lindberg, S. Suhonen, M. Vippola, E. Vanhala, J. Catalan, K. Savolainen, and H. Norppa. (2009). Genotoxic effects of nanosized and fine TiO2. Human & Experimental Toxicology 28:339–352.

Fenoglio, I., E. Aldieri, E. Gazzano, F. Cesano, M. Colonna, D. Scarano, G. Mazzucco, A. Attanasio, Y. Yakoub, D. Lison, and B. Fubini. (2012). Thickness of multiwalled carbon nanotubes affects their lung toxicity. Chemical Research in Toxicology 25:74–82.

Gini, C. (1912). Variabilità e mutabilità Memorie di metodologica statistica. Pages (E. Pizetti and T. Salvemini, Eds.). 1955thedition. Libreria Eredi Virgilio Veschi, Rome.

Di Giorgio, M. L., S. Di Bucchianico, A. M. Ragnelli, P. Aimola, S. Santucci, and A. Poma. (2011). Effects of single and multi walled carbon nanotubes on macrophages: Cyto and genotoxicity and electron microscopy. Mutation Research - Genetic Toxicology and Environmental Mutagenesis 722:20–31.

Golbamaki, N., B. Rasulev, A. Cassano, R. L. Marchese Robinson, E. Benfenati, J. Leszczynski, and M. T. D. Cronin. (2015). Genotoxicity of metal oxide nanomaterials: review of recent data and discussion of possible mechanisms. Nanoscale 7:2154–2198.

Greim, H., P. Borm, R. Schins, K. Donaldson, K. Driscoll, A. Hartwig, l E. Kuempe, G. Oberdörster, and G. Speit. (2001). TOXICITY OF FIBERS AND PARTICLES. Inhalation Toxicology 13.

Grosse, Y., D. Loomis, K. Z. Guyton, B. Lauby-Secretan, F. El Ghissassi, V. Bouvard, L. Benbrahim-Tallaa, N. Guha, C. Scoccianti, H. Mattock, and K. Straif. (2014). Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. The Lancet. Oncology 15:1427–8.

Gu, Z., Z. Yang, Y. Chong, C. Ge, J. K. Weber, D. R. Bell, and R. Zhou. (2015). Surface Curvature Relation to Protein Adsorption for Carbon-based Nanomaterials. Scientific reports 5:10886.

Guichard, Y., J. Schmit, C. Darne, L. Gaté, M. Goutet, D. Rousset, O. Rastoix, R. Wrobel, O. Witschger, A. Martin, V. Fierro, and S. Binet. (2012). Cytotoxicity and genotoxicity of nanosized and microsized

titanium dioxide and iron oxide particles in Syrian hamster embryo cells. The Annals of occupational hygiene 56:631–44.

Hamilton, R. F., Z. Wu, S. Mitra, P. K. Shaw, and A. Holian. (2013). Effect of MWCNT size, carboxylation, and purification on in vitro and in vivo toxicity, inflammation and lung pathology. Particle and fibre toxicology 10:57.

Heringa, M. B., L. Geraets, J. C. H. van Eijkeren, R. J. Vandebriel, W. H. de Jong, and A. G. Oomen. (2016). Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations. Nanotoxicology:1–11.

Jackson, P., K. Kling, K. A. Jensen, P. A. Clausen, A. M. Madsen, H. Wallin, and U. Vogel. (2015). Characterization of genotoxic response to 15 multiwalled carbon nanotubes with variable physicochemical properties including surface functionalizations in the FE1-Muta(TM) mouse lung epithelial cell line. Environmental and Molecular Mutagenesis 56:183–203.

Jacobsen, N. R., H. Wallin, W. De Jong, A. Oomen, E. Brandon, P. Krystek, M. Apostolova, I. Karadjova, F. Cubadda, F. Aureli, F. Maranghi, V. Dive, F. Taran, and B. Czarny. (2013). Towards a method for detecting the potential genotoxicity of nanomaterials; Deliverable 7: Identification of target organs and biodistribution including ADME parameters.

Jain, S., V. S. Thakare, M. Das, C. Godugu, A. K. Jain, R. Mathur, K. Chuttani, and A. K. Mishra. (2011). Toxicity of multiwalled carbon nanotubes with end defects critically depends on their functionalization density. Chemical Research in Toxicology 24:2028–2039.

Jensen, K. A., Y. Kembouche, and S. H. Nielsen. (2013). Nanogenotox deliverable 47: Hydrochemical reactivity, solubility, and biodurability of NANOGENOTOX nanomaterials.

Johnston, H. J., G. R. Hutchison, F. M. Christensen, S. Peters, S. Hankin, K. Aschberger, and V. Stone. (2010). A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. Nanotoxicology 4:207–246.

Jugan, M.-L., S. Barillet, A. Simon-Deckers, N. Herlin-Boime, S. Sauvaigo, T. Douki, and M. Carriere. (2012). Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. Nanotoxicology 6:501–513.

Kagan, V. E., Y. Y. Tyurina, V. A. Tyurin, N. V. Konduru, A. I. Potapovich, A. N. Osipov, E. R. Kisin, D. Schwegler-Berry, R. Mercer, V. Castranova, and A. A. Shvedova. (2006). Direct and indirect effects of single walled carbon nanotubes on RAW 2647 macrophages: Role of iron. Toxicology Letters 165:88–100.

Kakwere, H., M. P. Leal, M. E. Materia, A. Curcio, P. Guardia, D. Niculaes, R. Marotta, A. Falqui, and T. Pellegrino. (2015). Functionalization of strongly interacting magnetic nanocubes with (thermo)responsive coating and their application in hyperthermia and heat-triggered drug delivery. ACS applied materials & interfaces 7:10132–45.

Karlsson, H. L., S. Di Bucchianico, A. R. Collins, and M. Dusinska. (2015). Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity?. Environmental and Molecular Mutagenesis 56:82–96.

Kettiger, H., A. Schipanski, P. Wick, and J. Huwyler. (2013). Engineered nanomaterial uptake and tissue distribution: From cell to organism.

Kim, J. S., K. Lee, Y. H. Lee, H. S. Cho, K. H. Kim, K. H. Choi, S. H. Lee, K. S. Song, C. S. Kang, and I. J. Yu. (2011). Aspect ratio has no effect on genotoxicity of multi-wall carbon nanotubes. Archives of Toxicology 85:775–786.

Klimisch, H. J., M. Andreae, and U. Tillmann. (1997). A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regulatory toxicology and pharmacology

25:1–5.

Li, K., X. Zhao, B. K. Hammer, S. Du, and Y. Chen. (2013). Nanoparticles Inhibit DNA Replication by Binding to DNA: Modeling and Experimental Validation. ACS Nano 7:9664–9674.

Li, X., Y. Peng, and X. Qu. (2006a). Carbon nanotubes selective destabilization of duplex and triplex DNA and inducing B-A transition in solution. Nucleic Acids Research 34:3670–3676.

Li, X., Y. Peng, J. Ren, and X. Qu. (2006b). Carboxyl-modified single-walled carbon nanotubes selectively induce human telomeric i-motif formation. Proceedings of the National Academy of Sciences of the United States of America 103:19658–19663.

Liaw, A., and M. Wiener. (2002). Classification and Regression by randomForest. R News 2(3):18–22.

Liu, Y., Y. Zhao, B. Sun, and C. Chen. (2013). Understanding the toxicity of carbon nanotubes. Accounts of Chemical Research 46:702–713.

Liu, Z., X. Dong, L. Song, H. Zhang, L. Liu, D. Zhu, C. Song, and X. Leng. (2014). Carboxylation of multiwalled carbon nanotube enhanced its biocompatibility with L02 cells through decreased activation of mitochondrial apoptotic pathway. Journal of Biomedical Materials Research - Part A 102:665–673.

Luttrell, T., S. Halpegamage, J. Tao, A. Kramer, E. Sutter, and M. Batzill. (2014). Why is anatase a better photocatalyst than rutile? - Model studies on epitaxial TiO2 films. Scientific Reports 4:4043.

Magdolenova, Z., D. Bilaničová, G. Pojana, L. M. Fjellsbø, A. Hudecova, K. Hasplova, A. Marcomini, and M. Dusinska. (2012). Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. Journal of Environmental Monitoring 14:455.

Magdolenova, Z., A. Collins, A. Kumar, A. Dhawan, V. Stone, and M. Dusinska. (2014). Mechanisms of genotoxicity A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8:233–278.

Mano, S. S., K. Kanehira, S. Sonezaki, and A. Taniguchi. (2012). Effect of Polyethylene Glycol Modification of TiO 2 Nanoparticles on Cytotoxicity and Gene Expressions in Human Cell Lines. International Journal of Molecular Sciences 13:3703–3717.

Marchese Robinson, R. L., I. Lynch, W. Peijnenburg, J. Rumble, F. Klaessig, C. Marquardt, H. Rauscher, T. Puzyn, R. Purian, C. Åberg, S. Karcher, H. Vriens, P. Hoet, M. Hoover, C. Hendren, and S. Harper. (2016). How should the completeness and quality of curated nanomaterial data be evaluated?. Nanoscale.

Maruyama, K., H. Haniu, N. Saito, Y. Matsuda, T. Tsukahara, S. Kobayashi, M. Tanaka, K. Aoki, S. Takanashi, M. Okamoto, and H. Kato. (2015). Endocytosis of Multiwalled Carbon Nanotubes in Bronchial Epithelial and Mesothelial Cells. BioMed research international 2015:793186.

Mercer, R. R., A. F. Hubbs, J. F. Scabilloni, L. Wang, L. a Battelli, S. Friend, V. Castranova, and D. W. Porter. (2011). Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes. Particle and Fibre Toxicology 8:21.

Mercer, R. R., J. F. Scabilloni, A. F. Hubbs, L. a Battelli, W. McKinney, S. Friend, M. G. Wolfarth, M. Andrew, V. Castranova, and D. W. Porter. (2013). Distribution and fibrotic response following inhalation exposure to multi-walled carbon nanotubes. Particle and fibre toxicology 10:33.

Mitrano, D. M., S. Motellier, S. Clavaguera, and B. Nowack. (2015). Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products.

Mu, Q., W. Liu, Y. Xing, H. Zhou, Z. Li, Y. Zhang, L. Ji, F. Wang, Z. Si, B. Zhang, and B. Yan. (2008). Protein binding by functionalized multiwalled carbon nanotubes is governed by the surface chemistry

of both parties and the nanotube diameter. Journal of Physical Chemistry C 112:3300–3307.

Mulware, S. J. (2012). Trace elements and carcinogenicity: a subject in review. 3 Biotech:85–96.

Murphy, F. A., C. A. Poland, R. Duffin, and K. Donaldson. (2013). Length-dependent pleural inflammation and parietal pleural responses after deposition of carbon nanotubes in the pulmonary airspaces of mice.

Murray, A. R., E. R. Kisin, A. V Tkach, N. Yanamala, R. R. Mercer, S.-H. Young, B. Fadeel, V. E. Kagan, and A. a Shvedova. (2012). Factoring-in agglomeration of carbon nanotubes and nanofibers for better prediction of their toxicity versus asbestos. Particle and Fibre Toxicology 9:10.

MWCNT REACH Dossier. (2016). REACH registration dossier: Multi-Walled Carbon Nanotubes (MWCNT), synthetic graphite in tubular shape (EC number: 936-414-1) http://echa.europa.eu/registration-dossier/-/registered-dossier/13454/7/2/2/?documentUUID=d042b6e7-1608-420a-b644-475a6450fe43.

Nagai, H., Y. Okazaki, S. Chew, N. Misawa, Y. Yamashita, S. Akatsuka, T. Ishihara, K. Yamashita, Y. Yoshikawa, H. Yasui, L. Jiang, H. Ohara, T. Takahashi, G. Ichihara, K. Kostarelos, Y. Miyata, H. Shinohara, and S. Toyokuni. (2011). Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. Pnas 108:1330–1338.

Nanogenotox. (2010). Safety evaluation of manufactured nanomaterials by characterisation of their potential genotoxic hazard http://www.nanogenotox.eu/.

Nanogenotox. (2012). Towards a method for detecting the potential genotoxicity of nanomaterials D4 2 : Transmission electron microscopic characterization of NANOGENOTOX nanomaterials Final report Key intrinsic physicochemical characteristics of NANOGENOTOX nanomaterials WP.

NanoGenoTox Joint Action. (2013). NANOGENOTOX Final report Facilitating the safety evaluation of manufactured nanomaterials by characterising their potential genotoxic hazard.

Nanogenotox WP6. (2013). Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis.

Nel, A. E., W. J. Parak, W. C. W. Chan, T. Xia, M. C. Hersam, C. J. Brinker, J. I. Zink, K. E. Pinkerton, D. R. Baer, and P. S. Weiss. (2015). Where Are We Heading in Nanotechnology Environmental Health and Safety and Materials Characterization?. ACS Nano 9:5627–5630.

NIOSH. (2013). Occupational Exposure to Carbon Nanotubes and Nanofibers. 2013–145.

Norppa, H., K. Siivola, V. Fessard, A. Tarantini, M. Apostolova, N. Jacobsen, H. Wallin, M. Goetz, D. Fieblinger, M. Stepnik, S. Simar, S. Quarre, F. Nesslany, W. H. De Jong, R. Marcos, G. Vales, P. Troisfontaines, Y. Guichards, A. Tavares, H. Louro, and M. Silva. (2013). Nanogenotox deliverable 5 : In vitro testing strategy for nanomaterials including database Final report.

Oberdörster, G., V. Castranova, B. Asgharian, and P. Sayre. (2015). Inhalation Exposure to Carbon Nanotubes (CNT) and Carbon Nanofibers (CNF): Methodology and Dosimetry. Journal of toxicology and environmental health. Part B, Critical reviews 18:121–212.

OECD. (2014a). Genotoxicity of manufactured nanomaterials: Report of the OECD expert meeting Series on the safety of manufactured nanomaterials No 43. ENV/JM/WRPR(2014)51.

OECD. (2014b). Draft Report Of The OECD Expert Meeting on Genotoxicity of Manufactured Nanomaterials.

OECD. (2015). Titanium dioxide dossier (NM100-NM105). ENV/CHEM/NANO(2015)21/Part 1 to 5.

OECD WPMN. (2016). Multiwalled Carbon Nanotubes (Mwcnt): Summary of the Dossier ENV/JM/MON:1–48.

Oomen, A. G., P. M. J. Bos, T. F. Fernandes, K. Hund-Rinke, D. Boraschi, H. J. Byrne, K. Aschberger, S.

Gottardo, F. von der Kammer, D. Kühnel, D. Hristozov, A. Marcomini, L. Migliore, J. Scott-Fordsmand, P. Wick, and R. Landsiedel. (2014). Concern-driven integrated approaches to nanomaterial testing and assessment--report of the NanoSafety Cluster Working Group 10. Nanotoxicology 8:334–48.

Pantano, A., D. M. Parks, and M. C. Boyce. (2004). Mechanics of deformation of single- and multi-wall carbon nanotubes. Journal of the Mechanics and Physics of Solids 52:789–821.

Pauluhn, J. (2009). Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: Toxic effects are determined by density of agglomerate structures, not fibrillar structures. Toxicological Sciences 113:226–242.

Petersen, E. J., L. Zhang, N. T. Mattison, D. M. O'Carroll, A. J. Whelton, N. Uddin, T. Nguyen, Q. Huang, T. B. Henry, R. D. Holbrook, and K. L. Chen. (2011). Potential release pathways, environmental fate, and ecological risks of carbon nanotubes.

Poulsen, S. S., P. Jackson, K. Kling, K. B. Knudsen, V. Skaug, Z. O. Kyjovska, B. L. Thomsen, P. A. Clausen, R. Atluri, T. Berthing, S. Bengtson, H. Wolff, K. A. Jensen, H. Wallin, and U. Vogel. (2016). Multi-walled carbon nanotube physicochemical properties predict pulmonary inflammation and genotoxicity. Nanotoxicology 5390:1–13.

Poulsen, S. S., A. T. Saber, A. Williams, O. Andersen, C. Købler, R. Atluri, M. E. Pozzebon, S. P. Mucelli, M. Simion, D. Rickerby, A. Mortensen, P. Jackson, Z. O. Kyjovska, K. Mølhave, N. R. Jacobsen, K. A. Jensen, C. L. Yauk, H. Wallin, S. Halappanavar, and U. Vogel. (2015). MWCNTs of different physicochemical properties cause similar inflammatory responses, but differences in transcriptional and histological markers of fibrosis in mouse lungs. Toxicology and applied pharmacology 284:16–32.

Rasmussen, K., M. González, P. Kearns, J. R. Sintes, F. Rossi, and P. Sayre. (2016). Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme From exploratory testing to test guidelines. Regulatory toxicology and pharmacology 74:147–160.

Rasmussen, K., J. Mast, and P.-J. Temmerman. (2014a). Multi-walled Carbon Nanotubes, NM-400, NM-401, NM-402, NM-403: Characterisation and Physico-Chemical Properties.

Rasmussen, K., J. Mast, P. De Temmerman, E. Verleysen, N. Waegeneers, F. Van Steen, J. C. Pizzolon, L. De Temmerman, E. Van, K. A. Jensen, R. Birkedal, M. Levin, S. H. Nielsen, I. K. Koponen, P. Axel, V. Kofoed-sørensen, Y. Kembouche, O. Spalla, C. Guiot, D. Rousset, S. Bau, B. Bianchi, C. Motzkus, B. Shivachev, L. Dimowa, R. Nikolova, M. Tarassov, O. Petrov, D. Gilliland, F. Pianella, V. Spampinato, G. Cotogno, and N. Gibson. (2014b). Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico- Chemical Properties. Luxembourg.

Rice, Z., N. C. Cady, M. Bergkvist, P. E. Lobert, D. Bourgeois, R. Pampin, A. Akheyar, L. M. Hagelsieb, D. Flandre, J. Remacle, X.-H. Xu, H. C. Yang, T. E. Mallouk, and A. J. Bard. (2009). Terminal phosphate group influence on DNA - TiO2 nanoparticle interactions. MRS Proceedings 1236:1236-NaN-15.

Rittinghausen, S., B. Bellmann, O. Creutzenberg, H. Ernst, A. Kolling, I. Mangelsdorf, R. Kellner, S. Beneke, and C. Ziemann. (2013). Evaluation of immunohistochemical markers to detect the genotoxic mode of action of fine and ultrafine dusts in rat lungs. Toxicology 303:177–186.

RIVM, JRC, and ECHA. (2016). Usage of (eco)toxicological data for bridging data gaps between and grouping of nanoforms of the same substance Elements to consider.

Roller, M. (2011). In vitro genotoxicity data of nanomaterials compared to carcinogenic potency of inorganic substances after inhalational exposure. Mutation research 727:72–85.

Romoser, A. A. (2012). CYTOTOXICOLOGICAL RESPONSE TO ENGINEERED NANOMATERIALS: A PATHWAY-DRIVEN PROCESS. Pages Statewide Agricultural Land Use Baseline 2015.

Rushton, E. K., J. Jiang, S. S. Leonard, S. Eberly, V. Castranova, P. Biswas, A. Elder, X. Han, R. Gelein, J.

Finkelstein, and G. Oberdörster. (2010). Concept of Assessing Nanoparticle Hazards Considering Nanoparticle Dosemetric and Chemical/Biological Response Metrics. Journal of Toxicology and Environmental Health, Part A 73.

Sager, T. M., M. W. Wolfarth, M. Andrew, A. Hubbs, S. Friend, T. Chen, D. W. Porter, N. Wu, F. Yang, R. F. Hamilton, and A. Holian. (2014). Effect of multi-walled carbon nanotube surface modification on bioactivity in the C57BL/6 mouse model. Nanotoxicology 8:317–27.

Sakharova, N. A., A. F. G. Pereira, J. M. Antunes, and J. V. Fernandes. (2016). Numerical simulation study of the elastic properties of single-walled carbon nanotubes containing vacancy defects. Composites Part B: Engineering 89:155–168.

Sargent, L. M., A. F. Hubbs, S. H. Young, M. L. Kashon, C. Z. Dinu, J. L. Salisbury, S. A. Benkovic, D. T. Lowry, A. R. Murray, E. R. Kisin, K. J. Siegrist, L. Battelli, J. Mastovich, J. L. Sturgeon, K. L. Bunker, A. A. Shvedova, and S. H. Reynolds. (2012). Single-walled carbon nanotube-induced mitotic disruption. Mutation Research - Genetic Toxicology and Environmental Mutagenesis 745:28–37.

Sargent, L., A. Shvedova, A. Hubbs, J. Salisbury, S. Benkovic, M. Kashon, D. Lowry, A. Murray, E. Kisin, S. Friend, K. McKinstry, L. Battelli, and S. Reynolds. (2009). Induction of aneuploidy by single-walled carbon nanotubes. Environ Mol Mutagen. 50:708–717.

SCCS. (2013). Opinion on Titanium Dioxide (nano form). COLIPA n. S75.

Schins, R. P. F. (2002). Mechanisms of genotoxicity of particles and fibers. Inhalation toxicology 14:57–78.

Schins, R. P. F., and A. M. Knaapen. (2007). Genotoxicity of poorly soluble particles. Inhalation toxicology 19 Suppl 1:189–98.

Schinwald, A., F. A. Murphy, A. Prina-Mello, C. A. Poland, F. Byrne, D. Movia, J. R. Glass, J. C. Dickerson, D. A. Schultz, C. E. Jeffree, W. Macnee, and K. Donaldson. (2012). The threshold length for fiber-induced acute pleural inflammation: Shedding light on the early events in asbestos-induced mesothelioma. Toxicological Sciences 128:461–470.

Schröder, K., C. Pohlenz-Michel, N. Simetska, J. U. Voss, S. Escher, and I. Mangersdorf. (2014). Carcinogenicity and Mutagenicity of Nanoparticles – Assessment of Current Knowledge as Basis for Regulation.

Schultz, T. W., P. Amcoff, E. Berggren, F. Gautier, M. Klaric, D. J. Knight, C. Mahony, M. Schwarz, A. White, and M. T. D. Cronin. (2015). A Strategy for Structuring and Reporting a Read-Across Prediction of Toxicity. Regulatory toxicology and pharmacology.

Sellers, K., N. Deleebeek, M. Messiaen, M. Jackson, E. Bleeker, D. Sijm, and F. Broekhuizen. (2015). Grouping nanomaterials A strategy towards grouping and read-across. 2015–61.

Siegrist, K. J., S. H. Reynolds, M. L. Kashon, D. T. Lowry, C. Dong, A. F. Hubbs, S.-H. Young, J. L. Salisbury, D. W. Porter, S. A. Benkovic, M. McCawley, M. J. Keane, J. T. Mastovich, K. L. Bunker, L. G. Cena, M. C. Sparrow, J. L. Sturgeon, C. Z. Dinu, and L. M. Sargent. (2014). Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses. Particle and fibre toxicology 11:6.

Stone, U. K. V., D. Balharry, T. Fernandes, H. Johnston, L. Munro, M. Hartl, and H. W. and J. J. S.-F. V. Stone, S. Pozzi-Mucelli, L. Tran, K. Aschberger, S. Sabella, U.B. Vogel, C. Poland, D. Balharry, T. Fernandes, S. Gottardo, S. Hankin, M. Hartl, N. Hartmann, D. Hristozov, K. Hund-Rinke, H. Johnston, A. Marcomini, O. Panzer, D. Roncato, A.T. Saber. (2013). RESEARCH PRIORITISATION TO DELIVER AN INTELLIGENT TESTING STRATEGY FOR THE HUMAN AND ENVIRONMENTAL SAFETY OF NANOMATERIALS AS SE SM.

Sweeney, S., D. Grandolfo, P. Ruenraroengsak, and T. D. Tetley. (2015). Functional consequences for primary human alveolar macrophages following treatment with long, but not short, multiwalled

carbon nanotubes. International Journal of Nanomedicine 10:3115–3129.

de Temmerman, P., J. Mast, C. Guiot, O. Spalla, D. Rousset, B. Shivachev, and M. Tarassov. (2012). Nanogenotox deliverable 42 Transmission electron microscopic characterization of NANOGENOTOX nanomaterials Final report.

Toropov, A. A., and J. Leszczynski. (2006). A new approach to the characterization of nanomaterials: Predicting Young's modulus by correlation weighting of nanomaterials codes. Chemical Physics Letters 433:125–129.

Tournebize, J., A. Sapin-Minet, G. Bartosz, P. Leroy, and A. Boudier. (2013). Pitfalls of assays devoted to evaluation of oxidative stress induced by inorganic nanoparticles.

Trouiller, B., R. Reliene, A. Westbrook, P. Solaimani, and R. H. Schiestl. (2009). Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer research 69:8784–9.

US-EPA, and EPA. (2013). Comprehensive Environmental Assessment Applied to: Multiwalled Carbon Nanotube Flame-Retardant Coatings in Upholstery Textiles - A Case Study Presenting Priority Research Gaps for Future Risk Assessments.

Vietti, G., D. Lison, and S. van den Brule. (2016). Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). Particle and Fibre Toxicology 13:11.

Wang, X., T. Xia, S. Addo Ntim, Z. Ji, S. Lin, H. Meng, C. H. Chung, S. George, H. Zhang, M. Wang, N. Li, Y. Yang, V. Castranova, S. Mitra, J. C. Bonner, and A. E. Nel. (2011). Dispersal state of multiwalled carbon nanotubes elicits profibrogenic cellular responses that correlate with fibrogenesis biomarkers and fibrosis in the murine lung. ACS Nano 5:9772–9787.

Wohlleben, W., L. Ma-Hock, V. Boyko, G. Cox, H. Egenolf, H. Freiberger, B. Hinrichsen, S. Hirth, and R. Landsiedel. (2013). Nanospecific guidance in REACH: A comparative physical-chemical characterization of 15 materials with methodical correlations. Journal of Ceramic Science and Technology 4:93–104.

Ziemann, C., S. Rittinghausen, H. Ernst, A. Kolling, I. Mangelsdorf, and O. Creutzenberg. (2011). Genotoxic Mode of Action of Fine and Ultrafine Dusts in Lungs:107.

5   Recommendations for further research and development

5.1   Nanotoxicity modelling project landscape

5.1.1   European nanosafety research activities

European research projects addressing the safety of materials and technologies using nanomaterials are organised under the overarching umbrella of the NanoSafety Cluster (NSC) (http://www.nanosafetycluster.eu), in order to foster exchange and synergies and avoid duplication of work. Topics of the projects include safety assessment, assessment of toxicity to human health and the environment, exposure, mechanism of interactions and also definitions, standardisation and regulatory aspects.

Cross-NanoSafety Cluster working groups bring together researchers from the different projects to contribute to the discussion of specific topics and tackle issues common to the different projects. The original working groups were dedicated to:

- Materials   https://www.nanosafetycluster.eu/working-groups/materials-wg.html

- Hazard   https://www.nanosafetycluster.eu/working-groups/2-hazard-wg.html

- Exposure   https://www.nanosafetycluster.eu/working-groups/3-exposure-wg.html

- Database   https://www.nanosafetycluster.eu/working-groups/4-database-wg.html

- Risk   https://www.nanosafetycluster.eu/working-groups/5-risk-wg.html

- Modelling   https://www.nanosafetycluster.eu/working-groups/6-modelling-wg.html

- Dissemination   https://www.nanosafetycluster.eu/working-groups/7-dissemination-wg.html

- Systems Biology   https://www.nanosafetycluster.eu/working-groups/8-systems-biology-wg.html

- Safe by Design   https://www.nanosafetycluster.eu/working-groups/8-systems-biology-wg.html
  and Industrial
  Innovation

The status and activity are variable. In the recently presented NSC Action Plan 2017, it is foreseen to re-organise the working groups to match the planned transition of the NSC to an Innovation Governance platform (Cassee et al. 2017).

The NSC issues a yearly compendium describing the status of current EU projects on nanomaterial toxicity, exposure assessment and risk management, as well as giving an update on the NSC working groups. The focus is increasingly shifting towards safety-by-design considerations, predictive toxicology and high throughput / Tox21 type approaches (Lynch 2016). A biannual newsletter gives updates on the projects. Furthermore, the NSC has published a Strategic Research Agenda "Nanosafety in Europe 2015-2025" (Savolainen et al. 2013), and an update on research priorities in 2017 (Stone et al. 2017). The latter builds on proposals made by the ITS Nano project in their final report, which stressed the major topics of physicochemical characterisation, exposure identification, hazard identification and modelling approaches for the risk assessment of NMs (Stone et al. 2014).

For the scope of the Nanocomput project, the NSC Modelling and Database working groups are specifically relevant, since they discuss building of and guidance for models for nanomaterial characterisation and toxicity prediction, and databases and ontologies related to nanomaterials, respectively. The latter is particularly active and has carried out initiatives to provide a list of nanosafety related databases in Europe and globally (Mustad et al. 2014), a definition of data quality and recommendation of a minimal reporting standard, in particular which experimental details need to be recorded. Members have also contributed to an international effort of assessing data completeness and quality (Marchese Robinson et al. 2016). While data completeness is not explicitly defined in the paper, it generally "assesses the extent to which experimental details are described and associated experimental results are reported" (Marchese Robinson et al. 2016). This can be achieved for example by checking against a list of minimum information requirements which would need to be defined in the context of specific applications.

The NSC Database working group is in contact with the US-EU Communities of Research on Databases (http://us-eu.org/communities-of-research/search-communities-of-research/databases-ontologies/) and the US Nano WG (https://nciphub.org/groups/nanowg) (see 5.1.2).
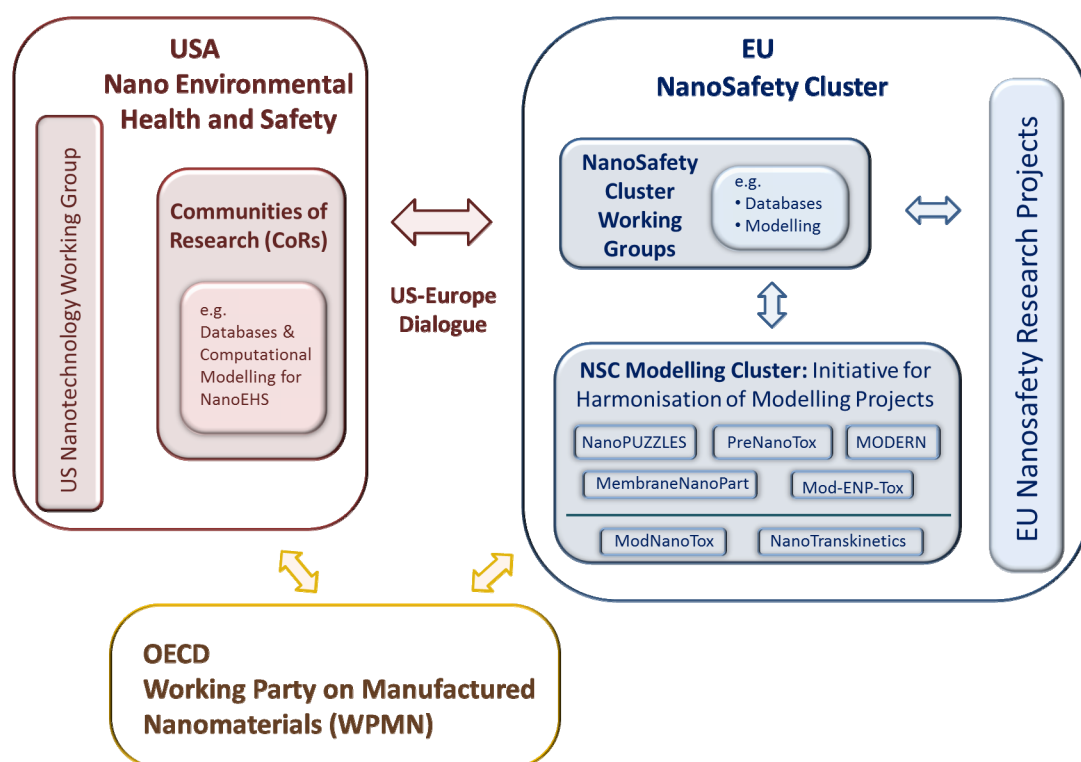

Figure 5.1. Schematic representation of the nanosafety related projects and working groups at European level and their interaction with international initiatives.

Table 5.1 lists the individual EC 7th Framework Programme research projects (including a LIFE+ project) that have been identified as related to computational modelling and grouping approaches for NMs.

Table 5.1. EU funded projects related to computational modelling and grouping approaches for nanomaterials

• eNanomapper	• NanoFATE
• ENPRA	• NanoMILE
• FutureNanoNeeds	• NanoPUZZLES
• ITS-NANO *(support action)*	• NANoREG
• MARINA	• NanoReTox
• MembraneNanoPart	• NANOSOLUTIONS
• MODENA *(COST initiative)*	• NanoTEST
• MOD-ENP-TOX	• NanoTranskinetics
• MODERN	• PreNanoTox
• ModNanoTox	• REACHnano *(LIFE+)*.
• NanoBRIDGES *(Marie Curie Action)*	

More details on the projects are given in Appendix IV.

Out of these projects, five have been dedicated to computational modelling and started at the same time. With two other modelling projects, already running, they formed the NSC Modelling Cluster (Table 5.2), agreeing in a Memorandum of Understanding to collaborate, share expertise and use common synergies. They held regular harmonisation meetings. eNanomapper joined this cluster later.

Table 5.2. Projects forming the NanoSafety Cluster (NSC) Modelling Cluster

MembraneNanoPart:	Modelling the mechanisms of nanoparticle-lipid interactions and nanoparticle effects on cell membrane structure and function
MOD-ENP-TOX:	Modeling platform to predict the toxicity of metal-based nanoparticles
MODERN:	Modelling the environmental and human health effects of nanomaterials
NanoPuzzles:	Modelling properties, interactions, toxicity and environmental behaviour of engineered nanoparticles
PreNanoTox:	Predictive toxicology of engineered nanoparticles
ModNanoTox:	Modelling nanoparticle toxicity: principles, methods, novel approaches
NanoTranskinetics:	Modelling the basis and kinetics of nanoparticle cellular interaction and transport

5.1.2 International activities

The European research projects are in contact with US colleagues through the Communities of Research (CoRs). Each CoR is dedicated to a specific topic and serves as discussion forum for researchers, interested stakeholders from academia, government, industry, and NGOs from both sides of the Atlantic in this US-Europe dialogue (http://us-eu.org/), bridging Nano Environmental Health and Safety (Nano EHS) research efforts:

- Databases & Computational Modeling for NanoEHS
  http://us-eu.org/communities-of-research/search-communities-of-research/databases-ontologies/

- EcoToxicity
  http://us-eu.org/communities-of-research/search-communities-of-research/ecotoxicity-testing-predictive-models/

- Risk Assessment
  http://us-eu.org/communities-of-research/search-communities-of-research/risk-assessment/

- Exposure through Product Life
  http://us-eu.org/communities-of-research/search-communities-of-research/exposure-through-the-life-cycle/

- Risk Management & Control
  http://us-eu.org/communities-of-research/search-communities-of-research/risk-management-control/

- Human Toxicity
  http://us-eu.org/communities-of-research/search-communities-of-research/predictive-modeling-for-human-health/

- Characterisation
  http://us-eu.org/communities-of-research/search-communities-of-research/characterisation/

On the US side, the Nanotechnology Working Group (NanoWG) is a working group of the National Cancer Informatics Program (NCIP, https://cbiit.nci.nih.gov/ncip/ncip-home) and is very active in the community. It included the Nanomaterial Data Curation Initiative (Hendren et al. 2015), relevant for the data-related issues for modelling. The NanoWG has close bonds to the NSC Database working group.

Furthermore, projects including aspects of modelling and grouping for NMs are funded by the National Center for Environmental Research (NCER) (see Appendix V).

On an international level, the OECD Working Party on Manufactured Nanomaterials (WPMN) promotes international co-operation in human health and environmental safety aspects of manufactured NMs.

5.2   Challenges and needs for the development and use of computational methods

There is consensus in the literature that computational models for prediction of nanotoxicity will be able to make an important contribution to nanosafety assessment, since it is not possible to test all existing nanoforms, which differ in particle size, shape, coating and other characteristics. It is also in line with general efforts in applying alternative methods in chemicals risk assessment. However several limitations and barriers to the development and use of computational prediction models for nanoparticles have been identified.

From the analysis of available models as described in Chapter 3, limitations on the availability and utility of computational models for NMs for regulatory purposes can be seen as:

- Limitation of existing models / applicability domains:
  Models available cover a limited number of endpoints and in some cases several have been derived from the same datasets. Most models were derived from small datasets.

- Limitation of use in the regulatory context:
  There is an insufficient number of QSPR and QSAR models for directly filling data gaps for REACH endpoints (physicochemical properties, ecotoxicity endpoints and human health endpoints). However available models can be used for screening or support grouping and read-across. On the other hand, the applicability and availability of environmental fate models is more promising.

In terms of challenges and knowledge gaps for the development of models, the overarching problems for the development of models can be summarised as follows, as described in the literature (Gajewicz et al. 2012)(Oksel et al. 2015) (Richarz et al. 2014)(Marchese Robinson et al. 2016). They are mostly linked to the underlying data and thus to experimental issues, apart from chemoinformatics problems and the general uncertainties of linking effects mechanistically to NMs:

- Lack of data (quantity) – large datasets needed for development of robust models

- Data completeness, data quality insufficient

- Lack of comparability – limiting creation of larger datasets by pooling data from different sources
  (comparability between labs, between different types of nanomaterials –variability, different protocols for characterisation)

- Validity for nanotoxicity (reliability, relevance) uncertain – link of NM properties with effect.

The knowledge gaps are detailed in Table 5.3.

Table 5.3. Limitations hampering the development of computational models for NMs and the specific related knowledge gaps.

Limitation	Specific related knowledge gaps
Data quantity: large datasets needed	o Standardised reporting format to enable pooling and comparison of data o Reference materials to allow comparison between studies (Eugenia Valsami-Jones 2016) o Existing data easily and freely accessible o Central data repository
Data quality	o Harmonised protocols (SOPs) for NM characterisation and toxicity measurements, also allowing for comparability of data measured in different laboratories o Polydispersity and heterogeneity of NMs o Batch to batch variability of nominal identical NMs (Marchese Robinson et al. 2016))
Data completeness	o Minimum requirements on parameters to measure and report
Validity for nanotoxicity prediction models	o Finding the appropriate (nanospecific) descriptors to link the complex, nonuniform NM properties to toxicity (Oksel et al. 2015) o Applicability of toxicity test guidelines to nanomaterials, i.e. validity of the results for nanosafety assessment; e.g. artefacts affecting reliability of NM biological assessment (Kroll et al. 2012), (Klaine et al. 2008)
Technical issues of modelling	o Transformation of NM structures into computer-usable representation (Oksel et al. 2015)

The quality and robustness of a computational prediction model depends indeed on the quality of the data used for the model development. Therefore the consideration and evaluation of the data, and steps to improve data availability and quality are a crucial for modelling. Key concepts of data completeness, minimum information checklist and data quality for example are highlighted in (Marchese Robinson et al. 2016). An important point is the aim to allow comparability of data from different sources and provided by different laboratories in order to create a more robust data basis, ideally in an overarching and comprehensive data repository, for analysis of property-effect relationships, building of predictive models and using grouping and read-across approaches (Richarz et al. 2017).

The uncertainties identified in the TiO_2 and CNT case studies (Tables 4.21 and 4.22) point at the same issues, i.e. uncertainty related to the nanoform identification, high variability in the experimental measurements, uncertainty in applying exiting test protocols and relevance for human health endpoints, lack of knowledge of the mechanism of toxic action.

As a way forward, recommendations have been given in the past, in the literature and e.g. OECD workshops (OECD 2016, 2017):

- Setting of minimum requirements on experimental parameters to record

- Standardisation of the data format, templates for data exchange and harmonisation

- Creation of a database framework for public availability and access of data

- Creation of ontologies for a harmonised terminology

- Implementation of standard operation procedures (SOPs) for experimental measurements, both for NM characterisation and toxicity measurements

- Clarification which test guidelines are applicable to nanomaterials, establishment of a set of caveats → adaption of test guidelines to nanospecific characteristics (Hansen et al. 2017)

- Elucidation of mechanisms underlying nanotoxicity and link to NM properties.

5.3   Recent progress against the challenges and needs

The research initiatives described in Section 5.1 have and are continuing to work towards solving the issues hampering model development and use. For example:

- The NSC Modelling Cluster aimed at harmonising data collection and recording between the projects by using a common standard (ISA-TAB-Nano)(Thomas et al. 2013, https://wiki.nci.nih.gov/display/ICR/ISA-TAB-Nano) in view of bringing all data together in one common database. However, while the envisaged cross-project data collection did not happen, some projects of the Modelling cluster did use the ISA-TAB-Nano format and made the collected and formatted data publicly available separately; e.g. NanoPuzzles (Marchese Robinson et al. 2015, all NanoPUZZLES ISA-TAB-Nano datasets available at Zenodo: https://doi.org/10.5281/zenodo.35493) and MOD-ENP-TOX (Vriens et al. 2017).

- The cross-NSC Database working group worked on the definition of minimum information requirements, based on the MINChar Initiative parameters list (https://characterizationmatters.wordpress.com/parameters/) and discussed data quality and completeness, together with US groups, e.g the NCIP Nanomaterial Data Curation Initiative (Hendren et al. 2015)

- The eNanomapper project set out to create a bespoke infrastructure to store data from EU research projects and also share predictive models for NMs. eNanomapper has indeed achieved to provide the technical framework to upload and store data from research projects in different formats). So far only few data have been uploaded:all data from NanoREG, plus data from MARINA (6 substances), Modena (59 substances), NanoWiki, Protein Corona Fingerprinting Predicts the Cellular Interaction of Gold and Silver Nanoparticles.csv (121 substances). This infrastructure can be used for further efforts to create a centralised data repository. Two current projects are using the infrastructure (NANoREG2, caLIBRAte) for internal data sharing at the moment. Regarding models: eNanomapper makes available cheminformatcs tools to create own models, including a nano-read-across application; but no models from EU projects are included.

- A dialogue between modellers and experimentalists was seen as essential, as stressed and for example in the NanoBRIDGES project.

An analysis of the ongoing work in European research projects has been carried out, in order to assess the (future) availability of more models, tools or knowledge contributing to close the gaps.

5.3.1 Review of deliverables from current EU-funded research projects

In the systematic review of EU projects, 18 projects under the 7th Framework programme related to modelling approaches were considered, plus a support action, a COST initiative and a LIFE+ project (Table 5.1 and Appendix IV). Most projects have reached their end since beginning of the analysis within Nanocomput, the last will end – as generally all FP7 projects – by 31 December 2017.

Tasks, work packages and deliverables in the projects relevant for the scope of NanoComput were identified by a keyword search of the project Descriptions of Work (DoWs). The keywords used were:

- group; grouping; category; read-across
- QSAR; QSPR; in silico
- Fate model; multimedia; kinetic
- Behaviour.

Steps followed in the evaluation of results of current EU projects:

1. Selection of projects relevant to NM computational modelling and grouping approaches
2. Identification of relevant tasks and deliverables by keyword search of the DoWs
3. Exclusion of deliverables not available within the time frame of Nanocomput (see section 5.3.2)
4. Request of deliverables from the project coordinators
5. Identification of relevant content: new models, tools, other knowledge possibly allowing progress towards the challenges and gaps
6. In case of not receiving the deliverables from the projects: consideration of other information e.g. from public project reports or the project websites.

Several projects only replied after several reminders or had concerns about intellectual property, which could be solved after discussions. For some projects, the coordinator did not reply, but material was available from JRC being a project partner. For two projects no reply or material at all was received.

This was also a lesson in how difficult it can be to assess research results which should ideally be public and be used to advance public knowledge and science.

Table 5.4 gives an overview of the results from current EU projects related to NM computational modelling and grouping approaches, as identified in the review exercise.

Table 5.4. Selected results and examples from current EU projects related to NM computational modelling and grouping approaches.

Project	Models (QSAR/fate/kinetic) or grouping approach [44]	Tools	Contribution to modelling e.g. related to data
eNanomapper		Jaqpot Quattro (JQ) web application, including model validation and optimal experimental design functionalities; Nano-Lazar extended for NMs, build toxicity predictions from a local nano-QSAR, based on eNanomapper database, supports grouping/read-across.	Publicly available database (Jeliazkova et al. 2015) able to importdata in different formats. Ontology. Annotated spreadsheet templates for capturing experimental data and software to convert the spreadsheets into different formats.
ENPRA	Development of structure-activity models for NP for binary oxides.		Led to publications by Enrico Burello (Burello and Worth 2011)
FutureNanoNeeds			Experimental data, e.g. degradation kinetics, nanoparticle-cell interactions
ITS-NANO			Framework of future research priorities (Stone et al. 2014)
MARINA	Grouping approach outlined (Oomen et al. 2015)	Decision tree construction tool (GPTree): application to nanoSAR modelling in case studies (Oksel et al. 2016)	Data for TiO_2, SiO_2, ZnO and MWCNT; reporting format based on OECD harmonised templates
MembraneNanoPart	Modelled dispersions of NP from 5 main groups of nanomaterials at physiological conditions.		Insights into interaction with lipid membranes.

---

[44] Models published by the project in the scientific literature were included in the Nanocomput model landscape review.

Project	Models (QSAR/fate/kinetic) or grouping approach [44]	Tools	Contribution to modelling e.g. related to data
MOD-ENP-TOX	Models developed predicting the effects of MeNPs on effects on mitochondria and their potential to damage DNA or otherwise interfere with genetic processes. PBK modeling of zinc oxide NPs and zinc nitrate in mice(Chen et al. 2015)	Modelling Assays Platform "MAP" for hazard ranking of engineered nanoparticles. Predicting the toxicity or hazard level of metal-based nanoparticles (MeNPs) - silicon, titanium, silver, zinc and their oxides.	Constructed a database with published experimental data on well-characterised silica and zinc oxide NPs (Vries et al. 2017).
MODERN	QNARs developed: (Kamath et al. 2015), (Liu et al. 2015), (Toropov et al. 2015), (Liu et al. 2013). NP categorisation criteria for category identification and formation. Data-driven grouping approaches: Multiscale bootstrap hierarchical clustering, Self-Organizing Maps, Complex Network analysis. Robust ranking hazard methodology for NMs using their toxicological and their physical-chemical data developed.	ISA-TAB-Nano Validator tools to validate ISA-TAB-Nano datasets based on compliance with the specifications. nanoDMS (Nanomaterial Data Manage-ment System) to facilitate data sharing via the ISA-TAB-Nano format. Anapath (Pathway Analisys): to identify pathways which are differentially expressed, from high-throughput transcriptomics or proteomics assays. Integration of NP categories and hazard ranking into a quantitative risk assessment scheme: framework implemented into an tool that will be made freely available.	Identification of NP bioactivity signatures from high-throughput omics data. Size-consistent nanodescriptors developed for QNPR modelling.

Project	Models (QSAR/fate/kinetic) or grouping approach [44]	Tools	Contribution to modelling e.g. related to data
NanoBRIDGES		Software tools with guidance documents made available at http://nanobridges.eu/software/: Standardisation of data sets, modelability of the dataset, data pre-treatment, Dataset Division, Kennard Stone Algorithm (Euclidean and Mahalanobis distance based), variable selection methods, applicability domain, Clustering - Modified k-Medoid Tool, NanoProfiler - Endpoint-dependent analogues identification software. Coral software for descriptor calculations for NMs (http://www.insilico.eu/coral/SOFTWARECORAL.html)	
NanoFATE			Insights into fate processes regarding behaviour of NPs
NanoPUZZLES	Conceptual framework for grouping NPs based on physicochemical and molecular properties		Standardised ISA-TAB-Nano templates to record data and submit them to databases (Marchese Robinson et al. 2015). Collected data made publicly available. Reviewed systems describing the structural characterisation of NPs. Considerations about data quality assessment. Discussion of validation criteria for nanoQSARs.

Project	Models (QSAR/fate/kinetic) or Tools grouping approach [44]		Contribution to modelling e.g. related to data
NANoREG	Pooling approach to analyse published *in vitro* data for grouping of NPs, balancing out the lack of standardisation of tests (Simkó et al. 2015)		Dispersion SOPs and minimum requirements for characterisation; data publicly available through eNanomapper; ISA-TAB Nano templates (Totaro et al. 2017). Harmonised terminology for NM environmental health and safety assessment.
NanoReTox			Approaches providing practical ways to compare nanomaterials, identify those physicochemical parameters that are relevant for toxicity assessment, and form the basis for an implementable risk assessment framework. Datasets and methodologies developed made extensively available in the scientific literature.

Project	Models (QSAR/fate/kinetic) or grouping approach [44]	Tools	Contribution to modelling e.g. related to data
NANOSOLUTIONS		Completed the development of the computational infrastructure of the ENM classifier. ENM safety classifier is intended to be a data-driven computational model derived from machine-learning algorithms. Multiple data layers, spanning from the physicochemical properties of the materials investigated, to their effects (mode of action) on living systems at the molecular, cellular, organismal and ecological levels will serve as the input. An easy to use graphic user interface is planned. - Development of a novel evolutionary algorithm explores the space of all the possible combination of features (solutions) to be used for prediction purposes. Method outperforms other existing multi-view clustering algorithms such as Tw-Kmeans and SNF.	Experimental translocation studies. To better understand the mechanisms of uptake of NMs via different routes into the body and into tissues/organs.
NanoTranskinetics			High quality data from previous experimental research projects and additional dedicated experiments in collaboration of experimentalists and modellers

Project	Models (QSAR/fate/kinetic) or grouping approach [44]	Tools	Contribution to modelling e.g. related to data
PreNanoTox	New category descriptors "collectors of eclectic information" instead of traditional descriptors; Monte Carlo technique used to build predictive models for the prediction of cell membrane damage of metal oxide nanoparticles; cellular viability (CV%) towards silica NPs. Suggestion of building predictions for NM related endpoints based on quasi-SMILES.		Database of collected data to be made available.

Overall the systematic review of the current EU project research into nanosafety assessment showed that:

- new models have been developed – the new models published in the scientific literature have been included in the Nanocomput model landscape review, including the evaluation of applicability in a regulatory context. Models still under development or not fully described or made public might add to the landscape in the future.
- new tools have been developed contributing to individual tasks related to computational modelling or designed as comprehensive modelling tools (see Table 5.4) – for example accessibility of cheminformatics tools on the eNanomapper website
- nanospecific descriptors were studied
- progress has been made towards data format standardisation, ontologies, data sharing, for example ISA-TAB-Nano based templates for data recording developed withing NANoREG and NanoPuzzles; the eNanomapper database technology to upload and store project data.
- SOPs for the experimental measurements. For example, within the MARINA project eight OECD TGs were adapted based on the testing of at least one ion-releasing NM (Ag) and two inert NMs (TiO2) (Hund-Rinke et al, 2016). Protocols have also been developed by the NanoValid project (http://www.nanovalid.eu/) and by NANoREG (Gottardo et al, 2017).
- new data have been generated and/or collected from the literature. Data from for example NANoREG, MARINA and NanoPUZZLES have been made publicly available.

Altogether, this contributes towards reducing the knowledge gaps and overcoming current limitations. Some examples are listed in Table 5.5.

Table 5.5 Mapping current project results against challenges

Limitation	New results filling specific related knowledge gaps
Data quantity: large datasets needed	o Standardised reporting format based on ISA-TAB-Nano developed in NAnoPUZZLES and NANoREG o New data created o eNanomapper public database to serve as repository, compatible with different data formats
Data quality	o SOPs developed in NANoREG, all measurements within the project carried out according to these SOPs
Data completeness	o Contribution to discussion about data quality, completeness and minimum requirements (Marchese Robinson et al. 2016)

Validity for nanotoxicity prediction models	o Descriptors studied and derived in several projects o New insights into mechanisms, NM behaviour and kinetics
Technical issues of modelling	o Representation of NM structures discussed

5.3.2 New Horizon2020 projects

In the framework of H2020, several new nanosafety projects have started. Since these projects started later than those described above (Section 5.3.1), deliverables were not available for review. These projects are listed in Table 5.6.

Table 5.6. New Horizon2020 projects related to NM computational modelling and grouping approaches.

Project	Aims
SmartNanoTox http://www.smartnanotox.eu Coordinator: University College Dublin	• To identify main pulmonary adverse outcomes induced by NMs, associated MIE, KEs and toxicity pathways • To establish relationships between physicochemical properties of NMs and KEs, suggest descriptors for grouping of NMs according to their toxicological mode-of-action • To create a database of bionano interactions that will enable development of read-across and QSAR tools • To develop a smart screening approach, with predictions of NM toxicity made on the basis of purely computational or limited *in vitro* screening test
NANoREG II www.nanoreg2.eu Coordinator: INERIS	• To develop supportive tools for Safe by Design, based on regulatory orientated grouping approaches, placed in a general ITS
NanoFASE http://www.nanofase.eu Coordinator: NERC	• Nanomaterial fate and speciation in the environment, the overarching objective is to deliver an integrated Exposure Assessment Framework.
ProSafe http://www.h2020-prosafe.eu Coordinator: Ministerie van Infrastructuur en Milieu	• To support the aims of EU and international efforts (OECD, COR, EU-USA) by streamlining data acquisition, collection and management

5.4 Conclusions and recommendations

The systematic review of the recent EU project research into nanosafety assessment showed that considerable progress has been made towards addressing the challenges of modelling nanomaterials. However there is still a fragmentation in the scientific results and a lack of coordination, and the lack of public access to the results and tools is preventing their uptake and use in regulatory decision making. To address these shortcomings, the Commission should consider implementing a number of recommendations. They are addressed and summarised in the Chapter 6.

5.4.1 Conclusions on the needs addressed

The following conclusions can be made after reviewing the scientific progress from current research progress:

- There is still fragmentation of the work. Similar developments have been conducted in parallel in several projects, e.g. ISA-TAB-Nano-based templates in NANoREG (Totaro et al. 2017) and NanoPUZZLES (Marchese Robinson et al. 2016).
- Public availability of scientific results, models and tools is not a given. While some results have been made available through the project websites or public repositories, others are not easily accessible (as also experienced in trying to obtain the deliverables from the project coordinators). Specifically, some new models have been disseminated as publications, others are only described in project deliverables, and not always in a usable form. Ideally models should be documented and made available in a suitable format (e.g. QMRF) in a public repository.
- Even though data templates and SOPs have been developed, they have not gained widespread use within projects, thereby creating a barrier to data sharing and dissemination.
- There are significant issues in terms of data quality and reproducibility of experiments. While new and future projects can be expected to produce higher quality data by following more standardised SOPs and harmonised reporting standards, this does not solve the problem of how to use existing data in a regulatory context. A strategy must be devised on how to interpret and integrate these data, while recognising their limitations.

5.4.2 Recommendations

Taking into account the experience from the reviews of the modelling landscape (Chapter 3), the read-across case studies (Chapter 4) and the review of recent EU project results (Chapter 5), the following recommendations can be given with a view to increasing the availability and utility of computational methods:

- Continued efforts should be made to address the already identified and still existing and knowledge gaps and technical challenges. The NSC organisation and structure could be exploited to support the scientific coordination across projects.

- Within the context of Horizon 2020 and related EU research programmes, the Commission should consider implementing quality guidelines to improve the overall consistency of reporting of data, models and tools. For example, the use of standardised SOPs and reporting formats for data and models, could be made a requirement in upcoming projects.

- In addition, the Commission should consider making the public dissemination of research and development outputs a contractual obligation.

- There is also a need to raise awareness in the research community that model development should be carried out with concrete regulatory applications in mind. For example, in our read-across case studies, we identified a need to develop QSPRs for the mechanical properties (e.g. rigidity) of carbon nanotubes, and to explore the utility of these (predicted) properties in supporting grouping and read-across.

- Models should be made available in a usable form. At the very least, they should be documented appropriately in a public repository so they can be reproduced, and ideally they should be implemented in the form of a software tool, to increase practical usability. Nanocomput has made a valuable contribution in creating/updating templates to record computational models, adapted for NMs. These templates should be made available for universal use. In addition, appropriately documented models could be disseminated via JRC information sources such as the QSAR Model Database (for QSPR/QSAR models), DB-ALM (for in vitro models and computational models other than QSPR/QSAR), as well as via project websites.

The creation of a "one stop" hub with all necessary information, linking to the respective tools and information sources, would be valuable. It seems that the planned ECHA Observatory for nanomaterials (EU-ON) could play this role. The overarching hub would include existing infrastructure technologies and refer to available repositories, such as the eNanomapper database to further integrate data, and the JRC QSAR Model Database and the OECD QSAR Toolbox for documenting and sharing models.

5.5 References

Burello, E., Worth A. P., (2011). A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology, 5, 228–235.

Cassee, F. et al. (2017). ACTION PLAN 2017 – 2021, pp.1–16.

Chen, W.-Y. et al. (2015). Physiologically based pharmacokinetic modeling of zinc oxide nanoparticles and zinc nitrate in mice. International journal of nanomedicine, 10, 6277–6292.

Eugenia Valsami-Jones, I.L. and C.A.C. (2016). Nanomaterial Ontologies for Nanosafety : A Rose by any Other Name …. Journal of Nanomedical Research, 3(5), 4–6.

Gajewicz, A. et al. (2012). Advancing risk assessment of engineered nanomaterials: application of computational approaches. Advanced drug delivery reviews, 64(15), 1663–1693.

Hansen, S.F. et al. (2017). A critical analysis of the environmental dossiers from the OECD sponsorship programme for the testing of manufactured nanomaterials. Environ. Sci.: Nano, 74, 147–160.

Hendren, C.O. et al. (2015). The Nanomaterial Data Curation Initiative: A collaborative approach to assessing, evaluating, and advancing the state of the field. Beilstein Journal of Nanotechnology, 6(1), 1752–1762.

Content

Hund-Rinke K et al (2016). Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. Nanotoxicology 10 (10), 1442-1447.

Jeliazkova, N. et al. (2015). The eNanoMapper database for nanomaterial safety information. Beilstein Journal of Nanotechnology, 6(1), 1609–1634.

Kamath, P. et al. (2015). Predicting cell association of surface-modified nanoparticles using protein corona structure - activity relationships (PCSAR). Current Topics in Medicinal Chemistry, 15(18), 175–182.

Klaine, S.J. et al. (2008). Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry, 27(9), 1825.

Kroll, A. et al. (2012). Interference of engineered nanoparticles with in vitro toxicity assays. Archives of toxicology, 86(7), 1123–1136.

Landsiedel, R. et al. (2012). Toxico-/biokinetics of nanomaterials. Archives of toxicology, 86(7), 1021–1060.

Liu, R. et al. (2013). Development of structure–activity relationship for metal oxide nanoparticles. Nanoscale, 5(12), 5644–5653.

Liu, R. et al. (2015). Quantitative structure-activity relationships for cellular uptake of surface-modified nanoparticles. Combinatorial Chemistry and High Throughput Screening, 18(4), 365–375.

Lynch, I. (2016). Compendium of Projects in the European NanoSafety Cluster. 2016 Edition. Available at: http://www.nanosafetycluster.eu/uploads/files/pdf/2016_NSC_Compendium.pdf..

Marchese Robinson, R.L., Cronin, M.T.D., Richarz, A.-N., Rallo, R. (2015). An ISA-TAB-Nano based data collection framework to support data-driven modelling of nanotoxicology. Beilstein Journal of Nanotechnology, 6(1), 1978–1999.

Marchese Robinson, R.L., Lynch, I., Peijnenburg, W., Rumble, J., Klaessig, F., et al. (2016). How should the completeness and quality of curated nanomaterial data be evaluated? Nanoscale, 8, 9919-9943.

Mustad, A.P. et al. (2014). Summary of the Spring 2014 NSC Database Survey. https://figshare.com/articles/Summary_of_the_Spring_2014_NSC_Database_Survey/1195888.

OECD (2017). Alternative testing strategies in risk assessment of manufactured nanomaterials: current state of knowledge and research needs to advance their use. Series on the Safety of Manufactured Nanomaterials No. 80, ENV/JM/MONO(2016)63, Paris.

OECD (2016). Grouping and read-across for the hazard assessment of manufactured nanomaterials. Report from the expert meeting. Series on the Safety of Manufactured Nanomaterials No. 76, ENV/JM/MONO(2016)59, Paris.

Oksel, C., et al. (2015). (Q) SAR modelling of nanomaterial toxicity : A critical review. Particuology, 21, 1–19.

Oksel, C., Winkler, D.A., Ma, C.Y., Wilkins, T., Wang, X.Z. (2016). Accurate and interpretable nanoSAR models from genetic programming-based decision tree construction approaches. Nanotoxicology, 10(7), 1001-1012.

Oomen, A. et al. (2015). Grouping and Read-Across Approaches for Risk Assessment of Nanomaterials. International Journal of Environmental Research and Public Health, 12(10), 13415–13434.

Richarz, A.-N., Avramopoulos, A., Benfenati, E., Gajewicz, A., Golbamaki Bakhtyari, N., Leonis, G., Marchese Robinson, R.L., Papadopoulos, M.G., Cronin, M.T., Puzyn, T. (2017). Compilation of Data and Modelling of Nanoparticle Interactions and Toxicity in the NanoPUZZLES Project. In L. Tran, M. A. Bañares, & R. Rallo, eds. Modelling the Toxicity of Nanoparticles. Cham: Springer International Publishing, pp. 303–324.

Richarz, A.-N., Madden, J.C., Marchese Robinson, R.L., Lubiński, Ł., Mokshina, E., Urbaszek, P., Kuz'min, V.E., Puzyn, T., Cronin, M.T.D. (2014). Development of computational models for the prediction of the toxicity of nanomaterials. Perspectives in Science, 3, 27-29.

Savolainen, K. et al. (2013). Nanosafety in Europe 2015-2025 : Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations Nanosafety in Europe Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations, Helsinki: Finnish Institute of Occupational Health. Available at: http://www.nano2all.eu/sites/default/files/NSC_SRA_2015_2025.pdf dx.doi.org/10.13140/2.1.3084.8969

Simkó, M., Tischler, S. & Mattsson, M.O. (2015). Pooling and analysis of published in vitro data: A proof of concept study for the grouping of nanoparticles. International Journal of Molecular Sciences, 16(11), 26211–26236.

Stone, A.V. et al. (2017). NanoSafetyCluster Research Regulatory Roadmap 2017: Research priorities relevant to development or updating of nano-relevant regulations and guidelines, Available at: https://www.nanosafetycluster.eu/uploads/files/pdf/RRR_Final_version_090317.pdf.

Stone, V. et al. (2014). ITS-NANO--prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Particle and fibre toxicology, 11, p.9.

Thomas, D.G., et al. (2013). BMC Biotechnology, 13, 2.

Toropov, A.A., Rallo, R. & Toropova, A.P. (2015). Use of Quasi-SMILES and monte carlo optimization to develop quantitative feature property/activity relationships (QFPR/QFAR) for nanomaterials. Current Topics in Medicinal Chemistry, 15(18), 1837–1844.

Totaro S., et al. (2017) Data logging templates for the environmental, health and safety assessment of nanomaterials. EUR 28137 EN, doi:10.2787/505397.

Vriens, H., et al. (2017). Case Study III: The Construction of a Nanotoxicity Database – The MOD-ENP-TOX Experience. In L. Tran, M. A. Bañares, & R. Rallo, eds. Modelling the Toxicity of Nanoparticles. Cham: Springer International Publishing, pp. 325–344.

6 Conclusions and Recommendations

This chapter presents the overall conclusions from the Nanocomput project, including lessons learned in conducting literature reviews and research-based case studies on grouping and read-across. A number of recommendations are also offered with a view to overcoming current shortcomings in our knowledge of NM behaviour, and in the availability of tools (such as databases and predictive models) and practical guidance to use such tools in the regulatory assessment of NMs.

Figure 6.1 summarises the major barriers to the development and regulatory use of predictive models in nanotoxicology. These play a role at different levels in the creation and application of knowledge: A) experimental methods for NM physicochemical and toxicity characterisation creating the knowledge needed for model development, and existing computational models; B) standardisation of the obtained information and tools for comparability; C) sharing and accessibility of both data and models; D) dissemination and awareness; E) actual use of data and models for application in regulatory decision-making.
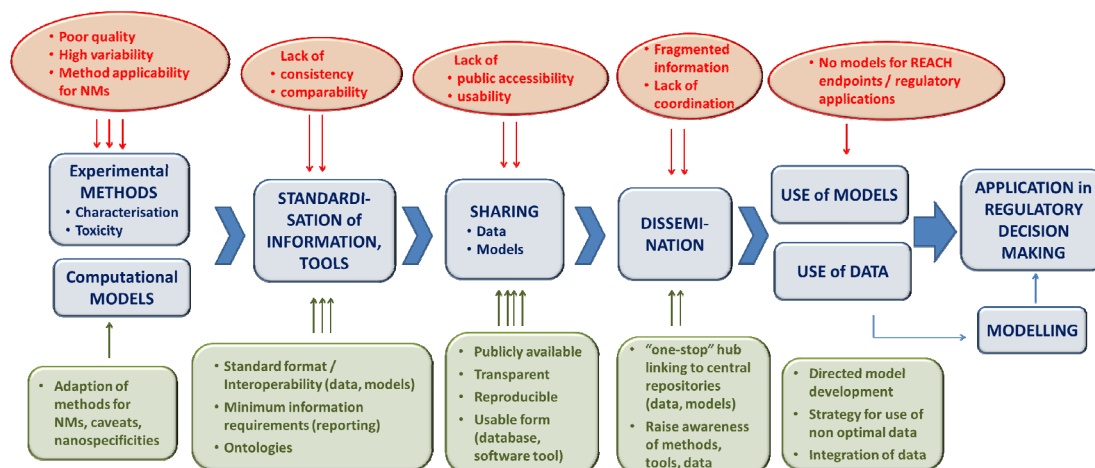

Figure 6.1. Summary of the issues hampering the successful development, uptake and use of prediction models to support regulatory decision-making on NMs. The problems identified (red) impact on computational nanotoxicology at different levels (blue): A) experimental methods for NM physicochemical and toxicity characterisation, existing models; B) standardisation of information and tools for comparability; C) sharing and accessibility; D) dissemination and awareness; E) use of data and models for application in regulatory decision-making. Possible measures to improve the situation are indicated (green).

6.1 Inherent scientific uncertainties

There are still significant knowledge gaps regarding the physicochemical characteristics of NMs and their influence on determining physicochemical, biokinetic and toxicodynamic properties of the materials. Even if non nano-specific mechanisms of toxicity have been identified (Gottardo et al, 2017), it is considered that the particle properties of NMs affect their kinetics / fate, as well as the nature of any Molecular Initiating Events in their toxicological pathways (Gerloff et al, 2017).

These knowledge gaps need to be filled. European research projects (Chapter 5) such as NANoREG, MARINA, NanoPUZZLES and MOD-ENP-TOX are generating new data and/or collecting literature data. However, these are data needed for the specific aims of the respective projects, and not a targeted approach to fill data gaps for regulatory assessments.

Furthermore, our review of the progress made and results obtained in European research projects has revealed the difficulty of actually finding and accessing publicly disseminated data from the projects.

In developing and applying computational models, it should also be borne in mind that NM behaviour depends on many parameters, and this dependence could be very sensitive to initial conditions. Therefore in some circumstances it could even be theoretically impossible to accurately predict the behaviour of NMs, especially over longer time periods. Therefore, modelling efforts should avoid false precision, such as for example the exact time-dependent composition of the protein corona. Despite this theoretical challenge, we believe that even relatively simple models can be useful aids for decision-making (e.g. Burello & Worth, 2013).

6.1.1 Recommendation

- Dedicated and focused research efforts are needed to elucidate the behaviour (toxicity and fate) of NMs of regulatory interest and to investigate which fundamental characteristics and properties are relevant for predictive modelling.

6.2   Data quality and availability

One of the major issues hampering the modelling of NMs is the lack of good quality data and the high variability of available data.

Reliable and relevant data are essential for deriving the underlying principles of NM behaviour, and for correlating physicochemical properties and activities/toxicity as the basis for building prediction models, grouping similar substances / materials and performing read-across.

For the Nanocomput read-across case studies, a reasonable amount of data was generally available for the selected NMs. However, closer inspection of the data and exclusion of inconsistent and highly variable data left only a limited selection of analogues for both TiO_2 and MWCNT with available data for the main physicochemical parameters and toxicological endpoints. This led to restricted datasets, and limited the endpoints that could be read across (*in vitro* Comet assay in the case of nano-TiO_2).

Moreover, an extensive data treatment (e.g. Appendix IX) was necessary to deal with the inhomogeneity in experimental measurements and to make the data sufficiently homogeneous to compare the analogues in the read-across process. One factor contributing to the inhomogeneity of available data is the lack of standardised and validated methods for physicochemical characterisation and toxicity assessment. The other factor is the low reproducibility of some measurements, such as zeta potential, due for example to dispersability problems (Appendix VIII). The importance of ensuring dispersion stability has been demonstrated by Cupi et al 2016.

Extensive data analysis and treatment will continue to be a significant step before modelling, as long as reliable and comparable methods for physicochemical characterisation and toxicological testing are not available, and existing data are not recorded in standardised formats, with agreed minimum data inclusion criteria. A lack of comparability between data from different studies means that individual datasets cannot always be merged and will therefore tend to be small, limiting their usefulness.

Data treatment such as averaging values from highly variable measurements - for example for crystallite size values obtained averaging different measures provided by different laboratories - has to be used with some caveats. The use of average values from highly variable measurements could result in different choices in the read-across process and potentially different conclusions. Therefore clear guidelines on how to select and pre-treat data should be developed for a transparent and consistent approach.

6.2.1 Recommendations

- To supplement ECHA's guidance on grouping and read-across (ECHA, 2017), which is written at a generic level, there is a need for more detailed and practical guidance on how to apply the grouping and read-across workflow. For example, this should include guidance on which experimental and modelling data to use, which should ideally be based on standardised procedures, on the avoidance of potential bias in analogue and data selection, on data treatment protocols, as well as the transparent recording of the results and conclusions.

- The collection of data should be a directed and systematic community effort to allow for targeted filling of data and knowledge gaps regarding physicochemical, biokinetic and toxicological properties of NMs. This process should take into account the reliability and relevance of available experimental methods, and apply suitable methods to NMs of regulatory interest.

6.3   Model landscape

As shown in the review of the model landscape (chapter 3), a number of QSPR, QSAR, PBK and environmental fate models have been developed for selected groups of NMs. However, these models cover a limited number of endpoints and have usually been derived from a limited number of small datasets. There are very few QSPR and QSAR models (physicochemical properties, ecotoxicity endpoints and human health endpoints) with regulatory relevance, e.g. which could directly be used to fill data gaps for REACH. For example many models (76 out of 152) have been developed for cytotoxicity which is not a regulatory endpoint. Furthermore, there are no models covering acute toxicity, repeated dose toxicity, skin and respiratory sensitisation, carcinogenicity and reproductive toxicity. The only endpoint of REACH relevance is the QSAR for "In vitro – Mutagenicity Bacterial Reverse Mutation Test (*Salmonella typhimurium*)", required under REACH Annex VII. However, the Ames test is not applicable to NMs (OECD, 2014). Development of many QSPR and QSAR models has been started in recent EU research projects, however not all have finalised or made them publicly available (yet). The applicability for REACH seems to be most promising for environmental fate models at the moment.

However, available models can be used for screening purpose and to support grouping and read-across (for example, properties such as solubility and cytotoxicity could be used to group NMs). In this context, some gaps in the model landscape were identified. For example, in the Nanocomput read-across case studies, a need to develop QSPRs for the mechanical properties (e.g. rigidity) of carbon nanotubes was identified, as a means of supporting category formation and read-across argumentation.

Apart from the current limited availability and relevance for regulatory applications, another barrier to the uptake of the models in decision-making is their limited public visibility and accessibility. Many models are "hidden" and have only been described in project reports so far. Even when models are eventually published in the scientific literature, their use is limited without a user-friendly software implementation for their application, such as the planned Nanosolutions project software tool for predicting health or environmental hazards of NMs. The step from model and tool development within individual research projects to their public availability and sustained maintenance after the end of the projects is not often made.

At the very least, an appropriate and complete documentation of the models should be made available in a public repository so they could be reproduced. Nanocomput has made a valuable contribution in reviewing the current state of existing nanomaterial models (QSPR / QSAR; modified QMRF; PBK, PBD, dosimetry models; environmental fate models) and creating/updating templates to record the computational models, adapted for NMs. These templates will be made available for universal use by the JRC. Similarly, information on all the models of the Nanocomput model inventory could be disseminated via JRC information sources such as the QSAR Model Database (for QSPR/QSAR models), DB-ALM (for *in vitro* models and computational models other than QSPR/QSAR). Ideally, the models should be implemented in the form of a software tool, to increase practical usability. Possible platforms include eNanoMapper (https://www.enanomapper.net/), the Estonian QSAR DB (https://qsardb.org/), and the OECD QSAR Toolbox (https://www.qsartoolbox.org/).

6.3.1   Recommendations

- To raise awareness in the research community that model development should be targeted: either to fill specific knowledge gaps or with concrete regulatory applications in mind. Calls for new EU projects should take these needs into account.

- Public dissemination of research and development outputs such as QSPR/QSAR models should be a contractual obligation for EC research projects and facilitated via publicly accessible platforms (e.g. JRC QSAR DB, Estonian QSAR DB, OECD QSAR Toolbox).

- Models should be documented in the bespoke model description templates from the Nanocomput project and made available in a public repository (see below).

- Models should ideally be implemented in a user-friendly form, e.g. as online tools or KNIME workflows, to facilitate their uptake and application.

6.4 Practicality of applying the ECHA guidance

As discussed above, there is a lack of data and models for regulatory endpoints, and knowledge gaps linking the NM properties to their potential toxicity. There is also a disconnection between legal information requirements and the minimal data set recommended to support grouping and read-across. Thus at the moment no incentives exist for filling the data gaps or for data sharing in view of regulatory applications.

The ECHA Guidance for applying read-across for NMs (ECHA, 2017) is generally useful to guide the user. However, it is more a general framework and may be difficult to apply in the light of the above. A concrete and detailed operational guidance is needed to facilitate the uptake of the read-across approach for nanomaterials in the regulatory context.

Application of the ECHA guidance framework within the Nanocomput case studies has shown some practical issues in following the proposed workflow. Suggestions are provided (Fig. 4.16) on how to deal practically with each step of the workflow. As a slight modification of the workflow, the data gathering step is considered first and the subsequent building of the hypothesis based on the analysis of all available data.

The Nanocomput case study could serve as a blue print for more concrete guidance on how to deal with available and often imperfect data and how to develop the read-across argumentation.

An important part has been the applicability of cheminformatics tools to support the grouping hypothesis: There was no agreement in the literature about the genotoxicity mechanism of nano-TiO_2 and MWCNTs, e.g. chemical reactivity, ROS generation, agglomeration and sedimentation. Furthermore, the variability of the measurement data of the NM characterisation hampered linking the characteristics to the observed effects. Therefore chemoinformatic tools have been used to analyse the NMs' physicochemical properties and find correlations for similar analogues and indications on how they may drive genotoxicity. The read-across hypothesis is based on or verified by this type of analysis.

Cheminformatics tools for modelling of NMs have been made publicly available for example from eNanomapper (e.g. Nano-Lazar extended for NMs) and NanoBRIDGES (e.g. tools for data pre-treatment, variable selection methods, applicability domain, endpoint-dependent analogue identification software) on the respective project websites. These tools are useful to complement read-across approaches, but should be used carefully and adapted to the specific case investigated.

6.4.1 Recommendations
- To provide a detailed practical guidance supplementing the ECHA guidance for NM read-across. The Nanocomput case study and adaptations of the ECHA workflow could serve as blue print for more concrete guidance on how to deal with available and often imperfect data and how to develop the read-across argumentation.
- To collect cheminformatics tools (software, web-applications), make them publicly available and raise awareness (e.g. through a one-stop-hub for NM modelling) and provide guidance for their application in supporting regulatory applications.
- A requirement or recommendation on the use of relevant and reliable *in vitro* and computational models to fill data gaps in regulatory assessments (e.g. in a WoE

approach), would be an incentive to generate adequate data. A focused data generation effort could be established to generate such data.

6.5 Utility of the ECHA Read-Across Assessment Framework (RAAF)

As for read-across of the properties of conventional chemicals, transparent consideration and documentation of all uncertainties is essential.

The ECHA RAAF was applied to the Nanocomput case studies for uncertainty analysis. It was shown that the RAAF structure and Assessment Elements are applicable also for nanomaterials, with some adaptations and explanations to accommodate nanospecific issues.

A key aspect is the concept of similarity, which in the RAAF is limited to chemical structure similarity, and for a more consistent application to NMs, other principles for similarity need to be included, i.e. the similarity of the group of analogues has to be based on the essential and relevant properties of the NMs, considering the physical form and key physicochemical properties. Moreover, the definition of the "identical or different compounds" in the RAAF should be adapted to nanoforms and take factors such as coating, size and length into consideration. In the case of nanomaterials it is not only a distinction between parent compounds and (bio)transformation products such as metabolites, but for example needs to consider either the nanomaterial as such, impurities/coating, released metals, and transformations through the life cycle. The underlying biokinetic and toxicodynamic mechanisms should also be taken into consideration.

6.5.1 Recommendations
* Overall the RAAF is applicable to nanomaterials and should be used to investigate and document uncertainties and confidence in the read-across argumentation.

* A note should be published in connection with the RAAF pointing out the nanospecific issues to be taken into consideration.

* As for the "normal" RAAF, more detailed practical guidance on how to apply the framework, including for example templates for data collection, should be provided.

6.6 Need for infrastructures

A centralised infrastructure is needed for information, data and models, to make them publicly available and accessible, and allow knowledge exchange. Even when information, data and models, are publicly available, it might be difficult to find them or know that they exist in the first place. Therefore a pointer is needed to the respective repositories and individual websites to build and assemble the community knowledge and reduce fragmentation of knowledge. This will also avoid duplication of work, which still exists to a certain extent in recent nanosafety projects (e.g. development of ISA-Tab-Nano templates).

Thus to ensure visibility and overcome the fragmentation of nanotoxicity related information (SOPs, data, models, tools), the creation of a "one stop" hub is essential. This does not necessarily need to store all information directly, but needs to assemble the

information in a structured manner, and link to the individual websites containing the information and/or tools. It should be maintained to accommodate all upcoming information and knowledge in the future, and could be filled also retrospectively with existing information, e.g. by systematic review of the literature and past EU project reports. This needs to be resourced and centrally coordinated. In view of the limited quality of data obtained with non-standardised experimental methods so far, a strategy should also be devised for the selection and use of sub-optimal data.

It seems that the planned ECHA Observatory for nanomaterials (EU-ON) could play this role, making use of and referring to existing technologies and compilations. A promising example is the eNanomapper project, which has provided a technical framework to upload and store data from research projects, with some flexibility of the data formats. eNanomapper is also a resource for chemoinformatic tools that could be used to support read-across. In addition, well established model repositories, such as the JRC QSAR database, Estonian QSAR DB and OECD QSAR toolbox should be used additionally to advance the uptake of computational methods in nanosafety assessment. It is important though, to link them all to a central hub to avoid fragmentation of knowledge. The NSC organisation and structure could be further exploited to support the scientific coordination across research projects.

6.6.1 Recommendations

- To evaluate the utility of data produced in EU projects and upload useful datasets into a centralised repository such as eNanomapper.

- To create an EU level "one-stop" hub with links to information sources, databases, modelling tools, and their websites.

- To establish an organisational mechanism for the targeted development of methods and their application to fill data gaps of regulatory relevance.

- To develop a knowledge sharing strategy to raise awareness in the research and regulatory communities of relevant initiatives, and facilitate access to available methods, tools, databases and guidance documents.

6.7 References

Burello, E. & Worth, A.P. (2013). A rule for designing safer nanomaterials: do not interfere with the cellular redox equilibrium. Nanotoxicology, 1–2.

Cupi, D., N. B. Hartmann & Baun A (2016). Influence of pH and media composition on suspension stability of silver, zinc oxide, and titanium dioxide nanoparticles and immobilization of Daphnia magna under guideline testing conditions. Ecotoxicology and Environmental Safety 127, 144–152.

Gerloff, K., Landesmann, B., Worth, A., Munn, S., Palosaari, T. & Whelan, M. (2017). The Adverse Outcome Pathway approach in nanotoxicology. Computational Toxicology 1, 3–11.

Gottardo et al.,2017. NANoREG framework for the safety assessment of nanomaterials, EUR 28550 EN, doi 10.2760/245972.

ECHA (2017). Appendix R 6-1 : Recommendations for nanomaterials applicable to the Guidance on

QSARs and Grouping.

OECD. (2014). Genotoxicity of Manufactured Nanomaterials: Report of the OECD Expert Meeting. Series on the Safety of Manufactured Nanomaterials No. 43. ENV/JM/MONO(2014)34.

7 Appendices

Appendix I. Glossary of specialised terms

Many terms commonly used in nanotoxicology are defined in Gottardo et al (2016): http://publications.jrc.ec.europa.eu/repository/handle/JRC100906

Term	Definition
Agglomerate	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. The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement. (ISO/TS 27687:2008)
Aggregate	Particle comprising 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. The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement. (ISO/TS 27687:2008)
Adverse Outcome Pathway	Conceptual construction that portrays existing knowledge concerning the link between a molecular initiating event (MIE) and an adverse outcome (AO), by capturing the sequential chain of causally-linked events at different levels of biological organisation. (Ankley et al., 2010)
Alternative method	A method that replaces, reduces or refines the use of animals in toxicity testing or biomedical research. Replacement alternatives include *in silico* and *in vitro* methods, as well as the use of read-across.
Chemical category (group)	A chemical category is a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity.
	The terms category approach and analogue approach are used to describe techniques for grouping chemicals. The term analogue approach is used when the grouping is based on a very limited number of chemicals, where trends in properties are not apparent (ECHA, 2008; OECD, 2014a). In a category approach, more members are generally present, enabling the detection of trends across endpoints. (ECHA 2008, OECD 2014)

Chemical descriptor	A molecular descriptor is a mathematical representation of chemical structure (Todeschini & Consonni Viviana, 2009). More generally a chemical descriptor can be considered as any numerical representation of a structural or physicochemical property of a substance. Descriptors are used as independent variables in QSAR models, and to support the grouping of chemicals. A list of descriptor types is given in Table 1.3.
DLVO theory	The classical DLVO (Derjaguin, Landau, Verwey and Overbeek) theory of colloidal stability has been proposed to address the kinetics of agglomeration processes. DVLO theory combines the opposing effects of the van der Waals attractive force and the electrostatic repulsive force due to the so called 'double layer' of counterions, i.e. zeta potential for NMs.
Engineered nanomaterial	Designed for specific purpose or function (ISO 2014).
Extensive property	A property that is independent on the amount or mass (e.g. volume).
Extrinsic property	Characteristics resulting from interactions occurring at the interface (i.e. boundary) and the surrounding medium (which may be an environmental or biological matrix or medium in an experimental test system).
Flocculation	Process of contact and adhesion where dispersed particles are held together by weak physical interactions leading to phase separation by the formation of precipitates larger than colloidal size. (IUPAC 1997)
In silico (method, tool, approach)	Computational (method, tool, approach)
In vitro (method, tool, approach)	Conducted using components of an organism that have been isolated from their usual biological surroundings, such as microorganisms, cells, or biological molecules. Wikipedia: https://en.wikipedia.org/wiki/In_vitro
Integrated Approach to Testing and Assessment (IATA)	An approach based on multiple information sources used for the hazard identification, hazard characterisation and/or safety assessment of chemicals. An IATA integrates and weights all relevant existing evidence and guides the targeted generation of new data, where required, to inform regulatory decision-making regarding potential hazard and/or risk. Within an IATA, data from various information sources (i.e. physicochemical properties, in silico models, grouping and read-across approaches, in vitro methods, in vivo tests and human data) are evaluated and integrated to draw conclusions on the hazard and/or risk of chemicals. (OECD 2016)
Intensive property	A property that is independent on the amount or mass (e.g. temperature, pressure).

Intrinsic property	Characteristics of the material itself and do not account for interactions with other components. (OECD, 2010a)
Manufactured nanomaterial	Intentionally produced for commercial purpose to have selected properties or specific composition. (ISO 2014)
Physicochemical property	Physical properties, solvation properties related to interactions with different media, and properties or molecular attributes that define intrinsic chemical reactivity (NAS, 2014) https://www.ncbi.nlm.nih.gov/books/NBK253965/pdf/Bookshelf_NBK253965.pdf A list of physicochemical properties is defined in Table 1.5
Quantitative Structure-Activity Relationship (QSAR)	SARs and QSARs, collectively referred to as (Q)SARs, are theoretical models that can be used to predict in a qualitative or quantitative manner the physico-chemical, biological (e.g. toxicological) and environmental fate properties of compounds from knowledge of their chemical structure. (ECHA 2008) In this report, QSAR is a theoretical model that can be used to predict in a quantitative manner biological (e.g. toxicological) or fate properties of compounds from knowledge of their chemical structure or physicochemical properties.
Quantitative Structure-Activity Relationship (QSPR)	In this report, QSPR is distinguished from QSAR, as a theoretical model that can be used to predict in quantitative manner the physicochemical or environmental fate properties of compounds from knowledge of their chemical structure. (ECHA 2008)
Read-across	Read-across is an approach for filling data gaps, either by using a category or an analogue approach. For the purposes of the REACH Regulation (Article 13(1)), read-across is considered by ECHA to be an alternative method. (Gottardo et al 2016)
Structure-Activity Relationship (SAR)	See QSAR
Smoluchowski- Friedlander theory	Theory describing the kinetics of dispersed spherical particles in fluids. Sometimes considered to represent the kinetics involved in aggregation and coagulation processes.
Supervised learning	Supervised learning is a machine learning approach that approximates the mapping function (relationship) between input (X) and output variables and an output variable (Y). The goal is to approximate the mapping function so well that when you have new input data (x) that you can predict the output variables (Y) for that data. Supervised learning methods can be used for developing both regression and classification methods A list of supervised methods that are commonly used to derive predictive models is given in Table 1.2.

Unsupervised learning	Unsupervised learning is a machine learning approach that discovers the underlying structure and patterns in the input data (X), but there are no output variables (Y), and thus no attempt to approximate a relationship between X and Y.
	In this report, principal components analysis (PCA) and cluster analysis were used as unsupervised methods to support the grouping and read-across case studies (Chapter 4).

Appendix II. Definitions of "Nanomaterial" in EU legislation

Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (European Parliament & Council, 2009)

"nanomaterial" means an insoluble or biopersistant and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm;

Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers   (European Parliament & Council, 2011)

"engineered nanomaterial" means any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale.

Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU) (EC, 2011a)

"Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.   In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

By derogation [...], fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

[...], "particle", "agglomerate" and "aggregate" are defined as follows:

"particle" means a minute piece of matter with defined physical boundaries;

"agglomerate" means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;

"aggregate" means a particle comprising of strongly bound or fused particles.

Where technically feasible and requested in specific legislation, compliance with the definition [...]- may be determined on the basis of the specific surface area by volume. A material should be considered as falling under the definition [...] where the specific surface area by volume of the material is greater than 60 m^2 /cm^3. However, a material which, based on its number size distribution, is a nanomaterial should be considered as complying with the definition [...] even if the material has a specific surface area lower than 60 m^2 /cm^3.

The EC Definition includes a revision clause which allows taking into account new scientific and technical insights by the time the definition is reviewed in 2014.

Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products (European Parliament and Council, 2012)

"nanomaterial" means a natural or manufactured active substance or non-active substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm.

Fullerenes, graphene flakes and single-wall carbon nanotubes with one or more external dimensions below 1 nm shall be considered as nanomaterials.

For the purposes of the definition of nanomaterial, "particle", "agglomerate" and "aggregate" are defined as follows:

- "particle" means a minute piece of matter with defined physical boundaries,

- "agglomerate" means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components,

- "aggregate" means a particle comprising strongly bound or fused particles;

Fullerenes, graphene flakes and single-wall carbon nanotubes with one or more external dimensions below 1 nm shall be considered as nanomaterials.

Appendix III. Overview of REACH Standard Information Requirements (Annexes VI-X)

The text and tables below summarise the Standard Information Requirements of REACH Annex VI and Annexes VII-X. Proposed changes for a revision of the REACH Annexes to better address nanomaterials are highlighted in *italics.*

REACH ANNEX VI

1. **GENERAL REGISTRANT INFORMATION**

2. **IDENTIFICATION OF THE SUBSTANCE**

2.1. Name or other identifier of each substance

2.1.1. Name(s) in the IUPAC nomenclature or other international chemical name(s)

2.1.2. Other names (usual name, trade name, abbreviation)

2.1.3. EINECS or ELINCs number (if available and appropriate)

2.1.4. CAS name and CAS number (if available)

2.1.5. Other identity code (if available)

2.2. Information related to molecular and structural formula of each substance

2.2.1. Molecular and structural formula (including SMILES notation, if available)

2.2.2. Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)

2.2.3. Molecular weight or molecular weight range

2.3 Composition of each substance

2.3.1. Degree of purity (%)

2.3.2. Nature of impurities, including isomers and by-products

2.3.3. Percentage of (significant) main impurities

2.3.4. Nature and order of magnitude (… ppm, … %) of any additives (e.g. stabilising agents or inhibitors)

2.3.5. Spectral data (ultra-violet, infra-red, nuclear magnetic resonance or mass spectrum)

2.3.6. High-pressure liquid chromatogram, gas chromatogram

2.3.7. Description of the analytical methods or the appropriate bibliographical references for the identification of the substance and, where appropriate, for the identification of impurities and additives. This information shall be sufficient to allow the methods to be reproduced.

3. INFORMATION ON MANUFACTURE AND USE(S) OF THE SUBSTANCE(S)

4. CLASSIFICATION AND LABELLING

5. GUIDANCE ON SAFE USE

6. INFORMATION ON EXPOSURE FOR SUBSTANCES REGISTERED IN QUANTITIES BETWEEN 1 AND 10 TONNES PER YEAR PER MANUFATCURER OR IMPORTER

REACH ANNEXES VII-X

Table 1a: REACH tonnage dependent information requirements: Physicochemical properties

Annex VII ≥ 1 t	Annex VIII ≥ 10 t	Annex IX ≥ 100 t	Annex X ≥ 1000 t
State of the substance at 20°C and 101,3 kPa			
Melting/freezing point			
Boiling point			
Relative density			
Vapour pressure			
Surface tension			
Water solubility			
Partition coefficient n-octanol/water			
Flash point			
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties			

Granulometry			
		Stability in organic solvents and identity of relevant degradation products - only for organic substances/when stability is critical	
		Dissociation constant	
		Viscosity	

Table 1b: REACH tonnage dependent information requirements: Toxicological Information

Endpoint	Annex VII ≥ 1 t	Annex VIII ≥ 10 t	Annex IX ≥ 100 t	Annex X ≥ 1000 t
Acute toxicity	Oral	Inhal/dermal		
Irritation/corrosivity	Skin/eye (*in vitro*)	Skin/eye (*in vivo*)		
Sensitisation	Skin (*in vivo*)			
Repeated dose toxicity	-	28 d	28 d 90 d	
Mutagenicity	Gene mutations bacteria (*in vitro*)	Gene mutations in mammalian cells (*in vitro*) Cytogenicity or micronucleus	If positive *in vitro,* then *in vivo* somatic cells If positive *in vivo*, then potential for germ cell	If positive *in vitro*, then *in vivo* somatic cells (2nd test) If positive *in vivo*, then potential for germ cell

			mutagenicity	mutagenicity
Carcinogenicity	-	-	-	2-yr
Reproductive toxicity	-	Screening repro/developm. tox	Pre-natal developm. tox 2-gen-study (if the 28 d and 90 d is positive)	Developm. tox 2-gen-study
Toxicokinetics	-	From relevant available information		

Table 1c: REACH tonnage dependent information requirements: **Ecotoxicological Information**

Endpoint	Annex VII ≥ 1 t	Annex VIII ≥ 10 t	Annex IX ≥ 100 t	Annex X ≥ 1000 t
Aquatic toxicity	Short term tox (invertebrates) Growth inhibition study (aquatic plants)	Short term tox (Fish) – long term testing may be considered Activated sludge respiration inhibition testing	Long term tox (invertebrates, e.g. Daphnia)) Long term tox (fish): fish early life stage test, test on embryo and sac-fry stages, juvenile growth test	

Degradation	Biotic: ready biodegradability - for organic substances	Abiotic (hydrolysis as a function of pH) .	Biotic: simulation testing in surface water, soil, sediment, and identification of degradation products.	Biotic: (additional studies depending on results of CSA
Fate and behaviour in the environment	-	Adsorption/desorption screening	Bioaccumulation in aquatic species (fish) Further adsorption/desorption studies (depending on results of previous studies)	Further studies depending on results of CSA
Effects on terrestrial organisms	-	-	Short term toxicity (invertebrates) Effects on soil micro-organisms Short term toxicity (plants)	Long term toxicity (invertebrates, plants, sediment organisms, birds - depending on results of CSA)

Appendix IV. List of EU projects including aspects of modelling (including grouping)

	Project Title	Project Acronym	Frame-work	Contact (coordinator)	Project website	Topics directly related to NanoComput	Duration
1	A Database and Ontology Framework for Nanomaterials Design and Safety Assessment	eNanomapper	FP7	Barry Hardy Douglas Connect GmbH Switzerland	http://www.enano mapper.net/	Creation of a platform that consists of an ontology addressing all standardisation and regulatory requirements supporting the exploration of SAR and of a data wharehouse for NMs	1/02/2014 – 31/01/2017
2	Risk Assessment of Engineered Nanoparticles	ENPRA	FP7	Lang Tran Institute of Occupational Medicine UK	http://www.enpra. eu	Identification of physicochemical properties that are useful as descriptors for building QSAR models for toxicologically relevant properties and effects	01/05/2009 – 31/10/2012
3	A framework to respond to the regulatory needs of future nanomaterials and markets	FutureNano Needs	FP7	Kenneth A. Dawson University College Dublin	http://www.future nanoneeds.eu/	Development of a novel framework to enable naming, classification, hazard and environmental impact assessment of the next generation nanomaterials	01/01/2014 – 31/12/2017
4	Intelligent testing strategies for engineered nanomaterials Project	ITS-NANO	FP7 (Support action)	Vicki Stone Herriot Watts University UK	http://nano.hw.ac. uk/research-projects/itsnano.ht ml	Definition of testing strategies based on protocols to support grouping and ranking of NMs	01/03/2012 – 31/05/2013
5	Managing Risks of Nanomaterials	MARINA	FP7	Lang Tran Institute of Occupational Medicine UK	http://www.marina -fp7.eu/	Harmonised database on Reference NMs fate-determining parameters in the environment and biota	01/11/2011 - 31/10/2015

6	Modelling the mechanisms of nanoparticle-lipid interactions and nanoparticle effects on cell membrane structure and function	MembraneNanoPart	FP7	Vladimir Lobaskin University College Dublin	http://www.membranenanopart.eu/	Development of physically justified models and computational tools to quantitatively describe and understand the molecular mechanisms of nanoparticle/cell membrane interactions, which are crucial in modelling of nanoparticle toxicity	1/1/2013 – 31/12/2015
7	Modelling Nanomaterial Toxicity	MODENA (COST initiative)	FP7	Lang Tran Institute of Occupational Medicine UK	http://www.modena-cost.eu/	MODENA COST Action has been established to promote and to co-ordinate these inter-disciplinary collaborations, with the ultimate aim of producing QNTR models for ENM. MODENA aims at The creation of transparent, validated and rigorous QNTR tools for regulatory purposes in the field of nanotoxicology according to OECD principles.	1/01/2014 - 31/12/2016
8	Modeling Platform to Predict the Toxicity of Metal-based Nanoparticles	MOD-ENP-TOX	FP7	Jean Pierre Locquet KU Leuven	http://fys.kuleuven.be/apps/modenptox/	Focussed on metal nanoparticles. It combines novel Computational Modelling Package (CMP) based on structural, mechanistic, as well as kinetic modelling tools and an innovative high content screening (HCS) strategy that allows performing multiplexed streamlined assays for calibration, refinement and validation of the computed models	01/01/2013 - 31/12/2015

9	MODelling the EnviRonmental and human health effects of Nanomaterials	MODERN	FP7	Robert Rallo Universitat Rovira i Virgili	http://modern-fp7.biocenit.cat	Establishment of new modelling approaches suitable for relating nanotoxicity with the intrinsic molecular and physicochemical properties of NMs. Definition of a categorization and hazard ranking protocol for NMs based on structural similarity principles and on the analysis of their toxicological profiles	01/01/2013 - 31/12/2015
10	Modelling nanoparticle toxicity: principles, methods, novel approaches	ModNanoTox	FP7	Eugenia Valsami-Jones University of Birmingham	http://www.birmingham.ac.uk/generic/modnanotox/index.aspx	Development of QSAR for NMs toxicity as far as current data availability allows. improving the accessibility and sustainability of the WP2 database by convert it into a format compatible with emerging standards (ISA-TAB NANO).	01/11/2011 - 31/10/2013
11	Building bridges between specialists on computational and empirical risk assessment of engineered nanomaterials	NanoBRIDGES	FP7 (Marie Curie Actions)	Tomasz Puzyn Uniwersytet Gdanski	http://nanobridges.eu/	Investigation on the relationship between cytotoxicity of 17 different types of nano-sized metal oxide nanoparticles to bacteria Escherichia coli and their structure	01/01/2012 - 31/12/2014
12	Nanoparticle Fate Assessment and Toxicity in the Environment	NanoFATE	FP7	Claus Svendsen NERC Centre for Ecology & Hydrology at Wallingford, UK	http://www.nanofate.eu	Nanoparticles ecotoxicology and bioavailability; environmental fate data and models	01/04/2010 - 31/03/2014

13	Engineered nanomaterial mechanisms of interactions with living systems and the environment: a universal framework for safe nanotechnology	NanoMILE	FP7	Eugenia Valsami-Jones University of Birmingham	http://www.nanomile.eu/	Development of quantitative structure (property) –activity relationship - QS(P)ARs - enabling predictive work to evolve and feed into risk assessment	01/03/2013 - 28/02/2017
14	Modelling properties, interactions, toxicity and environmental behaviour of engineered nanoparticles	NanoPUZZLES	FP7	Tomasz Puzyn Uniwersytet Gdanski	http://www.nanopuzzles.eu	Grouping and read-across; To create new computational methods for comprehensive modelling the relationships between the structure, properties, molecular interactions and toxicity of engineered nanoparticles	01/01/2013- 31/12/2015
15	A common European approach to the regulatory testing of Manufactured Nanomaterials	NANOREG	FP7	Tom van Teunenbroek Ministry of Infrastructure and the Environment NL	http://www.nanoreg.eu/	Work package 5 will develop a proposal for grouping of NMs in categories with similar biological, ecological and/or toxicological effects	01/03/2013 - 28/02/2017
16	The Reactivity and Toxicity of Engineered Nanoparticles: Risks to the Environment and Human Health	NanoReTox	FP7	Eugenia Valsami-Jones Natural History Museum UK	http://www.nanoretox.eu/	Study of molecular and cellular reactivity of sleected metal NMs in aquatic species and in cells and cell lines originating from different human target organs	01/12/2008 - 30/11/2012

17	Biological Foundation for the Safety Classification of Engineered Nanomaterials (ENM): Systems Biology Approaches to Understand Interactions of ENM with Living Organisms and the Environment	NANOSOLUTIONS	FP7	Kai Savolainen Finnish Institute of Occupational Health, (FIOH)	http://nanosolutionsfp7.com/	Development of reliable testing protocols for qualitative and semi-quantative approaches for protein corona detection by mass spectrometry Generated data on nanomaterials and biological specimens has been defined along the ISA-TAB Nano specifications A basic data repository has been created and tested A computer algorithm capable of simulating synthetic data, and a novel computational method for feature selection and prioritization based on fuzzy logic and random forests have been developed	01/04/2013 - 31/03/2017
18	Development of methodology for alternative testing strategies for the assessment of the toxicological profile of nanoparticles used in medical diagnostics	NanoTEST	FP7	Maria Dusinska Norwegian institute for Air Research	http://www.nanotest-fp7.eu/	Development of a theoretical model for predicting the reactivity of metal oxide NPs as well as their ability to cause oxidative stress through the generation of ROS	01/04/2008 - 31/03/2012

19	Modelling the basis and kinetics of nanoparticle cellular interaction and transport	NanoTranskin etics	FP7	Kenneth Dawson University College Dublin	http://www.nanotr anskinetics.eu/	Development of an user-friendly Bundle for Bio-nano Large-scale Efficient Simulations BUBBLES http://ovilanova.github.io/BUBBLES/ Data from FP6 NANOINTERACT project and the FP7 NEURONANO project plus new data	01/11/2011 - 31/10/2014
20	Predictive toxicology of engineered nanoparticles	PreNanoTox	FP7	Rafi Korenstein Tel-Aviv University	http://prenanotox. tau.ac.il/	Development of a database of in vitro and in vivo studies; development, adaptation and validation of novel methodologies for establishing QNAR modeling as a tool for predicting biological effects of selected NMs	01/01/2013 - 31/12/2015
21	Development of a web based REACH Toolkit to support the chemical safety assessment of nanomaterials	REACHnano	LIFE+	Carlos Fito ITENE, Spain	http://www.liferea chnano.eu/	Provide the industry and stakeholders with easy-to-use tools to support the risk assessment of nanomaterials along their lifecycle.	01/10/2012 – 30/09/2015

Appendix V. US EPA and OECD projects including aspects of modelling and grouping

	Project Title	Contact (coordinator)	Project website	Topics directly related to NanoComput	Duration
1	Genomics-based Determination of Nanoparticle Toxicity: Structure-function Analysis*	Bakalinsky, Alan T. Li, Qilin	http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/8449/report/0	Determination of the mechanisms by which manufactured nanomaterials cause cytotoxicity in realistic environments of exposure.	Ended (05/2007 – 05/2009)
2	Structure-function Relationships in Engineered Nanomaterial Toxicity*	Colvin, Vicki L. Rice University	http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/7888/report/0 Links to publications at: https://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.publications/abstract/7888	This project aimed to provide structure-function relationships for nanoparticle toxicology for the benefit of industry and regulators	Ended (12/2005 – 11/2008)
3	OECD Working Party on Manufactured Nanomaterials (WPMN) to promote international co-operation in human health and environmental safety aspects of manufactured nanomaterials. Steering groups (SGs): SG 347 (Testing and Assessment = TA); SG 56	Mar Gonzales (OECD secretariat) Jenny Holmqvist (chair SG TA)	www.oecd.org/env/nanosafety Link to OECD publication series on the Safety of Manufactured Nanomaterials: http://www.oecd.org/env/ehs/nanosafety/publications-series-safety-manufactured-nanomaterials.htm	Sponsorship Programme for the Testing of Manufactured Nanomaterial: Fullerenes (C60), SWCNTs, MWCNTs, Silver nanoparticles, TiO2, CeO, ZnO, SiO2, Dendrimers, Nanoclays and Gold nanoparticles	Since 2006

(Risk Assessment and Regulatory Program), SG8 (Exposure Measurement and Exposure Mitigation) SG 9: Environmentally Sustainable Use of Manufactured Nanomaterials			are tested for their physical chemicals properties, environmental fate and behaviour, ecotoxicity and toxicity using appropriate testing methods	

* Projects funded by the US EPA under the Extramural Nanotechnology Research http://www2.epa.gov/chemical-research/research-evaluating-nanomaterials-chemical-safety

Appendix VI. The revised QMRF

1.QSAR identifier

1.1. QSAR identifier (title):

Oxidative stress caused by metal oxides nanoparticles

1.2. Other related models:

None

1.3. Software coding the model:

None

2.General information

2.1. Date of QMRF:

November 2015

2.2. QMRF author(s) and contact details:

David Asturiol. David.asturiol-bofill@ec.europa.eu

2.3. Date of QMRF update(s):

2.4. QMRF update(s):

2.5. Model developer(s) and contact details:

Enrico Burello and Andrew Worth Joint Research Centre,Via Enrico Fermi 2749, Ispra, Varese, 21027 Italy

2.6. Date of model development and/or publication:

2011

2.7. Reference(s) to main scientific papers and/or software package: Burello, E., Worth, A.P., 2011. A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5, 228–35. doi:10.3109/17435390.2010.502980

2.8. Availability of information about the model:

The model is free

2.9. Availability of another QMRF for exactly the same model:

None to date

3.Defining the endpoint - OECD Principle 1

3.1.Species:

Various cell lines

3.2. Endpoint:

Reactive Oxygen Species (ROS) generation

3.3.   Comment on endpoint:

Metal oxides affecting relevant intracellular reactions.

3.4.   Endpoint units:

Not applicable

3.5.   Dependent variable:

Heat of formation (ΔH_f).which is obtained from ionization potential and electron affinity

3.6.   Experimental protocol:

The model is a theoretical framework based on a chemical hypothesis and confirmed with in vitro data from various sources.

3.7.   Endpoint data quality and variability:

No information available

4.Defining the algorithm - OECD Principle 2

4.1.   Type of model:

Classification QSAR

4.2.   Explicit algorithm:

Is a parametric model defined by the value of the band gap energy (Eg).

$E_g = A \cdot \exp(0.34\ \Delta H°)$.

Eg<-4.84 → non ROS generator

-4.84eV<=Eg<=-4.12 → ROS generator

Eg>-4.12 → non ROS generator

4.3.   Descriptors in the model:

The model uses reactivity descriptors to build the energy band structure of oxide nanoparticles, assuming a particle diameter larger than 20–30 nm and no surface states in the band gap, and predicts their ability to induce an oxidative stress by comparing the redox potentials of relevant intracellular reactions with the oxides' energy structure

4.4.   Descriptor selection:

Band gap energy of bulk material (obtained from ΔHf, enthalpy of formation, ionization potential, and electron affinity). See pag. 230 of Burello & Worth 2011 for further details.

4.5.   Algorithm and descriptor generation:

No information available

4.6.   Software name and version for descriptor generation:

No information available

4.7.   Chemicals/Descriptors ratio:

6/1=6

5.Defining the applicability domain - OECD Principle 3

5.1.   Description of the applicability domain of the model:

Metal oxides of sizes >20-30nm

5.2.   Method used to assess the applicability domain:

Not applicable

5.3.   Software name and version for applicability domain assessment:

Not applicable

5.4.   Limits of applicability:

No information available

6.Internal validation - OECD Principle 4

6.1.   Availability of the training set:

Yes

6.2.   Available information for the training set:

CAS: No

Chemical Name: not applicable

SMILES: not applicable

Formula: not applicable

INChI: not applicable

MOL file: not applicable

<u>Part extended for NPs.</u>

NP composition: Yes

NP size: Yes

NP surface chemistry: No

6.3.   Data for each descriptor variable for the training set:

Yes

6.4.   Data for the dependent variable for the training set:

Yes

6.5.   Other information about the training set:

The training set consists of 6 metal oxides of sizes 20-30nm.

6.6.   Pre-processing of data before modelling:

No information available

6.7.   Statistics for goodness-of-fit:

$R2=0.84$

6.8.   Robustness - Statistics obtained by leave-one-out cross-validation:

No information available

6.9.   Robustness - Statistics obtained by leave-many-out cross-validation:

No information available

6.10.   Robustness - Statistics obtained by Y-scrambling:

No information available

6.11.   Robustness - Statistics obtained by bootstrap:

No information available

6.12.   Robustness - Statistics obtained by other methods:

No information available

7.External validation - OECD Principle 4

7.1.   Availability of the external validation set:

Yes

7.2.   Available information for the external validation set:

CAS: No

Chemical Name: not applicable

SMILES: not applicable

Formula: not applicable

INChI: not applicable

MOL file: not applicable

Part extended for NPs.

NP composition: Yes

NP size: Yes

NP surface chemistry: No

7.3.   Data for each descriptor variable for the external validation set:

No

7.4.   Data for the dependent variable for the external validation set:

Yes

7.5.   Other information about the external validation set:

No information available

7.6.   Experimental design of test set:

No information available

7.7.   Predictivity - Statistics obtained by external validation:

No information available

7.8.   Predictivity - Assessment of the external validation set:

No information available

7.9.   Comments on the external validation of the model:

Band gaps of 64 untested metal oxides were predicted

8.Providing a mechanistic interpretation - OECD Principle 5

8.1. Mechanistic basis of the model:

The model uses reactivity descriptors to build the energy band structure of oxide nanoparticles, assuming a particle diameter larger than 20–30 nm and no surface states in the band gap, and predicts their ability to induce an oxidative stress by comparing the redox potentials of relevant intracellular reactions with the oxides' energy structure.

8.2. A priori or a posteriori mechanistic interpretation:

A priori

8.3. Other information about the mechanistic interpretation:

No additional information available

9.Miscellaneous information

9.1. Comments:

No additional information available.

9.2. Bibliography:

[1] Burello, E., Worth, A.P., 2011. A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5, 228–35

10.Summary (JRC QSAR Model Database)

10.1. QMRF number:

To be entered by JRC

10.2. Publication date:

To be entered by JRC

10.3.Keywords:

Oxidative stress, ROS, metal oxides, nanoparticle, nanomaterial, NM, QSAR, prediction, band gap energy.

10.4.Comments:

Appendix VII. The TK/TD/dosimetry/environmental fate model reporting template

List of the information collected in the model reporting template for toxicokinetic (TK), toxicodynamic (TD) and environmental fate models.

Model Metadata	Model	Model name
		Version
		Homepage
	Owner	Model ownership
		contact point
		email address
		License
	Reference	References associated
		DOI
Model description	Characteristics	Model output(s) (generic description)
		Level of organisation (cell, human body, organelle, environment, etc)
		Level of organisation (specific details)
		Time span
		Model type
	General description	Description (free text)
		Is a conceptual model (compartment diagram) given in the original reference?
	Process info	Processes considered
		Symbol (if applicable)
		Units
		Is a set of equations given corresponding to the process?
		Are the parameters relating to the model processes provided and defined?
		Assumptions/approximations related to the processes
	Other	Free Text
I/O	Information on NP (or chemical) dependent input parameters	NP (or chemical) dependent input parameters
		Symbol (if applicable)
		Units
		Protocol/assumptions
	Information on NP (or chemical)-independent input parameters	NP (or chemical)-independent input parameters
		Symbol (if applicable)
		Units
	Information on utput parameters	Output parameters
		Symbol (if applicable)
		Units
NPs description		Type NP
		Shape
		Coating
		Size (nm)

	Other/info on measured properties
	Used in reference
Model domain	General model assumptions
	Is the model nanospecific, i.e. capture proprties unique to the nano size range?
	Applicability domain
	Sources of uncertainty

Structure of the reporting template for the PBTK and environmental fate models.

Model reference

	Model Metadata								
	Model			Owner				Reference	
Ref	**Model name**	**Version**	**Homepage**	**Model ownership**	**contact point**	**email address**	**License**	**References associated**	**DOI**
Ref. number given by compilers	If the model has a name, report it, otherwise give a comprehensible one. Report also the acronym, if available.	If available	If available	If available	Related to the email address	Indicated on the website as contact point or in the publications as corresponding author	Is it free? Is there any information on the access?	Scientific publications or reports describing the model details	Index

398

Model description: characteristics, general description, process information, other.

Characteristics					General description	
Model output(s) (generic description)	**Level of organisation (cell, human body, organelle,**	**Level of organisation (specific details)**	**Time span**	**Model type**	**Description (free text)**	**Is a conceptual model (compartment diagram) given in the original reference?**
List of the model outputs	List of generic compartments: environment, biota, human body	List of specific ompartments/units/ organs considered	Refers to the input or output time scale; it can be dynamic or at steady state	Possible model types: multimedia mass balance model mass flow analysis Physical quantitative model …	Report a short text describing the model - what it does, how it works, hbow it can be used	Y/N

Process info						Other
Processes considered	**Symbol (if applicable)**	**Units**	**Is a set of equations given corresponding to the model processes?**	**Are the parameters relating to the model processes provided and defined?**	**Assumptions/approximations related to the processes**	**Free Text**
List the processes considered in the model	Symbol related to the process	measure unit related to the process	Indicate if equation(s) are identified for the description of the process. Indicate if the model code is available.	Indicate here the parameters necessary to describe the process	List the assumptions related to the process (if the assumption made is not related to the identified processes, shift the information to the last column "general assumptions")	Any relevant comment

399

Inputs and outputs of the models

I/O								
NP (or chemical) dependent input parameters	**Symbol (if applicable)**	**Units**	**Protocol/assumption**	**NP (or chemical)-independent input parameters**	**Symbol (if applicable)**	**Units**	**Output parameters**	**Units**
In these cells information on the (nano)particle-specific or chemical-specific parameters should be reported (parameter, symbol, units, protocol for measured values). Are the parameters mesaured or calculated? If there are assumptions on the NMs these should go under protocol/assumptions				In these cells information on the (nano)particle-independent or chemical-independent input parameters should be reported.			Model outputs should be reported here including units	

Nanomaterials description and considerations on model domain

NPs description				Model domain		
Type NP	**Shape**	**Coating**	**Size (n**	**General model assumptions**	**Is the model nanospecific, i.e. capture properties unique to the nano**	**Applicability dom**
Here input information on the (nano)parti the model simulation is reported. Please f adapt the list of properties included here Are the parameters mesaured or calculate If measured details are reported but are n as input parameters, then these should be under other/information on measured pro thid column also other properties reported shuld be reported (e.g. crystallinity, solubi				Here general assumptions on the model set up are reported	Are (nano)particle-specific parameters included in the model? Or is the model simply partcile-specific (irrespective of partcile size).	

Appendix VIII. TiO$_2$ case study - physicochemical data analysis

Physicochemical data collected from different sources (OECD dossiers, Nanogenotox deliverables, JRC repository) were analysed to build the data matrix for grouping and read-across (step 4 of the ECHA draft workflow for grouping and read-across) ad to support evaluation of uncertainty.

The properties that populate our dataset and that are analysed in this Annex are:

Total non-TiO2 content including coating and impurities: property corresponding to the %w/w of impurities and coating. This property was defined so as to include the large amount of impurities found in NM-101, which probably corresponded to coating, at the same level as the coating of NM-103 and NM-104.

Particle size distribution: the data represent the diameter of a sphere having the same physical property. This is known as the equivalent spherical diameter (ISO 9276-1). The measures reported under this property come from dynamic light scattering (DLS).

Zeta potential: the charge at the particles interfaces, measured by DLS.

Polydispersity index: measure done with the z-average instrument and gives the idea of a monomodal/bimodal distribution. According to NanoGenotox deliverable 4.5 (Guiot et al. 2012) when the PdI<0.25 then the size distribution is monomodal and z-average is calculated. When the PdI<0.8 then we have a bimodal distribution and we should take the Intensity main peak.

Solubility (dissolution) and biodurability: 0.05% BSA batch dispersion medium, low-Ca Gamble's solution, and in Caco 2 cell medium by 24-hour incubation at 37 °C and 5% CO2 air at 95% RH in a cell incubator. Solubility was assessed from elemental analyses of the solute adjusted for background concentrations in the three test media, and biodurability is also determined according to elemental analysis in the media after 24 h.

Dustiness: propensity of a material to generate airborne dust during its handling. Two different methods were available and reported in our dataset.

Primary particle size: this is the size of the particles, measured by Transmission Electron Microscope (TEM).

Specific surface area: surface area of the particle SA [m^2] per unit mass m [g] SSA=SA/m [m^2/g] and can be measured by Small-Angle X-Ray Scattering (SAXS) or by the Brünauer, Emmett And Teller (BET) method.

Crystallite size: Size of the crystal or grain.

Porosity: an indication of the fraction of the particle that is devoid of material (ISO, 2011e).

Isoelectric point: pH at which the NM has no net surface charge.

Data on particle size distribution

Figure A7.1 shows the average of all modes obtained for the size (in nm) per nanoform, treatment (sonication time and type) and method (biological medium). MQ stands for milli-Q water, DMEM for Dulbecco's modified eagle medium, PBS is phosphate-buffered saline medium, FBS stands for fetal bovine serum.

Figure A7.2 gives an overview of the data collected for each NM, treatment and medium. The different modes are also included.
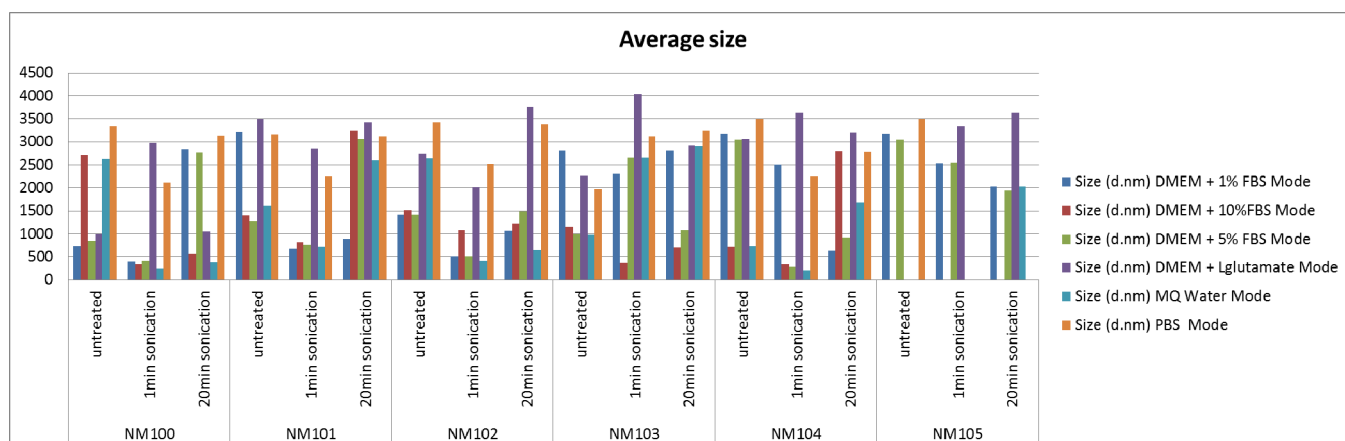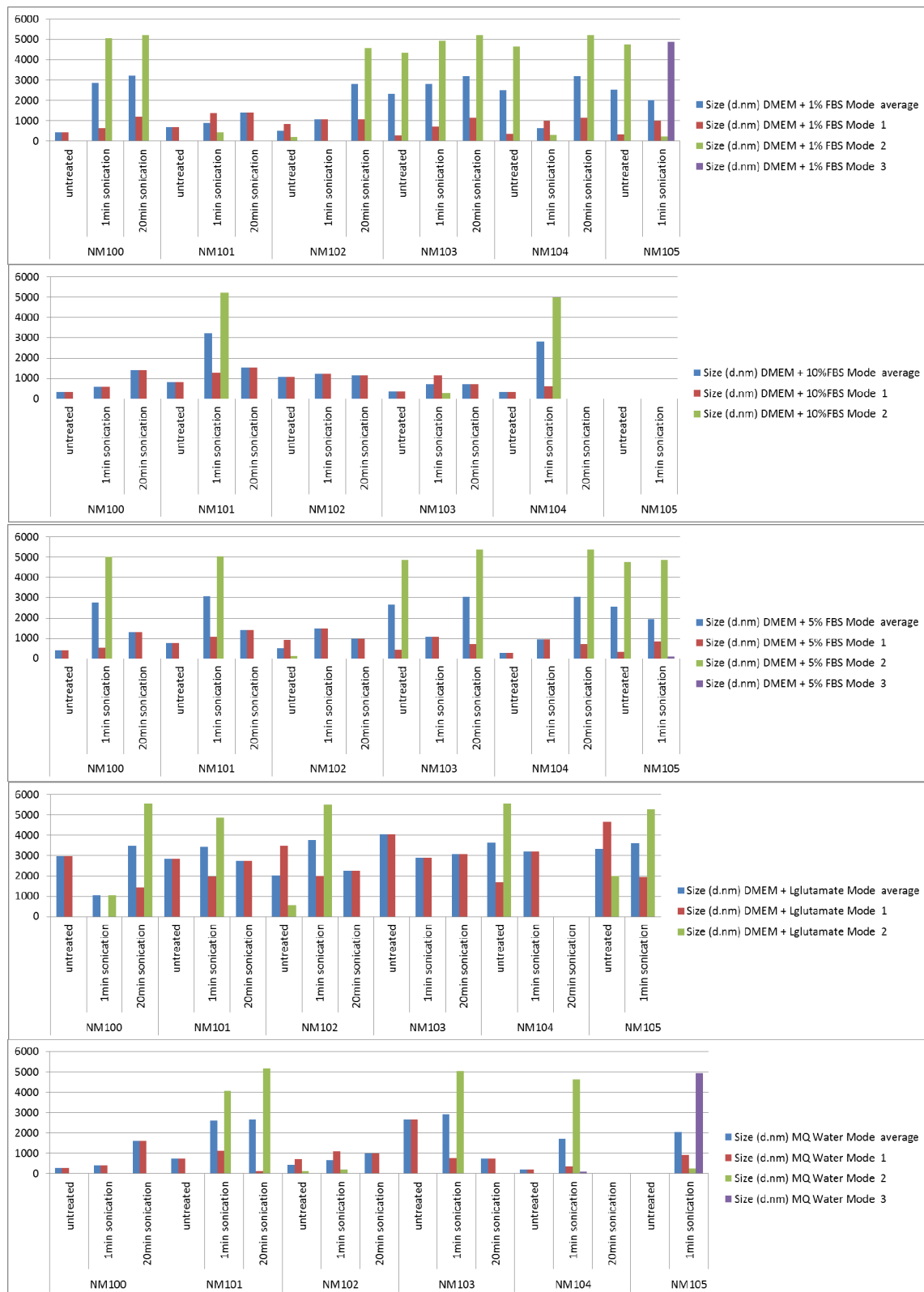

Figure A7.1. Average size distribution of different modes reported for the different nanoforms measured via dynamic light scattering (DLS).
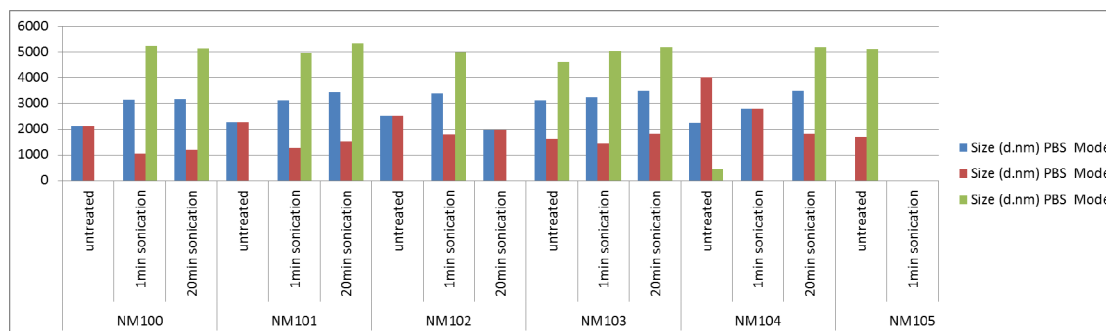
a)

b)

c)

d)

e)

f)

Figure A7.2. Modes 1 to 3 of the particle size distribution per mode 1 to 3 of NM-100 to NM-105 in different media: a) DMEM + 1% FBS; b) DMEM + 10% FBS; c) DMEM + 5% FBS; d) DMEM + L glutamate; e) in MQ water; f) in PBS

Figure A7.3 shows the presence of agglomerates in different dispersions and with different treatments. Data show that the different treatments (untreated or 1 min probe sonication and 20 min batch sonication) do not have a significant and equal impact on the measured size in the dispersion. In general it can be observed that the 20 min sonication treatment usually tends to correspond with large agglomerates.
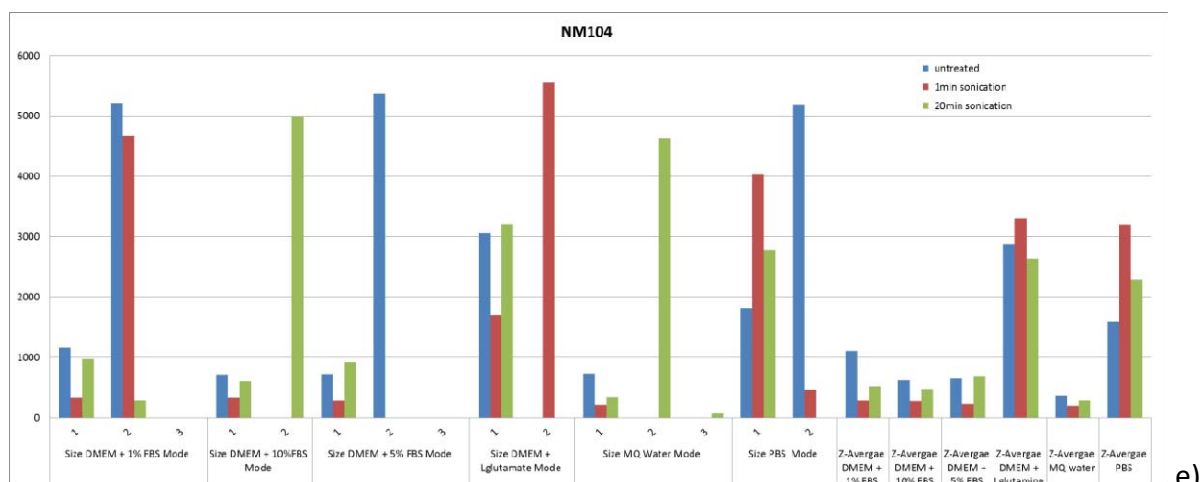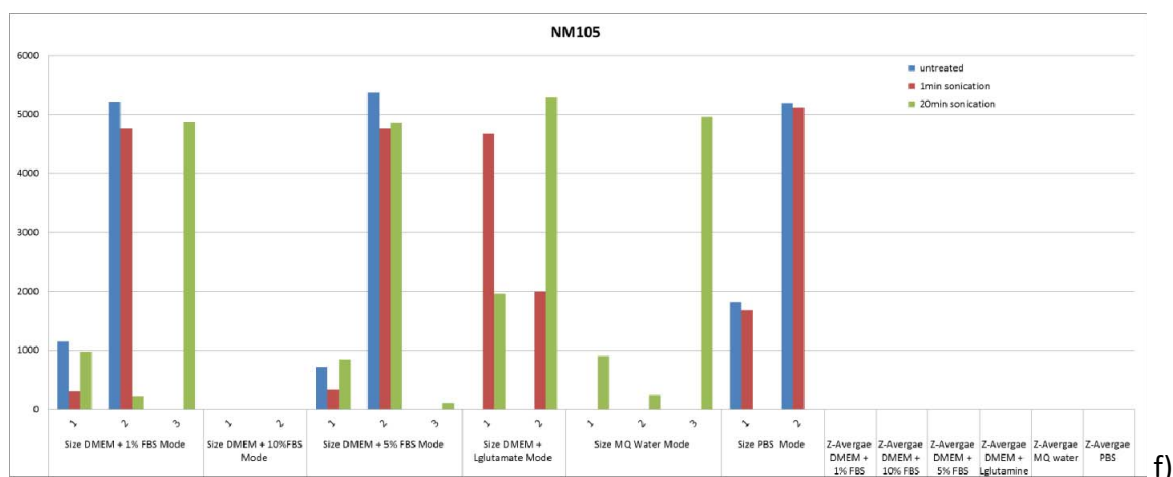

a)

b)

c)

d)

e)

f)
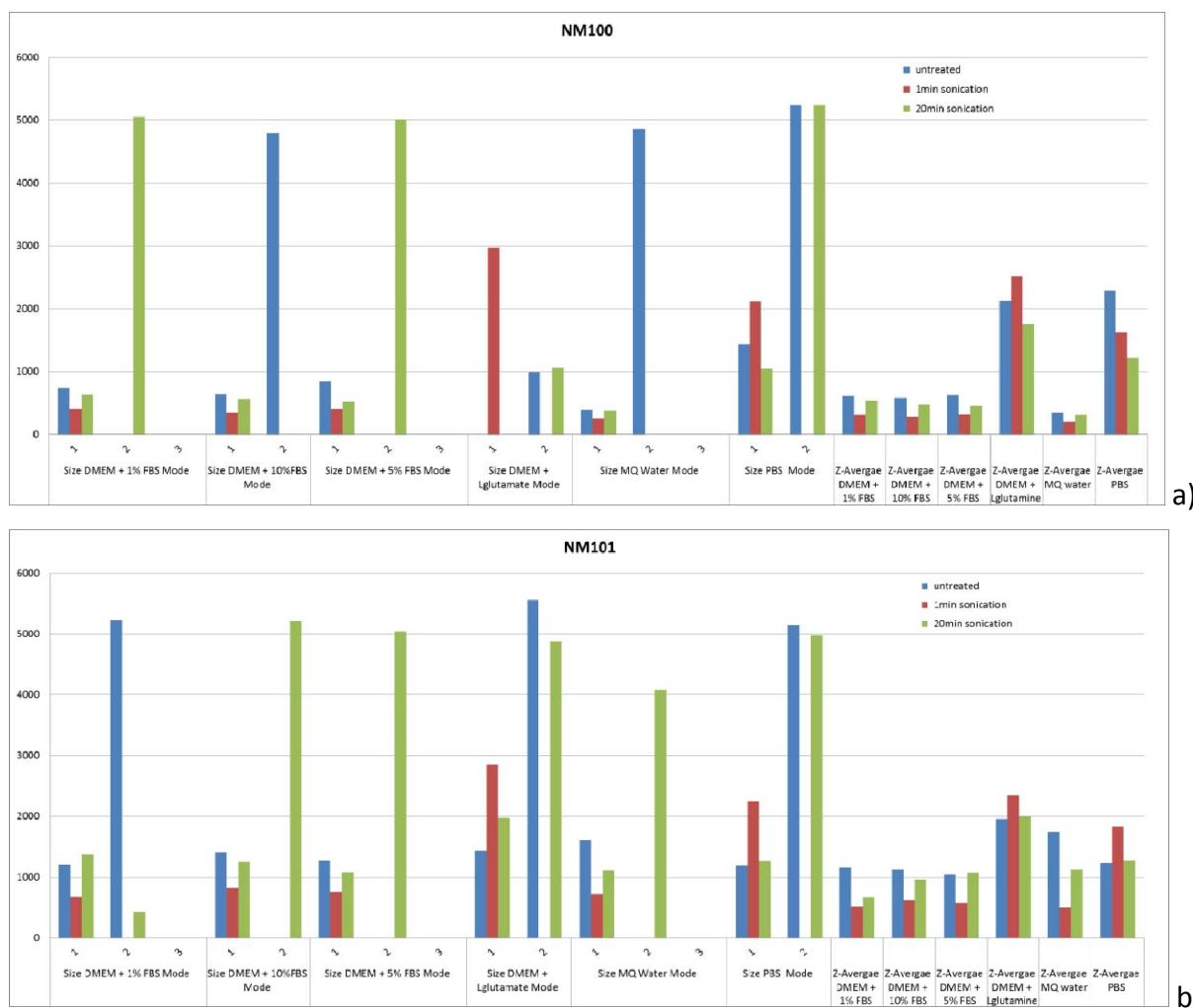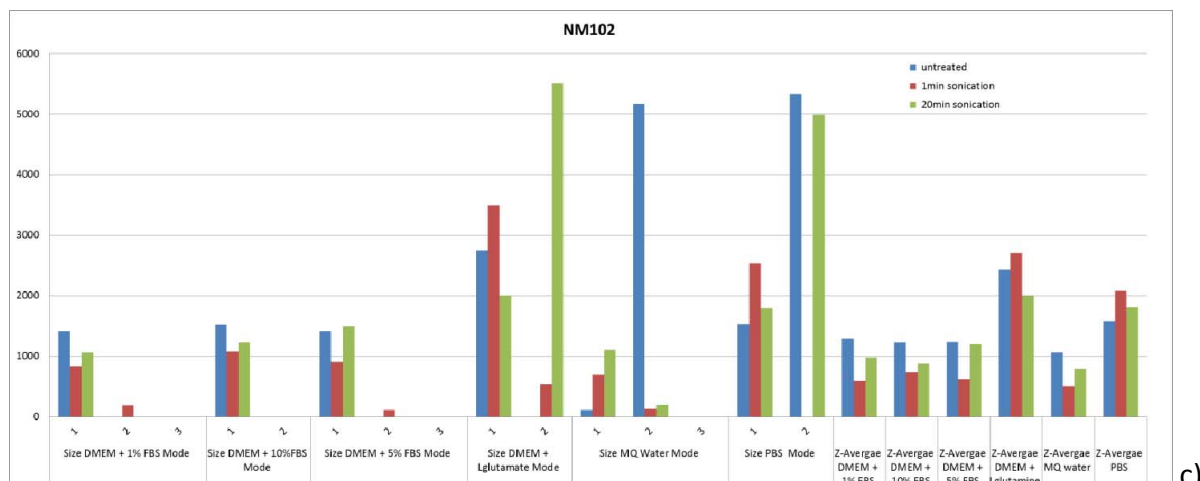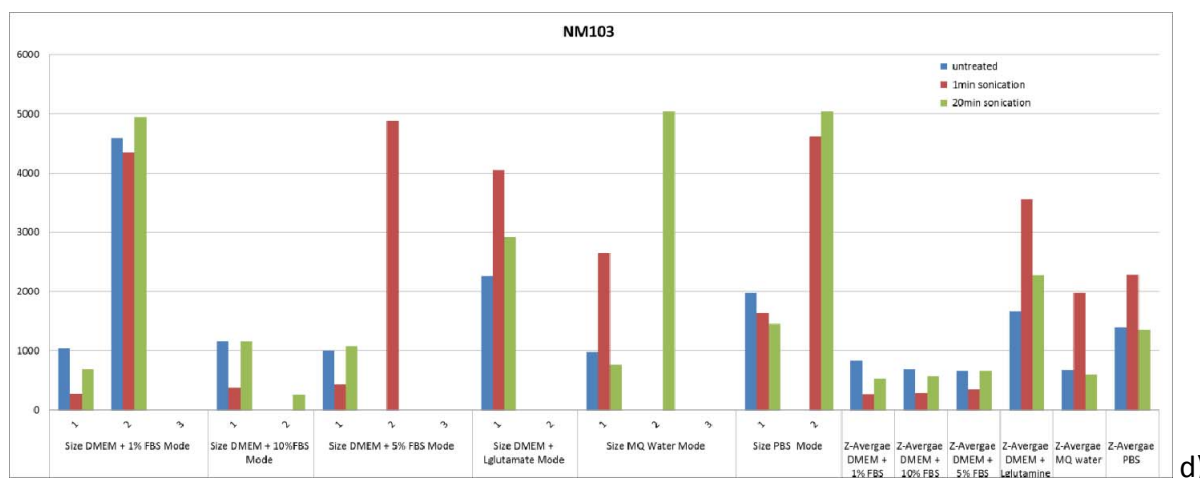
Figure A7.3. DLS measurements for the different NM at different sonication conditions and in different culture media; a) data for NM-100, b) data for NM-101; c) data for NM-102; d) reports data for NM-103; e) data for NM-104; f) data for NM-105.

Figure A7.4 shows the overall variability across the Zeta size of the different nanoforms (NM-102-105) in the different tested dispersion protocols. The plot corresponds to the mean values with the standard deviations obtained in each study, and shows that there is almost an overlap between them.

A measure based on the standard deviation shall capture the variance within a method over the Z-size. Whereas the between St.Dev (standard deviation of the averages) shall capture the variance between methods.

Since the average across studies may be used, the interesting measure to keep is the one describing the difference between methods to describe the uncertainty of the measure used.
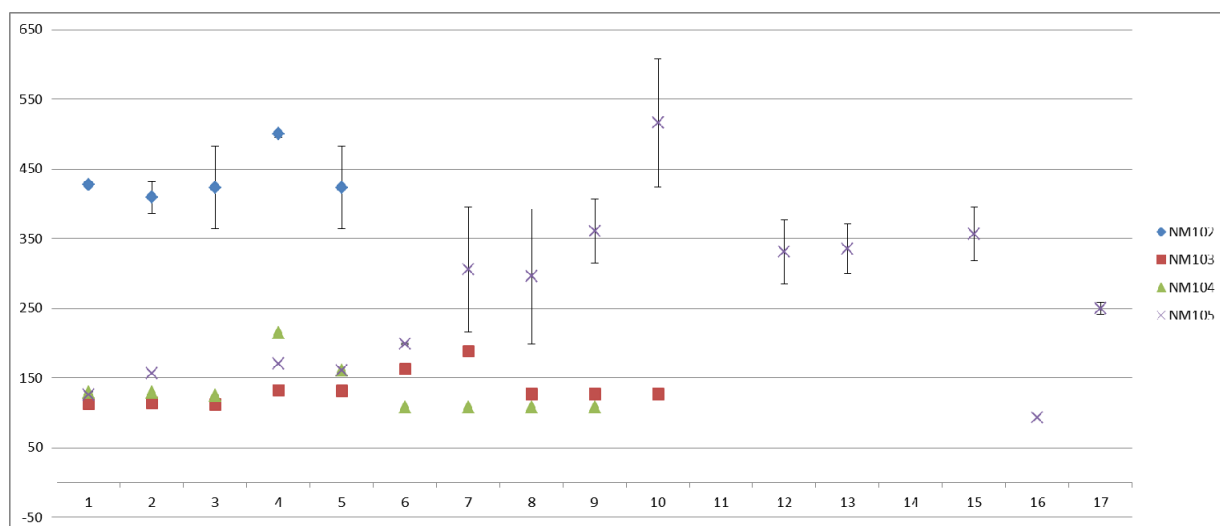

Figure A7.4. Plot of the overall variability in the size (nm) measured with different treatments and for different nanomaterials. Each measurement corresponds to an item of the x axis.

Data on zeta potential

Figure A7.5. shows the average of all modes of the Zeta potential per nanoform, per medium treatment (sonication time and type, exposure medium).
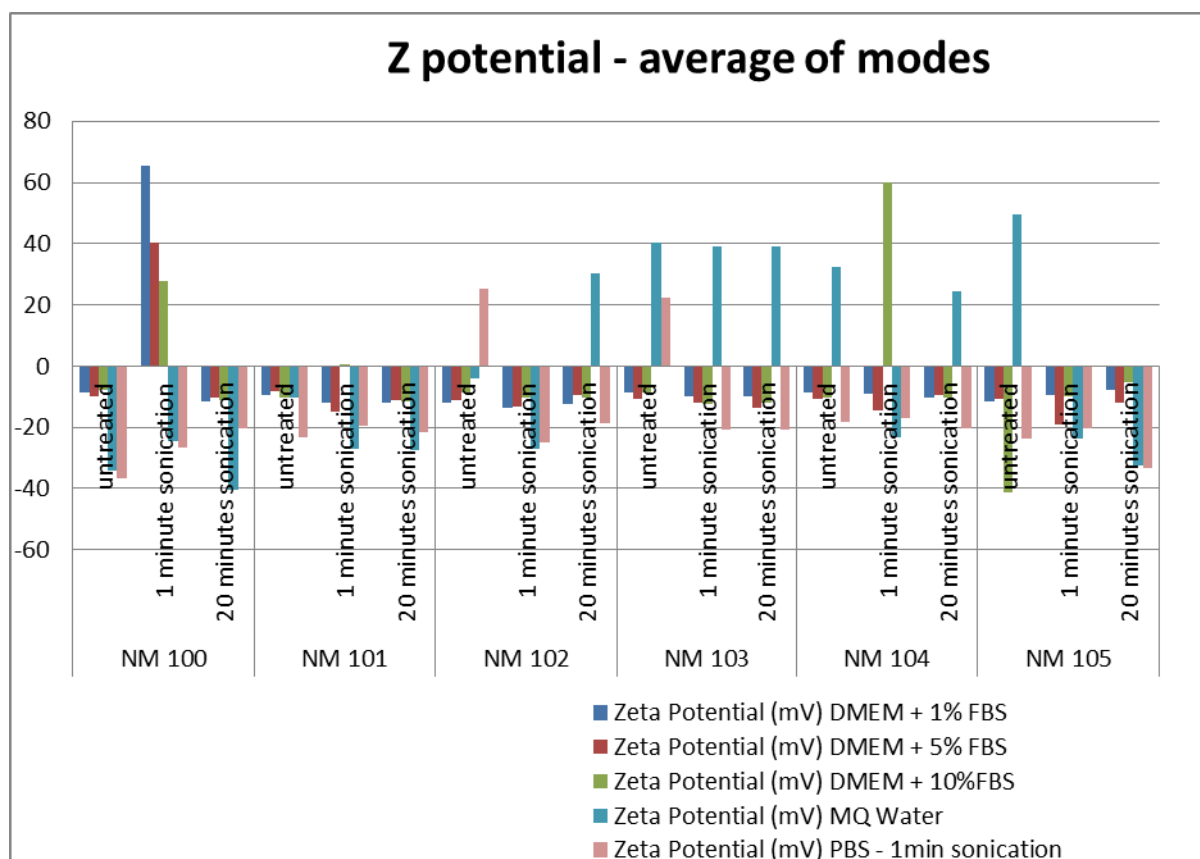
Figure A7.5. Zeta potential values collected from the OECD dossiers on the six nanoforms considered in the read-across case study (average of modes)

Figure A7.6 shows an overview of the Zeta potential modes for each nanoform, for each exposure medium. It is observed that when only one mode is reported, all nanoforms look alike, but when the solution is polydispersed, their behaviour is unpredictable as some modes are positive and some are negative; some are around ±20 mV (indicator of stability) and some are lower in modulus, what indicates low stability of the dispersion.

a)
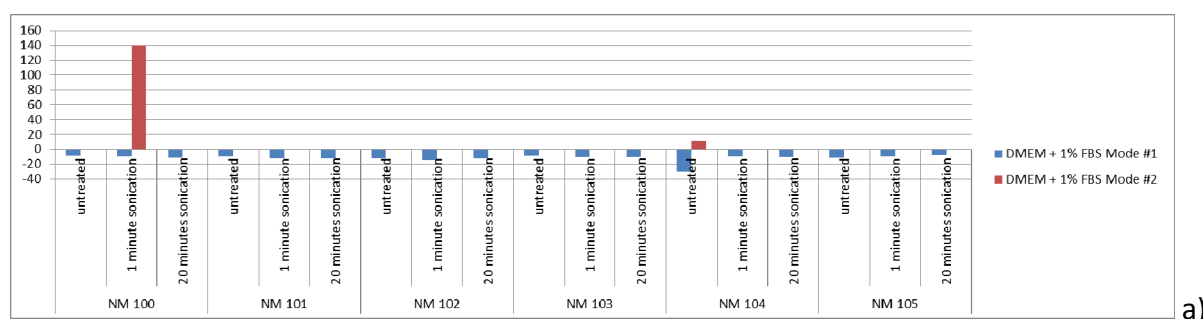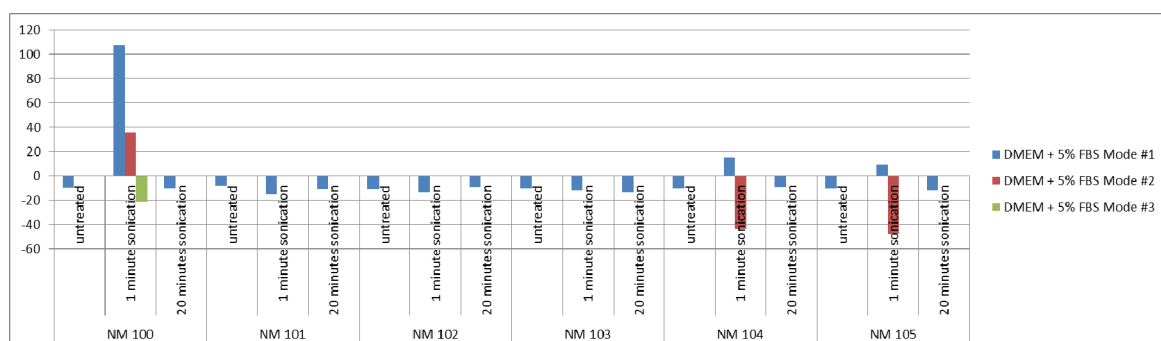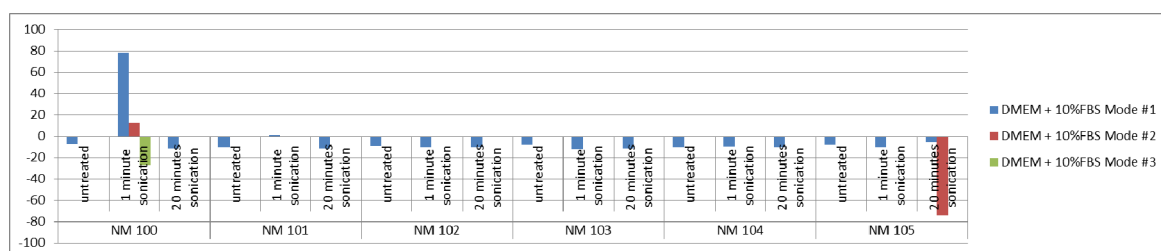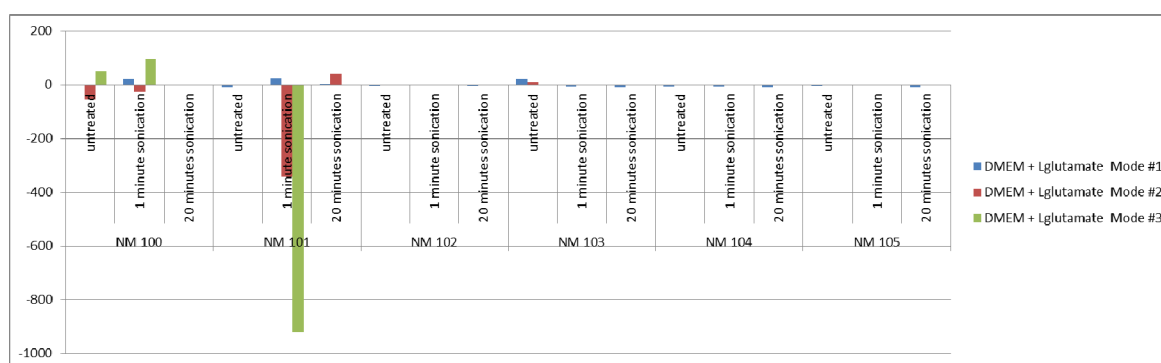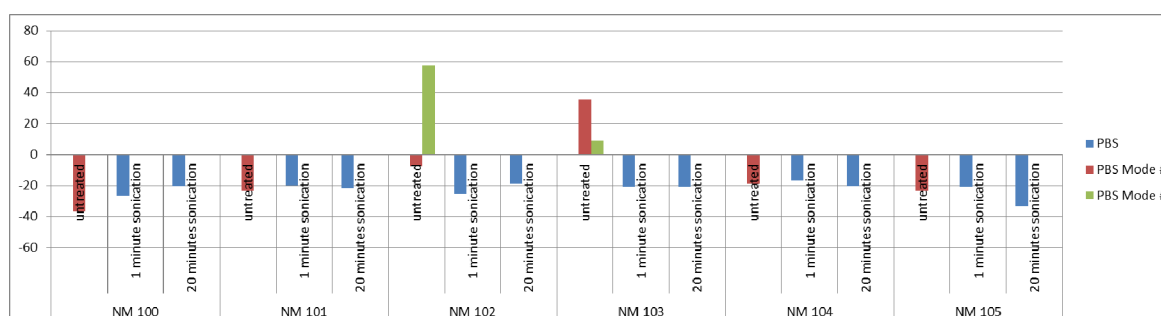
Figure A7.6. Zeta potential modes are reported per medium; different treatments (sonication times and types) are reported for each nanoform.

Figure A7.7 shows the modes reported for each nanoform, medium, and sonication treatment.
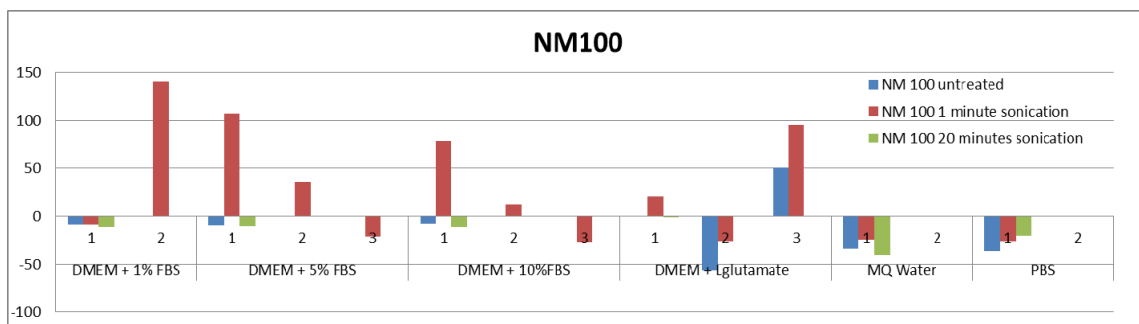
a)
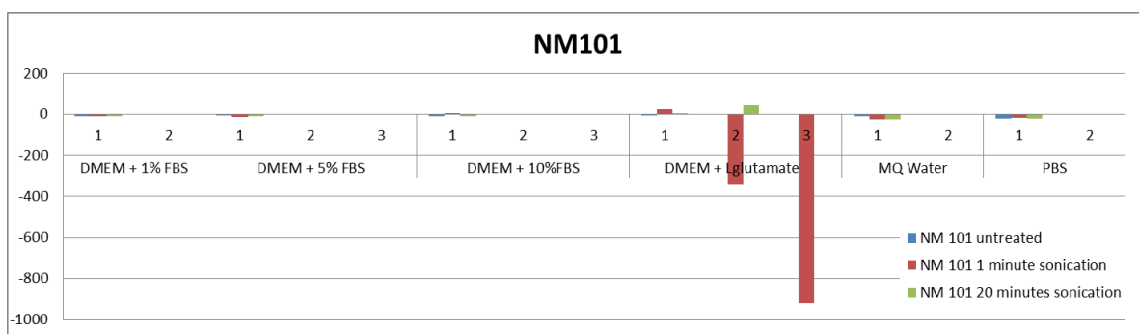
b)
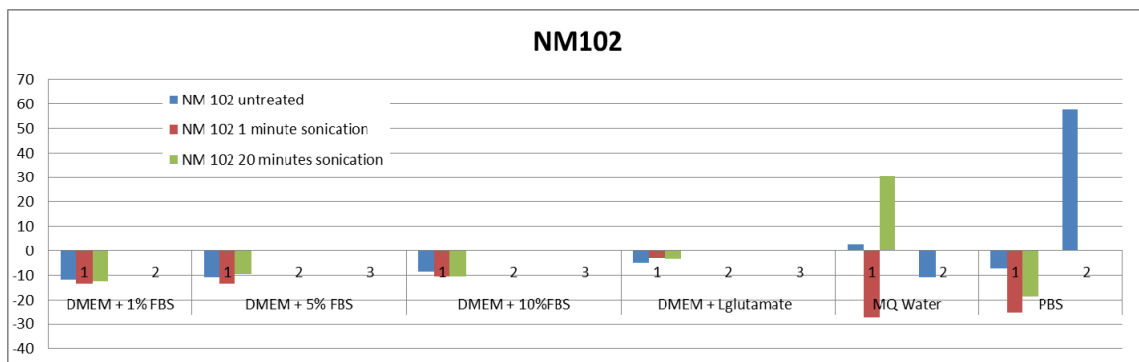
c)
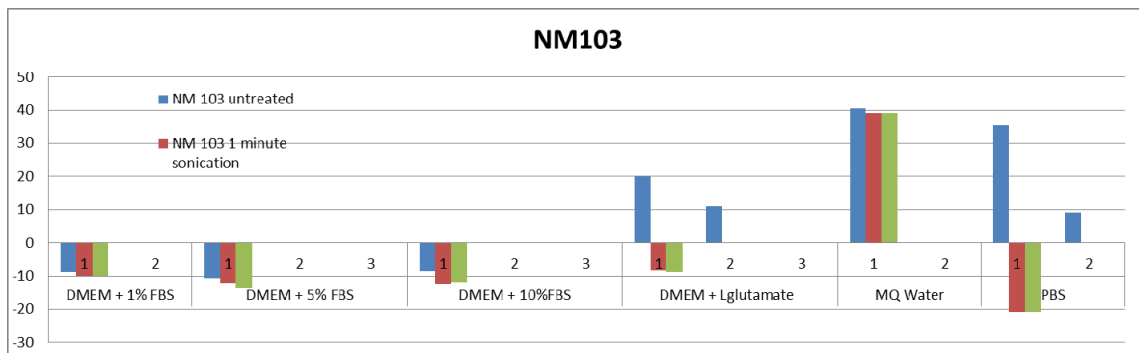
d)
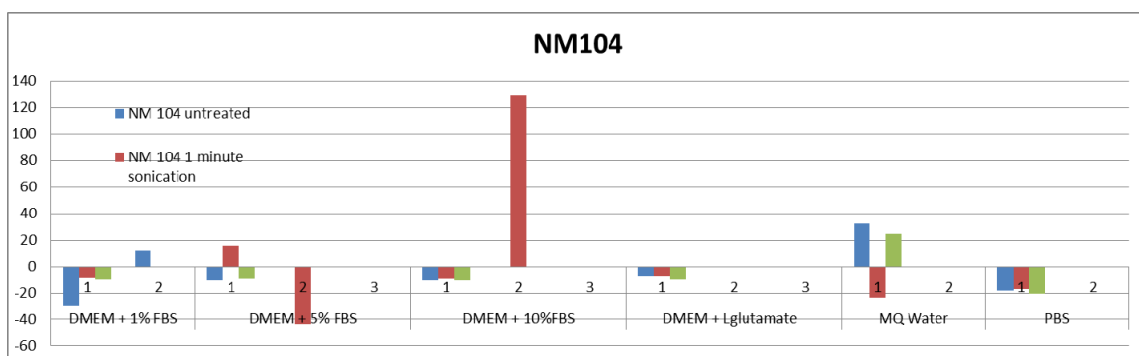
e)
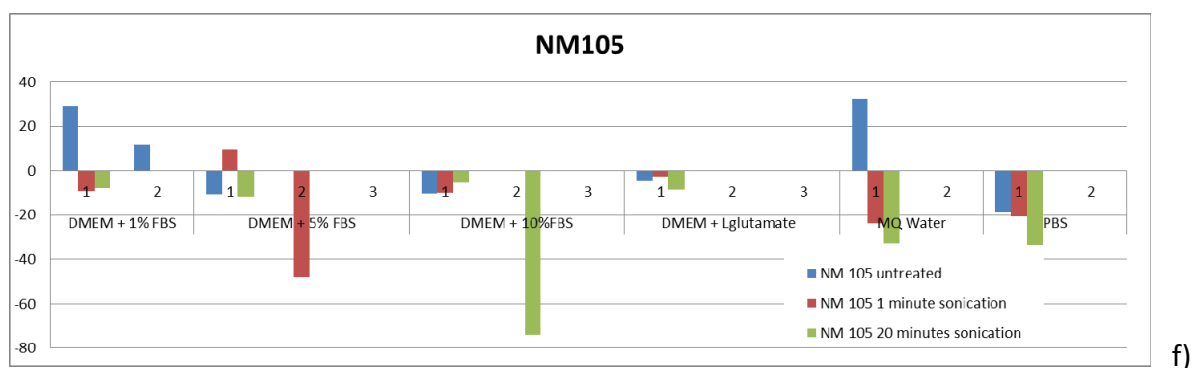
Figure A7.7. from a) to f) the modes are reported for each nanoform (NM-100 to NM-105) and for the different exposure media and tratments.

Figure A7.6 and Figure A7.7 shows that Zeta potential measures are not highly affected by treatment, medium or NM type. It is observed, though, that the Zeta potential measured in Milli-Q water is less stable (outside the stability interval -20<ζ<20) compared to the biological media.

Data on polydispersity index – PdI

PdI is a measure linked with the z-average measurement and it gives the idea of a monomodal/bimodal distribution. When the PdI<0.25 the size distribution is considered monomodal and z-average is calculated. When the PdI<0.8 the size distribution is considered bimodal and the Intensity main peak should be considered (Guiot et al., 2012). Figure A7.8 shows the values reported in the OECD dossier for this property. Considering the variability and the relevant range for identification of mono- or poly- dispersion, the resulting PdI per nanoform is too dependent on the media and treatments and, therefore, is not considered a reliable measure.

410
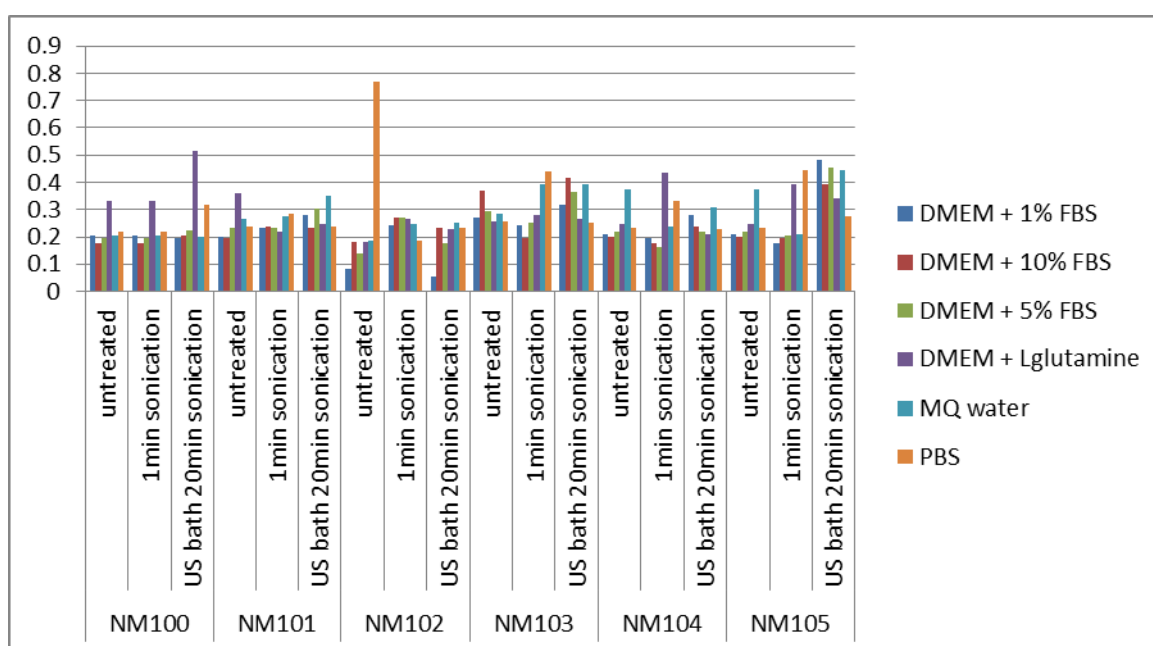
Figure A7.8. PdI for the NMs in different media and treatment (untreated, 1 min sonication, 20min sonication).

Data on biodurability

Biodurability of NMs is measured as the fraction of elements found in the media after 24h of incubation. Solubility of different elements in different media are reported below. Al concentration in NM-101 is due to the impurities (or not declared coating) whereas on NM-103-104 it is due to the coating. Si is also due to impurities in NM-105 and coating (NM-104).

a)

b)

c)
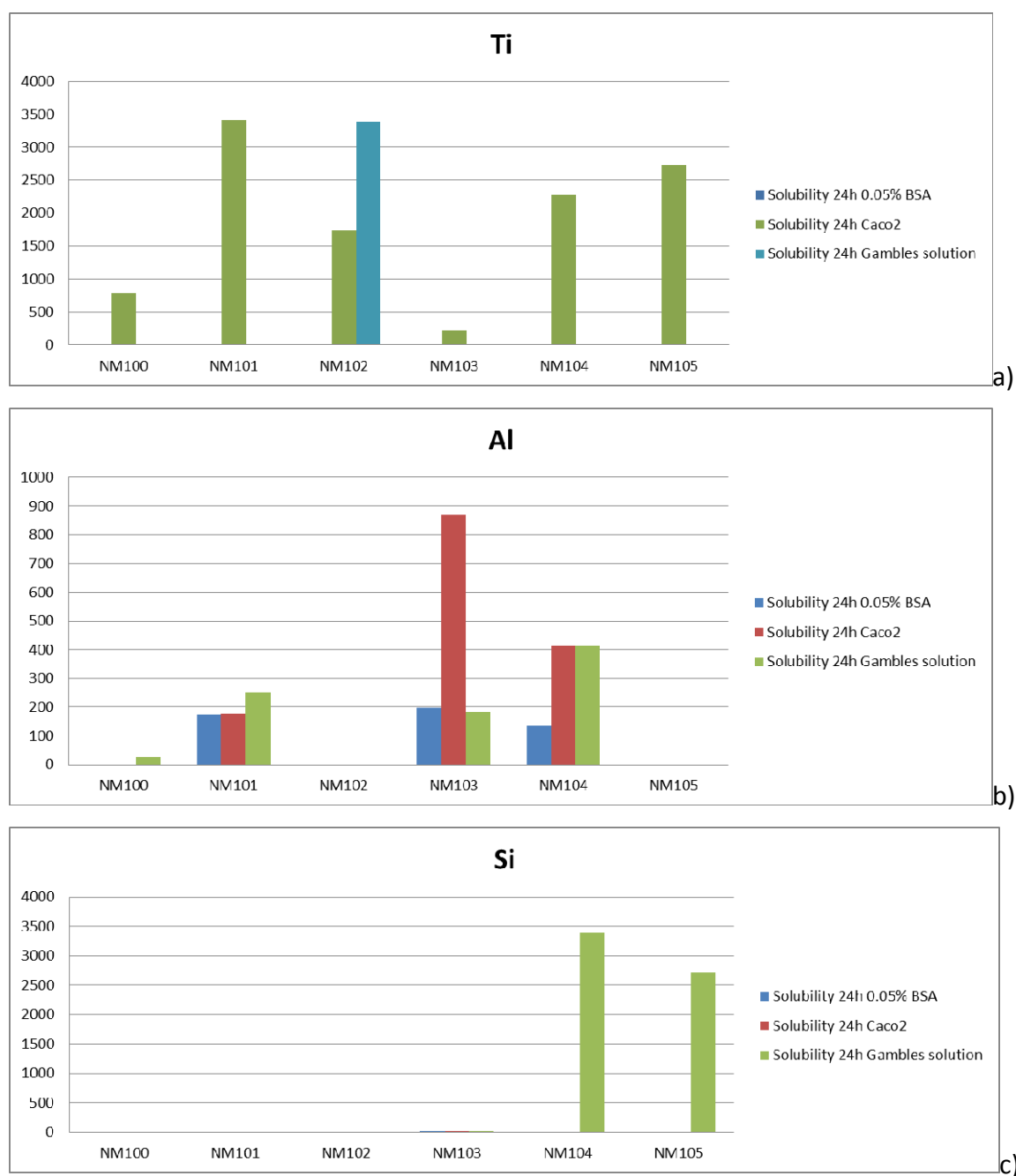
Figure A7.9. Elemental biodurability in different biological media; BSA: bovine serum albumin; Gambles solution: lung fluid; Caco2: Caco2 cell medium (acidic medium).

411

MDL			NM-100	σ	NM-101	σ	NM-102	σ	NM-103	σ	NM-104	σ	NM-105	σ
0.05% BSA														
1	mg/l	Si	-	-	-	-	-	-	0.9	1.3	-	-	-	-
30	µg/l	Al			175	49			198	116	137	25	-	-
5	µg/l	Ti	5.2	3.5	-	-	<	6.8	-	-	-	-	-	-
Gambles solution														
1	mg/l	Si	-	-	-	-	-	-	2.0	0.2	-	-	-	-
30	µg/l	Al	-	-	177	185	-	-	868	59	413	327	-	-
5	µg/l	Ti	-	-	-	-	3,388	3,900	-	-	-	-	-	-
Caco2													-	-
1	mg/l	Si	-	-	-	-	-	-	1.7	<	-	-	-	-
30	µg/l	Al	24	34	252	277	-	-	182	<	413	327	-	-
5	µg/l	Ti	796	2	3,414	1,683	1,741	683	222	337	3,386	3,900	2,724	3,846

MDL: Minimum detection limit in the raw analysis; - denotes not detected; < denotes background corrected concentration lower than 0.1 x MDL

Data on dustiness

Figure A7.10 shows two methods to report for dustiness, the small rotating drum (SD) and the vortex shaker (VS). Respirable and inhalable indexes[45] are measured. Only SD was considered for the respirable fraction as a measure for this property as it is given for all nanoforms. The other methods are lacking data for some of the NMs.
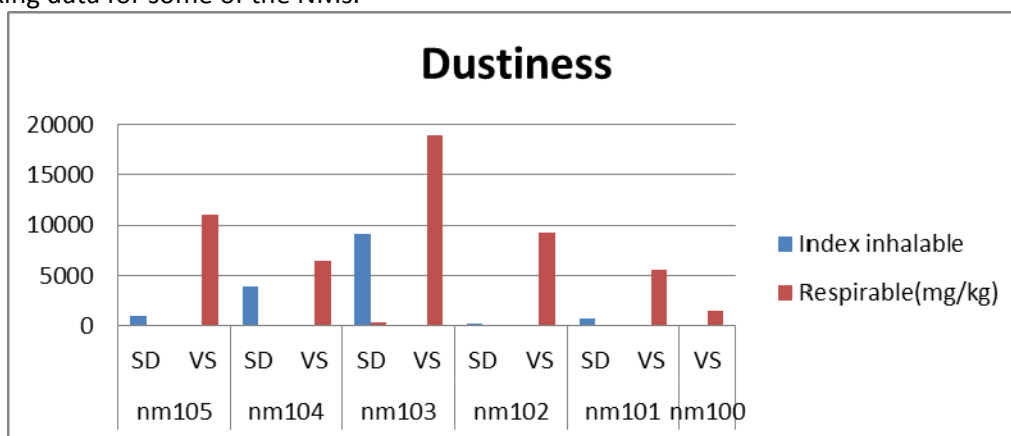
Figure A7.10. Dustiness measurement with vortex shaker (VS) and small rotating drum (SD).

Data on primary particle size

Figure A7.11 shows that the different methods used to measure primary particle size (TEM and USAX) give similar values for the small NMs. It shows that their values can be averaged.

---

[45] Inhalable index approximates to the fraction of airborne material which enters the nose and mouth during breathing and is therefore available for deposition in the respiratory tract; respirable index approximates to that fraction which penetrates to the gas exchange region of the lung (ISO 1995 Air Quality - Particle Size Fraction Definitions for Health-related Sampling ISO Standard 7708. International Organization for Standardization (ISO), Geneva.)
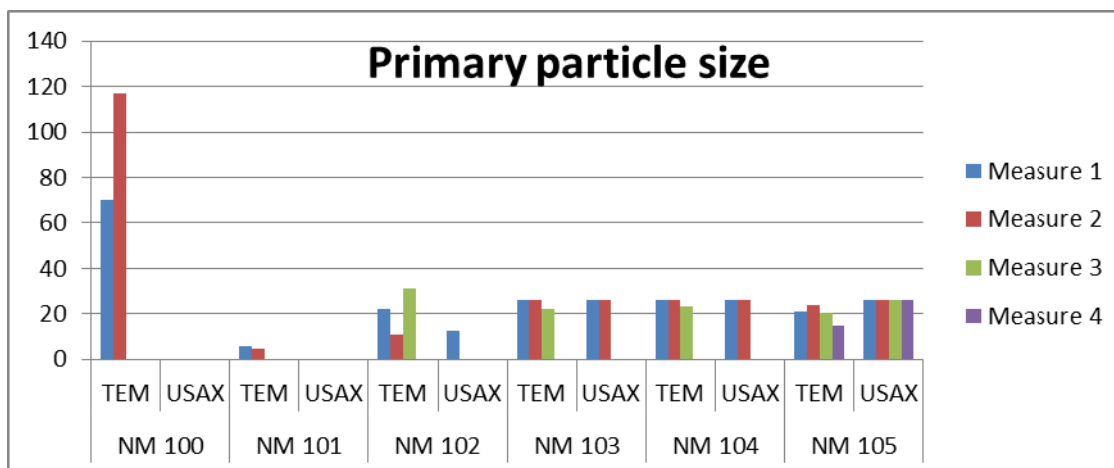
Figure A7.11. Primary particle size is measured through transmission electron microscopy (TEM) and ultra-small angle X-ray scattering (USAX).

Data on specific surface area (SSA)

Figure A7.12 shows that SSA measures with BET and USAX are in general similar, and therefore could be comparable. The smallest NM, NM-101, instead shows a higher variability.
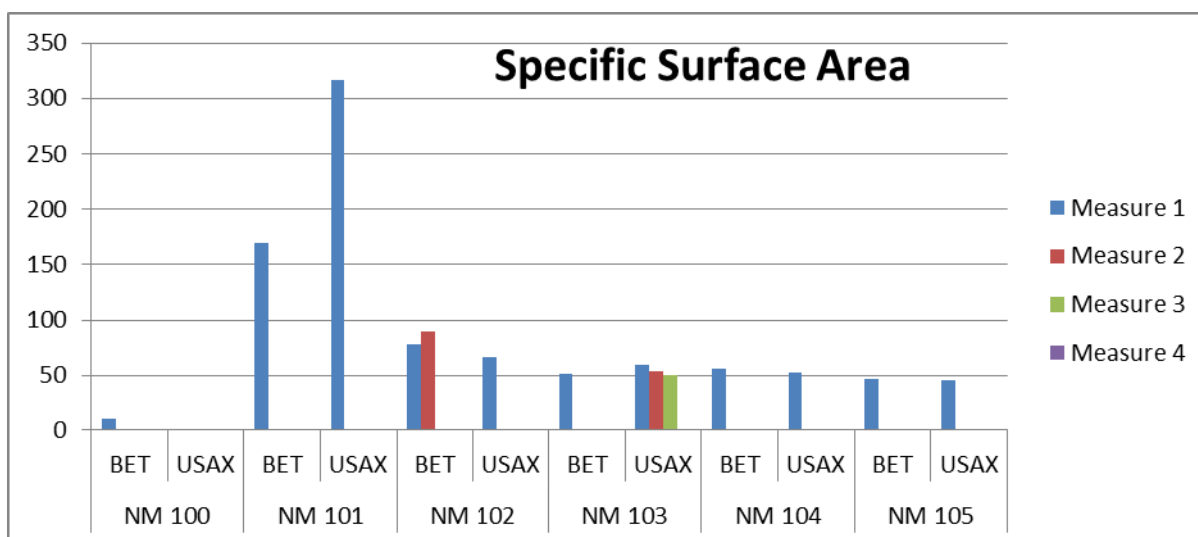
Figure A7.12. SSA measures with ultra-small angle X-ray scattering (USAX) and the Brunauer, Emmett and Teller method (BET)

Data on solubility

Solubility, intended as metal dissolved in media was considered as biodurability.

Data on total non-TiO2 content including coating and impurities

Figure A7.13 shows the elemental analysis (%w/w) of the NMs. These values correspond to impurities and coating, as they cannot be distinguished in the elemental analysis. The amounts of coating declared by the manufacturers (NM-103 and NM-104) are indicated in light blue.

413

The graph in Figure A7.13 is driven by the total amount of impurities, yielding the NM without coating at nearly 0. Only the amount of Al is significant with respect to other impurities and for NM-103 and NM-104.
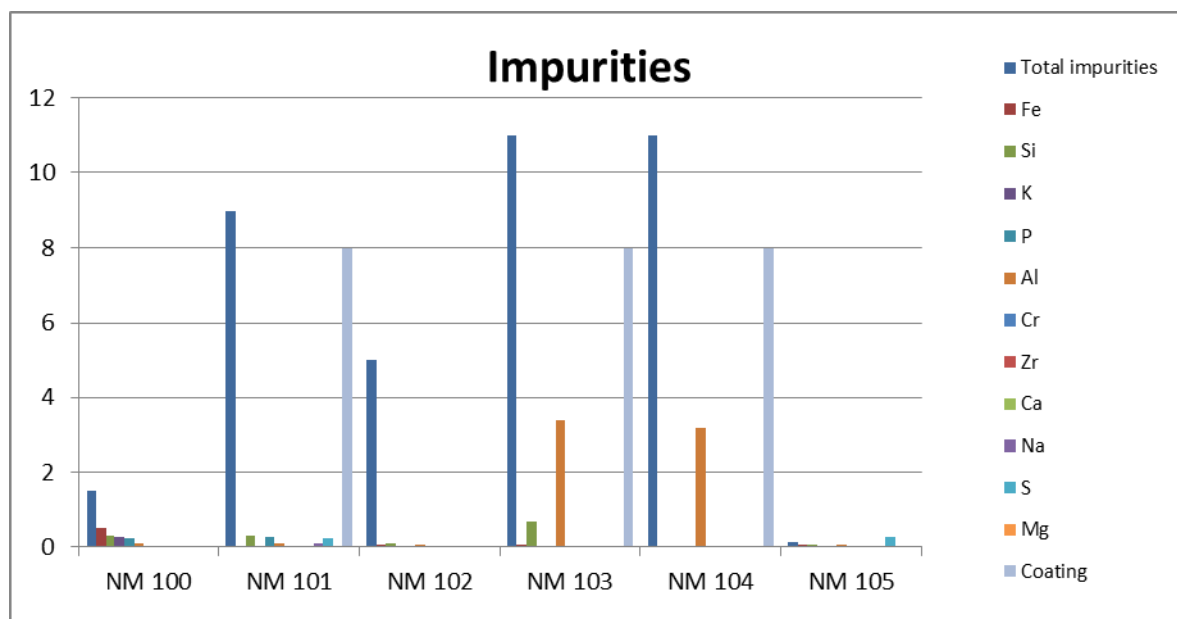
Figure A7.13. Elemental impurities for the different nanoforms (%w/w)

Data on crystallite size

Crystallite size is always measured with x-ray diffraction (XRD) and the measurements are not very variable, as shown in
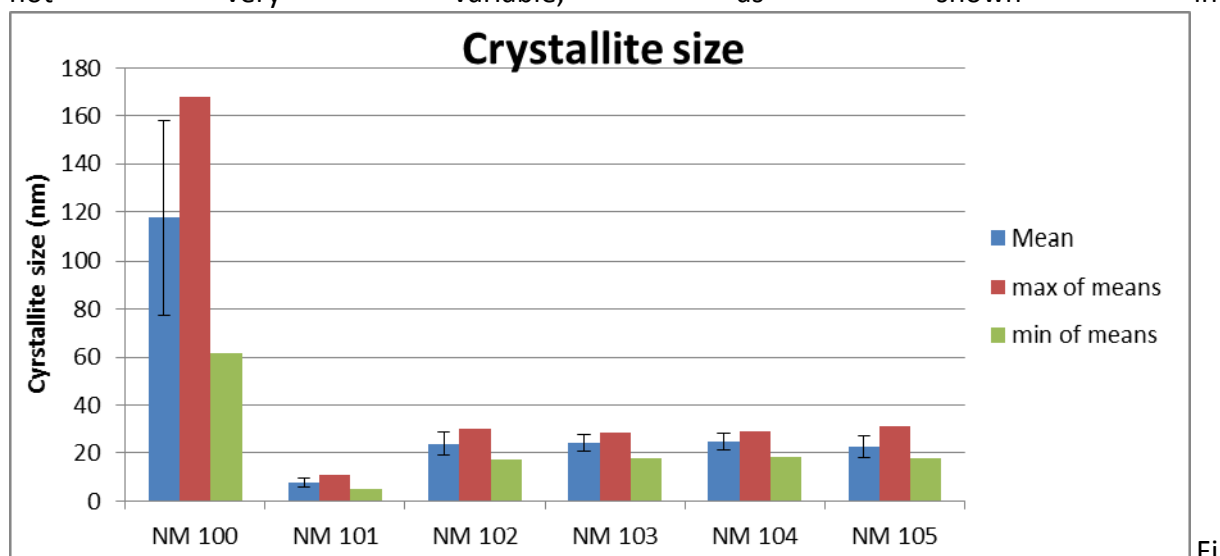
Figure A7.14. The mean will be considered in the data analysis.
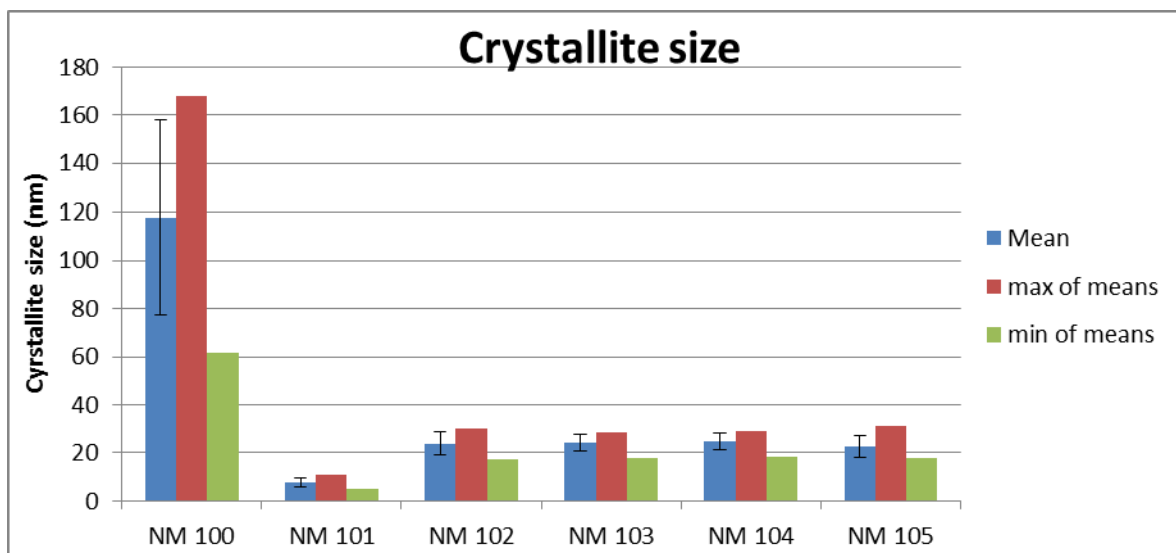
Figure A7.14. Crystallite size measured by XRD.

Data on porosity

Values for mean total pore volumes are presented in Figure A7.15.
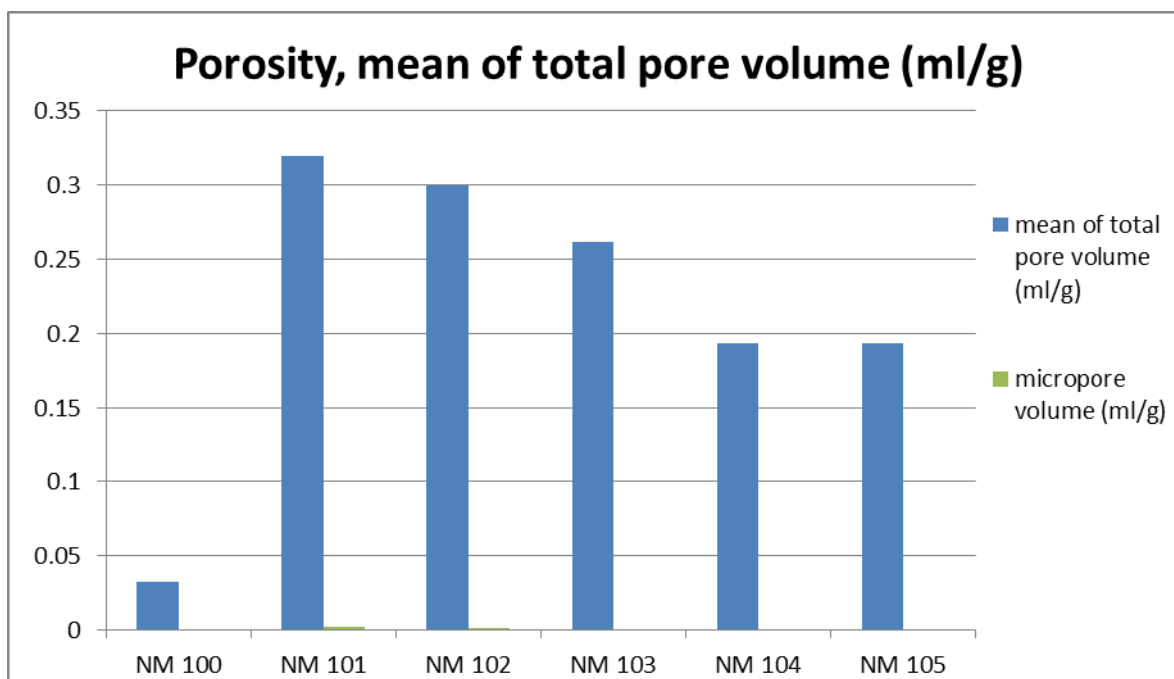
Figure A7.15. Porosity in the dataset is represented by mean of total pore volume.

Data on isoelectric point

Mean, maximum (max), and minimum (min) values are reported for isoelectric point in Figure A7.16. The measurement is not available for NM-100.

415
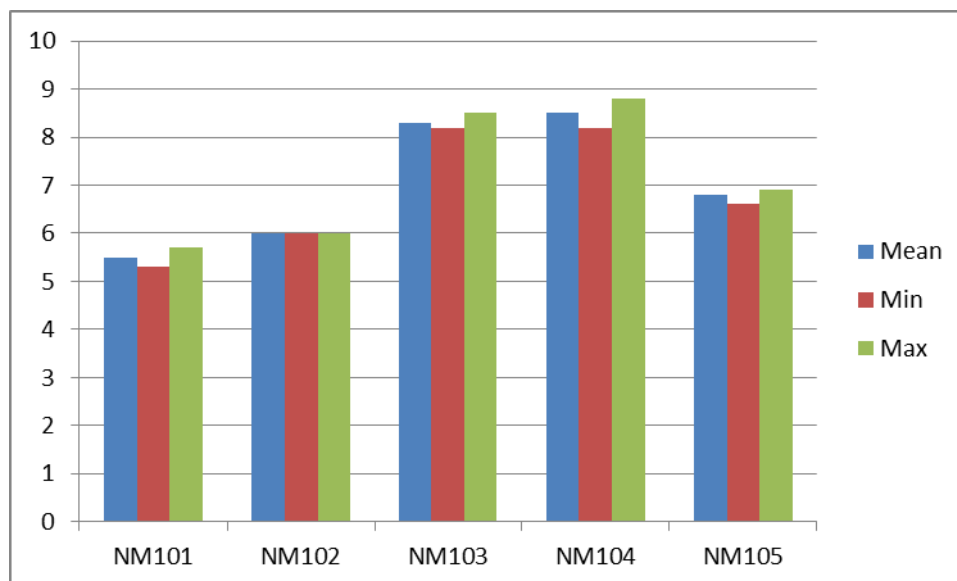
Figure A7.16. Isoelectric point.

Appendix IX. TiO$_2$ case study - data treatment

Two main problems arise when gathering data from already available sources: a) lack of data and b) availability of data generated with different methods, labs, technicians, or techniques. The latter situation is mitigated by the use of standard or guideline methods, which assures the comparability of the data and the mutual acceptance of data between administrations. In the case of nanomaterials, the reality is that there is lack of data and lack of guidelines and standards. This leads to a situation like the one that was encountered for nano-TiO$_2$, in which data obtained with two or more methods (e.g. EDS or ICP-OES for impurities, DLS and TEM for particle size), or data measured in different solvents (e.g. MQ water, DMEM + 1% FBS, DMEM + 5% FBS, DMEM + 10% FBS, PBS), or with different pre-treatments (e.g. not sonicated, 1min sonication with tip sonication, 20min bath sonication) are found and need to be merged. In such a situation, two options can be considered: a) each technique, instrument, media, and pre-treatment is considered as a different property or b) data from different origins is merged into a common value. Both options present advantages and disadvantages. Keeping each value as a different measure leads to a dataset plenty of data gaps that becomes unusable for modelling or read-across as the properties are not considered comparable and it becomes impossible to compare two substances. Instead, merging data from different techniques, studies, or pre-treatments enables the comparison of different substances but raises questions like: what is the best way to combine the data? (e.g. average, median, selection of a representative value), and more importantly how much will variability be affected by the inclusion of these data?

These questions do not have an easy answer and especially in a situation with a relatively low number of values (<5), as is the case of nano-TiO$_2$. Moreover, in many situations there are not right or wrong options.

In this section the way in which the nano-TiO$_2$ data that was obtained from different sources was merged into single values will be explained. This section is not intended to provide guidance on how to merge data from different sources, but rather to show the problems one may encounter when building a matrix of data to read-across and also to show how this step was carried out in the present report in order to render the read-across case study more transparent.

Compilation of the initial dataset

In order to compile all the available data for the case study a dataset for each nanoform (NM-100 –> NM-105) was created. The data was gathered in an excel file with columns defining properties (see Figure 17) such as impurities (Al, Si, P, etc.), crystal type, crystal size, surface coating, primary particle diameter, particle size distribution, etc., and rows corresponding to different entries of data.

Study	Source	Reference (Author, year, doi)	Method	um	Study guideline (OECD/US EPA) or remarks	Remarks	Total impurities	Impurity 1	Quantity1	Impurity 2	Quantity2	Impurity 3
Impurities	NanoGenotox D4.3	-	EDS	ppm	None	Table 5.5 (same as OECD Dossier)	2000	Al	500	Si	800	P
Impurities	NanoGenotox D4.3	-	ICP-OES	µg/g	SOP proposed by the project	Table 5.8	120	Al	-	Si	-	P
Impurities	NanoGenotox D4.7	-	Not reported	%	None	Table 2.1 (reported by suppliers)	5					

Figure 17. Example of the dataset created for NM-102. The first columns (pink) correspond to metadata and linkers to the data source, and the rest of columns to the data obtained.

The columns of the dataset were defined on-the-fly as the data was being processed, and the data type was defined by the type of data that was available in the sources. For instance, since the particle size diameter section was obtained from SAXS/USAXS data, the following columns were registered:

- Mean particle diameter (nm)

- Gyration radius of primaries (nm)

- Gyration radius of aggregates(nm)

- Fractal dimension

- Number particles/aggregate

- Aggregated diameter

Similarly, the particle size distribution section contained columns for the measures in each media, e.g. PBS, DMEM + X% FBS, MQ Water, etc.

Each row of the dataset corresponded to data obtained from one test. For instance, all impurities measured with EDS were inputted in the first row under the corresponding columns. Each new source of data was inputted as a new row. In case the same type of data was found in a different source, the data was inputted in a new row but under the same column that was created previously. In order to keep track of the sources of the data, the first columns of the dataset were dedicated to store metadata (see Figure 17) such as the test method (e.g. EDS, DLS, XRD, BET), source of data (e.g. Nanogenotox deliverable D4.3), units of the data (e.g. nm, %, ppm, µg/g), guideline used, or remarks.

This task yielded a dataset that was mostly empty as each new entry added a new row but only the property of interest was filled with data and the rest are left blank. The initial dataset of NM-102 was comprised of 7260 cells (44 rows x 165 columns), 464 of which contained some data, i.e. 6%.

Reducing the initial dataset to a single row of data

The next step to obtain a dataset for read-across that could be used, consisted of merging the data available in the columns into a row of data, which means that each column was reduced to a single value.

Impurities

Data on impurities was obtained from two different instruments, EDS and ICP-OES. In most cases, both instruments detected different atoms and there was no need to merge the data. However, for some cases like Impurity of K of NM-100 two values were present, 2500ppm (EDS) and 1000 µg/g (ICP-OES). In this case, the different precision of the instruments was not considered. Instead, the conservative principle was applied and only the largest value was considered, i.e. 2500ppm.

The same strategy was used for all impurities.

Crystal type

This data corresponded to the percentage of crystal. The decision for most of the NMs was not complicated as they were measured as 100% anatase or 100% rutile. Only for NM-105 two measures from different labs using the same technique were obtained, one with 86% anatase and the other one 81% anatase. It was decided to average the two values giving a final value of 83.68% of anatase and 16.32% of rutile.

Crystallite size

Crystallite size is one of the problematic measures because crystallite sizes are measured with XRD but different algorithms can be used to determine the sizes (see Nanogenotox 4.3 pag. 14 for further information), e.g. Scherrer equation, Peak fit FWHM, Topas, Fullprof. In addition, measures from three different labs were obtained for this property. The measures obtained by the different groups and algorithms are shown in Figure 18.

	NM100[€]	NM101	NM102	NM103	NM104	NM105 (Anatase)
Supplier information	200 - 220	< 10	-	20	20	21
IMC-BAS Peak fit	57	5	18	-	19	18
IMC-BAS TOPAS	62	5	16	19	20	18
IMC-BAS Fullprof	168	7	18	20	18	19
NRCWE Scherrer eq.	> 100	7	23	26	27	27
NRCWE TOPAS, IB	> 100	7	26	25	25	27
NRCWE TOPAS, FWHM	> 100	10	28	28	29	31
LNE Scherrer eq.	141	-	30	18	23	23

[€] Size-data not reliable due to large crystallite size.

Figure 18. Crystallite size measures obtained from the Nanogenotox report for TiO_2

The crystallite size measures for the small NMs, i.e. NM-101 - NM-105 showed a rather low variability independently of the laboratory or methods used to determine it. But some cases showed unexpected differences: For instance the laboratories IMC-BAS and NRCWE used the same algorithms and instrument to measure the "same" NMs, but the measures obtained by NRCWE were systematically larger than those of IMC-BAS, ~25% larger. The case of NM-102 is significant as although the larger size difference is only of 12nm, it is with respect to a measure of 18-30nm, which represents more than 50% variation. The authors acknowledge these differences and state that most of them can be explained by the differences of instrumental performance, which has an estimated standard deviation of 5 nm.

Different is the case of NM-100, whose values were rather variable (from 57-168 nm). The reason for this is probably the fact that XRD is not suitable to measure crystals of more than 100 nm as the authors stated in the report. In addition, the producers indicate a size of 200-220 nm, but one of the measures determined a crystal size of 57 nm. Therefore, it was difficult to determine what value was the right one. Like in most cases, the truth probably was somewhere in between the extremes. It is worth mentioning that NM-100

419

corresponded to a dry milled NM what could explain the presence of particles of different sizes.

When dealing with samples with large variability or having extreme values, it is usually advisable to use the median of these values. The median of the measures obtained for NM-100 was 120 nm. However, since the average was 117 nm and the average was used for the rest of NMs, it was considered appropriate to use for NM-100 the average value of 117 nm.

Surface chemistry

Surface chemistry is per se a difficult category because of the "legal" definition of surface chemistry and coating and the tests that are used to determine it. Surface chemistry and coating are defined as substances that were intentionally added to the surface of the nanomaterial and therefore it depends on the declaration of the manufacturer (see Figure 19). NM surface chemistry is usually determined by the w/w percentage of each of the constituents of the NM. The method used to determine the surface chemistry of NMs is usually Thermogravimetric analysis, which consists of the monitoring of the weight of the sample while increasing the temperature up to 1000 ºC. The thermogravimetric analysis is usually complemented with an elemental composition analysis that is carried out after calcination. As it stands, the thermogravimetric analysis cannot distinguish between substances that were intentionally added to the surface and those that were not intentionally added.

Code	impurity / coating
NM-100	-
NM-101	9%*
NM-102	-
NM-103	Al2O3 6%, silicone - Dimethicone 2%
NM-104	Al2O3 6% - Dimethicone 2%
NM-105	-

Figure 19. Impurities/coating of nano-TiO_2 as declared by manufacturers. * Indicates measures not declared by the producer

In the current case study, 2 NMs (NM-103 and NM-104) were declared as coated with Al_2O_3 and silicon dimethicone, and the rest of NMs were declared uncoated by the manufacturers.

As expected, the thermogravimetric analysis of NM-103 and NM-104 showed a mass loss that matched the declaration of coating by the producer, but NM-101 showed an unexpected similar mass loss (~8%) at the same temperature and of the same composition as NM-103 and NM-104. Since the raising of the temperature calcinates both surface coating and impurities, it is impossible to know if the mass loss of NM-101 corresponded to impurities or to undeclared coating. Therefore, since NM-101 was declared uncoated, the mass loss cannot be considered as surface chemistry but has to be considered as impurities.

In order to indicate this difference, a row consisting of "organic matter" impurities was added to the data matrix (see Table 5). Similarly, a row consisting of the sum of all non-TiO_2 content, which corresponded to the sum of surface chemistry and impurities was also added to the matrix.

Table 5 Data used in the grouping hypothesis and read-across of comet assay results of nano-TiO$_2$.

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
In vitro comet assay	**1**	**0**	**1**	**0**	**0**	**1**
Total non-TiO$_2$ content including coating and impurities (% w/w)	1.5	9	5	11	11	0.11
Surface coating (declared) (%)	0	0	0	8	8	0
Organic matter (%)	0	8	0	2	2	0
Impurity(% w/w Fe)	0.49	0	0.07	0.06	0	0.06
Impurity(% w/w Si)	0.28	0.29	0.08	0.68	0.018	0.07
Impurity(% w/w K)	0.25	0	0.001	0.001	0.001	0
Impurity(% w/w P)	0.21	0.27	0.001	0	0	0
Impurity – coating (% w/w Al)	0.09	0.09	0.05	3.4	3.2	0.04
Impurity(% w/w Cr)	0.03	0	0	0	0	0
Impurity(% w/w Zr)	0.005	0.01	0.005	0.001	0.001	0
Impurity(% w/w Ca)	0.001	0	0.005	0.005	0.01	0
Impurity(% w/w Na)	0.001	0.1	0.001	0.01	0	0.001
Impurity(% w/w S)	0	0.22	0.001	0.01	0.01	0.26
Impurity(% w/w Mg)	0	0	0	0.001	0.001	0

Primary particle diameter

Data from different sources and methods were obtained for the primary particle diameter. The most common technique to determine the primary particle size is TEM. In case different values from TEM were obtained, the average value was considered. In the present case study, however, data from SAXS/USAXS corresponding to the radius of gyration of primary particles was also found. But since data for NM-100 and NM-101 were missing, it was decided not to consider the data from SAXS/USAXS due to data gaps and keep only the TEM related data as data was available for all NMs.

Particle size distribution

Data on the particle size distribution and aggregation/agglomeration was gathered for the NMs from: a) different sources, b) different methods (DLS and PCS), c) different media (e.g. MQ Water, PBS, DMEM + 1-10% FBS, DMEM + L-Glutamine), d) different treatments (unsonicated, 1min probe-sonication, 20min bath sonication). In addition, different measures (e.g. Zeta-size, Intensity distribution main peak, and FWHM main peak) were obtained for some of the NMs, only.

Since NM-100 and NM-101 were missing data for the Intensity distribution main peak and FWHM main peak, these parameters were not considered in the analysis.

Data in different media and treatment

Data for DLS measured as Z average was averaged between the different sources. Instead, the data obtained from PCS in different solvents and treatments was treated individually. Figure 20 shows a snapshot of the data that was obtained for NM-102 from the OECD dossiers. The table contains different measures of particle size distribution, size, and Z-average with different solvents and treatments. Additionally, the data shows that measures such as the untreated sample in MQ water are bimodal as it contains sizes of 1115nm and

5170nm. In some cases, 3 modes were detected. This problem is reproduced in the corresponding measures of PdI and Zeta Potential. The differences of particle sizes indicate that the NMs agglomerate in a different way depending on the media and sonication treatment. In general they cannot be averaged between them unless a common behavior is observed. For instance, NM-102 shows very similar sizes in DMEM + 1,5,10% FBS as they correspond to 1415, 1414 and 1521 nm. However, NM-101 shows a bimodal of 1201/5232 for DMEM + 1% FBS, 1272 and 1406 for DMEM + 5 and 10% FBS, respectively. This shows that it is impossible to use a common merging technique and that the data needs to be treated individually.

NM102	0,4 mg/ml TiO2 particles (NM102, 22 nm, anatase, PC105) untreated					
Medium	Size (d.nm)	Z-Average (d.nm)	PdI	Zeta Potential (mV)	monomodal	Zeta Deviation
MQ Wasser	1115*/5170	1062	0,187	2,73*/-11,0		8,86
PBS	1528*/5330	1579	0,769	-7,21*/-57,5	-22,9	31,5
DMEM + L-Glutamine	2745	2427	0,181	-4,92	-5,77	25,2
DMEM + 1% FBS	1415	1295	0,081	-11,9	-10,8	37,2
DMEM + 5% FBS	1414	1234	0,139	-11		13
DMEM + 10% FBS	1521	1227	0,182	-8,69		17,7
0,4 mg/ml TiO2 particles (NM102, 22 nm, anatase, PC105) 1 min Sonifier (40% Amplitude)						
Medium	Size (d.nm)	Z-Average (d.nm)	PdI	Zeta Potential (mV)	monomodal	Zeta Deviation
MQ Wasser	703*/126,9	505,7	0,248	-27,1		7,29
PBS	2525	2079	0,188	-25,1		12,8
DMEM + L-Glutamine	3488*/543,7	2701	0,268	-3,14		9,89
DMEM + 1% FBS	837,5*/189,8	590	0,243	-13,6		20,4
DMEM + 5% FBS	901,8*/115,6	617,3	0,27	-13,4	-11,5	25,3
DMEM + 10% FBS	1077	732,2	0,27	-10,5		13,5
0,4 mg/ml TiO2 particles (NM102, 22 nm, anatase, PC105) 20 min US-bath						
Medium	Size (d.nm)	Z-Average (d.nm)	PdI	Zeta Potential (mV)	monomodal	Zeta Deviation
MQ Wasser	1103*/193,6	794,1	0,254	30,3		9,37
PBS	1789*/4988	1809	0,231	-18,5		15,6
DMEM + L-Glutamine	2001*/5517	1997	0,227	-3,46		14,9
DMEM + 1% FBS	1063	975,4	0,054	-12,4		17,6
DMEM + 5% FBS	1487	1197	0,179	-9,47		12
DMEM + 10% FBS	1228	874,8	0,235	-10,4		19,5

Figure 20. Particle size distribution data obtained for NM-102 from the OECD Dossiers.

An additional problem is the fact that not all media and treatments show bimodal or trimodal distributions. In order for the computer to be able to process the data, it was decided to include a row of data for each mode. In order to avoid blanks for the modes 2 and 3 of the monomodal cases, the value obtained was copied to mode 2 and mode 3. An example of the final data matrix is shown in **Table 6**

Table 6 Particle size distribution data in different media and treatments. Values in red indicate that the distribution was monomodal and that the same value was used for mode 1 and mode 2.

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
Particle size distribution in MQ Water, untreated, Mode #1 (nm)	391.2	1609	115	973.2	727.8	1102
Particle size distribution in MQ Water, untreated, Mode #2 (nm)	4862	1609	5170	973.2	727.8	204.7
Particle size distribution in PBS, untreated, Mode #1 (nm)	1440	1188	1528	1977	1817	4526
Particle size distribution in PBS, untreated, Mode #2 (nm)	5236	5148	5330	1977	5194	1150
Particle size distribution in DMEM + Lglutamate, untreated, Mode #1 (nm)	995.5	1438	2745	2255	3059	1881
Particle size distribution in DMEM + Lglutamate, untreated, Mode #2 (nm)	995.5	5560	2745	2255	3059	5372
Particle size distribution in DMEM + 1% FBS, untreated, Mode #1 (nm)	736	1201	1415	1040	1156	2454
Particle size distribution in DMEM + 1% FBS, untreated, Mode #2 (nm)	736	5232	1415	4593	5211	626.5

This strategy allowed the inclusion of all data but rendered a dataset with a number of particle size distribution values (54 for each NM). It is obvious that having 54 values for the particle size distribution of a NM is unnecessary and does not provide relevant information. In addition, it highly biases the dataset towards particle size distribution as it corresponds to more than 1/3 of the total number of physicochemical variables. In order to reduce the dimensionality of the particle size distribution part of the dataset, hierarchical clustering of the transposed dataset was used to find "similar" combinations of media-treatments. Figure 21 shows the resulting clustering with the values that were randomly selected from each cluster.

Such a dimensionality reduction is not expected to be representative of the original data or an example to follow to reduce the dimensionality. Since it was deemed necessary to reduce the weight of the particle size distribution part of dataset and there was no apparent rational, the solution of the hierarchical clustering and random selection was chosen as an objective way of doing it. The use of the hierarchical clustering assured that the measures chosen did not show similar behavior and contributed to capture differences between NMs.
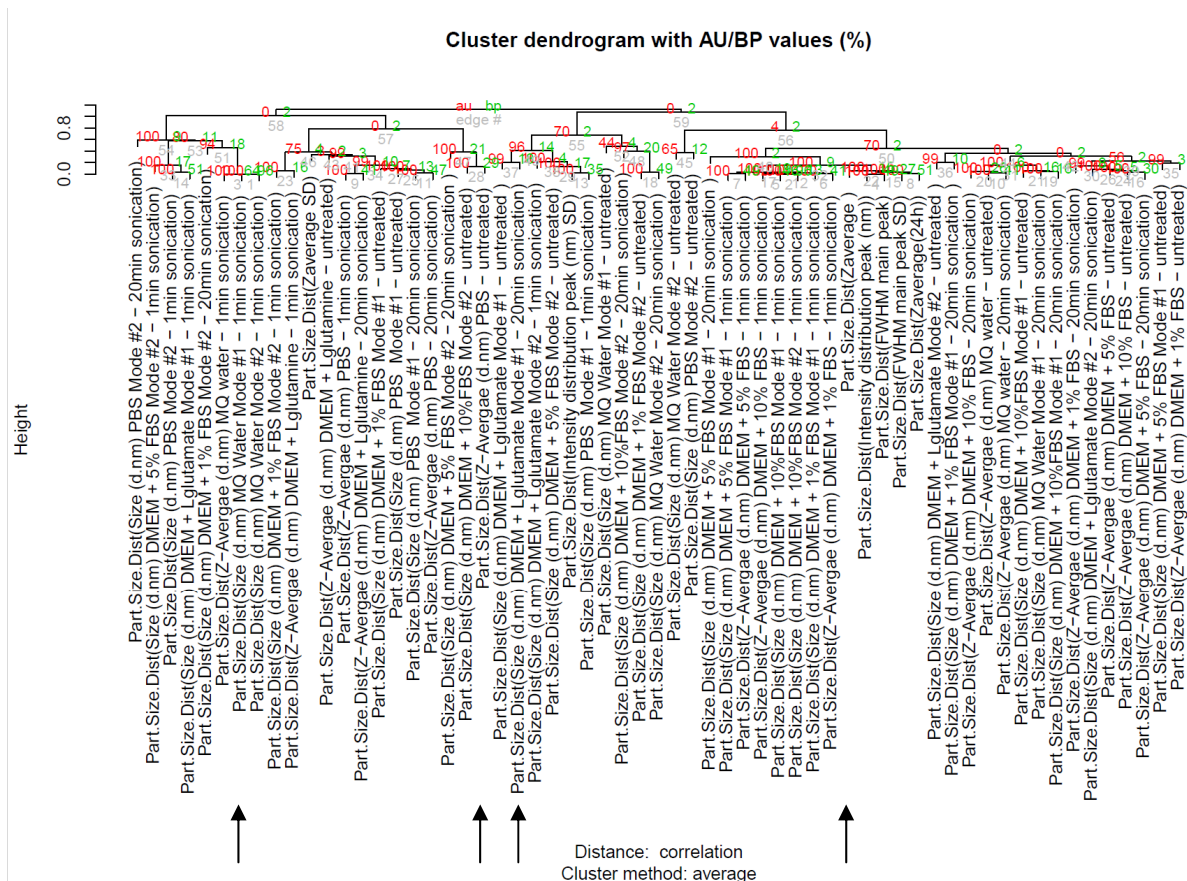
Figure 21. Hierarchical clustering of the transposed matrix of particle size distribution measures obtained for the nano-TiO$_2$. Arrows indicate the medium-treatment properties that were randomly selected from each cluster as representative.

Zeta Potential

Since the Zeta Potential data is obtained with the same instrument and test as the particle size distribution and PdI, the reader is referred to the particle size distribution section for further details. The hierarchical clustering of the transposed matrix of Zeta Potential with the randomly selected properties for each cluster is shown below.
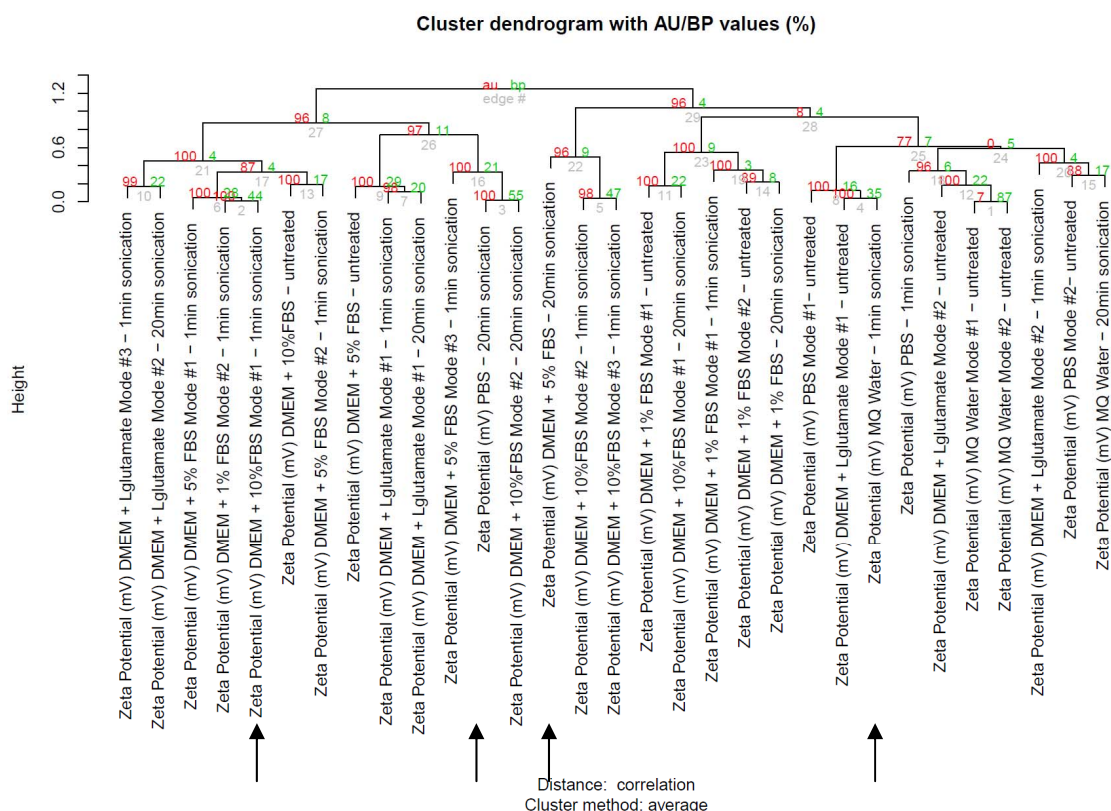
Figure 22. Hierarchical clustering of the transposed matrix of Zeta Potential measures obtained for the nano-TiO$_2$. Arrows indicate the medium-treatment values that were randomly selected for each cluster as representative.

Polydispersibility Index (PdI)

Since the PdI data is obtained with the same instrument and test as the particle size distribution, the reader is referred to the particle size distribution section for further details. The hierarchical clustering of the transposed matrix of PdI with the randomly selected properties for each cluster is shown below.

Figure 23. Hierarchical clustering of the transposed matrix of polidispersibility index measures obtained for the nano-TiO₂. Arrows indicate the medium-treatment values that were randomly selected for each cluster as representative.

Isoelectric point

Isoelectric point corresponds to the pH-value at which the charge on the surface of the NM, i.e. Zeta Potential, is reversed. The data gathered was generated in different media (water and 0.1mM CaCl$_2$), different conditions (acid-to-basic and basic-to-acid) and was found in three different sources (Nanogenotox, OECD dossiers, and in Cotogno et al. 2014). Some sources provided the exact values while in others the isoelectric point was given as an interval. In addition, no isoelectric point data could be found for NM-100. In order to take into account all these variations it was decided to consider the mean, minimum, and maximum isoelectric point data found for the NMs. This way, 3 rows with data relative to isoelectric point were added to the data matrix (see Table 7).

Table 7. Isoelectric point data included in the final dataset

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
Isoelectric Point (Mean) (pH)	NA	5.5	6.0	8.3	8.5	6.8
Isoelectric Point (Min) (pH)	NA	5.3	6.0	8.2	8.2	6.6
Isoelectric Point (Max) (pH)	NA	5.7	6	8.5	8.8	6.9

The fact that the nano-TiO$_2$ dispersions appear to be unstable at pH 5-9 and that in vitro experiments are generally carried out at pH 7.4 indicate that the dispersions will tend to sediment, which might affect the results.

Density

Density data was obtained from the OECD dossiers and the Nanogenotox project. Different methods were used to measure the density. Some of them declared the temperature at which the measure was carried out while others did not. Most of the methods rendered values of the order of 4.1 g/cm^3 but two measures corresponded to 0.5-1.2 g/cm^3 and 0.500-0.75 g/cm^3, the latter corresponding to *bulk density*. Since these two values were considered to correspond to "bulk density", they were discarded and the rest of the data was averaged.

Porosity

Data on porosity was obtained solely from a single source and, therefore, there was no problem with the data treatment. Three different measures were found: total pore volume (ml/g), micro surface area (m2/g), and micropore volume (ml/g).

Specific surface area

The data gathered for specific surface area corresponded to two different methods, SAXS and BET. The authors of Nanogenotox compared the two methods and observed a good agreement for values up to 130 m^2/g. The values obtained for the same NM that were smaller than 130 m^2/g were averaged. In the current dataset, only NM-101 was found to have values larger than 130 m^2/g (316 and 169.5 m^2/g for BET and SAXS, respectively). In such a situation, it is not recommended to average the values as they are not comparable, and the BET should be preferred.

Dustiness (respirable)

Data on dustiness was obtained from the Nanogenotox project. Two methods, small rotating drum and the vortex shaker, were used and different parameters were measured, e.g. number of particles in 180s or 3600s, inhalable dustiness index, and respirable dustiness index. The authors compared the two methods and observed no significant correlation between them. In addition, they acknowledged that dustiness values depended on the characteristic properties of the powders and the activation energy in the simulated handling. Therefore different values may be obtained by the different test methods (test apparatus, operation procedure, sampling and measurement strategy, etc.). In the current case, only respirable dustiness index measured by small rotating drum was considered in the read-across because it was found to be the only method with data for all NMs.

Biodurability

Data on biodurability corresponds to the amount of Ti, Al, and Si present in media after 24h of incubation of the NMs. Data on different media, i.e. water, bovine serum albumin 0.05%, Gambles solution, and Caco2 media was collected; but the data on water was discarded because all values were considered insoluble and data was not available for NM-100. The measures that were below the detection limit of the instrument were considered to be 0.

Redox potential

The redox potential of NMs corresponds to the variation of O$_2$ in the media during incubation. The data obtained from Nanogenotox was mainly qualitative and was

transformed into 1 for oxidative behavior, -1 for reductive behavior, and 0 for neutral. Data in 3 different media (BSA, Gambles, and Caco2) was obtained.

Appendix X. TiO$_2$ case study. Dataset for clustering, PCA, and variable selection (random forest)

Table A8.1 reports the full dataset considered in the data treatment (clustering, principal component analysis, random forest reported in paragraph 4.4.5).

Table A8.1. The full dataset. Data analysis is reported in *Appendix VIII. TiO2 case study - physicochemical data* analysis**Error! Reference source not found.**.

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
In vitro comet assay	**1**	**0**	**1**	**0**	**0**	**1**
Total non-TiO$_2$ content including coating and impurities (% w/w)	1.5	9	5	11	11	0.11
Impurity (% w/w Fe)	0.49	0	0.07	0.06	0	0.06
Impurity (% w/w Si)	0.28	0.29	0.08	0.68	0.018	0.07
Impurity (% w/w K)	0.25	0	0.001	0.001	0.001	0
Impurity (% w/w P)	0.21	0.27	0.001	0	0	0
Impurity – coating (% w/w Al)	0.09	0.09	0.05	3.4	3.2	0.04
Impurity (% w/w Cr)	0.03	0	0	0	0	0
Impurity (% w/w Zr)	0.005	0.01	0.005	0.001	0.001	0
Impurity (% w/w Ca)	0.001	0	0.005	0.005	0.01	0
Impurity (% w/w Na)	0.001	0.1	0.001	0.01	0	0.001
Impurity (% w/w S)	0	0.22	0.001	0.01	0.01	0.26
Impurity (% w/w Mg)	0	0	0	0.001	0.001	0
Organic matter (% w/w)	0	8	0	2	2	0
Crystal type (Anatase)	1	1	1	0	0	0.84
Crystal type (Rutile)	0	0	0	1	1	0.16
Crystal type (Cubic)	0	0	0	0	0	0
Crystallite size (mean)	117.81	7.69	23.93	24.32	24.71	22.44
Surface coating (declared) (%)	0	0	0	8	8	0
Specific surface area (m^2/g)	9.23	316.07	77.86	53.98	54.33	47.00
Shape (elongated=1, spherical=0)	0	0	0	1	0	1
Aspect ratio	1.53	1.53	1.53	1.70	1.53	1.36
Primary particle diameter (mean)	93.45	5.25	22.00	24.00	24.50	20.13
Particle size distribution (Z-average) (nm)	210	278	439.8	135.11	144.47	176.78
Particle size distribution-SD (Z-average) (nm)	10	0	36.66	25.27	35.21	38.99
Particle size distribution (Intensity distribution peak (nm))	NA	NA	685.55	146.62	193.94	180.75
Particle size distribution-SD (Intensity distribution peak (nm))	NA	NA	30.8	21.46	48.36	17.98
Particle size distribution (FWHM main peak)	NA	NA	444.5	82.36	100.52	75.47
Particle size distribution-SD (FWHM main peak)	NA	NA	94.9	12.74	31.37	10.20
Particle size distribution (after 24h) (nm)	NA	NA	969	198	NA	214
Particle size distribution-SD (after 24h) (nm)	NA	NA	7.65	NA	NA	NA
Particle size distribution in MQ Water, untreated, Mode #1 (nm)	391.2	1609	115	973.2	727.8	1102
Particle size distribution in MQ Water, untreated, Mode #2 (nm)	4862	1609	5170	973.2	727.8	204.7

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
Particle size distribution in PBS, untreated, Mode #1 (nm)	1440	1188	1528	1977	1817	4526
Particle size distribution in PBS, untreated, Mode #2 (nm)	5236	5148	5330	1977	5194	1150
Particle size distribution in DMEM + Lglutamate, untreated, Mode #1 (nm)	995.5	1438	2745	2255	3059	1881
Particle size distribution in DMEM + Lglutamate, untreated, Mode #2 (nm)	995.5	5560	2745	2255	3059	5372
Particle size distribution in DMEM + 1% FBS, untreated, Mode #1 (nm)	736	1201	1415	1040	1156	2454
Particle size distribution in DMEM + 1% FBS, untreated, Mode #2 (nm)	736	5232	1415	4593	5211	626.5
Particle size distribution in DMEM + 5% FBS, untreated, Mode #1 (nm)	845.4	1278	1414	991.1	719.3	1709
Particle size distribution in DMEM + 5% FBS, untreated, Mode #2 (nm)	845.4	1278	1414	991.1	5375	1709
Particle size distribution in DMEM + 10%FBS, untreated, Mode #1 (nm)	639.1	1406	1521	1156	711.2	1030
Particle size distribution in DMEM + 10%FBS, untreated, Mode #2 (nm)	4793	1406	1521	1156	711.2	4731
Particle size distribution in MQ water, untreated (Z-average) (nm)	343	1746	1062	671.6	367.8	720
Particle size distribution in PBS , untreated (Z-average) (nm)	2289	1229	1579	1397	1600	3342
Particle size distribution in DMEM + Lglutamine untreated (Z-average) (nm)	2129	1954	2427	1665	2869	2868
Particle size distribution in DMEM + 1% FBS, untreated (Z-average) (nm)	606.8	1166	1295	828.8	1111	1599
Particle size distribution in) DMEM + 5% FBS, untreated (Z-average) (nm)	621.3	1039	1234	653.2	657.5	1116
Particle size distribution in DMEM + 10% FBS, untreated (Z-average) (nm)	582.4	1127	1227	683.3	617.8	937.3
Particle size distribution in MQ Water, 1min sonication, Mode #1 (nm)	259.3	719.5	703	2649	207.7	352.6
Particle size distribution in MQ Water, 1min sonication, Mode #2 (nm)	259.3	719.5	703	2649	207.7	352.6
Particle size distribution in PBS, 1min sonication, Mode #1 (nm), Mode #1	2116	2254	2525	1629	4031	1682
Particle size distribution in PBS, 1min sonication, Mode #2 (nm)	2116	2254	2525	4619	465.2	5108
Particle size distribution in DMEM + Lglutamate, 1min sonication, Mode #1 (nm)	2973	2854	3488	4043	1701	4673
Particle size distribution in DMEM + Lglutamate, 1min sonication, Mode #2 (nm)	2973	2854	3488	4043	5560	1995
Particle size distribution in DMEM + 1% FBS , 1min sonication, Mode #1 (nm)	405.3	678.5	837.5	275.6	333.6	306.8
Particle size distribution in DMEM + 1% FBS, 1min sonication, Mode #2 (nm)	405.3	678.5	189.8	4344	4670	4755
Particle size distribution in DMEM + 5% FBS , 1min sonication, Mode #1 (nm)	408.8	755.5	901.8	432.4	278.2	336.9
Particle size distribution in DMEM + 5% , 1min sonication, Mode #2 (nm)	408.8	755.5	115.6	4881	278.2	4755
Particle size distribution in DMEM + 10%FBS, 1min sonication, Mode #1 (nm)	345.8	823.6	1077	370.9	334.4	349.8
Particle size distribution in DMEM + 10%FBS, 1min sonication, Mode #2 (nm)	345.8	823.6	1077	370.9	334.4	349.8
Particle size distribution in MQ water, 1min sonication (Z-average) (nm)	201.3	500.9	505.7	1977	194.3	227.5
Particle size distribution in PBS, 1min sonication (Z-average) (nm)	1624	1827	2079	2275	3197	3585
Particle size distribution in DMEM + , 1min sonication (Z-average) (nm)	2514	2350	2701	3551	3306	3507
Particle size distribution in DMEM + 1% FBS - 1min sonication) (Z-average) (nm)	310.4	521.2	590	263.5	278.5	265.3
Particle size distribution in DMEM + 5% FBS - 1min sonication) (Z-average) (nm)	315.2	569.2	617.3	345.8	225.8	286.3
Particle size distribution in DMEM + 10% FBS - 1min sonication) (Z-average) (nm)	283.9	623.4	732.2	286.9	267.8	281.2
Particle size distribution in MQ Water, 20min	378.8	1111	1103	765.3	344.5	902

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
sonication, Mode #1 (nm)						
Particle size distribution in MQ Water , 20min sonication, Mode #2 (nm)	378.8	4077	193.6	5041	4638	243.9
Particle size distribution in PBS , 20min sonication, Mode #1 (nm)	1042	1265	1789	1449	2779	4437
Particle size distribution in PBS Mode #2, 20min sonication, Mode #2 (nm)	5236	4976	4988	5037	2779	4437
Particle size distribution in DMEM + Lglutamate, 20min sonication, Mode #1 (nm)	1059	1974	2001	2916	3207	1956
Particle size distribution in DMEM + Lglutamate, 20min sonication, Mode #2 (nm)	1059	4881	5517	2916	3207	5290
Particle size distribution in DMEM + 1% FBS, 20min sonication, Mode #1 (nm)	631.9	1368	1063	684.1	975.4	969.6
Particle size distribution in DMEM + 1% FBS, 20min sonication, Mode #2 (nm)	5059	420.5	1063	4946	286.9	226.6
Particle size distribution in DMEM + 5% FBS, 20min sonication, Mode #1 (nm)	522.7	1073	1487	1079	925	848.3
Particle size distribution in DMEM + 5% FBS, 20min sonication, Mode #2 (nm)	5017	5046	1487	1079	925	4864
Particle size distribution in DMEM + 10%FBS, 20min sonication, Mode #1 (nm)	565.7	1255	1228	1155	605.2	1110
Particle size distribution in DMEM + 10%FBS, 20min sonication, Mode #2 (nm)	565.7	5222	1228	262.3	4991	1110
Particle size distribution in MQ water - 20min sonication)	307.6	1130	794.1	596.9	290.8	474.4
Particle size distribution in PBS,- 20min sonication (Z-average) (nm)	1217	1276	1809	1350	2284	4514
Particle size distribution in DMEM + Lglutamine, 20min sonication (Z-average) (nm)	1754	1992	1997	2268	2636	2938
Particle size distribution in DMEM + 1% FBS, 20min sonication (Z-average) (nm)	540.2	668.7	975.4	526.8	520.4	743.9
Particle size distribution in DMEM + 5% FBS, 20min sonication (Z-average) (nm)	450.4	1065	1197	656.9	696.2	921.8
Particle size distribution in DMEM + 10% FBS, 20min sonication (Z-average) (nm)	473.1	957.9	874.8	570.3	480.8	619.8
Zeta Potential in MQ Water, 1min sonication (mV)	-24.5	-27.2	-27.1	39.1	-23.4	-23.8
Zeta Potential in PBS, 1min sonication (mV)	-26.7	-19.7	-25.1	-20.8	-16.9	-20.5
Zeta Potential in DMEM + Lglutamate, 1min sonication, Mode #1 (mV)	20.5	22.3	-3.14	-8.44	-7.29	-2.55
Zeta Potential in DMEM + Lglutamate, 1min sonication, Mode #2 (mV)	-26	-34.3	-3.14	-8.44	-7.29	-2.55
Zeta Potential in DMEM + Lglutamate, 1min sonication, Mode #3 (mV)	95.4	-92	-3.14	-8.44	-7.29	-2.55
Zeta Potential in DMEM + 1% FBS, 1min sonication, Mode #1 (mV)	-9.14	-11.8	-13.6	-9.98	-8.88	-9.37
Zeta Potential in DMEM + 1% FBS, 1min sonication, Mode #2 (mV)	140	-11.8	-13.6	-9.98	-8.88	-9.37
Zeta Potential in DMEM + 5% FBS, 1min sonication, Mode #1 (mV)	107	-15	-13.4	-12	15.1	9.43
Zeta Potential in DMEM + 5% FBS, 1min sonication, Mode #2 (mV)	35.4	-15	-13.4	-12	-43.7	-47.9
Zeta Potential in DMEM + 5% FBS, 1min sonication, Mode #3 (mV)	-21.2	-15	-13.4	-12	-43.7	-47.9
Zeta Potential in DMEM + 10%FBS, 1min sonication, Mode #1 (mV)	78.4	0.13	-10.5	-12.4	-9.38	-9.92
Zeta Potential in DMEM + 10%FBS, 1min sonication, Mode #2 (mV)	12.2	0.13	-10.5	-12.4	129	-9.92
Zeta Potential in DMEM + 10%FBS, 1min sonication, Mode #3 (mV)	-27.1	0.13	-10.5	-12.4	129	-9.92
Zeta Potential in MQ Water - 20min sonication (mV)	-40.6	-27.5	30.3	39.1	24.6	-32.6
Zeta Potential in PBS - 20min sonication (mV)	-20.2	-21.7	-18.5	-20.9	-20.3	-33.2
Zeta Potential in DMEM + Lglutamate, 20min sonication, Mode #1 (mV)	-1.55	3.6	-3.46	-8.76	-9.98	-8.55
Zeta Potential in DMEM + Lglutamate, 20min sonication, Mode #2 (mV)	-1.55	-42.5	-3.46	-8.76	-9.98	-8.55
Zeta Potential in DMEM + 1% FBS, 20min sonication (mV)	-11.4	-12	-12.4	-10	-10.2	-7.76

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
Zeta Potential in DMEM + 5% FBS, 20min sonication (mV)	-10.4	-11.3	-9.47	-13.7	-9.38	-11.9
Zeta Potential in DMEM + 10%FBS, 20min sonication, Mode #1 (mV)	-11.3	-11.5	-10.4	-11.8	-10.5	-5.43
Zeta Potential in DMEM + 10%FBS, 20min sonication, Mode #2 (mV)	-11.3	-11.5	-10.4	-11.8	-10.5	-74
Polydispersity Index in MQ water - untreated	0.205	0.264	0.187	0.287	0.376	0.376
Polydispersity Index in PBS - untreated	0.219	0.239	0.769	0.255	0.232	0.232
Polydispersity Index in DMEM + Lglutamine – untreated	0.332	0.359	0.181	0.256	0.247	0.247
Polydispersity Index in DMEM + 1% FBS - untreated	0.207	0.201	0.081	0.269	0.208	0.208
Polydispersity Index in DMEM + 5% FBS - untreated	0.194	0.232	0.139	0.293	0.22	0.22
Polydispersity Index in DMEM + 10% FBS - untreated	0.176	0.194	0.182	0.369	0.201	0.201
Polydispersity Index in MQ water - 1min sonication	0.205	0.274	0.248	0.393	0.236	0.211
Polydispersity Index in PBS - 1min sonication	0.219	0.283	0.188	0.442	0.334	0.443
Polydispersity Index in DMEM + Lglutamine - 1min sonication	0.332	0.217	0.268	0.279	0.434	0.395
Polydispersity Index in DMEM + 1% FBS - 1min sonication	0.207	0.232	0.243	0.243	0.194	0.177
Polydispersity Index in DMEM + 5% FBS - 1min sonication	0.194	0.232	0.27	0.25	0.161	0.207
Polydispersity Index in DMEM + 10% FBS - 1min sonication	0.176	0.24	0.27	0.196	0.178	0.196
Polydispersity Index in MQ water - 20min sonication	0.199	0.351	0.254	0.393	0.306	0.443
Polydispersity Index in PBS - 20min sonication	0.317	0.238	0.231	0.25	0.227	0.274
Polydispersity Index in DMEM + Lglutamine - 20min sonication	0.515	0.247	0.227	0.264	0.209	0.341
Polydispersibility Index in DMEM + 1% FBS - 20min sonication	0.195	0.282	0.054	0.317	0.282	0.48
Polydispersibility Index in DMEM + 5% FBS - 20min sonication	0.223	0.302	0.179	0.367	0.221	0.456
Polydispersibility Index in DMEM + 10% FBS - 20min sonication	0.204	0.234	0.235	0.417	0.239	0.391
Polydispersity Index (PdI)	NA	0.323	0.427	0.292	0.227	0.245
IsoelectricPoint (Mean)	NA	5.5	6	8.3	8.5	6.8
IsoelectricPoint (Min)	NA	5.3	6	8.2	8.2	6.6
IsoelectricPoint (Max)	NA	5.7	6	8.5	8.8	6.9
Density (g/mL)	3.84	3.99	3.84	4.015	4.09	4.052
Mean of total pore volume (mL/g)	0.0324	0.319	0.2996	0.2616	0.1935	0.1937
Micro surface area (m^2/g)	0	13.625	1.108	0	0	0
Micropore volume (mL/g)	0	0.00179	0.00034	0	0	0
Specific surface area (mean)	9.23	242.785	77.864	53.983	54.331	47
Dustiness-Respirable(mg/kg)	1500	5600	9200	19000	6400	11000
Biodurability 24h 0.05% BSA (Ti content) (µg/l)	5.2	0	0	0	0	0
Biodurability 24h Gambles solution (Ti content) (µg/l)	0	0	3388	0	0	0
Biodurability 24h Caco2 (Ti content) (µg/l)	796	3414	1741	222	3386	2724
Biodurability 24h 0.05% BSA (Al content) (µg/l)	0	175	0	198	137	0
Biodurability 24h Gambles solution (Al content) (µg/l)	0	177	0	868	413	0
Biodurability 24h Caco2 (Al content) (µg/l)	24	252	0	182	413	0
Biodurability 24h 0.05% BSA (Si content) (mg/l)	0	0	0	0.9	0	0
Biodurability 24h Gambles solution (Si content)	0	0	0	2.0	0	0

Name	NM-100	NM-101	NM-102	NM-103	NM-104	NM-105
(µg/l)						
Biodurability 24h Caco2 (Si content) (µg/l)	0	0	0	1.7	0	0
Redox Caco2 medium [Ω]	1	-1	-1	1	-1	-1
Redox Gamble's solution [Ω]	1	0	-1	1	-1	-1
Redox BSA [Ω]	0	0	0	0	0	0

[Ω] values obtained from Nanogenotox 4.7 determined by measuring the content of O_2. Oxidising properties (1), neutral (0), reducing (-1)

Appendix XI. Literature search on genotoxicity studies

Our initial dataset on toxicological endpoints was collected from the OECD dossier on TiO$_2$ (OECD, 2015) that, although not aimed specifically at hazard assessment, is considered an updated NMs data repository. This starting toxicological dataset which content is reported in Table 4. was expanded for the selected endpoint to be read-across (genotoxicity) by searching available studies in the literature.

A bibliographic search was done in August 2016 in Scopus using the keywords genotox*, nano*, TiO$_2$. It resulted in 152 review and research papers. A first selection done depending on title and abstract contents: if it was not about genotoxicity testing, the papers were not included. Also, only toxicity studies relevant for human health hazard assessment were considered (studies on bacteria, plants, mussels, fish were excluded).

Table A9.1 reports the list of papers containing genotoxicity studies that were taken into consideration for the selection of the genotoxicity tests to read-across. According to the first criteria, related to NMs characterisation, the information necessary for identification was available in the reliable studies, hence the studied NM could be assigned to the corresponding analogue in our case study. The results coming from the reliable studies reported in the table are accounted for in Table 4..

Table A9.1. List of genotoxicity studies found in the literature search to extend the number of studies for building our grouping hypothesis for our set of source NMs.

	Assay type	Reference	Reliability
In vivo	Comet	(Dobrzyńska et al., 2014)	Unreliable
		(Trouiller et al., 2009)	Unreliable
		(Suzuki et al., 2016)	Unreliable
		(Louro et al., 2014)	Reliable
	Micronucleus	(Dobrzyńska et al., 2014)	Unreliable
		(Trouiller et al., 2009)	Unreliable
		(Suzuki et al., 2016)	Unreliable
		(Louro et al., 2014)	Reliable
	Chromosome aberration	(Louro et al., 2014)	Reliable
In vitro	Comet	(Prasad, Wallace, Daniel, Tennant, Zucker, Strickland, Dreher, Kligerman, Blackman, & Demarini, 2013)	Reliable
		(Vales et al., 2014)	Reliable

	Assay type	Reference	Reliability
		(Kansara et al., 2015)	Reliable
		(Armand et al., 2016)	Reliable
		(Jugan et al., 2012)	Reliable
		(Gerloff et al., 2012b)	Reliable
		(A Kermanizadeh et al., 2012)	Reliable
		(Ali Kermanizadeh et al., 2013)	Reliable
		(Stoccoro et al., 2016)	Unreliable
		(Guichard et al., 2012)	Reliable
		(Barillet et al., 2010)	Reliable
		(Guichard et al., 2012)	Reliable
	Micronucleus	(Prasad, Wallace, Daniel, Tennant, Zucker, Strickland, Dreher, Kligerman, Blackman, & Demarini, 2013)	Reliable
		(Prasad et al., 2014)	Reliable
		(Vales et al., 2014)	Reliable
		(Kansara et al., 2015)	Reliable
		(Jugan et al., 2012)	Reliable
		(Armand et al., 2016)	Reliable
		(Tavares et al., 2014a)	Reliable
		(Stoccoro et al., 2016)	Unreliable
		(Guichard et al., 2012)	Reliable
	Chromosome aberration	(L Browning et al., 2014)	Reliable

Reliability of the studies was assessed following the criteria reported in paragraph 4.4.3.2.

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.

EU Science Hub
ec.europa.eu/jrc

@EU_ScienceHub

EU Science Hub - Joint Research Centre

Joint Research Centre

EU Science Hub

Publications Office